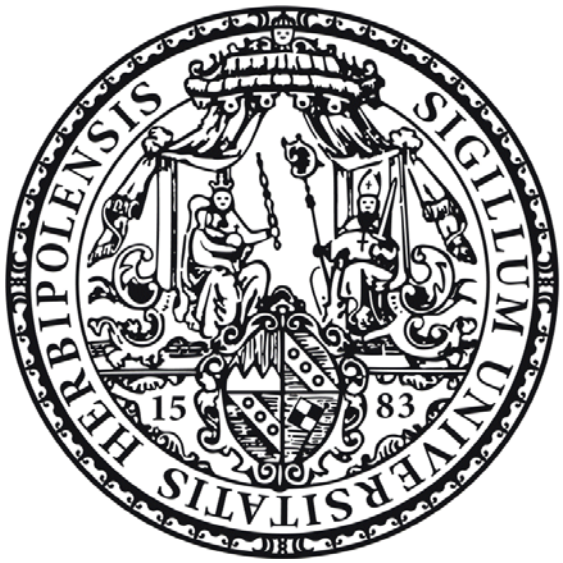
Effects of timing and herbivory on a grass-endophyte association and its trophic interactions

Auswirkungen von Timing und Herbivorie auf eine Gras-Endophyten Assoziation und ihre trophischen Interaktionen



Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences, Julius-Maximilians-Universität Würzburg, Section Integrative Biology

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Just because you cannot see something, does not mean it is not there

(Willard Wigan)

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Affidavit

I hereby confirm that my thesis entitled “Effects of timing and herbivory on a grass-endophyte association and its trophic interactions” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.
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Publication list

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Summary

I.) Plant associated microorganisms can affect the plant`s interaction with herbivores and higher trophic levels. For instance, endophytic fungi infecting aerial plant parts of grass species produce bioactive alkaloids that can negatively affect species from higher trophic levels, indicating a defensive mutualism between the grass and the endophyte. However, beneficial insects can also be negatively affected by the endophyte, which might question the mutualistic effect of endophytic fungi. On the other hand, grass-endophytes are affected by environmental conditions and species interactions. Grazing can increase endophyte frequencies in natural habitats. Furthermore, endophyte mediated effects on herbivores are most pronounced during warm summers following rainy springs. In this study, we investigated whether endophyte derived alkaloids cascade up a food chain (chapter II) and whether their concentrations depend on plant age and season (chapter III). Further we analysed, whether altered herbivore phenology affects the endophytic fungus (chapter IV) and whether endophyte derived alkaloid production is induced by different herbivore species (chapter V).

II.) In our first experimental study we analysed whether grass-endophyte derived alkaloids decreased the performance of two ladybird species feeding on aphids exclusively reared on endophyte infected grass (6 weeks young grass). Further, we screened species from three trophic levels (grass, herbivores and aphid predators) for their alkaloid content using two year old infected grass as diet for herbivores. We established an UPLC-MS method to detect and quantify the amount of the endophyte derived alkaloids peramine and lolitrem B extracted from the organic plant and insect material. Performance parameters of ladybirds revealed little differences between ladybirds fed on aphids reared on endophyte infected and non-infected grass, which probably resulted from low alkaloid concentrations in the young (6-weeks old) endophyte infected grass used in this part of the study. Alkaloid quantification of the two year old endophyte infected grass, herbivores and aphid predators revealed similar concentrations between grass and aphids, while aphid predators contained approximately half of that amount which still exceeded the bioactive threshold. We conclude that alkaloids produced by grass-endophytes cascade up the food chain and are responsible for fitness disadvantages of higher trophic levels.

III.) In the second study we investigated the impact of plant age and seasonal timing on grass-endophyte growth and alkaloid production. Plants were sown in April of 2013 and sampled monthly over 30 consecutive months. Endophyte growth was quantified with real-time PCR (qPCR) and alkaloid concentrations with UPLC-MS. We showed that alkaloid concentrations and fungal growth followed a seasonal rhythmicity and that alkaloid concentrations increased with plant age. Alkaloid concentrations peak during summer, when also herbivore abundances are high. Consequently, we conclude that plant age and season contribute to the toxicity of endophytes on grass herbivores

IV.) In the third study we simulated earlier spring arrival of aphids by enhancing aphid abundance on endophyte infected and endophyte-free grass in spring and analysed responses across three trophic levels. Enhanced aphid abundance in spring caused higher aphid abundances during the study period. Predators stayed unaffected by increased herbivore abundances; however they did level aphid numbers within two weeks after arrival on the plants, independent of aphid abundance. Grass-endophyte showed a time delayed growth, two weeks after aphid abundance peak and after predators already controlled aphid infestations on the plants. We conclude that phenology shifts of herbivorous insects can affect multi-trophic interactions leading to desynchronizations between phenologies of interacting species and mismatches in food-webs.

V.) In the fourth study we analysed whether herbivores induce endophyte growth and alkaloid production and whether different types of herbivores induce specific alkaloid production. We applied three different herbivore treatments on endophyte infected grass over 18 weeks. Locust herbivory increased the insect deterring alkaloid peramine and clipping of plants (simulation of grazing livestock) increased the vertebrate toxic alkaloid lolitrem B. Aphid herbivory did not affect endophyte derived alkaloid concentrations. Endophyte responses to herbivory were species specific which indicates a primarily plant protecting role of alkaloid synthesis in endophyte infected plants and a close chemical crosstalk between interacting species.

VI.) In summary, we showed that endophyte derived alkaloids affect higher trophic levels and that alkaloid concentrations in the plant depend on prevalent herbivore species, plant age and seasonal timing. Our results indicate a close chemical crosstalk between the host plant and the endophytic fungus which is susceptible to environmental changes altering the endophyte`s

alkaloid production in plants. We gained insights into the grass-endophyte symbiosis in ecological contexts and conclude that several factors determine the herbivore toxic potential of endophytic fungi and thereby their plant mutualistic or parasitic character. Future studies should investigate the mechanisms behind the herbivore induced alkaloid concentration increase, shown in this thesis, especially whether plant signals mediate the endophyte response. Furthermore it would be interesting to study the induction of indirect endophyte mediated defence and how it affects multi-trophic level interactions.

Zusammenfassung

I.) Mit Pflanzen assoziierte Mikroorganismen können die Interaktionen von Pflanzen mit Herbivoren und höheren trophischen Ebenen beeinflussen. Endophytische Pilze, die oberirdische Pflanzenteile von Gräsern infizieren, produzieren bioaktive Alkaloide, die negative Effekte auf pflanzenfressende Organismen haben können. Blattlaus parasitierende und räuberische Insekten hatten ebenso Fitnessnachteile, wenn sie mit Blattläusen gefüttert wurden, die auf *Epichloë festucae* var. *lolii* infiziertem *Lolium perenne* gezüchtet wurden. Umwelteinflüsse und Interaktionen zu anderen Arten beeinträchtigen das Wachstum und die Alkaloid Produktion von Endophyten. Die Häufigkeit von Endophyten in natürlichen Habitaten können von Herbivorie beeinflusst werden. Weiterhin sind Endophyten verursachte Effekte auf Pflanzenfresser am häufigsten in warmen Sommermonaten nach regenreichen Frühlingsmonaten. In dieser Studie analysieren wir, ob Endophyten produzierte Alkaloide in einer Nahrungskette aufsteigen und in höheren trophischen Ebenen gefunden werden (Kapitel II) und ob ihre Konzentrationen von Pflanzenalter und Saison abhängig sind (Kapitel III). Weiterhin analysieren wir, ob veränderte Phänologie von herbivoren Insekten den endophytischen Pilz beeinflussen (Kapitel IV) und ob Alkaloid Produktion von verschiedenen Pflanzenfressern induziert wird (Kapitel V).

II.) In der ersten Studie analysierten wir, ob Alkaloide, die von Gras Endophyten produziert werden, die Fitness von zwei Marienkäfer Arten verringern, wenn sie mit Blattläusen gefüttert werden, die ausschließlich auf Endophyten infiziertem Gras (6 Wochen alt) gezüchtet wurden. Weiterhin analysierten wir Arten aus drei trophischen Ebenen (Gras, Herbivore, Blattlaus Prädatoren) auf ihren Alkaloid Gehalt mit zwei Jahre altem Endophyten infizierten Gras als Futter für pflanzenfressende Insekten. Wir etablierten eine UPLC-MS Methode, um die Endophyten produzierten Alkaloide Peramin und Lolitrem B zu detektieren und zu quantifizieren, nach der Extraktion aus organischem Material von Pflanzen und Insekten. Fitness von Marienkäfern zeigte nur geringe Unterschiede zwischen Marienkäfern, denen Blattläuse gefüttert wurden, die ausschließlich auf Endophyten infizierten Grass oder nicht infiziertem Grass gezüchtet wurden. Die Ursache dafür, ist wahrscheinlich eine zu geringe Alkaloid Konzentration in dem jungen, Endophyten-infizierten Grass (6 Wochen alt), das für diesen Teil der Studie verwendet wurde. Die Quantifizierung der Alkaloide von zwei Jahre altem Endophyten infiziertem Gras, Herbivoren und Prädatoren zeigte ähnliche

Konzentrationen zwischen Gras und Blattläusen, wohingegen Prädatoren etwa eine halb so hohe Alkaloid Konzentration enthielten, die aber dennoch den Grenzwert für biologische Wirksamkeit überschritt. Wir folgern, dass Endophyten produzierte Alkaloide innerhalb der Nahrungskette weitergegeben werden und somit verantwortlich für Nachteile auf die Fitness höherer trophischer Ebenen sind.

III.) In der zweiten Studie untersuchten wir den Einfluss von Pflanzenalter und Saison auf Wachstum des Endophyten und dessen Alkaloid Produktion. Pflanzen wurden im April 2013 gesät und über einen Zeitraum von 30 Monaten monatlich beprobt. Endophyten Wachstum wurde mittels real-time PCR (qPCR) und Alkaloide mittels UPLC-MS quantifiziert. Wir zeigten, dass Pilzwachstum und Alkaloid Produktion einer saisonalen Rhythmik folgen und Alkaloid Konzentrationen zudem mit dem Pflanzenalter ansteigen. Demzufolge tragen Pflanzenalter und Saison zur Toxizität von endophytischen Pilzen bei. Alkaloid Konzentrationen sind am höchsten im Sommer, wenn auch die Abundanzen von Herbivoren besonders hoch sind.

IV.) In der dritten Studie simulierten wir frühzeitiges Blattlausvorkommen, indem wir Blattlausabundanzen auf Endophyten freien und infizierten Graspflanzen im Frühling erhöhten und die Reaktionen auf drei trophischen Ebenen analysierten. Top-Down Kontrolle durch Blattlausprädatoren wurde nicht von erhöhten Blattlausabundanzen beeinflusst, aber innerhalb von zwei Wochen nach ihrem Erscheinen, reduzierten Prädatoren die Anzahl an Blattläusen erheblich, unabhängig von den Blattlausabundanzen. Bottom-Up Kontrolle durch erhöhtes Wachstum der Grasendophyten war zeitlich verzögert, zwei Wochen nachdem Blattlausabundanzen ihre Maxima erreichten und bereits durch Prädatoren stark dezimiert waren. Daraus schlussfolgern wir, dass Phänologieverschiebungen von pflanzenfressenden Insekten multi-trophische Interaktionen beeinflussen und zu einer Desynchronisierung der Phänologien von interagierenden Arten und somit unausgeglichenen Beziehungen in Nahrungsnetzen führen können.

V.) In der vierten Studie analysierten wir, ob Herbivore Insekten das Wachstum und die Alkaloid Produktion von endophytischen Pilzen induzieren und ob verschiedene Arten von Herbivorie die Produktion spezifischer Alkaloide induzieren. Wir applizierten drei verschieden Arten von Pflanzenfraß Treatments an Endophyten infiziertem Grass über einen Zeitraum von 18 Wochen und fanden heraus, dass Heuschrecken eine Erhöhung der

Konzentration des insektenwirksamen Alkaloids induzieren und Schnitt der Pflanze (Simulierung von Weidevieh) eine Erhöhung des Vertebraten toxischen Alkaloids Lolitrem B. Herbivorie durch Blattläuse hatte keinen Einfluss auf Konzentrationen von Endophyten produzierten Alkaloiden. Reaktionen von endophytischen Pilzen auf Herbivorie sind demnach artspezifisch, was darauf hinweist, dass Alkaloide vorrangig zu Pflanzenschutz Zwecken gegen Fraßfeinde produziert werden.

VI.) Zusammenfassend zeigt die vorliegende Arbeit, dass die von endophytischen Pilzen produzierten Alkaloide, Organismen höherer trophischer Ebenen beeinflussen können und dass Alkaloid Konzentrationen im Wirtsgras von Herbivorietyp, Pflanzenalter und saisonbedingten Umwelteinflüssen abhängig sind. Schlussendlich deuten unsere Ergebnisse auf eine enge chemische Verständigung zwischen Wirtsgras und endophytischem Pilz hin, empfindlich gegenüber Umweltveränderungen, die folglich eine Veränderung in der Konzentration von Endophyten produzierten Alkaloiden auslösen. Zukünftige Studien sollten die Mechanismen hinter induziertem Anstieg von Alkaloid Konzentrationen analysieren, speziell, ob Pflanzensignale die Reaktion des Endophyten auf Herbivorie vermitteln. Weiterhin könnte eine Endophyten-vermittelte Induktion von indirekter Pflanzenabwehr zur Interaktion zwischen mehreren trophischen Ebenen beitragen.

Chapter I: General Introduction

Endophyte-grass symbiosis

Symbiotic relations between microorganisms like fungi or bacteria and plants or plant parts are widespread interactions in nature (Van Der Heijden, Bardgett & Van Straalen 2008). Symbiosis describes a close relation between species, including interactions from parasitic to mutualistic with many other forms of symbiosis between those extremes, depending on several species specific and environmental predictors (Hirsch 2004). In many cases these symbiotic relations are of mutualistic nature with benefits for both symbiotic partners. The most prominent example of plant-fungus mutualism is mycorrhizal association where the fungus benefits from photosynthetic products while providing the plant enhanced nutrient availability from the soil (Parniske 2008). Besides mycorrhizal fungi many other fungal species can infect plants, often without creating any noticeable phenotypic symptoms on the host (Clay & Schardl 2002). However, many fungal infections benefit their host plant. Among the most pronounced advantages are enhanced herbivore protection, higher stress tolerance or an increased water and nutrient uptake, which is of high scientific and agronomical interest (Hartley & Gange 2009). Indeed many agronomical important grass species often host endophytes with tremendous biological consequences. Endophytic living organisms infect their host and live inside the plant tissue, unlike epiphytic organisms living on the plant surface (Schardl, Leuchtmann & Spiering 2004).

Vertically transmitted endophytic fungi from the genus *Epichloë* infect the intercellular aerial plant parts of cool-season grass species and reproduce asexually via the plant seeds (Clay & Schardl 2002) (Fig I 1). Symbiotic endophytic fungi infecting cool-season grass species can be mutualistic or parasitic but mostly exist as a continuum between mutualism and parasitism depending on genetic predispositions, cultivar, endophyte strain, abiotic environmental factors (e.g. nutrient and water supply) and biotic interactions with other species (Müller & Krauss 2005). The focus of my thesis is the symbiosis between the cool-season grass species *Lolium perenne* in the family Poaceae with the foliar grass-endophyte *Epichloë festucae* var. *lolii* (formerly known as *Neotyphodium lolii* (Leuchtmann *et al.* 2014)), a fungus in the family Clavicipitaceae (phylum Ascomycota) (Fig I 1). Endophytic fungi from the genus *Epichloë* exist as sexually reproducing forms creating spores on the host grass surface which are transmitted by certain species of flies (Bultman & Leuchtmann 2008) and as asexual forms which only reproduce via distribution of the plants seeds like our study organism *Epichloë*

festucae var. *lolii* (Müller & Krauss 2005) (Fig I 1). Thereby, the transmission rate of the fungus to the next generation of grass plants can reach up to 100% (Clay 1997; Gundel, Rudgers & Ghersa 2011).

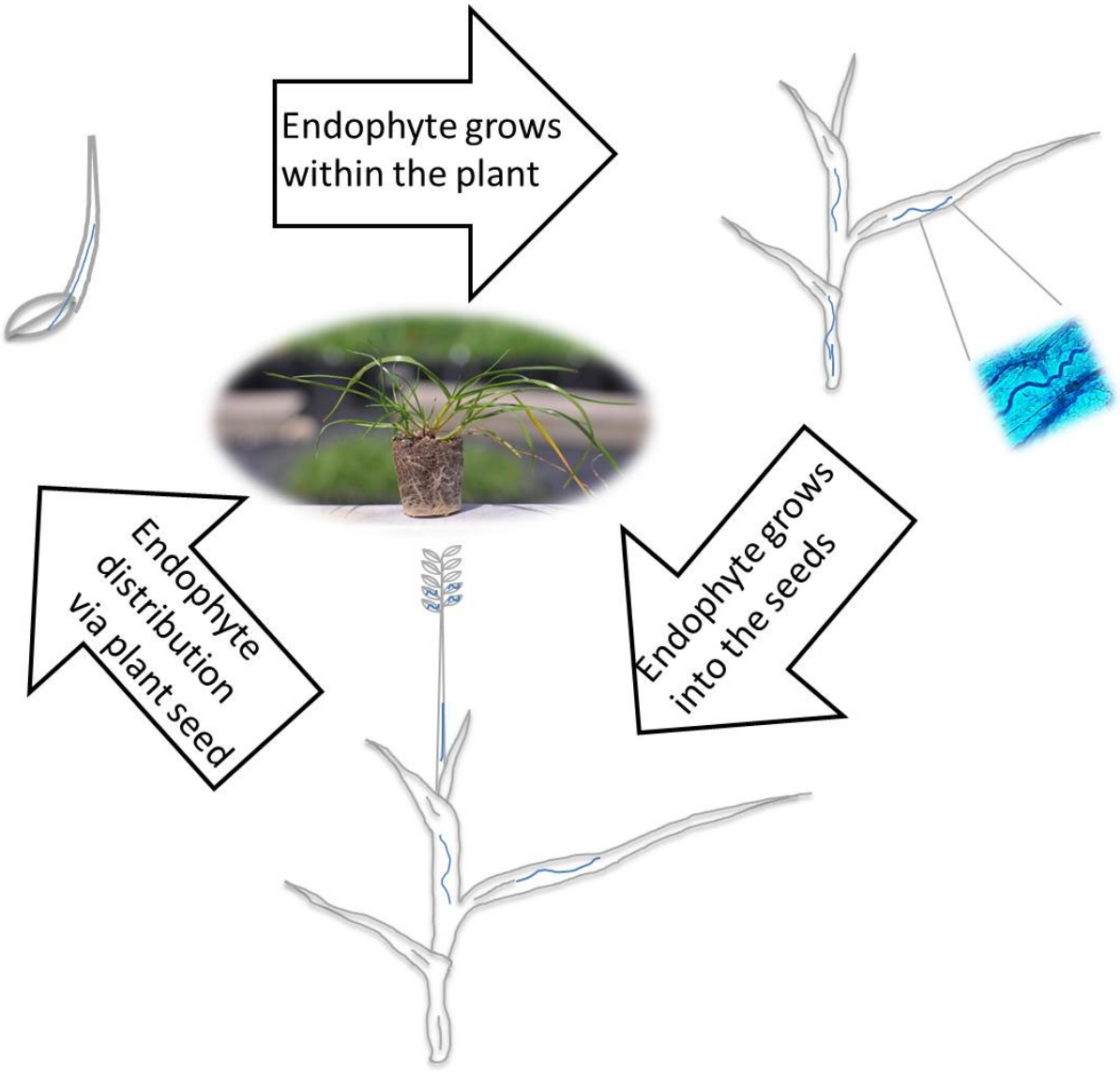

Fig I 1: Reproduction cycle of *Epichloë festucae* var. *lolii*. Intercellular growing fungal hyphae infecting the host plant *Lolium perenne* and are distributed via plant seed.

The grass-endophyte only grows intercellular within the aboveground plant tissue of the grass host where it can produce several biologically active compounds (Panaccione, Beaulieu & Cook 2014). Bioactive endophyte derived alkaloids alter the grass interaction with several species including neighbouring plants, other fungi, insects (Rudgers, Koslow & Clay 2004; Rudgers *et al.* 2016; Afkhami, Rudgers & Stachowicz 2014), herbivorous and parasitoid insects (Meister *et al.* 2006; Härri, Krauss & Müller 2008c) and herbivorous and granivorous birds and mammals (Clay 1990; Madej & Clay 1991). One of the major compound classes produced by *Epichloë* endophytes are alkaloids which received most scientific attention due

to their direct plant defensive features against herbivores (Bush, Wilkinson & Schardl 1997; Clay & Schardl 2002). The main focus of my PhD thesis are endophyte derived alkaloids, studied in a different ecological context in each chapter.

Grass-endophytes in multi-species interactions

Most endophytic fungi produce alkaloids, which show biological activity against different kinds of herbivores. Especially seed-transmitted endophytic fungi can produce several distinct groups of alkaloids supporting the host plant in defence against pest species (Clay 2014). Four major groups of alkaloids produced by Epichloë endophytes have been identified: Ergot alkaloids (Ergovaline), aminopyrrolizidine alkaloids (lolines), indolediterpene alkaloids (lolitrem B) and pyrrolopyrazine alkaloid (peramine) (Schardl *et al.* 2004) (Fig I 2).

Ergovaline and lolitrem B are toxic to vertebrate herbivores and can cause diseases to grazing livestock, such as "ryegrass staggers" and "fescue toxicosis" in concentrations from 0.3-0.8 µg/g plant material for ergovaline and 1.8-2 µg/g for lolitrem B (Tor-Agbidye, Blythe & Craig 2001; Hovermale & Craig 2001). The neurotoxin ergovaline is the cause of "fescue foot" and "fescue toxicosis" diseases in cattle, which led to tremendous agronomic costs in the past and still are a reason for losses in dairy production (Thom *et al.* 2012). Endophyte mediated livestock diseases occur especially in countries with predominantly monocultural pastures and highly grazed landscapes (Kauppinen *et al.* 2016). Countries like the USA and New Zealand were facing high agronomic losses due to endophyte intoxications until the introduction of symbiotically modified grass-endophyte combinations, which did not produce vertebrate toxic alkaloids (Easton *et al.* 2001; Young, Hume & McCulley 2013). Moreover, endophytic fungi can produce loline alkaloids and peramine which are rather harmless for vertebrate herbivores but are deterring to toxic for invertebrate herbivores feeding on the host plant (Panaccione *et al.* 2014). A New Zealand success story was the discovery that peramine prevents the Argentine stem weevil from

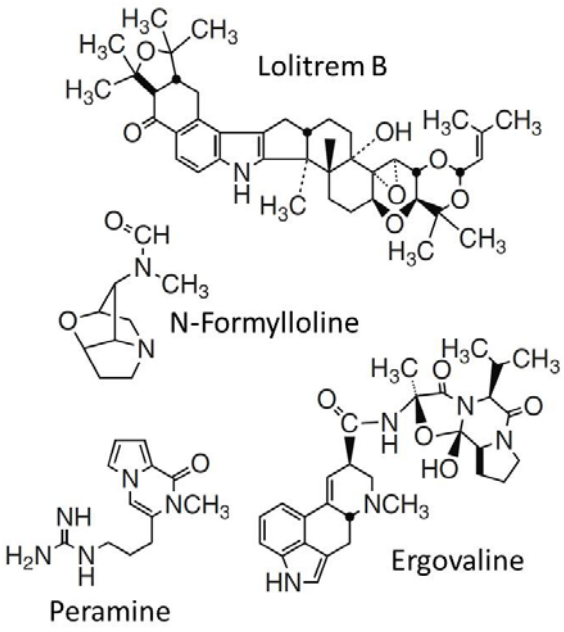

Fig I 2: Major alkaloids produced by vertically transmitted endophytic fungi of the genus *Epichloë* (Schardl *et al.* 2013a).

feeding on perennial ryegrass (Rowan, Dymock & Brimble 1990). Before the targeted application of endophyte infected grass on New Zealand pastures, larvae of the Argentine stem weevil caused high damage to pasture grass by tunnelling inside the ryegrass tillers and killing several tillers (McNeill, Knight & Baird 2001). Loline alkaloids are similar to peramine the most effective against insect herbivores, presumably with little effects on vertebrate grazers (Clay & Schardl 2002). Loline alkaloids are of little importance in this thesis since *Epichloë festuca* var. *lolii* does not produce them.

Besides the effects on the Argentine stem weevil, peramine affects the fitness of several other herbivores such as fall armyworm (*Spodoptera frugiperda*), locusts (*Schistocerca americana*) and aphids (*Rhopalosiphum padi*) (Salminen *et al.* 2005; Meister *et al.* 2006; Crawford, Land & Rudgers 2010). The symbiosis between endophytic fungi and cool-season grass is often entitled as defensive mutualism, where the plant provides nutrients and the endophyte provides herbivore defence (Clay 2014) (Fig I 3).

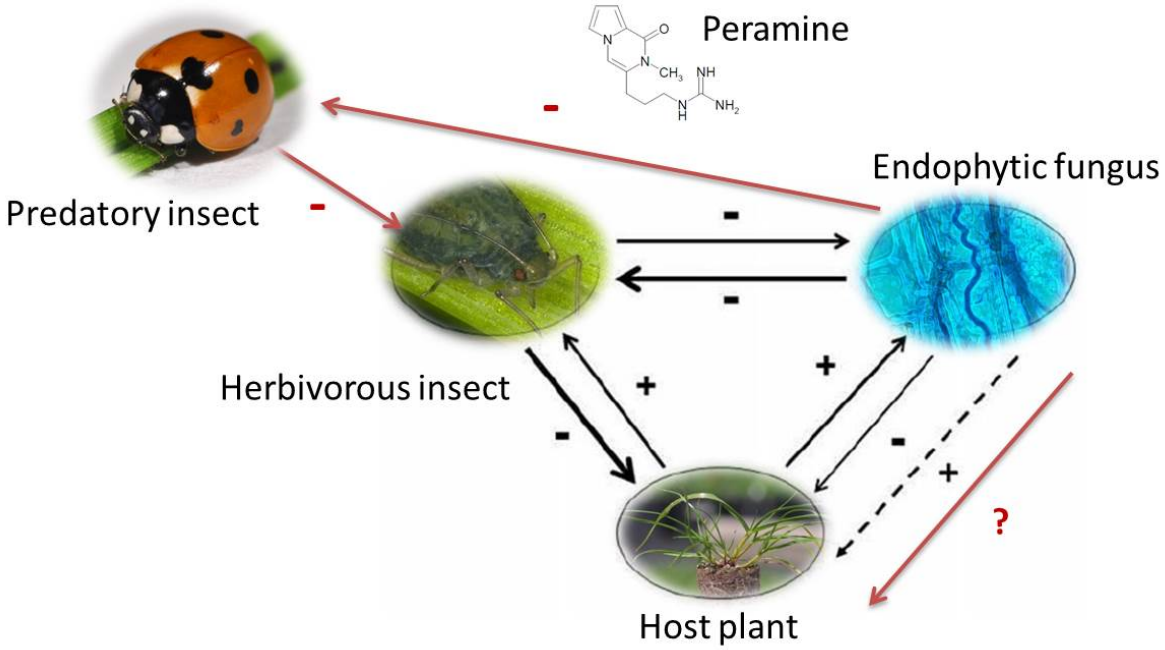

Fig I 3: Defensive mutualism shown with black arrows (Clay 2014): the endophyte harms the grass by taking nutrients, but deters herbivorous insects, probably by producing biologically active alkaloids (peramine). Endophyte harming higher trophic levels might question the beneficial function of endophytes (red arrows).

It is assumed that defensive mutualism played a major role in the evolutionary success of grass-endophyte associations, especially in environments with high herbivory pressure (Clay & Schardl 2002; Lehtonen, Helander & Saikkonen 2004; Koh & Hik 2007). However there are indications that several grass-endophytes in natural habitats have a parasitic nature, because of high costs for the host plants and little endophyte mediated protection (Saikkonen

et al. 1998; Cheplick & Faeth 2009). However, endophyte infected plants had an enhanced fitness in highly grazed areas compared to non-infected plants, which caused higher reproductive success and increased dispersal (Clay, Holah & Rudgers 2005). Thus, without herbivory pressure, endophyte infection might be lost or decrease in frequency (Clay 2014).

Effects on invertebrates mediated by endophyte derived bioactive metabolites harm not only herbivores but also enemies of herbivores, which might question the defensive mutualistic symbiosis between endophytic fungi and grass species (de Sassi, Müller & Krauss 2006) (Fig I 3). Aphids are common and very abundant herbivorous insects, feeding on plants by piercing through the plant cuticle and receiving nutrients by sucking mainly phloem sap of many plants including crop and pasture species (Van Emden & Harrington 2007). Thereby aphids cause productivity losses, enhance the chance of pathogen infection of plant surface by excretion of honey dew and transmit several plant viruses (Rabbinge *et al.* 1981; Dedryver, Le Ralec & Fabre 2010). Some species of aphid infested plants developed defence mechanisms to minimize herbivory damage (Züst & Agrawal 2016). One successful strategy is the production of secondary plant metabolites which are only produced with the purpose of herbivore deterrence (bottom-up control) (Leitner, Boland & Mithöfer 2005). Plant defence against aphids can further be accomplished by symbiotic microorganisms, like endophytic fungi with the production of bioactive metabolites (Meister *et al.* 2006; Panaccione *et al.* 2014). On the other hand, aphids can be top-down controlled by many predatory and parasitoid insects (Tscharntke & Hawkins 2002). In multi trophic food webs where herbivore deterrence is accomplished by endophytic fungi, the negative effect of endophyte derived alkaloids might also negatively affect higher trophic levels and decrease the fitness of predators and primary and secondary parasitoids of aphids (de Sassi *et al.* 2006; Härri, Krauss & Müller 2008a; Härri *et al.* 2008c; Härri, Krauss & Müller 2009) (Fig I 3). Affecting not only herbivores but also their enemies might question the implications of mutualistic defence with alkaloid production from the grass-endophytes (Fig I 3).

Effects of environmental factors on grass-endophytes

After outlining the effects of endophyte infection on plant interacting species, I will continue with effects from the environment on endophyte frequency, growth and alkaloid production. Every species on earth interacts with the environment and responds to environmental predictors (Herrera & Pellmyr 2002). Environmental predictors can be abiotic factors such as temperature, nutrition and water supply or biotic factors like symbionts, competitors and enemies (Mitchell *et al.* 2006) (Fig I 4). Higher frequencies of endophyte infected grass were found on pastures with rather hot and dry summers, probably explained by the endophyte mediated enhanced drought resistance compared to non-infected plants (Lewis *et al.* 1997; Leyronas & Raynal 2001). Outbreaks of livestock intoxications occurred especially in years with rainy spring and dry-hot summers (Reed *et al.* 2011a). Furthermore, the occurrence of vertebrate grazers increased the endophyte frequency in nature (Koh & Hik 2007). The endophyte growth itself is supposed to increase with plant growth and therefore depends on nutrient availability, precipitation and temperature among others (Schardl *et al.* 2004). Alkaloid concentrations correlate with endophyte growth and might increase with increasing endophyte concentration in the plant (Ryan *et al.* 2014).

Influences of biotic predictors on grass-endophytes range from other plant symbiotic microbes which can indirectly interact with endophytic fungi like root infecting mycorrhizae fungi (Vicari, Hatcher & Ayres 2002; Liu *et al.* 2011; Afkhami *et al.* 2014), to herbivorous insects and grazing livestock which directly interact by removing nutrients and damaging fungal and grass tissue (Cheplick & Faeth 2009; Tanentzap, Vicari & Bazely 2014). The assumption is that endophytic fungi respond to environmental changes similar as their host plant, since endophyte fitness is closely linked to nutrients provided by their host (Siegel, Latch & Johnson 1987). Many studies show performance effects on interacting species mediated by endophytic fungi but studies analysing the effects of biotic environment on endophyte growth and endophyte derived alkaloids are rare.

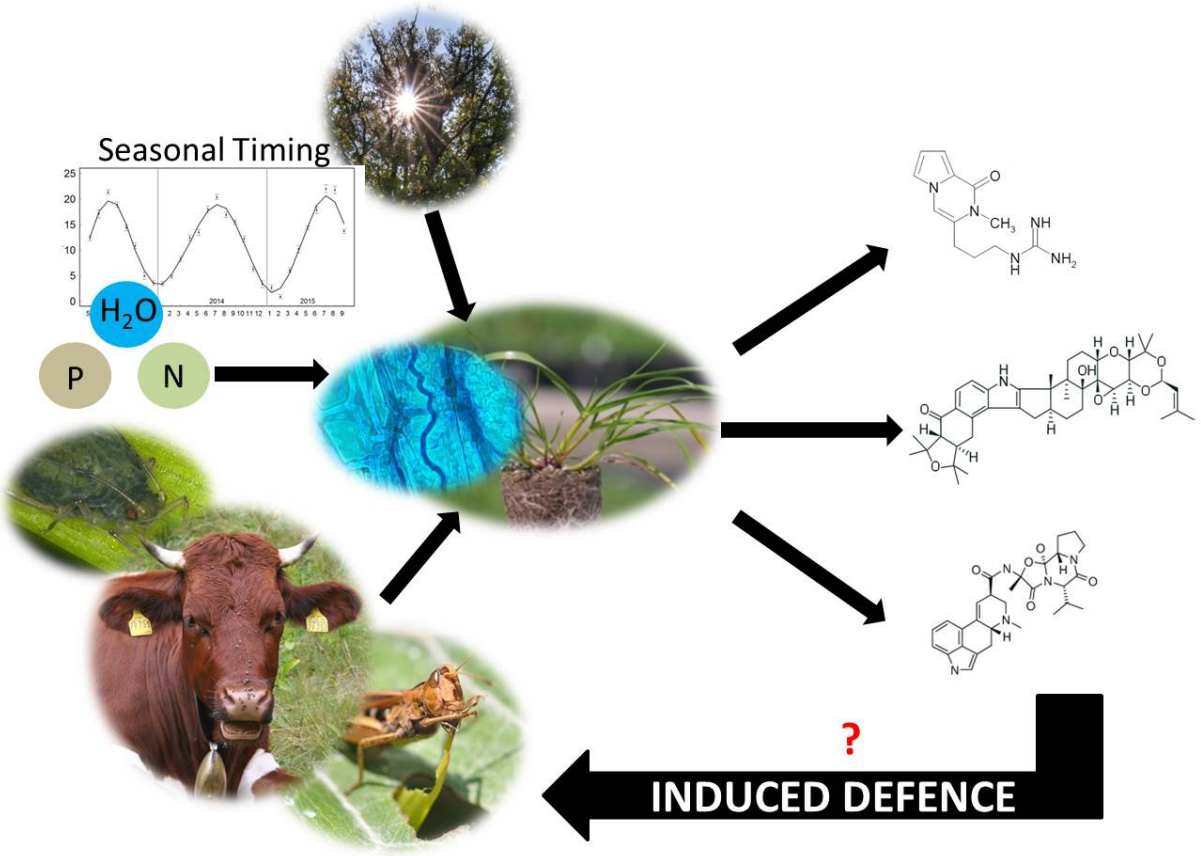

Fig I 4: Schematic representation of biotic and abiotic factors that affect the grass-endophyte symbiosis and thereby determine the alkaloid levels produced by the endophyte.

Plants often respond to herbivory with induced production of secondary metabolites directly targeting herbivores (direct defence) or attracting enemies of herbivores (indirect defence) (Agrawal 1998). For endophyte derived alkaloid production, induction is a possible mechanism, which received little attention in current literature. Endophyte derived alkaloids showed an increase in loline concentration after simulation of grazing (via clipping of the host plant) indicating an induced defence response (Bultman, Bell & Martin 2004). Furthermore alkaloid biosynthesis genes were upregulated after herbivory by *Spodoptera frugiperda* caterpillars. Contrary, Tanentzap, Vicari & Bazely (2014) showed an inhibition of endophyte growth and alkaloid production due to vertebrate saliva which indicates decreasing endophyte activity caused by active grazing.

Endophytic fungi in a changing world

Man-made climate change increased in the last decades, leading to temperature increase, higher CO_2 concentrations and an accumulation of extreme events. During the last 3 decades, temperature increased at a rate of ~0.2°C per decade and is predicted to increase to 3.7-4.8° C until the year 2100 compared to pre industrial levels (Edenhofer *et al.* 2014). Within

interspecific interactions species rely on their phenological synchronicity, especially in multi-trophic food webs (Visser, Holleman & Gienapp 2006) (Fig I 5). Consequences of higher temperatures are phenology shifts of species towards an earlier spring activity or emigration towards cooler regions. Not every species shifts in the same way following climate change, which affects species interactions, desynchronizes species relations and alters food web structures (Parmesan & Yohe 2003; Thackeray *et al.* 2016) (Fig I 5). For example, a mismatch occurred between a bird species and its main food source, a caterpillar, due to unequal phenology shifts of both species in spring following climate change (Visser *et al.* 2006). Another study showed ant aphid mutualism brake-down due to experimental warming (Barton & Ives 2014). Aphids are a widespread pest species for plants of nearly every species worldwide. Aphids feeding on crops arrive in spring after hibernation as eggs on their winter host plant (Leather 1993). Our studies were conducted with the bird cherry oat aphid *Rhopalosiphum padi*, whose winter host is the bird cherry tree (*Prunus padus*) (Dixon 1971). In summer, the aphid feeds on different crops and grass species and can transmit diseases like the Barley Yellow Dwarf Virus which causes substantial agronomic damages (Gray *et al.* 1991). The appearance of aphids on its summer host depends mostly on the temperature and earlier first flight trends were recorded during the last 50 years (Bell *et al.* 2015). It is assumed that the appearance of these aphids will further advance with climate change while the effects on interacting species are unknown. Especially interesting from an agronomical point of view is the biological control of aphids under climate change. Aphids can be bottom-up controlled by plant quality or top-down controlled by aphid predators and parasitoids (Boyer *et al.* 2003). In multi-trophic networks each interacting species might show a different phenological shift following climate change, which might lead to mismatch in herbivore appearance and herbivore control, and consequently pest outbreaks and crop production losses (Parmesan & Yohe 2003) (Fig I 5).

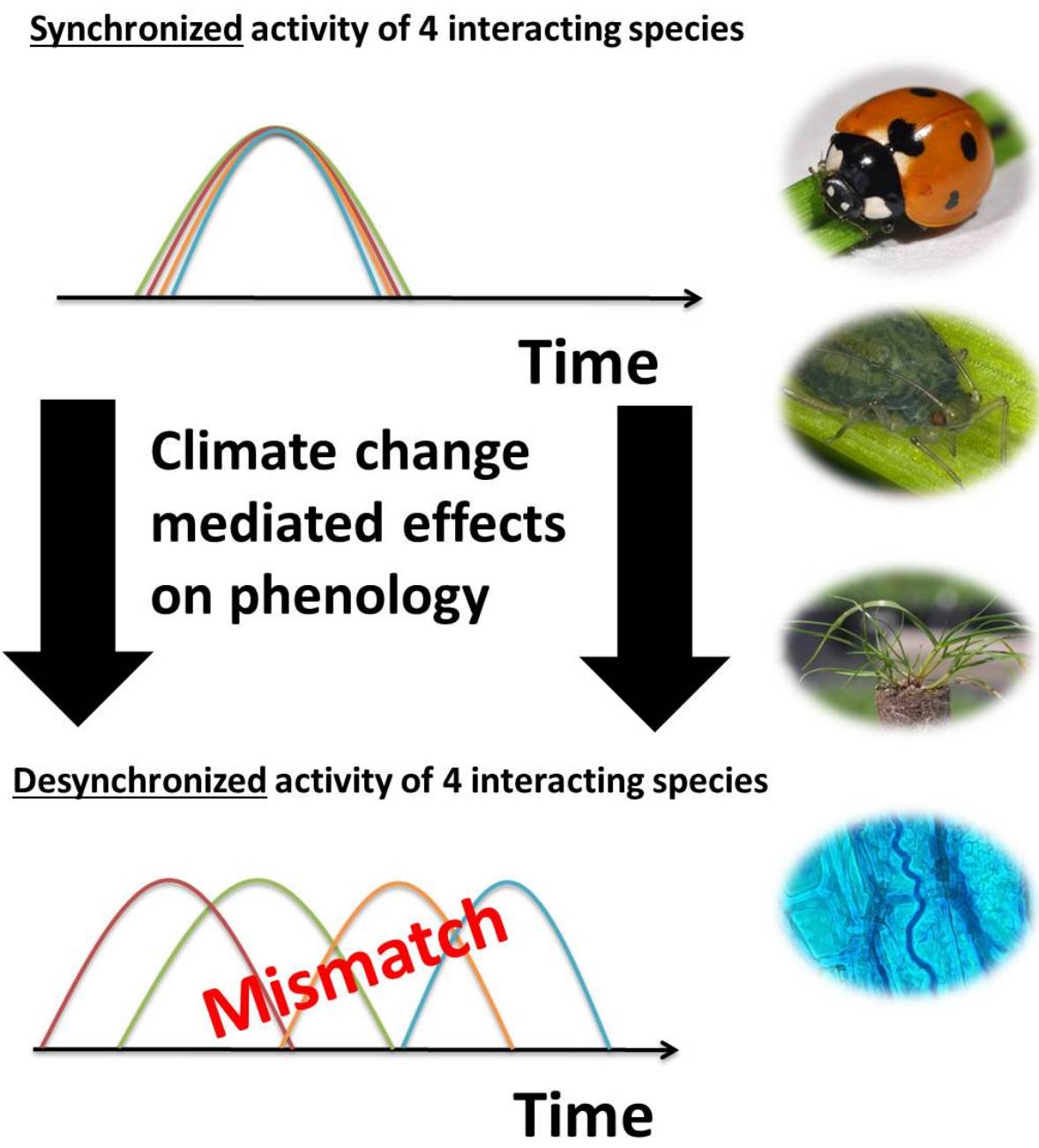

Fig I 5: Desynchronization in a multi-trophic system mediated by climate change. Scheme shows possible phenology shifts of four interacting species following climate change. In this example the interactions between endophytic fungus, host grass, herbivores and predators might desynchronize.

Aphid predators range from generalists such as spiders to more specialized predatory insects, for instance all stages of ladybirds and larvae of syrphid- and hoverflies (Symondson, Sunderland & Greenstone 2002; Schmidt *et al.* 2003). It is mostly unknown how and whether at all predatory insects shift their phenology following climate change and how this alters the interactions with aphids and their function as top-down control.

Furthermore, bottom-up control of aphids by plant-associated microorganisms might be influenced by altered climatic conditions as well. Altered herbivore appearance and abundance might have further effects on both, top-down and bottom-up control and might alter the phenology of predatory insects and plant associated microorganism growth and metabolite production.

In this dissertation we analysed grass-endophytic fungi and the amount of bioactive alkaloids produced under altered biotic and abiotic conditions and their persistence in trophic cascades. We considered multi-trophic level approaches, seasonal timing and age dependency, different herbivore treatments and altered herbivore phenology.

In chapter II of this thesis we show that endophyte derived alkaloid cascade up the food chain which can explain the fitness disadvantages found in herbivores and higher trophic levels (Fuchs *et al.* 2013).

In chapter III of this thesis we show that endophyte growth and the production of alkaloids is dependent on seasonal timing and that plant age has significant effect on the amount of produced alkaloids. These results come from a long-term study ranging over three summers.

In chapter IV of this thesis, we show that increased aphid abundances in spring can desynchronize predator-prey dynamics and cause a time delayed increase in endophyte growth.

In chapter V of this thesis, we show that the production of different endophyte derived herbivore toxic alkaloids is induced by specific herbivore application (aphids, locusts, clipping, control) on the host plant (Fuchs *et al.* 2016) (Fig I 4).

Chapter II: Peramine and lolitrem B from endophyte-grass associations cascade up the food chain

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Abstract

Endophytic fungi in cool-season grass species produce herbivore-toxic alkaloids, which are assumed to harm higher trophic levels along food chains. Previous studies have shown fitness disadvantages for higher trophic levels feeding on aphids that were exclusively reared on perennial ryegrass (*Lolium perenne*) infected with the endophytic fungus *Epichloë festucae* var. *lolii*. However, it is unknown whether the alkaloids produced by the fungus-grass association can be assimilated by plant sap-sucking insects like aphids. Using an ultra-high performance liquid chromatography method combined with mass spectrometry, we provide the first evidence that the alkaloids peramine and lolitrem B are present in aphids (*Rhopalosiphum padi*) and in aphid predators when the aphids were reared on endophyte-infected grass. We conclude that alkaloids can enter the plant sap of the grass and are responsible for longer pupal stages of the ladybird *Harmonia axyridis* and for fitness disadvantages of aphids and their predators as shown in previous studies.

Keywords: Microorganisms, multi-trophic interactions, predator-prey, chemical defence, herbivory, secondary metabolites.

Introduction

Cool-season grass species are sometimes infected by vertically transmitted endophytic fungi that live intercellularly within the plant tissue. These endophytes often change the fitness of the host plant, interact with other microorganisms in the host plant, and affect herbivores and higher trophic levels (Hartley & Gange 2009; Cheplick & Faeth 2009). Negative impact on herbivores is attributed to secondary metabolites like alkaloids, which are produced by the endophytic fungus-grass symbiosis (Schardl *et al.* 2004; Saikkonen, Gundel & Helander 2013). It is speculated that these alkaloids are also responsible for severe fitness reductions of aphids and of predators such as primary and secondary parasitoids of aphids, when the aphids were reared on endophyte-infected grass (de Sassi *et al.* 2006; Härri *et al.* 2008a; c). The endophyte hyphae are most abundant in leaf sheaths and absent in roots (Ball *et al.* 1997), but it is unknown if the alkaloids produced by the intercellular growing endophytic fungus can enter the plant sap. It is therefore questionable whether the alkaloids can be assimilated by herbivores feeding on plant sap of endophyte-infected grass (Saikkonen, Saari & Helander 2010).

In this study we worked with the host grass *Lolium perenne* (Poaceae) either infected with the endophytic fungus *Epichloë festucae* var. *lolii* (Clavicipitaceae) or endophyte-free. Three alkaloids produced by this endophyte-grass association have been identified. The alkaloids ergovaline and lolitrem B cause nervous diseases in cattle and sheep, while the alkaloid peramine can reduce the fitness or deter herbivorous insects (Schardl *et al.* 2004; Müller & Krauss 2005). In our study we chemically analysed samples of grass, three herbivore species including one aphid species, and five aphid predator species. We quantified the alkaloids peramine and lolitrem B. Additionally we recorded the effects of aphids as prey items, reared on *L. perenne* infected or uninfected with *E. festucae* var. *lolii*, on performance variables of two ladybird species.

We tested the following hypotheses:

Alkaloids produced by the endophyte-grass association cascade up the food chain and can be detected in the grass, in different herbivores and aphid predators. This chemical cascade may affect (1) larval developmental times, (2) mortality of larvae and pupae and (3) body mass of adult ladybird species.

Material and methods

Endophytic fungus and host grass.

For the experiments and alkaloid analyses we used the perennial ryegrass *Lolium perenne* uninfected or infected with the endophytic fungus *Epichloë festucae* var. *lolii*. Seeds of *L. perenne* belonged to the cultivar Grassland Samson and were either uninfected (identity number A 11104) or infected (identity number A 12038) with the wild type endophyte *E. festucae* var. *lolii*. In the following we will refer to "E+" as endophyte-infected *L. perenne* and "E-" as uninfected *L. perenne* grass.

The grass seeds were provided by David Hume, AG Research, NZ. The same cultivars were used in previous fitness experiments with ladybirds and aphid parasitoids (de Sassi *et al.* 2006; Härri *et al.* 2008a; c). We used two different ages of grass for the experiments, either (i) grass grown for two years in a greenhouse (abbreviated as old grass) or (ii) grass grown for six weeks in a climate chamber with L16:D8, 20-24°C and 70 % RH (abbreviated as young grass). For the alkaloid analyses, we sampled leaf stalks and leave sheaths of endophyte infected and endophyte free *L. perenne* plants on which we also reared the aphids for the analyses.

Herbivores and predators.

In our experiment, herbivores and predators were reared either (i) on old grass (Table II 1) and/or (ii) on young grass (Fig II 1). We used always the same number of individuals per species reared in an endophyte-infected (E+) and in an endophyte-free (E-) environment. As a plant-sap sucking insect we used the bird cherry-oat aphid *Rhopalosiphum padi* (Aphididae), a prevalent pest for several crop species (Blackman & Eastop 2000). These aphids were reared for at least seven days exclusively on either infected or uninfected grass before using them for alkaloid analyses or feeding them to aphid predators. Alkaloids were also analysed in two grasshopper species (*Schistocerca gregaria*, *Locusta migratoria*), which fed seven days on either endophyte-infected or endophyte-free old grass. Grasshoppers are plant chewers and, in contrast to aphids, they are in contact with the fungus and the alkaloids whether or not the alkaloids enter the plant sap.

We also tested the alkaloids peramine and lolitrem B in five species of aphid predators, which were fed *ad libitum* for at least seven days with aphids from infected or uninfected grass. Twice per day, cuttings of grass leaves (ca. 5cm pieces) densely packed with aphids were put

directly into the container of each predator. Predators were bought from Katz biotech AG, Germany and kept individually in containers (diameter 5 cm, height 10 cm) closed with foam.

Alkaloid analyses.

For alkaloid analyses, grass samples, grasshoppers, aphids, and aphid predators were kept frozen at -80°C until they were prepared for Ultra High Performance Liquid Chromatography-Tandem-Mass Spectrometry (UPLC-Tandem-MS). Alkaloids were extracted from the samples with methanol and dichlormethane in several steps before the determination with UPLC-Tandem-MS with argon as collision gas. We quantified the peramine with the internal standard compound homoperamine to the limit of detection of 5ng. Lolitrem B was quantified by reference to peramine (for additional information see Supplementary Material: Settings for UPLC-MS).

Ladybird performance.

We recorded performance variables for the two ladybird species, *Adalia bipunctata* and *Harmonia axyridis* (Coccinellidae). Both species were exclusively fed with either aphids reared on young endophyte-free grass (E- environment) or aphids reared on young endophyte-infected grass (E+ environment). We conducted the performance experiment in a climate chamber (L16:D8, 18-24°C, 70 % RH). We kept eggs of both species in petri dishes in the climate chamber until their emergence. Forty randomly chosen newly hatched larvae of *A. bipunctata* and 20 larvae of *H. axyridis* were fed individually with *R. padi* aphids reared on E+ young grass, while simultaneously 40 larvae of *A. bipunctata* and 20 larvae of *H. axyridis* were fed with *R. padi* aphids reared on E- young grass. We recorded the ladybird performance variables:

(1) Length of larval stages and larval developmental times in total (in days)

(2) Mortality rate of the larvae (in %)

(3) Weight of the hatched adult ladybirds (in mg)

Ladybird larvae and pupae were monitored daily to record the times to moult, pupation, and emergence, and their survival. The period of larval development was the time from emergence out of the egg to the emergence out of the pupae. Newly emerged adults were weighed before any further food was supplied.

Subsequently we related performance to endophyte-mediated alkaloids by analysing 24 adult *A. bipunctata* (E+ = 12; E- = 12) and 16 *H. axyridis* (E+ = 8; E- = 8) for alkaloids, directly after the hatching out of the approximately 7 day-long pupal phase that is devoid of food.

Furthermore, 24 adult *A. bipunctata* (E+ = 12; E- = 12) and 20 *H. axyridis* (E+ = 10; E- = 10) were fed *ad libitum* with aphids reared exclusively from E+ or E- grass for 7 consecutive days after hatching (Fig II 1).

Statistics.

We used one-way *ANOVA Type I SS* to compare performance variables of ladybirds fed with aphids reared on E+ young grass or with aphids of E- young grass. Model residuals were normally distributed and showed homogenous variances. We tested the alkaloid occurrence in plants, herbivores, and predators from the endophyte-free environment (E-) compared to the endophyte-infected environment (E+) using a generalized linear model for binomial values (Table II S 1). For data analyses we used the software R version 2.14.1. Means ± standard deviations of detected peramine are presented throughout the manuscript, unless otherwise stated.

Results

Alkaloid analyses.

We detected the alkaloid peramine only in trophic levels that had endophyte-infected (E+) grass in their food chain, independent of plant age (Table II 1, Fig II 1). We never detected peramine in herbivores or aphid predators that had E- grass in their food chain. Many individuals reared in an endophyte-infected environment contained detectable amounts of peramine. Therefore, the probability of the presence of peramine was higher in E+ compared to E- environments within each tested species (Table S II 1).

Aphids reared on E+ young grass (six weeks after germination) accumulated peramine (0.19 ± 0.03µg/g) at a concentration three times higher than that of their host grass (0.06 ± 0.02µg/g). Aphid predators showed mean peramine concentrations between 0.01µg/g and 0.10µg/g, except for the larvae of *H. axyridis* that had concentrations of 0.93 ± 1.77µg/g (Fig II 1). Furthermore, we detected peramine in ladybirds tested directly after hatching out of the pupae, with no ingestion of food during the approximately 7 day-long pupal phase (Fig II 1).

Peramine concentration from the infected old grass was more than 300 times higher (18.61 ± 5.17µg/g) than to the concentration of infected young grass (0.06 ± 0.02µg/g). Aphids fed on E+ old grass contained almost 100 times higher peramine concentrations (18.06 ± 7.28µg/g) (Table II 1) than aphids fed on E+ young grass (0.19 ± 0.03µg/g) (Fig II 1). We detected the alkaloid lolitrem B in the E+ old grass and in a low concentration in aphids feeding on this grass and in their aphid predators (Table II 1).

Table II 1 Peramine and lolitrem B concentration in 2-year old grass samples (old grass) either infected with *Epichloë festucae* var. *lolii* (E+) or uninfected (E-) and in higher trophic levels with old grass in their food chain. (/) means not tested

Sample	***N***	***Peramine[µg/g]***		***Lolitrem B[µg/g]***	
		Replicate 1	Replicate 2	Replicate 1	Replicate 2
Perennial Ryegrass					
Lolium perenne E -	1	0.00	/	0.00	/
Lolium perenne E+	2	23.77	13.45	6.06	2.65
Bird-cherry oat aphid					
Rhopalosiphum padi E-	2	0.00	0.00	0.00	0.00
Rhopalosiphum padi E+	2	25.33	10.78	0.03	0.02
Asiatic ladybird					
Harmonia axyridis adult *E-*	2	0.00	0.00	0.00	0.00
Harmonia axyridis adult *E+*	2	3.31	14.38	0.01	0.00
Two-spotted ladybird					
Adalia bipunctata Larva *E-*	1	0.00	/	0.00	/
Adalia bipunctata Larva *E+*	1	1.41	/	0.00	/
Common green lacewing					
Crysoperla carnea Larva E-	2	0.00	0.00	0.00	0.00
Crysoperla carnea Larva E*+*	2	3.79	4.01	0.02	0.02
Syrphid fly					
Episyrphus balteatus Larva E-	1	0.00	/	0.00	/
Episyrphus balteatus Larva E*+*	1	0.08	/	0.00	/
Grasshoppers:					
Schistocerca gregaria E-	5	Mean±SD:0.00±0.00		0.00	
Schistocerca gregaria E+	5	Mean±SD:1.41±1.45		0.00	
Locusta migratoria E-	8	Mean±SD:0.00±0.00		0.00	
Locusta migratoria E+	8	Mean±SD:0.13±0.28		0.00	

Ladybird performance.

All performance experiments were conducted with young grass with relatively low peramine concentrations compared to old grass.

Ladybirds of *H. axyridis* had a longer pupal stage when fed exclusively with aphids reared on E+ grass (mean ± SE; E+ = 6.00 ± 0.07 days; E- = 5.68 ± 0.11 days; ANOVA: $F_{1,37} = 5.89$, P = 0.02). The larval stages of the same ladybird species and larval and pupal stages of *A. bipunctata* and the total developmental time to adulthood of both species did not differ significantly between endophyte-infected (E+) and uninfected (E-) environments (all P > 0.05). Further, neither mortality nor weight of adult ladybirds of either tested species showed differences between E+ and E- grass in the food chain (all P > 0.05) (*ANOVA* results for mass and larval development of both ladybird species see Supplementary Material Table S II 2, Table S II 3).

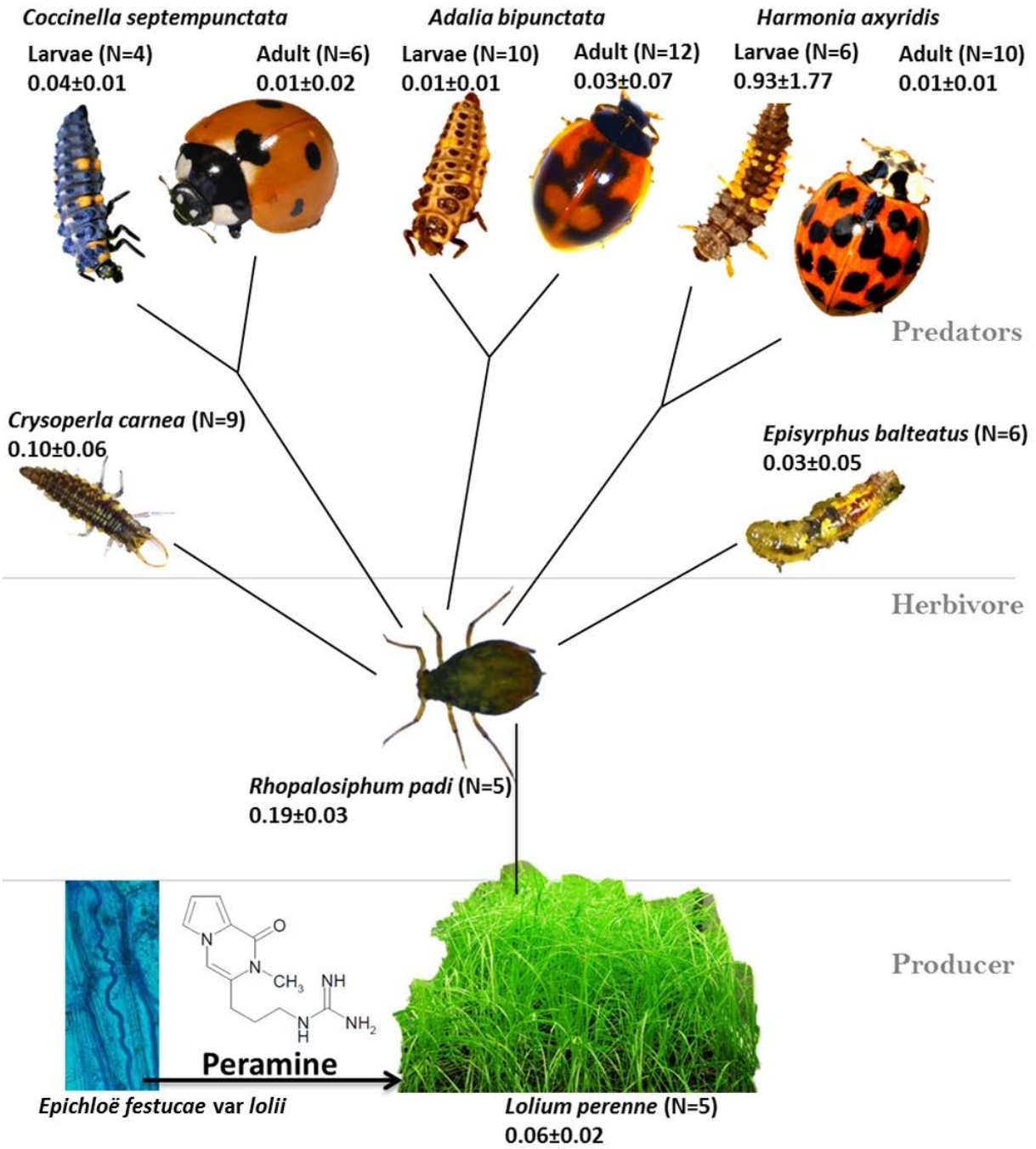

Fig II 1 Peramine concentrations (Mean±S.D. µg/g) in a trophic cascade based on 6-week old grass (young grass) infected with the endophytic fungus *Epichloë festucae* var. *lolii*. N= replication number of individuals tested with endophyte infected environment. The same number of individuals was tested with endophyte free environment, where we never detected alkaloids. Newly hatched: individuals analysed after hatching out of the pupae without further food consumption.

Discussion

Alkaloid analyses.

Previous studies have shown that plant secondary metabolites can cascade up the food chain (Pasteels 2007; Newton, Bullock & Hodgson 2009; Kos *et al.* 2011). In this study, we provide the first evidence that the alkaloids peramine and lolitrem B, produced by an endophyte-grass association, cascade up into higher trophic levels of insects. The detection of peramine in aphids indicates that peramine enters the plant sap of endophyte-infected *L. perenne* and thus gets ingested by aphids feeding on endophyte-infected grass. We conclude that insects feeding on plant sap of cool-season grass species and their aphid predators take up alkaloids of the endophyte-infected grass potentially leading to the changed insect community structures shown in other studies (Rudgers & Clay 2008).

We showed that 6-week old endophyte-infected grass has lower concentrations of peramine than two year old endophyte-infected grass. One explanation is that the endophytic fungus grows slower than the host grass (Tanaka *et al.* 2012) and hyphal density may increase with plant age. Alkaloid concentrations in endophyte-infected host grass correlates with the mass of fungal mycelia (Rasmussen *et al.* 2007).

Our results that demonstrate low alkaloid content in young grass and high alkaloid content in old grass indicate that studies are needed that further examine alkaloid concentrations in endophyte-grass associations as a function of age of the host plants and the timing and synchronisation of the interacting partners.

Our newly established protocol for alkaloid analyses using an UPLC-Tandem-MS will allow the quantification of alkaloids and further metabolites in the endophyte-grass symbiosis.

Ladybird performance.

The larvae of the ladybird *H. axyridis* contained higher peramine concentrations than the other tested insect larvae, which might be caused by their high consumption rate of aphids (Koch 2003). We found no indications for a performance reduction in ladybirds caused by the endophytic fungus; besides a prolonged pupal stage of *H. axyridis.* The prolongation can lead to a loss of fitness, because the pupal stage is a vulnerable developmental stage for ladybirds. One study shows that up to 38.9% of *H. axyridis* pupae were killed by cannibalism or parasitism (Osawa 1993). Our performance experiment was conducted with young grass, which had mean peramine concentrations <0.1 µg/g, which are too low to affect ladybirds. Previous studies showed that concentrations below 3µg/g peramine in the grass host were

non-toxic for invertebrates, while concentrations above 10µg/g acted as feeding deterrence for insects (Siegel & Bush 1996; Keogh, Tapper & Fletcher 1996).

Our study design does not allow us to exclude the possibility that alkaloids detected in aphid predators originated in the remains of ingested aphids remaining in the predator`s midgut. However, ladybirds analysed directly after the pupation period also contained alkaloids, which confirms at least the stability of peramine during ladybird pupation. Further studies are needed to proof whether aphids and predators take up the alkaloids from their food in the midgut or if alkaloids are excreted.

In contrast to the relatively few performance disadvantages that we identified for ladybirds on endophyte-infected grass, (de Sassi *et al.* 2006) showed strong fitness disadvantages for the ladybird *Coccinella septempunctata* leading to higher mortality rates and a reduced reproduction success.

Conclusion

The detection of the alkaloid peramine in aphids and aphid predators indicate the stability of the alkaloid within the food chain, and may explain fitness disadvantages of the endophytic fungus in aphid predators and parasitoids (de Sassi *et al.* 2006; Härri *et al.* 2008a; c). Assuming that alkaloids from endophytes harm aphid predators raises issues of the ecological consequences of endophytic fungi, because it is unknown whether the protective function against herbivores is reduced or even eliminated by the parallel negative effect on predators (Faeth & Saari 2012).

Supporting Information to Chapter 2

Settings for UPLC-Tandem-MS

Extraction

Samples were mixed with 150 µL methanol and 125 µL methylene chloride, 500 ng of homoperamine was added as internal standard. The mixture was shaken for 3 min using a ball mill. After centrifugation, the tissue residue was re-extracted with 150 µL methanol and 125 µL methylene chloride, the organic phases were combined and 25 µL were separated and evaporated. The residue was dissolved in 25 µL 80% methanol and analysed using UPLC-MS/MS.

LC-MS/MS analyses

LC-MS/MS analyses were performed using a Waters Acquity ultra-high-performance liquid chromatograph coupled to a Waters Micromass *Quattro Premier* triple quadrupole mass spectrometer (Milford, MA, USA) with a electrospray interface (ESI). All aspects of system operation and data acquisition were controlled using MassLynx V 4.1 software.

Chromatographic conditions

Separation of alkaloids was achieved using an Acquity BEH C18 column (2.1 x 30 mm, 1.7 µm particle size with a 2.1- x 5 mm guard column; Waters) with the following solvent systems: solvent A consisted of aqueous formic acid, solvent B contained acetonitrile with 0.1% of formic acid. A gradient elution was performed at a flow rate of 0.3 mL min^{-1} from5% to 100% B in 5 min, followed by 100% B for 2 min, and reconditioning at 5% B for 3 min.

Mass spectrometric conditions

For the analysis of alkaloids the electrospray source was operated in the positive electrospray mode (ESI^+) at 120°C and a capillary voltage of 3 kV. Nitrogen was used as the desolvation and cone gas with flow rates of 800 L h^{-1} at 350°C and 50 L h^{-1}, respectively, the cone voltage (CV) was adjusted to 25 V. Alkaloids were analysed by multiple reaction monitoring (MRM) using Argon as collision gas at a collision energy (CE) of 18 eV for peramine, 20 eV for homoperamine and 32 eV for lolitrem B.

Table S II 1 Generalized linear model for binomial values. We tested the individuals in an endophyte infected environment (E+) where we often detected peramine, compared to individuals in an uninfected environment (E-) where we never detected peramine.

Species	**Df**	**Deviance**	**Residual Df**	**N**	**Residual Deviance**	**P(>Chi)**
Young grass environment						
L. perenne	1	13.86	8	10	0	<0.001
R. padi	1	13.86	8	10	0	<0.001
A. bipunctata larvae	1	2.99	18	20	24.73	0.084
A. bipunctata adult newly hatched	1	6.35	22	24	26.92	0.012
A. bipunctata adult	1	2.95	22	24	30.32	0.086
H. axyridis larvae	1	3.18	10	12	13.46	0.075
H. axyridis adult newly hatched	1	4.86	14	16	17.32	0.028
H. axyridis adult	1	4.69	18	20	23.04	0.03
C. septempunctata larvae	1	11.09	6	8	0	<0.001
C. septempunctata adult	1	5.18	10	12	11.46	0.023
E. balteatus larvae	1	3.18	10	12	13.46	0.075
C. carnea larvae	1	18.45	16	18	6.5	<0.001
Old grass environment						
S. gregaria	1	11.09	6	8	0	<0.001
L. migratoria	1	6.9	14	16	15.28	0.009

Table S II 2 Mean with standard error, F-Value, degrees of freedom and p-Values from the larval development of the ladybird species *Adalia bipunctata* and the mass of the hatched adult *A. bipunctata* ladybirds. Sex was tested as an additional categorial Variable.

Adalia bipunctata	**E- (Mean±S.E.)**	**E+ (Mean±S.E.)**	**F**	**n**	**p**
Length of larval development (days)	18.06±0.2	18.45±0.22	$F_{1,60}$=1.50	64	0.23
Sex			$F_{1,60}$=0.21	64	0.65
Interaction			$F_{1,60}$=0.08	64	0.78
Male	18.07±0.34	18.33±0.41			
Female	18.06±0.23	18.50±0.27			
Length of Instar I	2.34±0.08	2.38±0.08	$F_{1,75}$=0.15	77	0.70
Length of Instar II	2.11±0.05	2.11±0.05	$F_{1,69}$=0.00	71	0.97
Length of Instar III	2.21±0.11	2.26±0.08	$F_{1,65}$=0.15	67	0.70
Length of Instar IV	5.67±0.13	5.71±0.18	$F_{1,65}$=0.03	67	0.86
Length of Pupal stage	5.84±0.09	6.00±0.12	$F_{1,62}$=1.07	64	0.31
Mass of adults after hatching (mg)	9.15±0.28	9.05±0.26	$F_{1,60}$=0.17	64	0.68
Sex			$F_{1,60}$=0.64	64	0.43
Interaction			$F_{1,60}$=0.77	64	0.38
Male	9.14±0.50	8.51±0.42			
Female	9.16±0.33	9.25±0.31			

Table S II 3 Mean with standard error, F-Value, degrees of freedom and p-Values from the larval development of the ladybird species *Harmonia axyridis* and the mass of the hatched adult *H. axyridis* ladybirds. Sex was tested as an additional categorial Variable. Significant p-values are in bold.

Harmonia axyridis	**E- (MW±S.E.)**	**E+ (MW±S.E.)**	**F**	**n**	**p**
Length of larval development (days)	15.74±0.21	16.15±0.15	$F_{1,35}$=0.19	39	0.67
Sex			$F_{1,35}$=2.85	39	0.10
Interaction			$F_{1,35}$=1.02	39	0.32
Male	15.88±0.40	16.00±0.24			
Female	15.64±0.24	16.27±0.19			
Length of Instar I	2.10±0.07	2.05±0.05	$F_{1,38}$=0.35	40	0.56
Length of Instar II	1.85±0.08	1.85±0.08	$F_{1,38}$=0.00	40	1.00
Length of Instar III	1.63±0.11	1.65±0.11	$F_{1,37}$=0.01	39	0.91
Length of Instar IV	4.47±0.14	4.60±0.11	$F_{1,37}$=0.50	39	0.48
Length of Pupal stage	5.68±0.11	6.00±0.07	$F_{1,37}$=5.89	39	**0.02**
Mass of adults after hatching (mg)	30.45±0.65	30.08±0.45	$F_{1,37}$=0.19		0.67
Sex			$F_{1,37}$=2.85		0.10
Interaction			$F_{1,37}$=1.02		0.32
Male	29.23±1.00	29.78±0.97			
Female	31.34±0.79	30.32±0.58			

Chapter III: Plant age and seasonal timing determine endophyte growth and alkaloid biosynthesis

This chapter has been submitted as: Fuchs, B., Mueller, MJ., Krischke, M., Krauss, J. Plant age and seasonal timing determine endophyte growth and alkaloid biosynthesis

Abstract

The systemic endophytic fungus *Epichloë festucae* var. *lolii* produces three alkaloids in the grass *Lolium perenne* which can cause diseases to livestock or deter invertebrate herbivores. The amount of alkaloids depends on the genotypic predisposition of grass and endophyte, influenced by their abiotic and biotic environment, but seasonal timing and plant age received little attention. We investigated the effects of plant age and seasonal timing on endophyte growth and alkaloid production in a common garden experiment.

We monitored endophyte and alkaloid concentrations in endophyte infected perennial ryegrass (cultivar "Samson") planted in Southern Germany over a period of three summers.

Plant age did not affect the fungal concentration but alkaloid concentrations increased with plant age. Further fungal and alkaloid concentrations were correlated with growing degree day showing the impact of temperature on the endophyte. Thus, alkaloid levels vary with plant age and seasonal timing which determines the herbivore toxicity of endophyte derived alkaloids and could explain disease outbreaks in livestock during certain times of the year.

Livestock intoxications caused by grass-endophyte derived alkaloids might increase in European grasslands when temperature further increases due to climate change. Besides genotypic predisposition and nutrition supply, seasonal timing and plant age play a key role for the toxicity of systemic endophytic fungi in grass species.

Key- words: grasslands, symbiosis, defensive metabolites, herbivory, microbial ecology, UPLC-MS, secondary metabolites, pest control, seasonal timing;

Introduction

Grass endophytes affect their host grasses and alter their relations to above- and belowground interacting species (Kauppinen *et al.* 2016). Thereby the effects of grass endophytes can range from changes in physiological plant processes like promoted growth (Krauss *et al.* 2007), higher seed production (Saari *et al.* 2010a) and enhanced drought stress tolerance (Hesse *et al.* 2003), to alterations of the symbiosis to root associated fungi (Liu *et al.* 2011), up to a change in the whole plant composition surrounding endophyte infected grasses (Rudgers & Clay 2007). The benefit of fungal plant infection is most defined during extreme abiotic conditions, like low nutrient availability and water deficiency (Saikkonen *et al.* 1998; Young *et al.* 2014).

Grass endophytic fungi can negatively affect vertebrate and invertebrate herbivores feeding on the host grass (Shymanovich *et al.* 2014; Philippe 2016). Herbivore intoxications are caused by several endophyte derived alkaloids and are of high agronomic and scientific interest. This indicates a defensive mutualistic relationship between grass and endophyte (Clay, 2014, but see Cheplick & Faeth, 2009).

The cool-season grass *Lolium perenne* (perennial ryegrass) is distributed all over the globe with a high agronomic value. *L. perenne* can be associated with the endophytic fungus *Epichloë festucae* var. *lolii,* a vertically, via the plant seed, transmitted fungus of the *Clavicipitaceae* family which produces three main alkaloids affecting herbivore fitness (Schardl *et al.* 2004). The pyrrolizidine alkaloid peramine has toxic effects on invertebrate herbivores and confers resistance to insect pests like the argentine stem weevil, which is a serious pasture pest species in New Zealand (Rowan *et al.* 1990). The indole-diterpene alkaloid lolitrem B and the ergot alkaloid ergovaline are neurotoxins for vertebrate herbivores, causing diseases like ryegrass staggers and fescue toxicosis to grazing livestock (Guerre 2015).

Meta analyses on effects of grass endophytes on trophic and community levels displayed a huge variation in results (Saikkonen *et al.* 2006; Larimer, Bever & Clay 2010). Varying alkaloid concentrations might be the reason for these contrasting results, as alkaloids vary in quantity depending on grass and endophyte genotype combination (Schardl *et al.* 2013b; Ryan *et al.* 2015). Additionally, abiotic factors, like nutrition (Malinowski *et al.* 1998; Helander *et al.* 2016), temperature (Salminen *et al.* 2005; McCulley *et al.* 2014) and drought (Bush *et al.* 1993, 1997) affect the alkaloid concentrations. Years with enhanced outbreaks of fescue toxicosis in Australia correlated with rainy spring and dry-warm summer conditions (Reed *et al.* 2011a). Consequently herbivore performance from feeding on grass is indirectly determined by abiotic factors affecting endophytic fungi, as shown for the increased

resistance to fall army worm at higher temperatures due to higher alkaloid levels (Salminen *et al.* 2005). Further, alkaloid concentrations showed fluctuations following the yearly temperature curve (Ball *et al.* 1991; Repussard *et al.* 2014). Additionally to temperature, age of the host grass-endophyte association might determine alkaloid concentrations. Aphids showed a better survival on young plants (Eichenseer, Dahlman & Bush 1991) and alkaloid concentrations from endophyte infected young grass is lower than in older grass (Fuchs *et al.* 2013). Low alkaloid concentrations might derive from a low fungal concentration in the plant, as alkaloid and fungal concentrations are correlated (Ryan *et al.* 2014).

To be toxic for herbivores, the alkaloid concentrations require a certain threshold in the plant. The toxicity or deterring threshold concentration of peramine for insect herbivores is about 2 µg/g plant material (Siegel & Bush 1996). Ergovaline caused symptoms in vertebrates starting from concentrations between 0.3 and 0.8 µg/g and lolitrem B causes symptoms from concentrations about 1.8 µg/g (Tor-Agbidye *et al.* 2001; Hovermale & Craig 2001). Studies on the ecological role of endophyte infected grass in multi-species interactions often ignore the impact of plant age and seasonal timing, which might affect alkaloid concentrations and therefore species interactions. Consequently questions were raised regarding the effect of endophytic fungi on herbivores under natural conditions and their plant mutualistic function (Faeth 2002).

We determined endophyte growth and alkaloid production of the endophytic fungus *Epichloë festucae* var. *lolii* in the grass *Lolium perenne* over a growth period of three summers after sowing. We addressed the question whether plant age and season affects endophyte and alkaloid concentrations.

Our hypotheses are:

(1) Endophyte and alkaloid concentrations are fluctuating annually, following the four seasons in a temperate region with different temperatures
(2) Alkaloid concentrations correlate with endophyte concentration
(3) Alkaloid and fungal concentrations increase with plant age

Material and Methods

Study Design

We sowed 350 seeds of *Lolium perenne* infected with the endophytic fungus *Epichloë festucae* var. *lolii* (cv. Grassland Samson) on 15th of April 2013 into round propagation trays (5x5 cm) and repotted the germinated plants in June 2013 into bigger pots (18x18x18 cm) filled with common garden soil (Einheitserde classic CL ED73, Profi Substrat). Pots were

placed in a garden outside the University of Würzburg, Germany for the whole study period from April 2013 until September 2015.

The grass cultivar Samson with endophyte infection was used in previous studies and the effects on aphids and their enemies have been frequently reported (e.g. (Meister *et al.* 2006; de Sassi *et al.* 2006; Härri *et al.* 2008c; Fuchs *et al.* 2013). Plants were watered regularly and NPK fertilizer was added at the beginning of March 2014 and 2015 (Compo 20-5-10, equivalent to 200 N kg*ha^{-1}*yr^{-1}). In March 2014 and March 2015 we manually removed all wilted tillers from the pots to provide space and light for fresh tillers to grow before we added fertilizer. At every 21st of each month we sampled 10 plants randomly by cutting the whole aboveground plant material with a sharp gardening scissor. Sampling started one month after seeds germinated, respectively. We never sampled the same plant twice. Immediately after harvesting, aboveground plant material was frozen in liquid nitrogen. Subsequently plant material was ground meshed and circa 50 mg plant material was used each for qPCR and UPLC-MS analysis.

Climate Data

Climate data were provided by the "Deutscher Wetterdienst", recorded hourly in approximately 1km distance to the study site at a similar altitude (Latitude: 49.77°, Longitude: 9.96° absolute altitude: 272 m above sea level). We used a growing degree day (GDD) model to show the influence of temperature and time of the year on fungal and alkaloid concentrations, using published protocols of (Kreuser & Soldat 2011; Repussard et al. 2014). Thereby GDD was calculated for cool-season grass by summing up mean daily air temperatures (°C) with a base temperature of 0°C. We calculated GDD from 01st of January until 31st of August (plant maturity with fully developed seeds) of the years 2014 and 2015 (Saiyed *et al.* 2009).

Endophyte and Alkaloid Analysis

Endophyte concentration was determined by quantitative PCR (qPCR) analysis (detailed protocol see Fuchs *et al.* 2016). Genomic DNA (gDNA) was extracted from circa 50µg powdered grass material. The amount of endophytic gDNA was quantified by relation to amplified grass gDNA. For quantification of the endophyte gDNA we used a fungal specific primer (*Chitinase A*) and for the plant gDNA quantification we used a grass specific primer (*β tubulin*).

Alkaloid concentrations were determined with UPLC-MS (detailed protocol see Fuchs *et al.* 2013). Extraction was performed with dichloromethane and methanol. The alkaloids peramine and lolitrem B were both quantified with reference to the internal standards homoperamine, ergovaline was quantified with reference to the internal standard ergotamine. Detection limit for all alkaloids was 5ng.

Statistics

Statistical analyses were conducted using the statistical software R (Version 3.2.3). Correlation between the alkaloid concentrations and the fungus concentration over the whole study period was shown with linear regression analysis. Further we used linear regression to analyse the dependency of cumulative degree day and alkaloid concentrations from January until August of the years 2014 and 2015 (Crawley 2012). We compared the average fungal and alkaloid concentrations from January until August between the year 2014 and 2015 with a one-way ANOVA. To test differences between the mean of the fungal and alkaloid concentrations from the emergence of the inflorescence until the fully ripe stage (June-August) across the three years, we conducted multiple comparison of means analyses following a Tukey posthoc test (Hothorn, Bretz & Westfall 2008).

Results

The fungal and the alkaloid concentrations show a seasonal rhythmicity with peak concentrations in summer and minimal concentrations in winter across all three years (Fig III 1a). Fungal growth and alkaloid concentrations are following the ambient temperature curve (© Deutscher Wetterdienst) (Figs III 1a, III 2). Thereby we found low fungal and alkaloid concentrations in winter month during plant dormancy (Fig III 1a). High fungal and alkaloid concentrations were recorded with the emergence of the inflorescence and the ripening of the plant seeds in summer months from June until September (Fig III 1a-d).

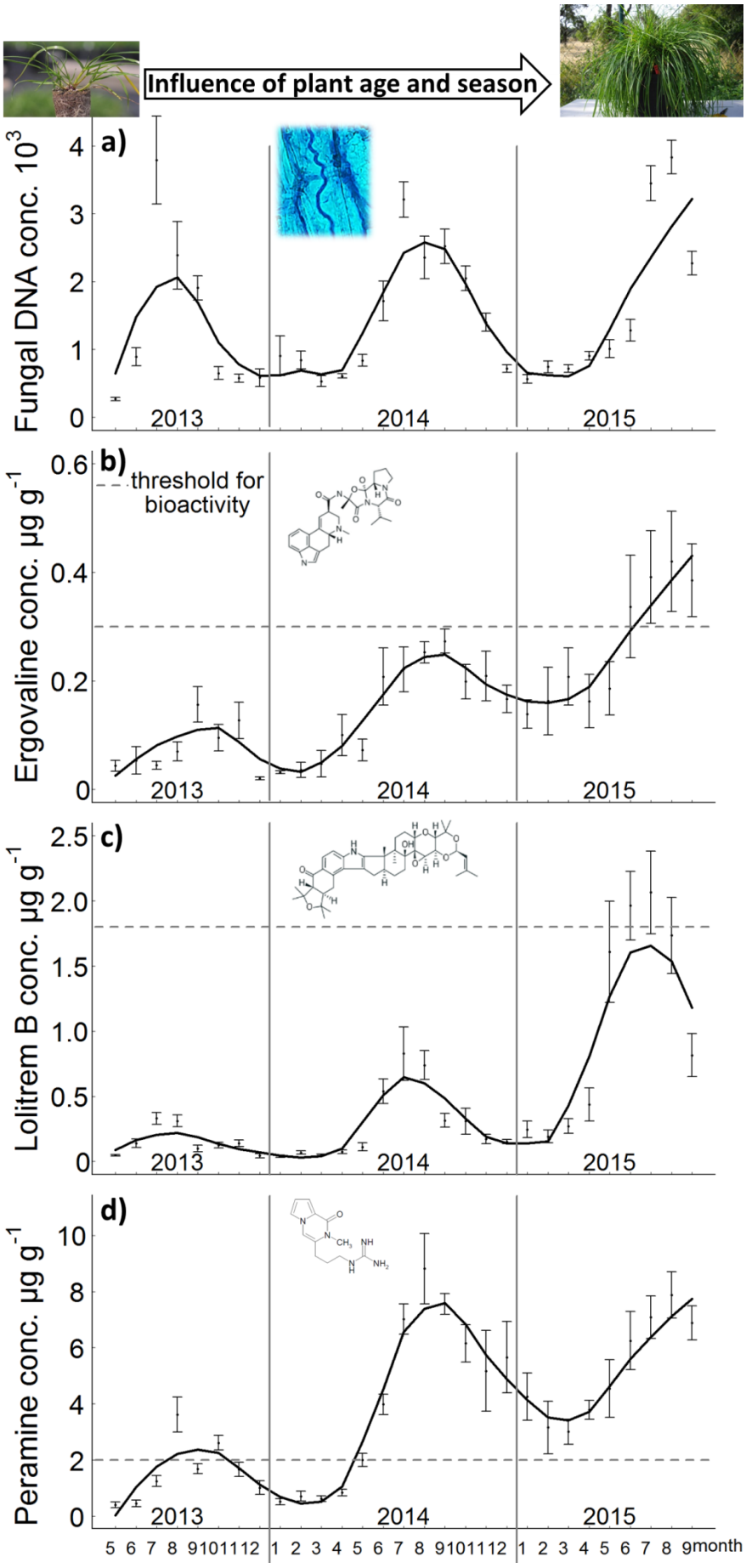

Fig III 1
Endophyte (*E. festucae* var. *lolii*) and alkaloid concentrations follow seasonal rhythmicity. Alkaloid concentrations increase with plant age in three consecutive years. Toxicity thresholds are shown with dotted lines.

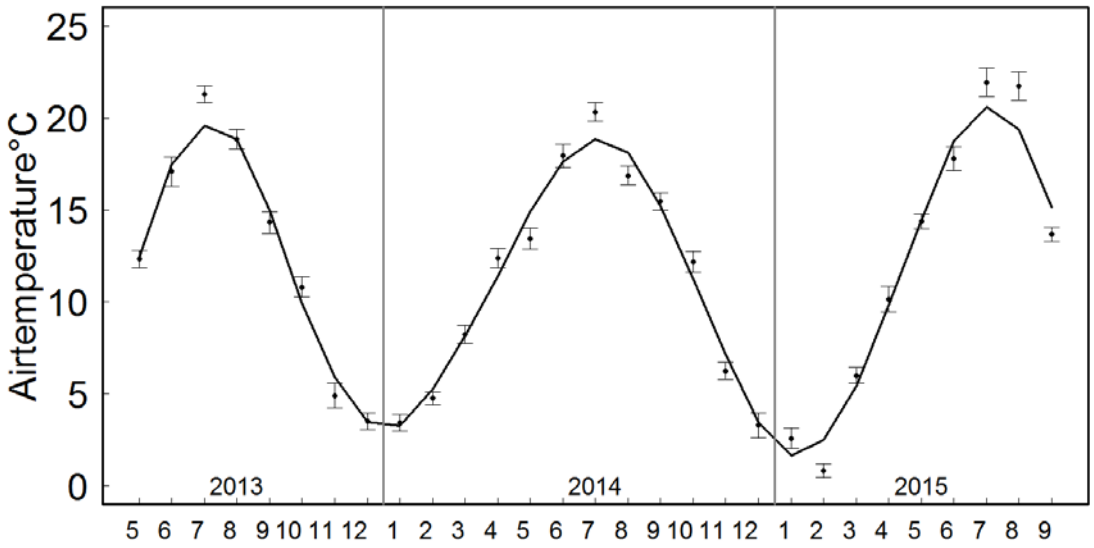

Fig III 2 Monthly air temperature (mean) recorded in proximity to the study site (©Deutscher Wetterdienst).

Growing degree day analyses show a linear increase of all concentrations with increasing degree day from January to August in the years 2014 and 2015 (Fig III 3a-d) with on average higher alkaloid concentrations in 2015 compared to 2014 (Table III 1). Ergovaline concentrations increased at 100%, lolitrem B at 250% and peramine at 60% from 2014 to 2015 (Table III 1).

Table III 1 Comparison of average fungal and alkaloid concentrations during the growing season from January until August between the years 2014 and 2015. Fungal concentration shows no significant difference, but all alkaloid concentrations are higher in 2015 than in 2014. Significant effects are highlighted in bold

	mean±s.e. concentration		*ANOVA results*	
	2014	2015		
Fungal DNA*10^3	1.42±0.13	1.59±0.15	$F_{1,142}$=0.64	p=0.43
Ergovaline µg/g	0.13±0.01	0.26±0.03	$F_{1,129}$=15.33	**p<0.001**
Lolitrem B µg/g	0.34±0.05	1.20±0.12	$F_{1,121}$=31.67	**p<0.001**
Peramine µg/g	3.22±0.39	5.05±0.34	$F_{1,141}$=11.35	**p<0.001**

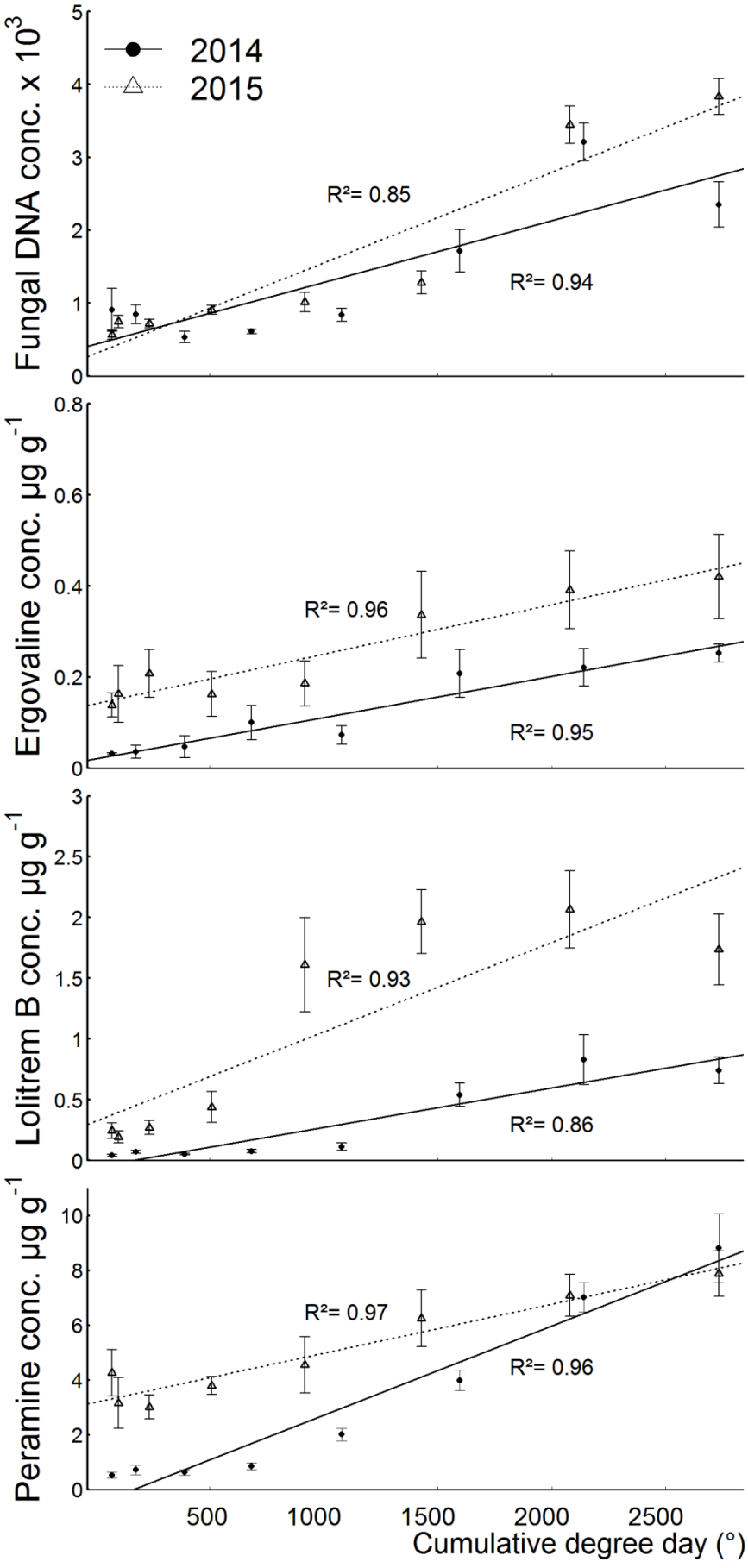

Fig III 3
Fungal and alkaloid concentrations for the years 2014 and 2015 from January until August showing a linear increase with increasing temperature in a growing degree day model.

Fungal DNA concentrations from June to August (emergence of the inflorescence until the fully ripe stage) across all three years did not differ significantly (ANOVA: $F_{2,83}$=0.99, p=0.376) (Table III 2). Alkaloid concentrations from June to August across all three years showed differences for ergovaline (ANOVA: $F_{2,74}$=20.27, p<0.001, increase by 280% between 2013-2014, 65% between 2014-2015), lolitrem B (ANOVA: $F_{2,76}$=56.58, p<0.001, increase by 180% between 2013-2014, 175% between 2014-2015) and peramine (ANOVA: $F_{2,76}$=35.21, p<0.001, increase by 260% between 2013-2014). A Tukey post hoc test (multiple comparison analyses) revealed that mean values from June to August of all alkaloid concentrations differed significantly across the three tested years, except for peramine which did not differ significantly between the years 2014 and 2015 (second and third summer) (Fig III 1a-d, Table III 2).

Table III 2 Multiple comparison analysis from a Tukey contrast test comparing the mean concentrations in the summer months (June to August: emergence of inflorescence to fully ripe stage) between three years show no significant differences in fungal concentration but all alkaloid concentrations differ between all three years, except of peramine between 2014 and 2015. Significant effects are highlighted bold

	mean± s.e.(June-August)			*Multiple comparison (p-values)*		
	2013	*2014*	*2015*	*2013-2014*	*2013-2015*	*2014-2015*
Fungal DNA*10^3	2.36±0.35	2.45±0.20	2.88±0.24	0.967	0.381	0.537
Ergovaline µg/g	0.06±0.01	0.23±0.02	0.38±0.05	**0.004**	**<0.001**	**0.007**
Lolitrem B µg/g	0.25±0.03	0.70±0.08	1.93±0.16	**0.024**	**<0.001**	**<0.001**
Peramine µg/g	1.82±0.33	6.62±0.58	7.07±0.50	**<0.001**	**<0.001**	0.798

The concentration of ergovaline exceeded the toxicity level for livestock from June to September and the concentration of lolitrem B from July to August of the third growing year in 2015, while peramine exceeded the toxicity threshold for invertebrates in August and October of the first growing year in 2013 and from May of the second year 2014 until the end of the study period in September 2015 (Fig III 1b-d).

Enhanced fungal concentration increased the alkaloid concentrations of all three alkaloids over all samples (Fig III 4), but these relationships were not significant when each year was calculated separately, apart from lolitrem B concentrations in 2013 (Table III 3).

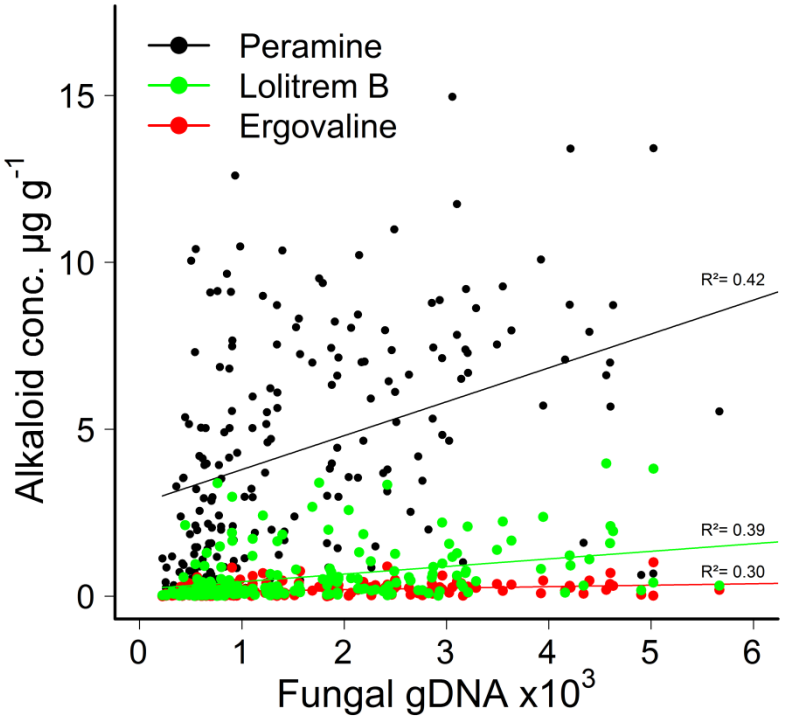

Fig III 4
Peramine, ergovaline and lolitrem B (alkaloids) linearly increase with increasing fungal concentration over the whole study period.

Table III 3 Regression analyses between fungal concentration and alkaloid concentrations for each year. Significant results ($p<0.05$) are highlighted with bold R^2 values. All model coefficients were positive (See Fig III 4 for regression analyses over the whole study period).

	Year					
	2013		2014		2015	
	R^2	F	R^2	F	R^2	F
Ergovaline	< 0.01	$F_{1,59}$<0.01	**0.23*** **	$F_{1,93}$= 27.4	**0.139*** **	$F_{1,74}$= 12.0
Lolitrem B	**0.17** **	$F_{1,51}$= 10.7	**0.32*** **	$F_{1,85}$= 39.4	**0.255*** **	$F_{1,71}$= 24.3
Peramine	0.02	$F_{1,70}$= 1.74	**0.33*** **	$F_{1,104}$= 50.7	**0.292*** **	$F_{1,79}$= 32.6

Discussion

We showed that the defensive compounds produced by an endophytic fungus - grass symbiosis increases with plant age in three consecutive years after sowing, while the fungal concentration remains at similar levels across the three years. Thereby, both, fungal and alkaloid concentrations showed a seasonal rhythmicity following the seasonal temperature curve.

Grass age

We confirm our hypothesis that age of the *Lolium perenne* and *Epichloë festucae* var. *lolii* association affects the alkaloid concentrations produced by the endophyte. During the summer of 2013 and 2014 (first and second year of plant growth), neither lolitrem B nor ergovaline reached a toxic level for grazing livestock. Increasing alkaloid concentrations within the first three years of grass growth might explain that some studies did not show endophyte mediated effects on higher trophic levels where other studies showed effects (Saikkonen *et al.* 2006). Our study indicates that young endophyte infected plants (first year) contained alkaloid concentrations below toxicity levels for livestock. Thresholds for invertebrate herbivores were only exceeded in two month, according to reported bioactivity thresholds for grass herbivores (Siegel & Bush 1996; Tor-Agbidye *et al.* 2001; Hovermale & Craig 2001). In plants of the second year of growing only the concentration of the insect deterring alkaloid peramine exceeded the bioactive threshold. Experimental studies using plants in the second growing year would show the beneficial effect against insect herbivores, often pest species, without showing signs of livestock intoxications.

Our results show a lower alkaloid production in younger plants but fungal concentration did not differ across years. Endophytic fungal growth has long been assumed to only grow at the hyphal tip, but Christensen *et al.*, (2008) showed an intercalary division and extension of fungal tissue, which is connected to enlarging host plant cells and enables fungal extension at the same rate as host growth. The host grass *Lolium perenne* is fully mature in the first growth year and its phenotype in summer did not differ across the three years (personal observations). Similar plant phenotype together with constant endophyte concentrations across years indicates an endophyte growth parallel to grass growth by intercalary division (Christensen *et al.* 2008).

Alkaloid concentrations correlate with fungal concentration (Ryan *et al.* 2014), a finding which we can confirm across all samples. However, yearly separate analysis indicate that ergovaline and peramine do not linearly depend on fungal concentration in the first year of

growing showing that high fungal growth does not necessarily imply high alkaloid production.

Low alkaloid concentrations in young plants indicate that alkaloid biosynthesis might be costly. In young plants nutrient resources are primarily used for primary metabolism like plant and endophyte growth and establishing of the symbiosis to the grass host instead of secondary metabolite synthesis (Faeth & Fagan 2002). Further, chemical complexity and biosynthetic cost might be a reason for the timing and amount of produced alkaloids. Similar to plant secondary metabolite synthesis (Nishida 2002), endophyte derived alkaloid synthesis might follow a trade-off between cost and benefit. Peramine is produced in higher concentrations than lolitrem B and ergovaline, and already exceeds the bioactive threshold in two month of the first year. Biosynthetic pathways of the alkaloids reveal that peramine is a chemical compound originated from only three precursors produced from a single gene (Tanaka *et al.* 2005; Schardl *et al.* 2013b) while ergovaline and lolitrem B synthesis require pathways of much higher complexity originated from gene clusters (Schardl *et al.* 2012; Panaccione *et al.* 2014). Another reason for increasing alkaloid concentrations over time might be alkaloid accumulation in the plant (Siegel *et al.* 1990), since peramine, lolitrem B and ergovaline are molecules with high chemical stability, which even persisted in dead plant tissue at high concentrations within several weeks after cutting the plant material (Hume, Hickey & Tapper 2007).

Seasonal timing

Besides the plant age, seasonal timing determined the course of the alkaloid concentrations with peaks during summer (July-September) along with high temperatures and plant seed maturation. High alkaloid concentrations during seed maturation decrease the probability of seed predation (Madej & Clay 1991), enhancing endophyte dispersal together with grass seeds. Enhanced alkaloid concentrations can further be induced herbivore-specific (Fuchs *et al.* 2016), which was rather unlikely due to exclusion of vertebrate herbivores and only occasionally occurring locusts on our study pots without noticeable plant damage.

The impact of seasonal predictors on increasing alkaloid levels was demonstrated by an enhanced number of livestock intoxications during long and hot summers (Young *et al.* 2013). Experimental warming on a grass-endophyte system further showed an increase of ergot alkaloid concentrations (e.g. ergovaline) with temperature, but other group of alkaloids required both, temperature increase with additional precipitation (McCulley *et al.* 2014). Environmental conditions can exceed the effects of genetic background of the grass-

endophyte association (Oldenburg 1997). An intercontinental reciprocal transplantation experiment showed an opposite temperature effect on alkaloid production explained by geographically specific abiotic conditions overriding the effect of higher temperature (Helander *et al.* 2016), which illustrates the multiple conditions determining alkaloid concentrations, where CO_2 concertation, nitrogen availability and genetic predisposition interact with regional climatic conditions (Hunt *et al.* 2005; Schardl *et al.* 2012; Ryan *et al.* 2015).

High diversity of grass species and relatively low frequencies of endophyte infections paired with interacting abiotic and biotic factors further limits the threat of endophyte transmitted diseases to become a concerning problem for Central European agronomy (Saikkonen *et al.* 2000; Leyronas & Raynal 2001; Kauppinen *et al.* 2016). Nevertheless single signs for intoxications in Europe have been reported showing that high alkaloid levels are possible under natural conditions (Canty *et al.* 2014).

Climate change associated predictors like enhanced temperature, CO2 concentration and the increasing succession of extreme weather events lead to a higher probability to increase alkaloid concentrations than to a decrease (Brosi *et al.* 2011). Our study provides an approach to unravel the biology of grass endophyte symbiosis with plant age and under ambient seasonal climatic conditions. Therefore we present evidence for a plant age and season dependent endophyte productivity which can explain some of the variability on findings of effects on herbivores mediated by endophyte infected grass. Besides grass and endophyte genotype, age and season must be considered in future laboratory and field studies on the effects of fungal grass endophyte associations on herbivores.

Chapter IV: Enhanced aphid abundance in spring desynchronizes predator-prey and plant-microorganism interactions

This chapter is under review (*Oecologia*) as: Fuchs, B., Breuer, T., Findling, S., Mueller, M.J., Krischke, M., Holzschuh A., Krauss, J. Enhanced aphid abundance in spring desynchronizes predator-prey and plant-microorganism interactions.

Abstract

Climate change leads to phenology shifts of many species. However, not all species shift in parallel, which can desynchronize interspecific interactions. Within trophic cascades herbivores can be top down controlled by predators or bottom up controlled by host plant quality and host symbionts, like plant associated microorganisms. Synchronization of trophic levels is required to prevent insect herbivore (pest) outbreaks. In a common garden experiment we simulated an earlier arrival time (approximately two weeks) of the aphid *Rhopalosiphum padi* on its host grass *Lolium perenne* by enhancing the aphid abundance during the colonization period. *Lolium perenne* was either uninfected or infected with the endophytic fungus *Epichloë festucae* var. *lolii*. The plant symbiotic fungus produces insect deterring alkaloids within the host grass. Throughout the season we tested the effects of enhanced aphid abundance in spring on aphid predators (top down) and grass-endophyte (bottom up) responses. Higher aphid population sizes earlier in the season lead to overall higher aphid abundances, as predator occurrence was independent of aphid abundances on the pots. Nonetheless, after predator occurrence aphids were controlled within two weeks on all pots. Possible bottom up control of aphids by induced increased endophyte concentrations occurred time delayed. Endophyte derived alkaloid concentrations were not significantly affected by enhanced aphid abundance but increased throughout the season. We conclude that phenology shifts in a herbivorous species can desynchronize predator - prey and plant - microorganism interactions and might enhance the probability of pest outbreaks with climate change.

Key-words: multi trophic interactions, pest control, herbivory, insect timing

Introduction

Climate change causes shifts in the timing of seasonal events (phenological shift) in many species (Van der Putten, Macel & Visser 2010; Stevenson *et al.* 2015; Thackeray *et al.* 2016). In a climate change meta-analyses on 677 species, 62% of species shifted their phenology towards spring advancement while 38% of the species did not shift or shifted even towards a delay of spring events (Parmesan & Yohe 2003). Such different responses to climate change can cause desynchronizations between interacting species which can lead to a breakdown of mutualistic interactions (Kiers *et al.* 2010). Climate change driven shifts in species phenology are expected to impact species abundances and interactions (Tylianakis *et al.* 2008) as shown e.g. in a disruption of predator-prey dynamics due to climate driven temporal desynchronization of peak abundances between prey and predators (Visser *et al.* 2006). The majority of studies on phenological mismatches have focused on just two trophic levels while studies considering three or more species levels are rare (Rafferty *et al.* 2013). Thereby primary consumers are more susceptible to climate change than primary producers and secondary consumers which can cause mismatches along trophic cascades (Thackeray *et al.* 2016). In two laboratory experiments on multi-trophic interactions involving insect crop pest species, higher temperature caused an increase in aphid abundance (Bezemer, Jones & Knight 1998; Marquis, Del Toro & Pelini 2014). In parallel host plant biomass decreased and top down control by parasitoids increased in one study (Bezemer *et al.* 1998), whereas predator abundance decreased in the other study (Marquis *et al.* 2014). Despite the importance of such studies for biological pest control under climate change, similar studies under more realistic field conditions are missing.

Aphids are good model organisms to study multi-trophic interactions (Härri, Krauss & Müller 2008b). Many aphid species are serious crop pests that cause damage by feeding on plant sap or act as vectors of virus diseases (Van Emden & Harrington 2007). Phenotypic plasticity, rapid growth rate and multivoltine life cycles in aphids are an advantage in responding to climatic changes (Ward & Masters 2007). Due to their parthenogenetic reproduction during the summer months, aphid populations can grow exponentially (Costamagna *et al.* 2007). First flight trends of aphids advanced in the last five decades (Bell *et al.* 2015) and will further advance by approximately 8 days per 1 °C higher spring temperature (Kindlmann, Dixon & Michaud 2010). Aphid populations are top down controlled by predators or parasitoids (Schmidt *et al.* 2003; Chen 2008), but in contrast to aphids, predators have lower population growth rates and 5 to 20 times longer development times than aphids (Snyder & Ives 2003), which might be a constraint in responding to climate change (Reed, Schindler &

Waples 2011b). A recent meta-study showed that phenology shifts following climate change were larger for primary consumers like aphids compared to secondary consumers (Thackeray *et al.* 2016).

Aphid populations are not only top down controlled but can also be bottom up affected by chemical defence mechanisms of plants or microbial symbionts in plants (Müller & Krauss 2005; Chen 2008). Microbial symbionts like endophytic fungi in grass species can play an important role in plant defence against herbivores (Clay 2014, but see Saikkonen et al. 1998; Cheplick and Faeth 2009). Vertically transmitted grass-endophytes of the *Epichloë* group affect the grass physiology depending on biotic and abiotic conditions by increasing biomass (Müller & Krauss 2005), enhancing drought resistance (Hesse *et al.* 2003) or altering plant compositions (Rudgers & Clay 2007). *Epichloë* endophytes produce herbivore toxic alkaloids in the plant, which decrease the fitness of invertebrate herbivores or cause intoxications of grazing livestock (Müller & Krauss 2005; Philippe 2016). On the other hand it is under debate whether endophyte and alkaloid concentrations increase with herbivory (Hartley & Gange 2009; Zhang, Nagabhyru & Schardl 2009). Herbivores can drive plant-endophyte dynamics which can lead to higher infection levels in grass populations with high herbivore pressure (Koh & Hik 2007). Whether endophytic growth and alkaloid production increases with increasing herbivory is mostly unknown.

Our field experiment contained four interacting species along a tri trophic level food chain, (i) grass-endophyte (*Epichloë festucae* var. *lolii*), (ii) host plant (*Lolium perenne*), (iii) aphid (*Rhopalosiphum padi*) and (iv) aphid predators. We investigated the effects of enhanced aphid abundance in spring on aphid population development, predator occurrence and abundance, plant biomass and endophyte growth and alkaloid production. The key question of this study is whether organisms that interact with aphids have the phenological plasticity to respond to the simulated aphid shift. Fertilizer is often used on grasslands with our host plant *Lolium perenne* and fertilization can enhance plant growth, endophyte derived alkaloids and aphid and aphid predator abundances (Gastal and Nelson 1994; Krauss et al. 2007; Rasmussen et al. 2007, Ryan et al. 2014). We therefore included a fertilizer treatment in our study to verify our results for differently fertilized grasslands.

Our main predictions are:

(1) Experimental setting: Enhanced aphid abundance in spring (aphid shift) leads to overall higher aphid abundances.

(2) Top down control: Enhanced aphid abundance in spring (aphid shift) leads to a desynchronization between aphid and predator phenology.

(3) Induced defence: Endophyte and alkaloid concentrations increase in the host grass due to enhanced aphid abundance in spring (aphid shift).
(4) Bottom up control (endophytes): Aphid population growth is reduced on endophyte infected plants, due to the production of insect toxic compounds.

Material and Methods

Experimental design

In a common garden experiment with a randomized block design we tested the effects of (i) enhanced herbivore abundances in spring (aphid: *Rhopalosiphum padi*), (ii) endophytic fungus infection (*Epichloë festucae* var. *lolii*) in host plants (*Lolium perenne*), and (iii) fertilization of host plants on trophic interactions along a food chain. Every treatment combination was represented once per block resulting in 8 randomly arranged pots per block, replicated 10 times. A block design was chosen to control for unexplained variances, if block has a significant influence. Altogether we used 80 pots (18cm x 18cm x 18cm) with common garden soil (Einheitserde classic CL ED73, Profi Substrat) with a nitrogen availability of 250 N mg*L^{-1}. Each pot was sown with 10 *Lolium perenne* seeds of the grass cultivar Samson at the end of March 2013. Seeds were provided by David Hume, AG Research, NZ. The same cultivar was used in previous experiments and alkaloid studies including aphids and aphid predators (Meister *et al.* 2006; de Sassi *et al.* 2006; Fuchs *et al.* 2013). In 40 pots, the seeds were infected with the endophytic fungus *Epichloë festucae* var. *lolii*, which is formerly known as *Neotyphodium lolii* Glenn, Bacon and Hanlin (identity number A 12038) (Leuchtmann *et al.* 2014). In 40 other pots, seeds were not infected by the endophytic fungus (identity number A 11104). In the following we abbreviate *E. festucae* var. *lolii* infected plants with “E+” and uninfected with “E-”.

After 40 days of rearing the plants in a greenhouse, plants were transferred to the field, where the distance between grass pots was 30 cm to avoid contact between plants. In the field, in half of the pots aphid abundance was experimentally increased (simulated phenology shift see below), and half of the pots were treated with additional fertilizer in a crossed design. We used NPK fertilizer (Compo 20-5-10) equivalent to 400 N kg*ha^{-1}*yr^{-1} in eight doses between May 15th and July 3rd. We abbreviate fertilized plants with “F+” and non-fertilized with “F-”.

The study site was fenced to exclude vertebrate herbivores, mainly rabbits. We trimmed the plants three times at the beginning of the field experiment to a height of 20cm to avoid contact to surrounding plants. We finished the field experiment at 14th of August 2013 with taking the

aboveground biomass of the plants at an age of 140 days. Only aboveground biomass was taken, as *E. festucae* var. *lolii* only infects aerial plant parts (Cheplick & Faeth 2009).

Aphid addition

Grass plants were 56 days old when we started our experiment on May 22nd by adding 60 adult aphids *Rhopalosiphum padi* in each of 40 pots (Aphid supplier: Katz Biotech www.katzbiotech.de). Due to a low survival rate of the added aphids due to the changeover from rearing conditions in the supplier's lab to common garden conditions another 60 aphids per pot were added on June 4th. *R. padi* aphids overwinter on the bird-cherry tree and change their host in spring to cereal plants (Dixon 1971). We were checking grass plants daily for natural aphid arrival from beginning of May to have exact dates to adjust our aphid addition. As aphid arrival is not exactly predictable, an actual shift of two weeks earlier was not possible but our enhanced aphid abundances in spring simulated an advancement of aphid phenology compared to natural aphid phenology by approximately two weeks (treatment abbreviated as "A+"). We achieved this by (i) recording the time of natural occurrence of single individuals of *R. padi* on our pots; (ii) we estimated that 2 (to 3) adult aphids occur two weeks earlier when they shift their phenology; (iii) we estimated the overall fecundity of *R. padi* with approximately 30 offspring within 2 weeks averaged for endophyte free and endophyte infected host grass of the cultivar Samson (Meister *et al.* 2006) and finally (vi) we estimated a 50% mortality of added aphids caused by the changeover from rearing conditions in the laboratory to outdoor conditions (personal observations). We chose to simulate two weeks as it represents an aphid shift with an increasing spring temperature by approximately 2°C which represents a range between the SRES climate change predictions for 2099 (IPCC WG III. 2000; Kindlmann *et al.* 2010). We are aware that climate warming can also change endophyte and host grass growth (Vega-Frutis, Varga & Kytöviita 2014; McCulley *et al.* 2014), but the focus of our study was to uncover effects of increased aphid abundances in spring on interacting trophic levels.

The other 40 grass pots received no additional aphids (treatment abbreviated as "A-"), but all 80 pots were exposed to naturally immigrating aphids under natural common garden conditions.

Surveys

We counted aphids for five minutes per pot once a week for a total of eight weeks during the summer. We started at a plant age of 77 days at June 12th counting every seven days up to a

plant age of 126 days at 31st of July 2013 (calendar weeks 24-31). In few cases in the calendar weeks 26-28 five minutes were not enough to count the whole pot. In such cases only half or a quarter of the pot was counted and aphid numbers were extrapolated for the whole pot. Due to a symmetrical plant phenotype and a homogenous aphid distribution all over the plant we multiplied the recorded aphid number by two (in case of counting half of the pot) or multiplication by four (in case of counting quarter of the pot). Predators of aphids, including hoverfly larvae and pupae, lacewing larvae, all ladybird stages and spiders were counted for three minutes per pot, which was always sufficient time to count the whole pot. After eight weeks of the experiment aphid abundance and predator abundance were too low for meaningful counts.

Plant samples to quantify endophyte concentration and alkaloid concentrations were taken in parallel to aphid and predator counts, but were taken for two additional weeks until a plant age of 140 days at 14th of August 2013 (calendar weeks 24-33). We collected plant material for quantitative PCR (qPCR) and Ultrahigh Performance Liquid Chromatography- Mass Spectrometry (UPLC-MS) analyses by cutting a 3cm piece from the plant, around the oldest leaf sheath. Withered parts were removed from the sampled material to ensure similar sample quality. We sampled one tiller per pot per week of all 80 pots. After grounding the samples in liquid nitrogen, we split the grass material onto two tubes, one for UPLC-MS analysis and one for qPCR analysis. At the 14th of August we harvested the total aboveground biomass of all pots. Biomass was dried for 3 days in a 60 °C tempered drying oven (Memmert GmbH) before weighing.

Alkaloid extraction and analysis

We quantified the alkaloids peramine, lolitrem B and ergovaline produced by the endophytic fungus, while peramine is the most insect deterring alkaloid (Tanaka *et al.* 2005). We sampled weekly about 3cm plant material from leaf stalks and leave sheaths of endophyte infected and endophyte free *L. perenne* plants which was immediately frozen after sampling. We analysed weekly taken plant material from E+ pots (see above) while from E- pots samples were analysed at the end of the study period to confirm that all E- pots are free from endophyte infection and alkaloids. We weighed the grass material with a micro scale (Mettler-Toledo Intl. Inc.) before alkaloids were extracted from the samples with methanol and dichloromethane in several steps. Afterwards, alkaloids were determined and quantified with Ultrahigh Performance Liquid Chromatography (UPLC-MS) with argon as collision gas. Using our previous published UPLC-MS method developed to detect and quantify alkaloids

produced by *E. festucae* var. *lolii* (Fuchs *et al.* 2013), we quantified the peramine concentration with reference to the internal standard compound homoperamine. Lolitrem B concentration was quantified semi-quantitative by reference to homoperamine. Ergovaline was quantified with the internal standard compound ergotamine. Detection limit for all alkaloids was 5 ng.

Endophyte quantification by qPCR

We established a quantitative PCR (qPCR) system for quantification of *Epichloë festucae* var. *lolii* in the plant tissue of endophyte infected *Lolium perenne* based on quantitative PCR of genomic DNA (gDNA) isolated from aboveground plant material. Genomic DNA was extracted from about 50 mg fresh weight of homogenized plant material. Taking the exact sample weight was not necessary due to calculation of total fungal DNA to total grass DNA transcripts. DNA extraction was performed with 750 µL CTAB-extraction buffer and 750 µL Chloroform. Genomic DNA was precipitated with Isopropanol [1:1; v/v] followed by two washes with Ethanol (500 µL [70%]). Total DNA concentration was estimated by using Nano-Drop 1000 (Thermo-Scientific, Hamburg, Germany) and adjusted to 10 ng/µL. Quantitative PCR was performed with 20 ng genomic DNA, SYBR-Green Capillary Mix (ThermoFisher Scientific, Hamburg, Germany) with a CFX96 Touch™ qPCR-machine (Bio-Rad, Munich, Germany). Primers (TIB MOLBIOL, Berlin, Germany) suitable for qPCR amplification were designed for a fragment of an *E. festucae* var. *lolii* specific gene encoding *Chitinase A* (forward: 5'-AAgTCCAggCTCgAATTgTg- 3'; reverse: 5' -TTgAggTAgCggTTgTTCTTC- 3'). Primers suitable for qPCR were chosen for a fragment of *L. perenne* specific gene encoding *β- tubulin* (forward: 5' -gCTgCCTAAggTTCCCTgg- 3'; reverse: 5' -gCAgAggCggTgAggTAA- 3') (Rasmussen *et al.* 2007). The qPCR protocol was as follows: pre-incubation: one cycle of 95°C for 15 min; amplification: 44 cycles of 95°C for 15 s, 61°C for 20 s, 72°C for 25 s. All presented fungal gDNA results refer to 10,000 copies of amplified grass *β- tubulin* transcripts.

Uninfected samples showed up to 200 copies of fungus per 10,000 copies of grass *β- tubulin* transcripts which is caused by primer dimers and general background noise. Consequently we set samples as endophyte free below 200 copies of fungal gDNA. Non infection was also confirmed by the absence of endophyte produced alkaloids.

Statistics

All statistical analyses were conducted using the software R version 3.0.2. We used ANOVAs with the three explanatory variables aphid shift (A+/A-), endophyte infection (E+/E-) and fertilizer (F+/F-). The treatments were arranged in a randomized balanced block experiment (Crawley 2012), resulting in eight treatment combinations, which we replicated each ten times. Response variables were (i) plant biomass, (ii) aphid abundance and (iii) aphid predator abundance. Total aphid and predator abundances were estimated by summing up the numbers of aphids and predators, respectively, per pot during 8 weeks of counting. Block was tested as a fixed factor in the models but was never significant and never changed our results. We therefore simplified our models and omitted "block" from the analyses (Crawley 2012). None of the treatment interactions showed significant results (all $p > 0.05$) and were therefore omitted from the final models and are not further discussed. We also used ANOVAs to analyse separately the 40 pots with endophyte infected plants (E+) to detect the effects of aphid shift (A+/A-) and fertilizer (F+/F-) on (iv) alkaloid concentrations and (v) endophyte concentration. We used mean of alkaloid concentration and endophyte concentration per pot during 10 weeks of sampling to test for differences between our treatments over the whole study period. We also analysed ANOVAs separately for each week to detect if the endophyte concentration, aphid abundance and predator abundance differs between treatments in single weeks. We did not apply repeated measure analyses because it is not an appropriate method to detect differences in time but to correct for temporal pseudo replication (Crawley 2012). Residuals in all models had homogenous variances and were normally distributed. Means ± standard errors are presented throughout the manuscript. Alkaloid concentrations over time were illustrated with a local polynomial regression fitting model (Cleveland, Grosse & Shyu 1992).

Results

We recorded 158,385 aphids on the 80 pots within the eight week study period. *Rhopalosiphum padi* was with 98.57% the most dominant aphid species followed by *Aphis fabae* 1.38% and *Sitobion avenae* 0.05%. We considered only *R. padi* in our analyses as we added this species to simulate phenology shift. A total of 1,307 aphid predators were recorded during the eight weeks, however, none was detected in the first two weeks of our study. Hoverfly larvae (644 individuals) were the most abundant predators.

In total we recorded 2.5 times more aphids per pot with aphid shift (A+) than on pots without aphid shift (A-) (Table IV 1, Fig IV S1b). Endophyte infection and fertilization had no

significant effect on aphid abundance (Table IV 1). Predator abundance was neither significantly affected by aphid shift nor by endophyte infection, but was higher on fertilized pots (Table IV 1, Fig IV S1a). Aboveground plant biomass at the end of the experiment was higher in endophyte infected pots compared to uninfected pots and in fertilized pots compared to unfertilized pots (Table IV 1). Aphid shift increased the endophyte concentration in endophyte infected plants by 25% (Table IV 1, Fig IV S1c), but did not significantly influence alkaloid concentrations (Table IV 1). Fertilization affected the concentration of the alkaloid lolitrem B, but not significantly the concentrations of peramine and ergovaline (Table IV 1).

Aphid abundance increased in both A+ pots (aphid shift) and A- pots (no aphid shift) over the first weeks of the experiment (Fig IV 1). Until calendar week 27 (first four weeks of the experiment), the weekly recorded aphid abundance was significantly higher in A+ than in A- pots. The aphid abundance in A+ pots in calendar week 26 corresponded approximately to the aphid abundance in A- pots reached in calendar week 28 indicating that the treatment A+ successfully simulated an advancement of aphid population growth by about two weeks (Fig IV 1 “shift”).

Table IV 1 ANOVA table showing the effects of aphid shift (enhanced abundance), endophyte infection and fertilization on aphid abundance, predator abundance and plant biomass; and of aphid shift and fertilization on endophyte concentration (gDNA $x10^4$ referred to 10^4 copies of grass gDNA) and alkaloid concentrations (µg g^{-1}) tested only for endophyte infected host plants. Interaction terms of predictor variables were never significant and were removed from the models

Response	Predictor	*df*	F	P	mean± s.e.	mean± s.e.
All pots						
Aphid abundance	Aphid shift	1,76	38.60	**<0.001**	A-: 1152±134	A+: 2888±243
	Endophyte infection	1,76	0.64	0.43	E-:1908±207	E+:2132±269
	Fertilization	1,76	0.31	0.58	F-:1942±263	F+:2098±215
Predator abundance	Aphid shift	1,76	1.01	0.32	A-: 14.9±1.38	A+: 17.2±1.85
	Endophyte infection	1,76	0.83	0.36	E-: 15.0±1.81	E+: 17.1±1.44
	Fertilization	1,76	7.34	**0.007**	F-: 13.0±1.88	F+: 19.1±1.18
Biomass (g)	Aphid shift	1,76	0.99	0.32	A-: 41.6±2.1	A+: 39.9±2.0
	Endophyte infection	1,76	4.09	**0.046**	E-: 39.0±2.1	E+: 42.5±2.0
	Fertilization	1,76	146.1	**<0.001**	F-: 30.4±1.3	F+: 51.1±1.1
Only endophyte infected pots						
Endophyte conc.	Aphid shift	1,37	7.05	**0.012**	A-: 1.21±0.06	A+: 1.62±0.14
	Fertilization	1,37	0	0.99	F-: 1.41±0.12	F+: 1.41±0.11
Peramine (µg/g)	Aphid shift	1,37	2.75	0.11	A-: 3.94±0.21	A+: 3.53±0.13
	Fertilization	1,37	0	1	F-: 3.73±0.17	F+: 3.73±0.19
Lolitrem B (µg/g)	Aphid shift	1,37	0.06	0.80	A-: 1.87±0.16	A+: 1.83±0.09
	Fertilization	1,37	4.90	**0.035**	F-: 2.04±0.14	F+: 1.66±0.09
Ergovaline (µg/g)	Aphid shift	1,37	1.27	0.27	A-: 0.07±0.01	A+: 0.08±0.01
	Fertilization	1,37	0.85	0.36	F-: 0.07±0.01	F+: 0.08±0.01

P<0.05 highlighted in bold

Temporal dynamics

Predators were nearly absent in both treatments until calendar week 27 (week four of the experiment). In that week, aphid abundance in A+ pots was three times as high as in A- pots (Fig IV 1). Despite the advancement of the prey phenology in A+ pots by two weeks, predator abundances increased simultaneously in both treatments in calendar week 28 and reached similarly high values in A+ and A- pots (Fig IV 1, Fig IV S1). In other words, predators on A+ pots did not increase their abundance in parallel to their prey. With high predator abundances the aphid abundances decreased within two weeks by more than 92 % independent of the aphid abundance (Fig IV 1).

Endophyte concentration in A- plants reached its maximum in calendar week 28 and was thus perfectly synchronized with the maximum of aphid abundances on the host plants (Fig IV 1). Endophyte concentration in A+ plants reached its maximum in calendar week 30, which was three weeks after aphid abundance reached its maximum on the host plants. In calendar weeks 30-32, the endophyte concentration in A+ plants was higher than in A- plants. At this time aphid populations were already controlled by top-down effects (predation and potentially emigration) and aphid abundances were very low in both treatments (Fig IV 1). The alkaloids produced by the endophytic fungus did not significantly differ between A+ and A- plants during the study period, but increased in both treatments from mid-June to mid-August when we stopped recording concentrations (Fig IV 2)

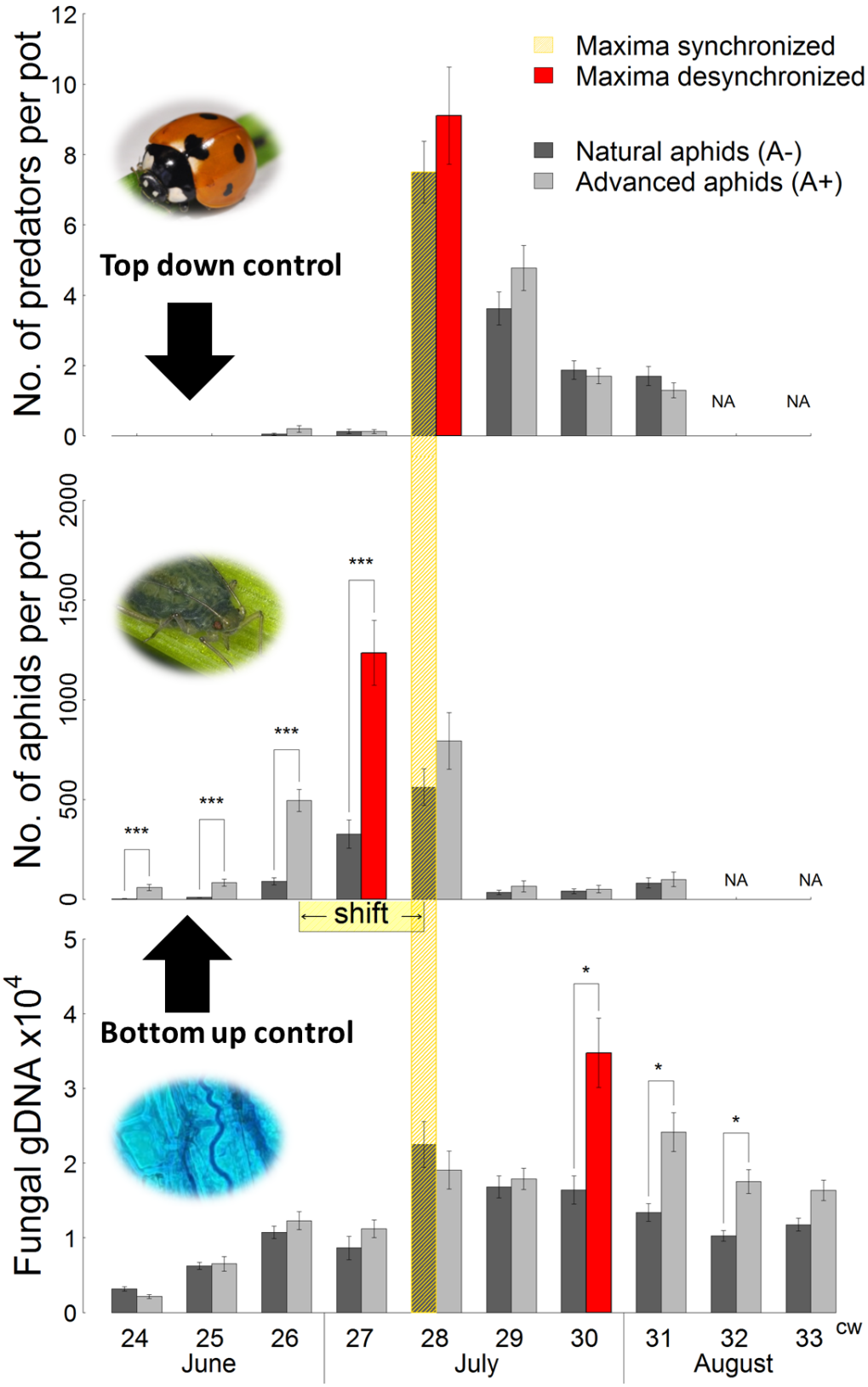

Fig IV 1 Temporal dynamics of aphids, predators, fungal gDNA depending on aphid shift (A+, A-) over the study period. Fungal gDNA is only presented for endophyte infected pots. Mean ± S.E per calendar week (cw) of aphid abundance, predator abundance and concentration of fungal gDNA. Yellow bar: Synchronized maxima with naturally occurring aphids (A-); Red bars: Desynchronized maxima with simulated aphid shift (A+).
NA: not available; *** P≤ 0.001, ** P≤ 0.01, * P≤ 0.05

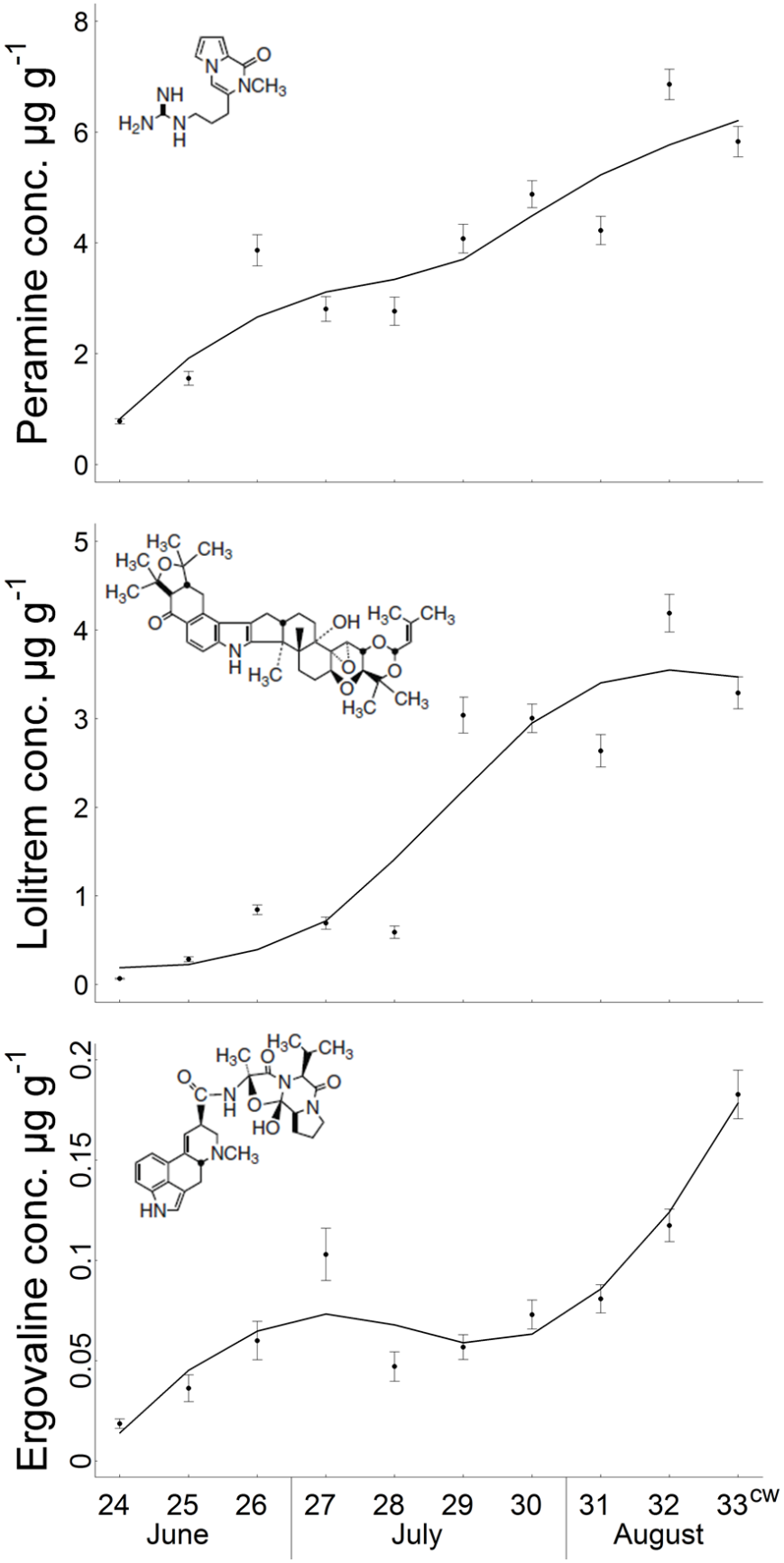

Fig IV 2 Change of alkaloid concentrations from late spring (cw 24) to summer (cw 33) in endophyte infected pots (N=40). Mean ± S.E per calendar week (cw) of alkaloid concentrations in µg g^{-1}.

Discussion

Enhanced aphid abundances in spring lead to overall higher aphid abundances, because neither aphid predators (top down control) nor chemical defence of the endophyte (bottom up control) had the plasticity to shift their phenology or to increase their abundance when aphid abundances reached high levels. However, when predators occurred, aphid abundances dropped within two weeks, independently of aphid population size which displays the impact of predators as pest control. Endophyte concentration increased time-delayed to enhanced aphid abundance at a time when bottom up control had no effect on aphid control because aphids were already top-down controlled. Phenology shift of aphids therefore caused desynchronization between the trophic levels resulting in (i) larger herbivore populations before top down control by predators and (ii) time delayed bottom up response by the endophytic fungus.

Increased aphid abundance in spring leads to overall higher aphid abundances and simulated a phenology shift of aphids. This result is not surprising, as aphids have a fast parthenogenetic life cycle (Leather & Dixon 1984) and the addition of aphid individuals allowed a larger starting population. We conclude that our experimental setting simulated a shift in aphid phenology of approximately two weeks, even though it remains difficult to separate effects between shift and abundance of aphids on the other trophic levels. As the top-down control by predators occurred independent of aphid abundance, higher aphid abundances early in the season are likely to cause stronger plant damage (Van Emden & Harrington 2007).

We predicted that enhanced aphid abundance in spring leads to a temporal desynchronization with their predators (top down control). We confirmed our prediction because predators did not shift their phenology to an earlier arrival but occurred at the same time in both aphid treatments regardless of high differences in aphid abundances between the treatments. In contrast to a desynchronized caterpillar-bird system, where birds missed the peak abundance of their prey with possible fitness disadvantages for predators (Visser et al. 2006), aphid predators might even benefit from enhanced prey abundances. Surprisingly plants with high prey abundances did not show higher predator abundances. Prevalent aphid predators were mostly larvae of hoverfly species. Hoverflies can choose their egg deposition sites by semiochemical cues, emitted by aphids and their host plants (Almohamad *et al.* 2007). We speculate that aphid predators were attracted by plant cues and general aphid presence rather than primarily distinguished between pots with higher or lower aphid abundance. If aphids occur earlier on plants, predators could also arrive earlier, but the phenological plasticity of aphid predators is assumed to be lower than of aphids (Reed *et al.* 2011b). With our study

design we cannot exclude that predator arrival can adapt to earlier aphid arrival, but our results indicate a plant quality and photoperiod triggered predator arrival. Plant biomass increased with fertilization in our study and higher predator abundances were recorded on fertilized pots which indicates that egg deposition is determined by plant cues rather than by aphid abundance. Further aphid predators occurred in the second week of July with high abundances, presumably determined by photoperiod. Photoperiod triggers diapause and migration of aphid predators like hoverflies (Dingle 1972; Saunders 1981; Hondelmann, Borgemeister & Poehling 2005), while the arrival of aphids is triggered by temperature (Zhou *et al.* 1995). Ongoing climate change increases temperature unlike photoperiod which indicates desynchronizations between aphids and their predators in future climate change scenarios. Even though we detected a desynchronization between aphids and aphid predators in our study, aphid populations were top- down controlled within two weeks, independent of prey abundance highlighting their efficiency in top-down controlling in such trophic cascades (Symondson *et al.* 2002).

As predicted, the concentration of the endophytic fungus was increased in plants with enhanced aphid abundances in spring which indicates an induction due to enhanced herbivory. However, endophyte concentration increased time delayed, when aphid abundances were already controlled by predators and when no further herbivore species were present in considerable numbers. Defence strategies of plants and plant associated symbionts can be costly and are often induced by prevalent herbivory (Strauss *et al.* 2002). Hosting the endophytic fungus can turn into a costly association for the plant, when endophyte concentration increases after high herbivory but without affecting herbivory (Cheplick & Faeth 2009), especially when nitrogen is a limiting resource for the host plant and endophytic metabolite synthesis competes with basic plant growth and reproduction success (Faeth & Fagan 2002). Our study shows evidence that enhanced herbivore abundance in spring can desynchronize the symbiotic relationship in a plant-endophyte association. This is in line with desynchronizations found in ant-aphid (Barton & Ives 2014), plant-mycorrhiza (Vega-Frutis *et al.* 2014) and plant-pollinator symbioses (Hegland *et al.* 2009).

The endophytic fungus-grass association used in our study produces different alkaloids which are toxic for herbivores (Clay 2014). We expected increased alkaloid concentrations on plants with higher aphid abundances, but our results showed similar alkaloid concentrations independent of aphid abundances. One explanation could be that alkaloids accumulate in the plant and alkaloid production might follow time delayed after endophytic growth. Another reason might be that alkaloid concentrations depend on the type of herbivory. Chewing

herbivores and mechanical plant damage increased alkaloid concentrations in endophyte infected plants (Zhang *et al.* 2009), but plant sap sucking herbivores might not induce alkaloid production. Unlike earlier findings (Krauss *et al.* 2007), fertilization did not enhance endophyte and alkaloid concentrations, probably because soil used in our study already contained a high amount of nitrogen.

In contrast to our fourth prediction, aphid abundance was not significantly affected by the presence of the endophytic fungus (E+ vs. E- plants). This is contrary to laboratory studies showing a reduced herbivore performance on host grass with an *Epichloë festucae* var. *lolii* symbiosis (Breen 1994; Meister *et al.* 2006) but is in accordance with no differences found in alkaloid concentration levels. An increase in endophyte concentration might be a first step towards induced bottom up control by increased aphid abundance earlier in time. Nevertheless endophyte growth was time delayed to high aphid abundance which strongly reduced the potential beneficial effect against herbivores as herbivores were already levelled by top down effects. Further, the missing bottom up control of aphids in our study might be caused by low alkaloid concentrations when aphid abundances peaked at the beginning of July. All three tested alkaloids increased over the study period probably due to accumulation in the plant. With an aphid shift to an earlier arrival in spring (Bell *et al.* 2015), an increasing temporal desynchronization between high aphid abundances in spring and high alkaloid concentrations in summer might be the consequence.

Conclusions

We showed with our common garden experiment that enhanced herbivore abundance in spring affected interacting species in a multi trophic system, which can desynchronize trophic cascades. In our multi-trophic level approach the possible bottom up control for herbivores (grass associated microorganism) was enhanced when herbivores were already controlled by predators. Without the function of herbivore deterrence by the plant associated microorganism, the mutualistic symbiosis could turn antagonistic with possible fitness costs for the symbiotic association which can alter the relations of interacting species (Saikkonen *et al.* 1998; Müller & Krauss 2005). Further we showed that aphid predators did not shift their phenology when aphid abundances were higher in early summer. Predator control of aphids was very efficient after the occurrence of predators. Nevertheless, plants were exposed to stronger herbivore pressure before the occurrence of predators. This can lead to higher plant damage and an increase in vector transmitted crop diseases (Fand, Kamble & Kumar 2012). To uncover effects of climate change on pest outbreaks in crops it needs more than one study,

where one pest species was manipulated but our study provides a first indication for desynchronized predator-prey and plant-microorganism interactions within one food chain.

Supporting Information to Chapter IV

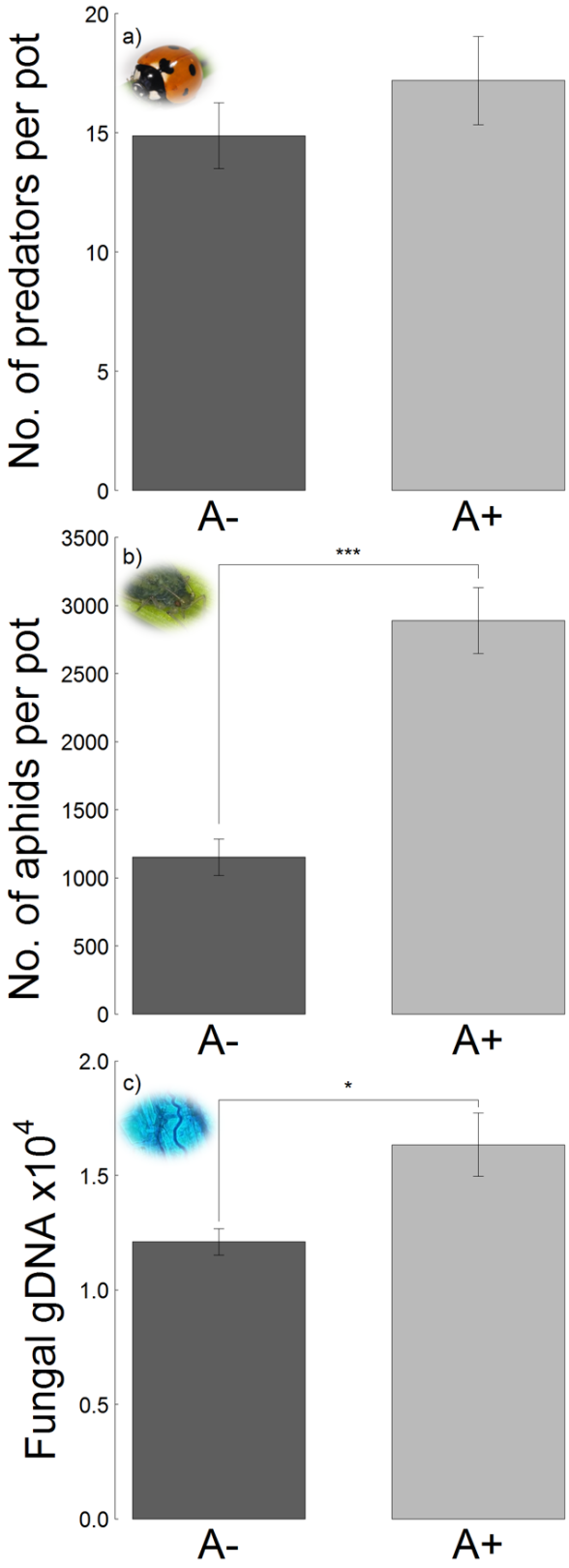

Fig IV S1 Total number of aphids (a), average number of predators (b) and average concentration of fungal gDNA per grass DNA (c) shown for all pots (a,b) and only E+ pots (c). Values see Table IV 1.

*** P≤ 0.001, ** P≤ 0.01, * P≤ 0.05

Chapter V: Herbivore-specific induction of defence metabolites in a grass-endophyte association

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Abstract

Plants have developed a variety of defence strategies against herbivores. One possible strategy is the induced production of metabolites following herbivore attack. Plant-associated microorganisms can be the source of such defensive compounds. For example, cool-season grasses can be associated with systemic endophytic fungi of the genus *Epichloë*, which produce herbivore-toxic alkaloids.

In a controlled common garden approach we tested the hypothesis that different types of herbivory induce endophyte growth and increase the endophyte-mediated production of three bioactive alkaloids which can deter or toxify herbivores. During 18 weeks we analysed biweekly endophyte and alkaloid concentrations in the grass *Lolium perenne* infected with the endophytic fungus *Epichloë festucae* var. *lolii.* The experiment was conducted throughout the field season and compared three different herbivore treatments to the control treatment (herbivory exclosure)

We showed that the concentration of the vertebrate toxic alkaloid lolitrem B increased following clipping (a simulation of grazing herbivores), while the insect deterring alkaloid peramine increased following locust herbivory (biting-chewing herbivores). The endophyte concentration increased slightly following clipping ($p=0.09$). Sap sucking aphids altered neither endophyte nor alkaloid concentrations.

Our study provides evidence for a herbivore-specific induction of endophyte-mediated responses following herbivore attack on its host grass. Our results suggest that the grass-endophyte symbiosis involves a close chemical crosstalk between the interacting partners.

Key-words: chemical crosstalk, constitutive defence, defensive mutualism, endophytic fungi, induced resistance, multi-trophic interactions, plant defence, secondary metabolites

Introduction

Plant defence strategies to minimise herbivory can determine plant fitness (Agrawal 1998). Such defence mechanisms can be constitutive (always present in the plant) or induced (produced or mobilized when herbivory occurs) (Herrera & Pellmyr 2002). In order to minimise the costs and maximise the benefits of defensive mechanisms plants have developed adaptive plasticity in their ability of inducing defence mechanisms under herbivore attack (Agrawal 1999). One such defence mechanism is the production of toxic or deterring metabolites (secondary metabolites) that harm herbivores (Raguso *et al*. 2015), with different plant defence pathways being induced depending on herbivore type (Walling 2000). Mechanical plant damage from grazing cattle or chewing insects induces defence signalling pathways that differ from those induced by pathogens and piercing-sucking insects such as aphids (Walling 2008). This is followed by the synthesis of specific defensive chemicals (Leitner *et al.* 2005). Furthermore, certain microorganisms within the plant tissue can support the plant by altering plant defence or by producing herbivore-toxic compounds (Pineda *et al*. 2010; Zamioudis & Pieterse 2011; Clay 2014; Li *et al*. 2014). A rising number of studies demonstrate that induced systemic resistance against diseases and herbivores are mediated by belowground or aboveground plant symbiotic fungi (Pineda *et al*. 2010; Zamioudis & Pieterse 2011; Li *et al*. 2014).

Cool-season grass species within the family Poaceae regularly tolerate herbivory through a rapid regrowth of plant tissue and not necessarily through chemical defence strategies (Hawkes & Sullivan 2001). The symbiosis with microorganisms, such as the intracellularly growing fungi of the *Epichloë* group, can alter the plant's relation to herbivory through the synthesis of herbivore deterring alkaloids (Saikkonen *et al.* 2013). Asexual fungi of the genus *Epichloë* form a close symbiosis with their grass host as their existence and dispersal is completely plant-dependent (Saikkonen *et al*. 2015). *Epichloë* belong to the clavicipitaceous fungi and are growing intercellularly in the above ground plant parts of estimated 20-30% of all grass species worldwide (Leuchtmann 1992). *Epichloë* endophytes can produce bioactive metabolites in their host grass, which can have detrimental effects on grass herbivores (Schardl *et al.* 2012). Thereby the extent of harmful effects on herbivores depends on herbivore species and the genotypic predisposition of host and endophyte (Faeth & Saikkonen 2007; Ryan *et al.* 2015).

The asexual endophytic fungus *Epichloë festucae* var. *lolii* grows asymptomatically and intracellularly within the agronomical relevant host grass *Lolium perenne* and harms vertebrate and invertebrate herbivores by producing defensive metabolites (Müller & Krauss

2005; Cheplick & Faeth 2009). *Epichloë festucae* var. *lolii* produces three main alkaloids: peramine, lolitrem B and ergovaline. The pyrrolizidine alkaloid peramine deters or toxifies invertebrate herbivores, while the indole-diterpene alkaloid lolitrem B and the ergot alkaloid ergovaline are neurotoxins with severe consequences for vertebrate herbivores (Philippe 2016). It is currently under debate if and how strong ergovaline is toxic for invertebrates (Hartley & Gange 2009).

Such effects of endophyte derived alkaloids on herbivores and higher trophic levels have already been shown in several studies (reviewed by Saikkonen, Gundel & Helander 2013); however, it remains mainly unclear as to whether herbivores can affect the grass-endophyte and induce the production of alkaloids. One study on another grass-endophyte system showed an increase in invertebrate toxic loline alkaloids in regrown plant tissue following mechanical plant damage from clipping after two weeks, indicating an induction of loline alkaloids (Bultman *et al.* 2004).

We predict that different types of herbivory induce the production of different alkaloids, as suggested by the plant defence theory of herbivore specific induction of defensive compounds (War *et al*. 2012; Pineda *et al*. 2013; Li *et al*. 2014). In a common garden experiment we tested the effect of clipping (simulation of grazing vertebrates), aphids and locust herbivory on the growth of the endophytic fungus *Epichloë festucae* var. *lolii* and the subsequent production of alkaloids in the host grass *Lolium perenne*.

Our hypotheses are:

(1) Herbivory increases endophyte concentration and defensive alkaloid concentrations in its host plant.

(2) Insect deterring or toxifying alkaloids are enhanced by chewers and sap sucking insects, while vertebrate toxic compounds are enhanced by vertebrate grazing (simulated by clipping).

Material and Methods

Experimental design

In a common garden experiment we tested the effects of three different herbivores plus the exclusion of herbivores (control) on the growth of an endophytic fungus and the concentration of chemical defence compounds. Our study system was the grass species *Lolium perenne* which was infected with the endophytic fungus *Epichloë festucae* var. *lolii*, formerly known as *Neotyphodium lolii* Glenn, Bacon and Hanlin (identity number A 12038) (Leuchtmann *et al*. 2014). We used 48 pots which were each sown with one seed of endophyte infected *L.*

perenne. Plants were in their second growing year when we started the experiment. We chose two year old plants, as a previous study had shown that alkaloid concentrations are above the toxicity level for vertebrates and invertebrates in two year old infected plants, but not in 6 week old infected plants (Fuchs et al. 2013). Pots had a diameter of 30 cm and a height of 30 cm and were filled with common garden soil (Einheitserde classic CL ED73, Profi Substrat). We installed gauze (Rantai S48 Firma Schachtrupp KG) with a mesh width of 0.8 x 0.8 mm, surrounding each pot, which prevented the plant from invasion by the majority of herbivores, predators and parasitoids (see Fig V S1 in Supporting Information). Pots were burrowed in the soil to a depth of 30 cm to protect the pots from storms (Fig V S1). The plant pots were placed in four rows with 12 pots per row before treatments were assigned randomly to each pot. Distance between pots was 1.2 meters on a plane field (Fig V S1). The plants were regularly watered and once cut to a height of 30 cm one month before we started the experiment to achieve similar sized plants. The experiment started at the beginning of summer on the 2nd of June 2014 by taking plant samples from each pot, before the herbivore treatments started. On the same day we randomly assigned 12 pots to each of the following treatments (n=12 per treatment): (1) clipping of plants weekly to a height of 10 cm, an established method to simulate grazing (Saari *et al.* 2010b), (2) adding 50 adult *Rhopalosiphum padi* aphids (piercing-sucking herbivore) (3) adding eight larvae (L3) of the desert locust *Schistocerca gregaria* (biting-chewing herbivore), (4) herbivore exclusion (control treatment). We removed six pots from the analyses (two pots each with locust and clipping and one pot each with aphids and control), because fungal infection of the host grass after the experiment could not be confirmed. Such imperfect growing of the fungus into their host plant despite the infection of the seeds is common in this symbiosis (Hume, Card & Rolston 2013). We sampled grass material for alkaloid and endophyte analyses every second week until 6th of October 2014 (18 weeks in total). For each sampling a separate green tiller of the pots was randomly chosen. Approximately 3 cm of the tiller around the collar and including parts of the leaf sheath and leaf blade of the oldest leaf was selected (see Supporting Information Fig V S2), as this area of the infected grass contains high endophyte concentrations (Spiering *et al.* 2005). We did not remove withered plant material in general, but did not use withered plant material for our analyses, as withered plant parts differ in their alkaloid concentrations from living plant material (Hume *et al.* 2007). If the oldest leaf was strongly withered, the tiller around the collar of the second oldest leaf was chosen. Where the sampled grass material still contained withered plant parts, they were removed before analyses (removed parts < 5 % of plant

material). An advantage of this sampling procedure was that all sampled grass material was of similar age and condition in all plant pots.

In parallel to the plant sampling we recorded the herbivore abundances of aphids (2 min counting per pot) and locusts. Aphid populations increased exponentially in the aphid enclosures for 6 weeks and decreased afterwards (Table V S1), probably because of the reduction in plant quality at high aphid abundances. Locust numbers decreased slowly from 8 individuals to an average of 2 individuals (Table V S1). We also estimated herbivore-induced damage to the plants qualitatively and show typical phenotypes of the four treatments (Fig V 1a). The damage from aphid and locust treatments peaked approximately at study week 6 but probably due to constant herbivore presence plant quality remained low with minimal regrowth/improvement in plant quality during the experiment.

Alkaloid extraction and analysis

The alkaloids peramine, lolitrem B and ergovaline produced by the endophytic fungus were quantified by Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). We analysed the powdered material from tillers with leaf sheath and blade of endophyte infected *L. perenne* plants. Grass samples were kept frozen at -20°C until preparation for UPLC-MS/MS detection. We weighed the grass material with a micro scale (Mettler-Toledo Intl. Inc.) before alkaloids were extracted from the samples with methanol and dichlormethane. We used our previously published UPLC-MS/MS method developed to detect and quantify alkaloids produced by *E. festucae* var. *lolii* (Fuchs *et al.* 2013), to determine the concentrations of ergovaline, lolitrem B and peramine by reference to the internal standard compounds ergotamine and homoperamine.

Endophyte quantification by qpcr

We established a quantitative PCR (qPCR) system for quantification of *Epichloë festucae* var. *lolii* in the plant tissue of endophyte infected *Lolium perenne* based on quantitative PCR of genomic DNA (gDNA) isolated from the sampled plant material. Genomic DNA was extracted from about 50 mg fresh weight of homogenized plant material. Exact weight of the sample was not necessary because total fungal DNA was expressed relative to the total number of grass DNA transcripts and not relative to plant weight. DNA extraction was performed with 750 µL CTAB-extraction buffer and 750 µL Chloroform. Genomic DNA from the organic phase was precipitated with Isopropanol [1:1; v/v] followed by two washes with Ethanol (500 µL [70%]). Total DNA concentration was estimated by using a Nano-Drop

1000 (Thermo-Scientific, Hamburg, Germany) and adjusted to 10 ng/µL. Quantitative PCR was performed with 20 ng genomic DNA, SYBR-Green Capillary Mix (ThermoFisher Scientific, Hamburg, Germany) using a CFX96 Touch™ qPCR-machine (Bio-Rad, Munich, Germany). Primers (TIB MOLBIOL, Berlin, Germany) suitable for qPCR amplification were designed for a fragment of an *E. festucae* var. *lolii* specific gene encoding *Chitinase A* (forward: 5'-AAGTCCAGGCTCGAATTGTG- 3'; reverse: 5' -TTGAGGTAGCGGTTGTTCTTC- 3'). Primers suitable for qPCR were chosen for a fragment of a *L. perenne* specific gene encoding *β- tubulin* (forward: 5' -GCTGCCTAAGGTTCCCTGG- 3'; reverse: 5' -GCAGAGGCGGTGAGGTAA- 3') (Rasmussen *et al*. 2007). The qPCR protocol started at pre-incubation with one cycle of 95°C for 15 min, followed by amplification with 44 cycles of 95°C for 15 s, 61°C for 20 s, 72°C for 25 s. All presented fungal gDNA results refer to 10,000 copies of amplified grass gDNA.

Statistics

All statistical analyses were conducted using the software R version 3.2.3. We used the lme4 package to perform linear mixed effect models for temporal pseudo-replication followed by an ANOVA (repeated measure ANOVA) (Crawley 2012; Bates *et al*. 2015). Repeated measure analyses contained 9 time points from week 2 to week 18. When the repeated measure ANOVA was $p < 0.1$ we compared the three herbivore treatments with the control using a Dunnett's test (Dunnett 1955). To show temporal changes of the treatment effects we also conducted ANOVAs for each sampling time separately. Response variables of all models were concentrations of (i) fungal gDNA, (ii) ergovaline (iii) lolitrem B and (iv) peramine. Residuals in all models had homogenous variances and were normally distributed. Means ± standard errors are presented in the figures.

Results

Different types of herbivory caused different plant damage. After four weeks, clipped plants were green but small, whereas aphids caused a yellow plant phenotype and locusts lead to high tissue losses in the plant (Fig V 1a). Clipping increased the concentration of the vertebrate toxic alkaloid lolitrem B, whereas locusts increased the peramine concentrations in the plants (Fig V 1d, e, Table V 1). Fungal concentration was only marginally affected by the herbivore treatments ($p = 0.09$); a Dunnett`s posthoc test showed higher concentrations in clipped compared to control plants (Fig V 1b, Table V 1). The alkaloid ergovaline was not significantly affected by any herbivory treatment and aphids had no significant effect on any

measured alkaloid or the endophyte concentration (Fig V 1, Table V 1). Alkaloid and endophyte concentrations were similar in all plants before the application of herbivore treatments in week 0 (Fig V S3, Table V 1).

Separate analyses for each week showed that the fungal DNA concentration was only enhanced by clipping in week 10 compared to the control, while lolitrem B was enhanced by clipping from July to August (Fig V S3, Table V 1). Locusts consistently enhanced the peramine concentration in August and September starting 2 weeks after we observed the largest feeding damage from locusts on the plants (Fig V S3, Table V 1). No overall effect on ergovaline was found, except for an enhancement due to clipping in one single week (Fig V 1c, Fig V S3, Table V 1). We found a herbivore independent increase in alkaloid concentrations from June to July for lolitrem B, from June to August for peramine and from June to September for ergovaline (Fig V S3).

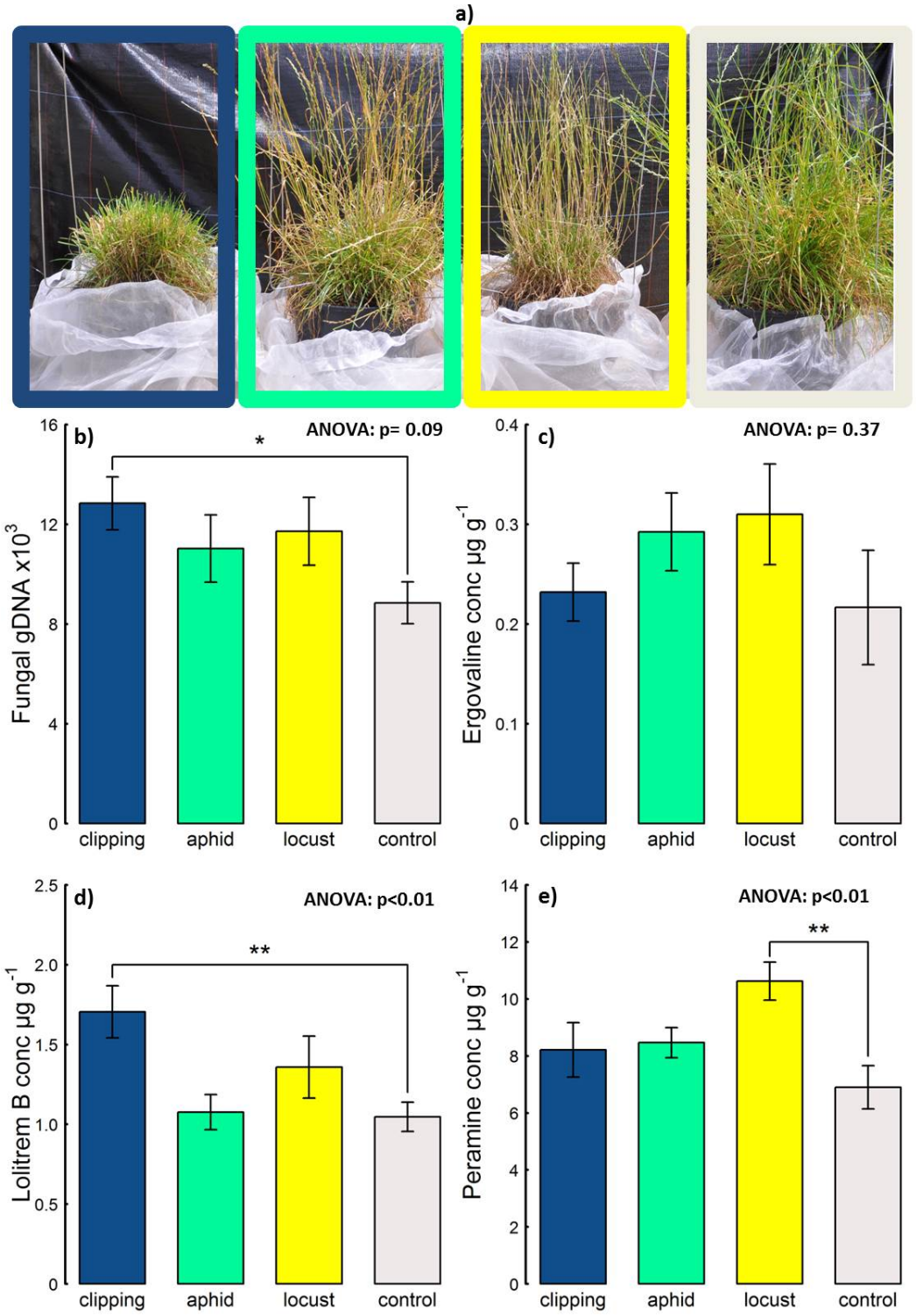

Fig V 1 a) Physical conditions of the plants four weeks after herbivore introduction. Weekly clipped plants show fresh green leaf material after cutting. Aphid treated plants turn towards a yellowish colour, while locust treated plants show reduced leaf material compared to control plants. Concentrations of fungal gDNA (transcript abundance relative to the transcript abundance of plant gDNA) (b) and three alkaloids (µg/g leaf dry weight) (c-e) produced by *Epichloë festucae* var. *lolii* in the grass host *Lolium perenne.* Means ± S.E is shown across 18 study weeks per treatment. Dunnett`s posthoc test vs. control ** $p \leq 0.01$, * $p \leq 0.05$ are shown when the treatment effect in the repeated measurement ANOVA is significant at $p<0.1$.

Table V 1 ANOVA table showing differences between the herbivore treatments clipping, aphid, locust and control separately for each sampling time (weeks 0-18) and in total, as analysed by repeated measurement ANOVA (bottom). In week 0 (grey), plants were analysed before the herbivory treatments, showing no significant differences. See Fig V 1 for a repeated measure analysis with Dunnett`s posthoc test and Fig V S3 for post-hoc analyses separately per week.

		Fungal DNA		*Ergovaline*		*Lolitrem B*		*Peramine*	
week	df	F	p	F	p	F	p	F	p
0	3,38	1.18	0.33	0.42	0.74	0.45	0.72	0.50	0.69
2	3,38	0.83	0.48	0.75	0.53	0.23	0.88	0.22	0.88
4	3,38	1.30	0.29	1.01	0.40	0.17	0.92	0.64	0.60
6	3,38	1.82	0.16	0.90	0.45	1.06	0.38	1.69	0.19
8	3,38	2.32	0.10	0.72	0.54	4.80	**<0.01**	1.28	0.29
10	3,38	3.56	**<0.05**	0.38	0.78	7.01	**<0.001**	2.84	**0.05**
12	3,38	0.36	0.78	0.87	0.47	7.42	**<0.001**	6.32	**<0.01**
14	3,38	1.11	0.36	0.56	0.64	0.47	0.7	4.00	**<0.05**
16	3,38	0.98	0.41	2.91	**<0.05**	0.54	0.65	3.60	**<0.05**
18	3,38	1.32	0.28	1.17	0.33	0.29	0.83	5.77	**<0.01**
repeated measure	3,38	2.26	**0.09**	1.07	0.37	4.70	**<0.01**	5.00	**<0.01**

P<0.1 highlighted in bold

Discussion

Induced defence is a successful plant strategy to minimise herbivory (Agrawal 1998). Our results showed a herbivore-induced increase in the production of defensive alkaloids by a grass symbiotic endophytic fungus. This study presents, to our knowledge, the first evidence for herbivore-specific induction of defensive metabolites in a grass-endophyte system.

Furthermore, we showed that clipping, which simulated grazing livestock, induced an increase in the concentration of the vertebrate toxic alkaloid lolitrem B while an increase in the insect deterring alkaloid peramine was induced by locust herbivory. The increase in endophyte growth after clipping was marginally significant and this effect must remain preliminary. Aphid herbivory affected neither the endophyte growth nor alkaloid concentrations in our study. We found a herbivory-independent increase in alkaloid concentrations in all treatments which might be caused by higher temperature in summer or seasonal timing. Temperature dependent alkaloid increase has been shown in several studies (Ball *et al.* 1991; Repussard *et al.* 2014; Hennessy *et al.* 2016). Another explanation for increasing alkaloid concentrations through time might be an accumulation in the plants due to the chemical stability of alkaloids (Siegel *et al.* 1990).

Compared to aphids and locusts, clipping caused the highest plant damage. To deal with such a strong herbivory, grass species have mostly developed a compensatory rapid regrowth strategy (Belsky *et al*. 1993). However, in many cool-season grass species the symbiosis with endophytes results in the production of the neurotoxin lolitrem B which decreases vertebrate health and productivity (Cheplick & Faeth 2009). Consequently, endophytes can alter the grass strategy against vertebrate herbivores from compensation to defence (Sullivan *et al.* 2007). Higher alkaloid levels induced by herbivores could be a defensive advantage against vertebrate herbivores and might explain higher endophyte infection frequencies found in grazed landscapes (Gwinn *et al*. 1998; Koh & Hik 2007). An increase in both, marginally in endophyte growth and significantly in the neurotoxin lolitrem B concentrations in our study highlights the potential for a defensive mutualistic role of endophytes for the fitness of agronomic cool season grass species in grazed landscapes.

Aphids feed on plants by piercing-sucking herbivory, with generally little mechanical plant damage. Therefore, aphid herbivory is perceived by some plant species similar to pathogen infections and does not induce the same secondary plant metabolites as mechanical damage (Walling 2000). Nevertheless, some studies have shown that aphid herbivory can affect the composition of defensive compounds of plants and subsequently induce different defence pathways than chewing herbivorous insects (Züst & Agrawal 2016). Aphid herbivory altered

the volatile profile of an endophyte infected grass (Li *et al.* 2014) but alkaloid induction in endophyte infected grass has never been shown for aphid herbivory. A reason for the lack of alkaloid induction by aphids might be the generally low plant damage from piercing-sucking herbivores. It has been postulated that the feeding strategy of aphids is to avoid high plant damage and triggering defence mechanisms in plants (Walling 2008), which might have impeded the induction of defensive alkaloids in our experiment.

In contrast to aphids, locusts caused high mechanical damage due to chewing-biting herbivory. Consequently, locusts induce pathways in plants which are responsible for the synthesis of the majority of insect herbivore deterring or toxic substances (Walling 2000). In endophyte-infected grass the insect toxic alkaloid peramine was induced by locust herbivores, which indicates a perception of locust herbivory perhaps mediated by plant signals. Plant responses to locust herbivory can be triggered by locusts oral secretion (Schäfer *et al.* 2011). Moreover, peramine production by the endophyte might also be induced by locust specific triggers, as the pure mechanical damage due to clipping did not increase the peramine concentration in our study. Another study which analysed a different grass-endophyte symbiosis found an increase in insect toxic loline alkaloids following clipping (Bultman *et al.* 2004).

Similar to herbivore-induced defence in plants, we present evidence that induction of endophyte-mediated plant defence is herbivore-specific. However, the timing of herbivore-induced secondary metabolite production appears to be different when it is endophyte-mediated and when it is not. Plant responses to herbivores often appear within hours or days after herbivory (Baldwin 1989; War *et al*. 2012) while endophyte-mediated enhanced alkaloid synthesis appears only weeks to months after herbivore addition to the plants. This might be related either to endophyte intrinsic processes, such as a slow growth or slow alkaloid production or the cost-benefit ratio between the cost of producing alkaloids and the benefit to deter herbivores. To gain further insights into the determining processes of induced alkaloid production, further analysis of the chemical crosstalk between herbivores, plants and symbiotic microorganisms is required.

In conclusion, endophytic fungi in grasses can not only affect the herbivore species, but herbivores can also affect endophytes and even induce their growth and alkaloid production. Thus, communications between herbivores, endophytic fungi and plants appear to be close and targeted. Our results indicate a close chemical crosstalk, in which herbivore presence and type is perceived either by the grass which induces endophyte activity, or by the endophytic fungus which responds with the production of herbivore active alkaloids. Our results highlight

the importance of studying context dependent interactions across more than two trophic levels. It is necessary to understand the mechanisms behind herbivore-specific induction of defensive compounds in more detail. For this, it requires a multidisciplinary approach focusing on interspecific signal pathways across trophic levels in an ecological context.

Supporting Information to Chapter V

Table V S1 Mean ± S.E. numbers of aphids and locusts at sample dates. In week 0, when the experiment started 50 adult *Rhopalosiphon padi* (aphids) and 8 juvenile (L3 larvae) *Schistocerca gregaria* (locusts) were placed on the experimental pots.

Week	0	2	4	6	8	10	12	14	16	18
Aphid numbers	50	396±37	1242±38	1300±32	539±21	408±20	339±20	242±14	122±14	34.2±4.3
Locust numbers	8	7.8±0.1	6.9±0.1	6.1±0.1	5.7±0.2	5.2±0.2	5.0±0.2	4.2±0.2	2.5±0.2	2.1±0.1

Figure V S1

Fig V S1 Experimental set-up: 48 pots were burrowed in the soil to a depth of 30 cm. Four treatments (clipping, locusts, aphids, control) were randomly assigned to 12 pots each. All pots were enclosed with mesh bags.

Figure V S2

Fig V S2 Sampling procedure: One tiller per sampling interval was randomly chosen. The sample contained about 3 cm of tiller around the collar including 1.5 cm of leaf sheath and leaf blade of the oldest leaf.

Figure V S3

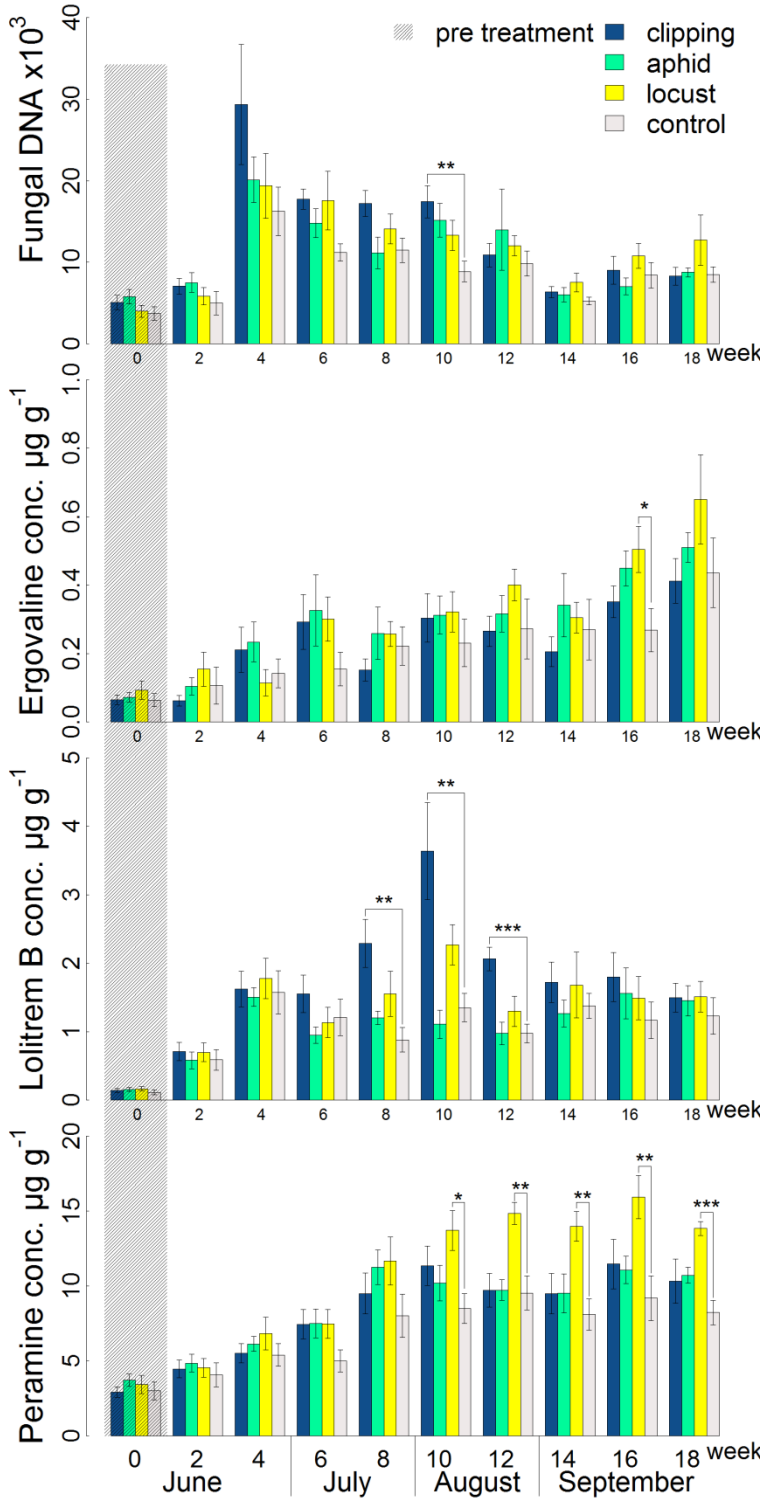

Fig V S3 Dependence of fungal gDNA and alkaloid concentrations on herbivory (N=42). Means ± S.E are presented for every two weeks during overall study period of four month. Dunnett`s posthoc test vs. control *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ are shown when the treatment effect in the repeated measurement ANOVA is significant at $p<0.1$.

Chapter VI: General Discussion

In my PhD thesis, we were investigating whether grass-endophyte derived alkaloids cascade up the food chain and how herbivore species, their spring arrival timing, seasonal timing and plant age affect the growth and alkaloid production of the endophytic fungus *Epichloë festucae* var. *lolii* in the host grass *Lolium perenne*.

First, we analysed alkaloid concentrations in three trophic levels: endophyte-infected grass, several herbivore species and aphid predators feeding on aphids reared on endophyte infected grass (chapter II). In all tested species, we found the insect deterring, endophyte derived alkaloid peramine. Aphids showed similar alkaloid concentrations as the host grass and aphid predators contained less alkaloid concentrations, which still exceeded the bioactive threshold. Further, this study revealed that 6 weeks young endophyte infected grass contained 300-fold less alkaloid concentrations compared to 2 year old grass. Second, to unravel the effects of plant age and seasonal timing, we conducted a long-term experiment with monthly monitoring endophyte growth and alkaloid concentrations (ergovaline, lolitrem B, peramine), from sowing, up to a plant age of 30 month (chapter III). We found an age dependent increase of alkaloid concentrations following a seasonal rhythmicity with peak concentrations in summer. Fungal concentrations did not significantly increase with plant age but followed seasonal rhythmicity peaking in summer month. Peaks in summer might be explained by high temperatures, however they also coincide with high herbivore abundance. Third, we analysed whether a phenological shift (enhanced abundance in spring) of herbivores (aphids) can cause phenological responses in bottom-up (endophytic fungus) and top- down (aphid predatory insects) control and whether it leads to desynchronizations in multi-trophic interactions (chapter IV). We showed that with natural occurring aphids, peak abundances of aphids, predators and endophyte concentrations appear synchronized, while with enhanced aphid abundance in spring, aphid abundance peaks one week advanced with twice the abundance compared to natural aphid occurrence. Aphid predators were unaffected and endophyte concentration increased two weeks delayed to high aphid abundances indicating herbivore mediated endophyte growth. Fourth, we investigated whether different types of herbivores (clipping, biting-chewing insects and piercing-sucking insects) induce endophyte growth and alkaloid production (chapter V). We found an induced increase in concentrations of the insect deterring alkaloid peramine following locust (biting-chewing) herbivory, and of the neurotoxin lolitrem B following clipping of the plants.

Endophyte mediated effects on herbivores and higher trophic levels

The expression “the enemy of my enemy is my friend” is used in the context of plant defence mechanisms against herbivores, where the third trophic level helps the plant to control herbivorous insects, by parasitizing or feeding on them (Kaplan 2012). In chapter II we included a grass symbiont which harmed the plant`s enemy and the enemy of the enemy (friend). To pick up the above mentioned quote and extend it including the grass symbiont, we conclude: “If my symbiont harms the enemy of my enemy, my friend is weakened and the benefit of my symbiont against my enemy is questioned”. The grass *Lolium perenne* infected with endohytic fungus *Epichloë festucae* var. *lolii* is suggested to be a defensive mutualism, but discussions are ongoing doubting the beneficial effect of endophytic fungi of the *Epichloë* family, especially regarding their effects in non-agronomical relevant grass species (Faeth & Saikkonen 2007; Saikkonen *et al.* 2010; Clay 2014). Our results from chapter II provide further reasons to discuss *Epichloë festucae* var. *lolii* in *Lolium perenne* as defensive mutualism due to their negative effects on beneficial insects like aphid predators and parasitoids (de Sassi *et al.* 2006; Härri *et al.* 2008b, 2009). We showed that alkaloid levels in aphid predators can exceed the insect harming threshold and are therefore the reason for fitness disadvantages found in previous studies (Fuchs *et al.* 2013). In a healthy ecosystem high herbivory is prevented by bottom-up and top-down control with beneficial effects on plant fitness (Herrera & Pellmyr 2002). However, in a system where bottom-up control negatively affects the performance of top-down controlling organisms, such as predators, the benefits of bottom-up control itself are reduced (Pasteels 2007). Besides the negative direct effects on predatory insects, endophyte infection might indirectly affect predators by altering the plant volatile composition (Li *et al.* 2014), which can guide predatory insects (indirect defence) (Agrawal 2011). Since learning of plant odours has been shown as a successful strategy for predatory insects while foraging for food (Glinwood *et al.* 2011), they might favour foraging on uninfected over endophyte infected plants by reference to their emitted odour. Consequently endophyte mediated changes in direct and indirect plant defence might contribute to altered insect community structures in landscapes with high abundances of endophyte infected grass (Rudgers & Clay 2008)

Environmental and timing effects on endophytic fungi and trophic level interactions

One result from chapter II was that young endophyte infected plants contain less alkaloid concentrations than old plants. This was confirmed by our second study (chapter III) where

we showed age and season dependent alkaloid concentrations. Symbiotic grass-endophytes are obligate grass symbionts and their biology is closely linked to the plant biology (Cheplick & Faeth 2009). Consequently, the growth and alkaloid production of grass-endophytes depend on nutrition, water supply, light and temperature (Schardl *et al.* 2004; Dobrindt *et al.* 2013; Hennessy *et al.* 2016). Since we supplied nutrients and regularly watered the plants, temperature was determining factor in plant and presumably endophyte growth, as well as for alkaloid synthesis. This is supported by several other studies showing higher alkaloid concentrations with higher temperatures (e.g. Hennessy *et al.* 2016). Further we showed a strong seasonal dependency of endophyte and alkaloid concentrations peaking during late summer month, in times of plant seed maturation. As *Epichloë festucae* var. *lolii* reproduces asexually and is distributed via the plant seeds (Schardl *et al.* 2004), high fungal growth activity during seed maturation might increase transmission rates into the next generation and therefore determine endophyte fitness. In this context, high alkaloid concentrations indicate a protective function, especially against seed predators. In chapter V we showed increased alkaloid concentrations induced by herbivory, indicating alkaloid synthesis as a response to herbivory rather than a preventive production. Nevertheless, plants excluded from herbivores in chapter V showed an increase in endophyte and alkaloid concentrations during summer. We conclude from chapter III and V that endophyte and alkaloid concentrations increase with temperature and plant growth until seed maturation but enhanced alkaloid synthesis can be induced herbivore specific (Fuchs *et al.* 2016).

Under climate change, species show temporal shifts towards earlier spring events and primary consumers tend to be more susceptible to such changes compared to primary producers and secondary consumers (Thackeray *et al.* 2016). In chapter III, we showed alkaloid concentrations following seasonal rhythmicity with peak concentrations during summer when herbivore abundances are also high (Boyer *et al.* 2003). In chapter IV we showed a desynchronization in a tri-trophic food chain involving four interacting species, following enhanced aphid abundance in spring, which simulated climate change mediated earlier aphid arrival on plants in spring (Bell *et al.* 2015). Enhanced aphid numbers in spring resulted in high aphid numbers in summer, temporally advanced to natural conditions, but aphid predators arrived unaffected while endophyte growth increased time delayed. High aphid abundances increase aphid transmitted plant diseases and result in decreased crop production (Fand *et al.* 2012). In contrast to predators which might even benefit from higher food availability, plants suffer from increased herbivore abundances and energetic costs for delayed endophyte growth. Without herbivore presence, endophyte growth is rather costly for

the plant than beneficial. Without providing benefits to the host plant, endophyte infected plant fitness may be reduced with the consequence of decreasing frequency in natural habitats (Faeth & Fagan 2002). Nevertheless advancing aphid herbivory seemed to cause a (delayed) response in endophyte growth, which indicated a herbivore induced response in a plant associated microorganism, which is further discussed in the following sub-chapter.

Herbivore mediated effects on the endophyte

A rising number of studies show that plant defence mechanisms are mediated by plant associated microorganisms (Pineda *et al.* 2010; Zamioudis & Pieterse 2011; Clay 2014). Thereby, microorganisms are involved in induced plant defence responses following herbivory (Bezemer & van Dam 2005). Since induced defence is a successful plant strategy and the results from chapter IV indicated delayed induction of endophyte growth following aphid herbivory, we showed in chapter V that different herbivore species induce an increase in different endophyte derived alkaloid concentrations (Fuchs *et al.* 2016). In contrast to chapter II, where our results question the mutualistic role of grass-endophytes, the results from chapter V indicate a defensive mutualistic symbiosis, where the endophyte responded to grass herbivory by increasing the production of herbivore specific metabolites in the host plant. The production of herbivore specific metabolites is known from plants, where different kinds of herbivores induce different plant hormonal pathways determining specific defence responses (Walling 2000). For instance, plant responses to chewing herbivory are mainly mediated by Jasmonic Acid pathways, in contrast to piercing-sucking herbivory which induces Salicylic acid pathways (Leitner *et al.* 2005). Locust herbivory has been shown to induce specific plant defence response, especially due to saliva transmitted signals (Schäfer *et al.* 2011). Comparing systemic plant defence with endophyte mediated plant defence response to different herbivore species shows similar pattern. Locusts increased invertebrate deterring alkaloid peramine, however aphid herbivory did not induce any endophyte derived alkaloid indicating perception of distinct herbivore types. Thereby the endophyte response might be directly triggered by herbivore species or indirectly by plant released hormones that are induced by a specific herbivore species. Our results further indicate that locust saliva might trigger specific alkaloid production, since pure mechanical damage from clipping (simulation of grazing) did not increase peramine concentrations but increased concentrations of the vertebrate toxic alkaloid lolitrem B. In contrast to clipping and locust herbivory, aphid herbivory caused least mechanical plant damage. Aphids might not interact with the endophytic fungus and consequently did not increase any alkaloid concentration or endophyte

growth, even though we showed in chapter IV that fungal growth responds delayed to high aphid abundances. In chapter IV aphid abundances peaked in spring, while they peaked in summer in chapter V, which might be too late to cause endophyte mediated plant response. Plant defence responses to herbivory vary depending on plant and leaf age (Bowers & Stamp 1993; Boege & Marquis 2005), which might affect endophyte response to aphid herbivory. Plant age differed between both chapters. Plants in the first growing year (chapter IV) might translocate resources from plant primary processes to endophyte growth following enhanced aphid abundances. This adaptive phenotypic plasticity might be lost in plant-endophyte associations in their second growing year (chapter V). Piercing-sucking feeding strategy of aphids aims to avoid plant damage induced defence responses (Walling 2008). Nevertheless many plants respond to aphid herbivory with defence mechanisms (Züst & Agrawal 2016). A successful plant defence against aphids is indirect defence by emitting volatiles to attract predators or parasitoids (Züst & Agrawal 2016). Endophytic fungi can alter plant volatiles (Li *et al.* 2014), indicating that endophytes respond to aphid herbivory but our study tested only for endophyte derived alkaloids and not for endophyte mediated changes in emitted plant volatiles.

Synthesis

We found that alkaloid levels from the endophytic fungus *Epichloë festucae* var. *lolii* in the host grass *Lolium perenne* vary with season and plant age and can be induced by specific herbivores. This thesis shows that additional to genetic predispositions of the endophyte and the host grass (Schardl *et al.* 2013a), several seasonally determined predictors and biotic interactions can affect endophyte growth and alkaloid production in the plant which is a key feature determining the position of grass-endophytes in the mutualism-parasitism continuum (Saikkonen *et al.* 1998; Müller & Krauss 2005).

Furthermore, natural ecosystems consist of species interaction dynamics constantly affected by abiotic factors. We conclude that many environmental predictors and their succession affect the success of endophyte persistence and their distribution in diverse grass populations. Thereby the classification of grass-endophytes in the parasitism-mutualism continuum is shifted constantly depending on plant and endophyte genotype paired with changing environmental conditions.

Following up this thesis, further research is needed for understanding the mechanisms behind herbivore induced alkaloid synthesis, especially whether plant signals mediate increased endophyte derived alkaloid production. In the same context endophyte mediated indirect plant

defence is little understood and might contribute to the diverse repertoire of plant defence mechanisms.

To understand alkaloid increase related to the plant age, further studies that analyse the symbiotic coexistence of grass and endophyte in the first two years after germination are needed. Analysis of plant parameters that might limit energy expenses from secondary metabolite production in young plants might reveal reasons for plant age related alkaloid production.

Concluding, we suggest that grass-endophytes play an important role in trophic interactions. Their deterring effect against herbivorous insects is a key component for the mutualistic symbiosis with their host grasses, which might be of value to future sustainable agricultural applications and research (Kauppinen *et al.* 2016).

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Author contributions

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Chapter II: **Fuchs, B.,** Krischke, M., Müller, M.J., Krauss, J., (2013) Peramine and lolitrem B from endophyte-grass associations cascade up the food chain *J Chem Ecol* 39, 1385-1389					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	BF	JK	MK	MJM	
Data Collection	BF				
Data Analysis and Interpretation	BF	JK	MK		
Manuscript Writing	BF	JK	MK	MJM	

Chapter III: **Fuchs, B.,** Krischke, M., Müller, M.J., Krauss, J., (manuscript) Age and seasonal timing of an endophytic fungus: A long-term study of growth and alkaloid concentrations					
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I also confirm my primary supervisor's acceptance.

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