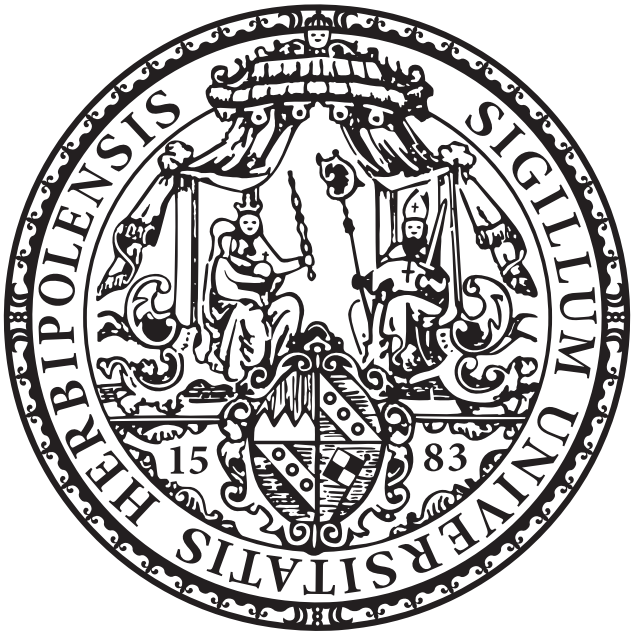
Fungal grass endophytes and their dependence on land-use intensity

Gras-Endophyten und ihre Abhängigkeit von der Landnutzungsintensität



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"The big things you can see with one eye closed. But keep both eyes wide open for the little things. Little things mark the great dividing line between success and failure."

Jacob M. Braude

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Affidavit

I hereby confirm that my thesis entitled “Fungal grass endophytes and their dependency on land-use intensity” 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.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Hiermit erkläre ich an Eides statt, die Dissertation “Gras-Endophyten und ihre Abhängigkeit von der Landnutzungsintensität” eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Publication list

König J., Papp L., Fuchs B., Krischke M., Mueller M.J., Krauss, J. (in revision) Seeking for a treasure – Predicting the potential of *Epichloë* endophytes in protecting meadow fescue from herbivory on managed grasslands. *Fungal Ecology*.

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Summary

Chapter I - General Introduction

Plant-associated fungi can affect the plants‘ interaction with herbivores and other microorganisms. For example, many common forage grasses are infected with *Epichloë* endophytes. The endophytes systemically colonize the aerial parts of the plants. They produce bioprotective alkaloids that can negatively affect insects and livestock feeding on the grasses, and interact with other fungal species which living from the plants‘ nutrients. Environmental conditions strongly influence *Epichloë* endophytes. Endophyte-mediated effects on herbivores are more pronounced under increased temperatures and the endophytes may benefit from land use in managed grasslands. Under the framework of the large-scale German project “Biodiversity Exploratories”, I investigated whether infection rates and alkaloid concentrations of *Epichloë festucae* var. *lolii* in *Lolium perenne* (Chapter I) and *Epichloë* endophytes (*E. uncinata, E. siegelii*) in *Festuca pratensis* (Chapter II) depend on land use and season. Further I analysed, whether foliar fungal assemblages of *L. perenne* are affected by the presence of *Epichloë* endophytes (Chapter IV).

Chapter II - Infection rates and alkaloid concentrations of *Epichloë festucae* var. *lolii* in *Lolium perenne* along a land-use gradient in Germany

In the first study, I determined infection rates and quantified alkaloid concentrations of *E. festucae* var. *lolii* in *L. perenne*. I analysed, whether infections and alkaloid content increased with the intensity of land use and whether alkaloid concentrations depend on seasonal changes. I used commercially available immunoblot kits for endophyte detection and quantified the amount of the vertebrate toxic alkaloids lolitrem B and ergovaline, and insect toxic alkaloid peramine by UPLC-Tandem-MS method. Endophyte infection rates were generally low (13 % of individuals) but highly variable depending on different environmental conditions on local scale. The content of endophyte-derived alkaloids increased with season and single plants with extremely high concentrations were detected in summer. The intensity of land use did not affect the *Epichloë* endophytes. I conclude, the current risks for endophyte-mediated livestock intoxications is low, as alkaloid concentrations per grassland were always below toxicity thresholds and *L. perenne* never dominated the grasslands vegetation.

Chapter III - Predicting the potential of *Epichloë* endophytes in protecting meadow fescue from herbivory on managed grasslands

In the second study, I analysed infection rates and alkaloid content of *Epichloë* endophytes in *F. pratensis*. In two seasons I tested, whether infections and alkaloid content change along a land-use intensity gradient. Endophytes were detected by microscopic investigation and immunoblot assays. Concentrations of the insect toxic/deterring loline alkaloids were quantified by UPLC-Tandem-MS method. Plants were highly endophyte-infected (75 % of individuals), but differed among regions and decreased with land-use intensity and fertilization. The content of endophyte-derived alkaloids changes with season. Depending on different environmental conditions of the study regions, loline alkaloids either increased or decreased in summer, but were generally below (< 14 µg/g) typical *in planta* levels reported from other studies. I conclude, effects on insect herbivores are rather low and that intensive managed grasslands can be a disadvantage for *Epichloë* endophytes associated with *F. pratensis*.

Chapter IV - The effects of *Epichloë* endophytes on foliar fungal assemblages in perennial ryegrass in dependence of season and land-use intensity

In the third study, I tested if *E. festucae* var. *lolii* determine species composition of fungal assemblages isolated from *L. perenne* leaves. Furthermore, I analysed whether fungal communities are affected by land use or seasonal changes. The *Epichloë* endophyte was detected by immunoblot assays, the fungal assemblages of *L. perenne* leaves were assessed by Next Generation Sequencing of ITS rRNA gene region. Dissimilarities in species composition were found between the studied regions and fungal communities changed with season. In contrast, I found no significant differences between assemblages in dependence of land-use intensity and the presence/absence of *Epichloë* endophyte. I conclude, that local and seasonal variability in real-world grasslands masks the effects on foliar fungal endophytes caused by land use and *Epichloë* infection.

Chapter V – General Discussion

Epichloë endophytes can largely affect grassland ecosystems. *Epichloë* endophytes of the studied host grass species *L. perenne* and *F. pratensis* frequently appeared in the grasslands of the Biodiversity Exploratories. Depending on the endophyte-host genotypes, effects of intensive land use were different, but never beneficial. Responses of *Epichloë* endophytes are strongly determined by environmental factors such as seasonal changes and local abiotic and biotic conditions, and possibly benefit from climate warming. The effects of the two studied associations were diluted in the diverse vegetation of mixed grasslands in Germany as native habitats. However, the introduction of *Epichloë* endophyte-infected seeds knowingly or accidentally, can shift relations of the endophyte, their host grasses and other interacting species, and may change their role within grassland ecosystems. Furthermore, still undiscovered mechanisms have to be clarified to understand *Epichloë* endophytes, their consequences at community level and the role of endophyte-derived alkaloids.

Zusammenfassung

Kapitel I – Allgemeine Einleitung

Mit Pflanzen assoziierte Pilze können die Interaktionen von Pflanzen und Herbivoren, als auch die Kommunikation mit anderen Mikroorganismen beeinflussen. Viele Futter- und Weidegräser sind beispielsweise mit endophytischen Pilzen der Gattung *Epichloë* infiziert, die die oberirdischen Pflanzenteile der Gräser systemisch besiedeln. Diese Endophyten produzieren bioaktive Alkaloide, die sich negativ auf Fraßfeinde wie Insekten, aber auch Weidetiere, auswirken, und mit anderen pflanzen-assoziierten Pilzarten interagieren. *Epichloë* Endophyten werden von ihrer äußeren Umwelt stark beeinflusst. So treten die von den *Epichloë* Endophyten ausgehende Effekte auf Herbivore meist unter erhöhten Temperaturen auf. In agrar-genutzten Grünflächen profitieren die Endophyten möglicherweise auch von der Landnutzung. Im Rahmen des deutschlandweiten Großprojekts „Biodiversitätsexploratorien“ untersuchte ich die Infektionsfrequenzen und Alkaloidkonzentrationen von *Epichloë festucae* var. *lolii* in *Lolium perenne* (Kapitel II) und den *Epichloë* Endophyten in *Festuca pratensis* (Kapitel III) in Abhängigkeit von der Landnutzung und Jahreszeit. Des Weiteren untersuchte ich, ob das Auftreten bzw. die Abwesenheit von *Epichloë* Endophyten einen Einfluss auf die Zusammensetzung der endophytischen Pilzgemeinschaften in Blättern von *L. perenne* hat (Kapitel IV).

Kapitel II – Infektionsraten und Alkaloidkonzentrationen von *Epichloë festucae* var. *lolii* in *Lolium perenne* entlang eines Landnutzunggradienten in Deutschland

In meiner ersten Studie bestimmte ich Infektionsraten und Alkaloidkonzentrationen von *E. festucae* var. *lolii* in *L. perenne*. Ich untersuchte, ob die Infektionsraten, als auch der Alkaloidgehalt, mit zunehmender Intensität der Landnutzung steigen, und ob die Alkaloidkonzentrationen von saisonalen Änderungen abhängen. Für die Detektion der *Epichloë* Endophyten wurde ein Immunoblot Kit verwendet. Die Konzentrationen der vertebraten-toxischen Alkaloide Lolitrem B und Ergovalin, als auch die Konzentration des insekten-toxischen bzw. abschreckenden Alkaloids Peramin, quantifizierte ich mittels Ultra-Hochleistungsflüssigkeitchromatographie (UPLC). Die allgemein geringen Infektionsraten des *Epichloë* Endophyten (ca. 13 % der Individuen) variierten stark auf Grund unterschiedlicher lokalen Umweltbe-

dingungen. Der Gehalt der Alkaloide stieg über das Jahr, wodurch einzelne Pflanzen im Sommer extrem hohe Alkaloidkonzentrationen aufwiesen. Im Gegensatz dazu, hatte die Intensität der Landnutzung keinen Einfluss auf die *Epichloë* Endophyten. Da sich die allgemeinen Alkaloidkonzentrationen auf die gesamte Graslandschaft bezogen, unterhalb der Toxizitätsschwelle bewegten und die Vegetation niemals von *L. perenne* dominiert wurde, folgere ich, dass das aktuelle Vergiftungsrisiko von Weidetieren durch *Epichloë* Endophyten als gering einzuschätzen ist.

Kapitel III – Abschätzung des Potentials von *Epichloë* Endophyten des Wiesen-Schwingels im Schutz vor Herbivorie auf bewirtschafteten Graslandschaften

In der zweiten Studie, untersuchte ich die Infektionsraten und den Alkaloidgehalt von *Epichloë* Endophyten in *F. pratensis*. Ich testete für zwei unterschiedliche Jahreszeiten, ob sich die Rate der Infektionen und die Konzentration der Alkaloide entlang eines Landnutzungsintensitäts-Gradienten verändern. An Hand histologischer Untersuchungen und den Einsatz von Immunoblot Kits, wurden die Endophyten detektiert. Mittels Ultra-Hochleistungsflüssigkeitchromatographie (UPLC) quantifizierte ich anschließend die Konzentration der insekten-toxischen bzw. abschreckenden Loline Alkaloide. Ich stellte eine hohe Endophyten Infektionsrate in *Festuca pratensis* fest (75 % der Individuen). Die Infektionsraten nahmen mit zunehmender Landnutzungsintensität und mit der Düngung ab. Die Infektionsraten und saisonale Akkumulation der Loline Alkaloide unterschieden sich zwischen den Regionen. Insgesamt war die Konzentration der Loline wesentlich geringer (< 14 µg/g) als *in planta*-typische Werte aus anderen Studien. Daraus folgere ich, dass die Loline Konzentrationen in deutschen Graslandschaften zu gering sind, um einen effektiven Schutz gegen Herbivore zu bieten, und eine intensive Bewirtschaftung der Graslandschaften für die *Epichloë* Endophyten-Gras Symbiose von Nachteil sein kann.

Kapitel IV – Die Auswirkungen von *Epichloë* Endophyten auf die endophytischen Pilzgemeinschaften in Blättern des Deutschen Weidelgrases in Abhängigkeit von Jahreszeit und Landnutzungsintensität

In der dritten Studie untersuchte ich, ob *E. festucae* var. *lolii* die Artzusammensetzung der Pilzgemeinschaften in Blättern des Deutschen Wei-

delgrases, *Lolium perenne,* bestimmt, und ob sie von der landwirtschaftlichen Nutzung und/oder saisonalen Veränderungen beeinflusst wird. Mit Immunoblot Kits wurde die Infektion von *Epichloë* Endophyten in *L. perenne* festgestellt. Die Pilzgemeinschaften in den Blättern von *L. perenne* wurden mit Next Generation Sequencing der ITS rRNA Genregion ermittelt. Die Pilzgesellschaften wurden stark von der Region und der Jahreszeit beeinflusst. Im Gegensatz dazu, konnte ich keine Unterschiede der Pilzgemeinschaften in Abhängigkeit von der Landnutzungsintensität und dem Auftreten bzw. der Abwesenheit von *Epichloë* Endophyten feststellen. Ich folgere daraus, dass die Auswirkungen der Landnutzung und *Epichloë* Endophyten durch die variierenden lokalen und saisonalen Bedingungen in echten Graslandschaften verschleiert werden.

Kapitel V – Allgemeine Diskussion

Epichloë Endophyten können Grasland Ökosysteme stark beeinflussen. Eine Infektion mit *Epichloë* Endophyten in den Gräsern *L. perenne* und *F. pratensis* trat in den untersuchten Graslandschaften der Biodiversitätsexploratorien häufig auf. Abhängig von dem Genotypus des Endophyten und Graswirts, wirkte sich eine intensive Landnutzung unterschiedlich auf die Symbiosen aus. Die Endophyten werden stark von Umweltfaktoren wie z.B. saisonale Änderungen und lokal bedingte Einflüsse bestimmt, und profitieren möglicherweise vom Klimawandel. Durch die artenreiche Vegetation der gemischten Graslandschaften in Deutschland als ursprüngliches Habitat, waren die Effekte der zwei untersuchten Endophyten-Gras Assoziationen in dieser Studie gering. Allerdings kann die absichtliche oder unabsichtliche Ausbringung von Endophyten-infizierten Samen die Interaktionen zwischen Endophyten, ihren Graswirten und anderen interagierenden Organismen, wie z.B. Herbivore, beeinflussen, und dadurch möglicherweise ihre Rolle innerhalb einer Pilz- und Pflanzengesellschaft von Grasland Ökosystemen verändern. Dennoch, um die *Epichloë* Endophyten, ihre Konsequenzen auf Populationsebene und die Ökologie der Endophyten-produzierten Alkaloide verstehen zu können, müssen bislang noch unerkannte Mechanismen aufgedeckt und geklärt werden.

Chapter I

General Introduction

Plant-associated fungi can affect the plants‘ interaction with herbivores and other microorganisms. For example, many common forage grasses are infected with *Epichloë* endophytes. The endophytes systemically colonize the aerial parts of the plants. They produce bioprotective alkaloids that can negatively affect insects and livestock feeding on the grasses, and interact with other fungal species which living from the plants‘ nutrients. Environmental conditions strongly influence *Epichloë* endophytes. Endophyte-mediated effects on herbivores are more pronounced under increased temperatures and the endophytes may benefit from land use in managed grasslands. Under the framework of the large-scale German project “Biodiversity Exploratories”, I investigated whether infection rates and alkaloid concentrations of *Epichloë festucae* var. *lolii* in *Lolium perenne* (Chapter I) and *Epichloë* endophytes (*E. uncinata, E. siegelii*) in *Festuca pratensis* (Chapter II) depend on land use and season. Further I analysed, whether foliar fungal assemblages of *L. perenne* are affected by the presence of *Epichloë* endophytes (Chapter IV).

I.1 Fungal grass endophytes – The genus *Epichloë*

Fungi are ubiquitous and highly diverse microorganisms which form symbiotic relations with a large number of plant species in all terrestrial ecosystems (Dix and Webster, 1995). In symbioses, both associated species are closely linked to each other (Hirsch, 2004). Based on the benefits to the host plant and fungal symbiont, a variety of symbiotic lifestyles have been defined and ranges from antagonistic to mutualistic (Lewis, 1985; Petrini, 1986; Sánchez Márquez et al., 2012). While in antagonistic or parasitic symbioses only one partner benefit from the association, mutualistic symbioses provide benefits for both symbiotic partners. The most prominent example of a mutualistic fungus-plant symbiosis is the association of mycorrhizal fungi with plant roots. The fungi enhance nutrient availability in soil for their host plants and in return, the fungi are provided with plant-derived photosynthetic products (Parniske, 2008).

Fungal symbionts play an important role in structuring plant communities and composition and can have profound effects on the plants‘ fitness and associated organisms such as herbivores (Bundrett, 2006; Clay and Holah, 1999; Omacini et al., 2001). Most plants in natural ecosystems are symbiotic with mycorrhiza fungi and/or other fungal endophytes (Arnold, 2007; Petrini, 1986; Stone et al., 2004). Unlike epiphytic fungi living on the plant surface, fungal endophytes live inside the plant tissue often without noticeable phenotypic symptoms on the host plant (Schardl et al., 2004). Among the most pronounced advantages are an enhanced stress tolerance, increased water and nutrient uptake, and an improved resistance against herbivores, which is of high scientific and agronomic interest. Many worldwide common forage grasses, e.g. ryegrass (*Lolium* L.) and fescue (*Festuca* L.), have evolved as associates with the systemic endophyte of the genus *Epichloë* (Fr.) Tul. & C. Tul. with tremendous biological consequences.

The association of *Epichloë* endophytes (Ascomycota, Clavicipitaceae) with cool-season grasses of the Poaceae (R.Br.) Barnhart family is, besides mycorrhiza, some of the best-known plant-fungus interaction (Schardl et al., 2004; Tanaka et al., 2012). Possibly up to 900 pooid grass species harbour these fungal endophytes (Faeth, 2002; Leuchtmann, 1992). Today, 34 *Epichloë* species, as well as several subspecies and varieties, are described, which are specific to their host grass species (Leuchtmann et al., 2014). *Epichloë* endophytes systemically colonize the intercellular space of above ground tissue of their host grasses (Schardl et al., 2004; Figure 1). Depending on biotic and abiotic factors, the *Epichloë* endophyte-grass association can be mutualistic or antagonistic, but mostly exist as a continuum between mutualism and parasitism (Müller and Krauss, 2005).

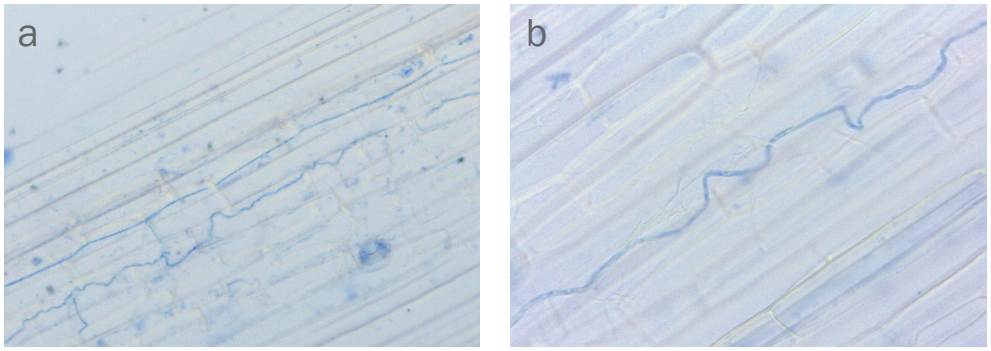

Figure I.1 (a) Fungal mycelia of an *Epichloë* endophyte localized in the intercellular space of the host grass, (b) typically forming elongated and curved hyphae.

Several species of the genus *Epichloë* reproduce sexually by the production of spores on the grasses' surface, which suppress the maturation of the grass inflorescences (Schardl et al., 2004; Figure I.2 a, b). The spores are transmitted and dispersed by symbiotic flies (*Botanophila* Lioy) and cross-fertilized the fungal opposite mating types. In my thesis, I focused on asexual *Epichloë* endophyte species of perennial ryegrass (*Lolium perenne* L.) and meadow fescue (*Festuca pratensis* Huds.). While *L. perenne* is associated with *Epichloë festucae* Leuchtm., Schardl & M.R. Siegel var. *lolii* (Latch, M.J. Chr. & Samuels) C.W. Bacon & Schardl, *F. pratensis* can be infected with *Epichloë uncinata* (W. Gams, Petrini & D. Schmidt) Leuchtm. & Schardl or *Epichloë siegelii* (K.D. Craven, Leuchtm. & Schardl) Leuchtm. In contrast to the sexual *Epichloë* species, these *Epichloë* endophytes cause no negative effects on their host grasses and are vertically transmitted (Schardl et al., 2004). During seed ripening, hyphae of the endophytes grow into the ovules of the developing seeds. The fungal material of the

Figure I.2 (a) Early and (b) mature state of sexually produced spores of *Epichloë* endophytes occurring on the surface of the host grass stems (*Dactylis glomerata* L.).

endophytes becomes dispersed by the mature grass seed and develops with the emerging seedling (Figure I.3).

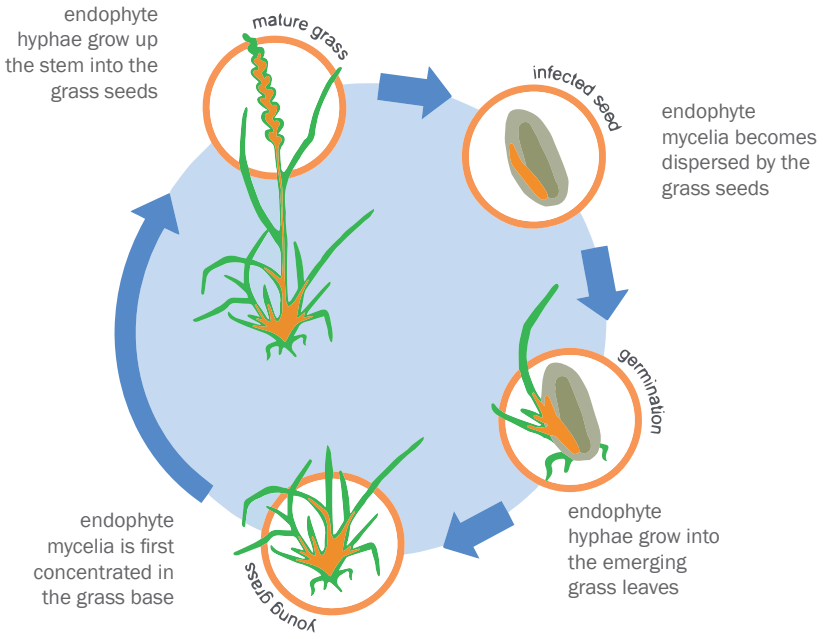

Figure I.3 The asexual life cycle of *Epichloë* endophytes. Endophytes transmit vertically due to mycelia growing into the developing seeds of their host grasses (Schardl et al., 2004).

Since the *Epichloë* endophytes were recognised for enhancing plant fitness and grassland persistence, they are of high agronomic interest (Hoveland, 1993; Hume et al., 2016). Furthermore, they provide resistance to several insect pest species due to the production of bioprotective alkaloids (Schardl et al., 2013a). However, some of these alkaloids are responsible for poisoning grazing livestock and already caused huge economic losses with costs of up to two billion dollars per year in the United States, Australia and New Zealand (Aiken and Strickland, 2013; Bluett et al., 2005; Fletcher, 1999; Hume and Sewell, 2014). Although, the *Epichloë* endophytes and their host grass species originated in Europe (Malinowski and Belesky, 2006) and infections of different host grass species are known from several European countries (Do Valle Ribeiro et al., 1996; Dobrindt et al., 2013; Jensen and Roulund, 2004; Leyronas and Raynal, 2001; Oldenburg, 1997; Saikkonen et al., 2000; Zabalgogeazcoa et al., 2003; Zurek et al., 2012), reports about livestock intoxication in Europe are rare (Lewis, 1997; Zabalgogeazcoa and Bony, 2005). In order to predict negative/positive effects of the *Epichloë* endophyte-grass associations on grazing livestock and insect pests in a native habitat, the main focus of my PhD thesis was, to record the distribution and alkaloid content of the different *Epichloë* endophyte species of *L. perenne* and *F. pratensis* in Germany.

I.2 Boone or bane? — Bioprotective alkaloids of the *Epichloë* endophytes

Living organisms are able to possess a range of different defence mechanisms against their natural enemies (Clay, 2014). Plants often grow under strong herbivore pressure, but are not able to escape of them. One effective way to defend is the production of defensive chemical metabolites, but production is accompanied by the cost of plant material or energy (Iwasa et al., 1996). Microbial symbionts are able to provide this service to their host plants, as the host is not capable of it, or it is more cheaply than for the host themselves (Clay, 2014). In the association of *Epichloë* endophytes and their host grasses, diverse secondary metabolites are detected, which are not typically produced in the plant family Poaceae (Clay and Schardl, 2002). Four major groups of *Epichloë* endophyte-derived alkaloids are of principle interest: Ergot alkaloids (ergovaline), indolediterpene alkaloids (lolitrem B), the pyrrolopyrazine alkaloid peramine and aminopyrrolizidine alkaloids (lolines; Figure I.4).

Ergot alkaloids are complex compounds which affect the vertebrate central and peripheral nervous system (Schardl et al., 2006). Ergovaline has been recognised as most abundant and most toxic in the *Epichloë coenophiala* (Morgan-Jones & W.Gams) C.W. Bacon & Schardl-infected tall fescue, and is responsible for fescue toxicosis in livestock (Guerre, 2015). Concentrations of 0.3-0.8 µg/g ergovaline are described as toxic to livestock and can induce symptoms such as poor weight gain, increased respiration rate and salivation, and loss of blood flow to the extremities resulting in necrosis of the hooves and tail (Hovermale and Craig, 2001; Schardl et al., 2006; Tor-Agbidye et al., 2001).

Ergot alkaloids are also found in ryegrass, but in lower concentrations than the neurotoxic lolitrem B (Hovermale and Craig, 2001). Lolitrem B has been identified as the main causative agent of ryegrass staggers, a disorder affecting livestock grazing *E. festucae* var. *lolii*-infected perennial ryegrass (Gallagher et al., 1981; Gallagher et al., 1984). Concentrations of 1.8-2.0 µg/g lolitrem B are responsible to cause muscular incoordination in livestock displaying symptoms of ataxia and sustained tremors (Hovermale and Craig, 2001). However, when the animal is removed from the source, symptoms can be reversed (Gallagher et al., 1982). Nevertheless, these *Epichloë* endophyte-mediated diseases led to tremendous agronomic costs and are reason for losses in dairy and meat production (Thom et al., 2012). To prevent those problems, especially in high affected countries such as the United States and New Zealand, grass cultivars with “novel”

Epichloë endophyte strains (e.g. AR37, AR1) are introduced to agricultural practice (Easton et al., 2001; Young et al., 2013). These *Epichloë* endophytes produce only minimal or no concentrations of the vertebrate toxic alkaloids, and mainly the metabolite peramine and loline alkaloids (Woodfield and Easton, 2004).

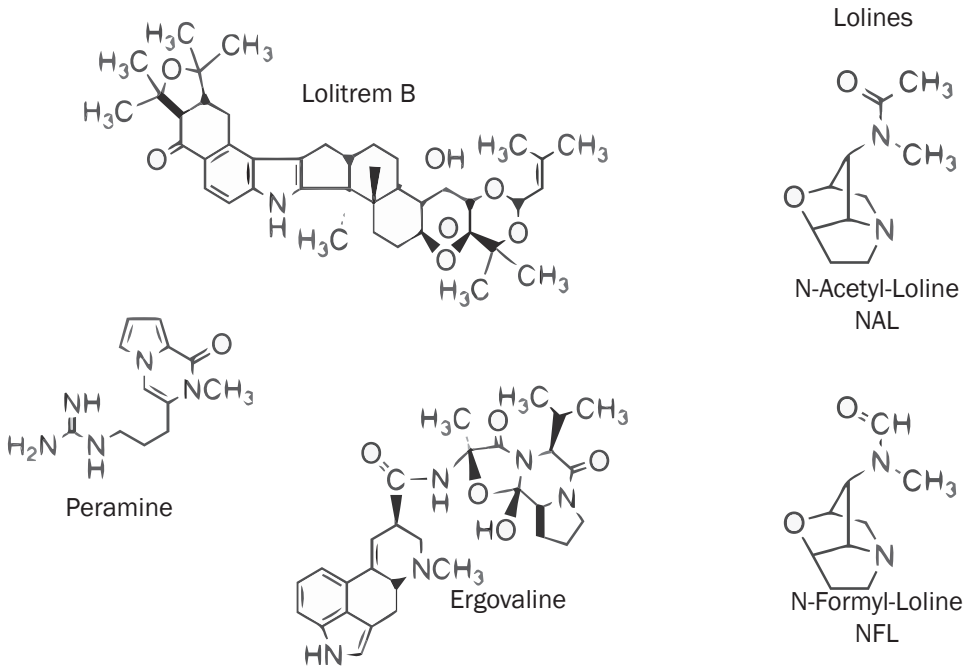

Figure I.4 The four major groups of alkaloids produced by the *Epichloë* endophytes: Ergot alkaloids (ergovaline), aminopyrrolizidine alkaloids (lolines), indolediterpene alkaloids (lolitrem B) and pyrrolopyrazine alkaloid (peramine; Schardl et al., 2013a).

Peramine and loline alkaloids are rather harmless for vertebrate herbivores, but are toxic or deterring to invertebrates feeding on the host grasses (Panaccione et al., 2014). The production of peramine is unique to the association of *Epichloë* endophytes and grasses (Tanaka et al., 2005). Peramine is the most widely distributed alkaloid of the four groups (Clay and Schardl, 2002; Schardl et al., 2012) and was identified as strong feeding deterrent of Argentine stem weevil (*Listronotus bonariensis* Kuschel; Prestidge et al., 1982). Since the discovery of these defensive effects (Rowan et al., 1986; Rowan et al., 1990; Siegel et al., 1989), *E. festuca* var. *lolii*-infected ryegrass is applied to New Zealand's pastures to prevent the grasslands from the pest species (Hume et al., 2016). Although the anti-feeding effects of peramine are not universal (Johnson et al., 1985; Panaccione et al., 2014), several other insects, such as fall armyworm (Clay et al., 1985), are negatively affected by this alkaloid.

Loline alkaloids occur almost exclusively in the *Epichloë* endophyte-grass association (Clay and Schardl, 2002; Wilkinson et al., 2000), but were also

isolated from few non-grass species, e.g. Convolvulaceae (Steiner et al., 2006). Lolines can be detected in above and below ground plant tissue of the host grasses (Bush et al., 1997) and can be abundantly found in *Epichloë* endophyte-infected meadow and tall fescue (Schardl et al., 2007). They are known for their broad spectrum of deterrence and insecticidal activity (Schardl et al., 2007), including several aphid species (Siegel et al., 1990; Wilkinson et al., 2000), root aphids (Schmidt and Guy, 1997), fall armyworm and European corn borer (Riedell et al., 1991), Japanese beetle (Patterson et al., 1991), black beetle and crickets (Barker et al., 2015a; b), as well as Argentine stem weevil (Jensen et al., 2009) and grass grub (Schmidt and Guy, 1997). In grasses, loline alkaloids mostly occur in concentrations up to 1000 times higher compared to the other *Epichloë* endophyte-derived alkaloids (Schardl et al., 2013a; Siegel and Bush, 1996; Spiering et al., 2005b; Zhang et al., 2009). The loline derivatives N-methyl- and N-formyl-loline (Figure I.4) accumulate in the highest concentrations between 1000 µg/g to 4000 µg/g in vegetative shoots. Their insecticidal activities are comparable to nicotine (Leuchtmann et al., 2000; Riedell et al., 1991; Siegel and Bush, 1996).

Mutualistic associations in which one symbiont provides protection from natural enemies to its symbiotic partner, as in the association of *Epichloë* endophytes and grasses, are often described as defensive mutualism (Clay, 2014). This mechanism is assumed to play an important role in the evolution of *Epichloë* endophyte-grass associations, especially in environments with high herbivory pressure such as managed grassland ecosystems (Clay and Schardl, 2002; Koh and Hik, 2007). In general, the bioprotective alkaloids are most frequent in strictly seed-transmitted (asexual) species of *Epichloë* endophytes and can produce much higher concentrations than horizontally (sexual) transmitted species (Schardl et al., 2013a; 2013b). The chemical profiles of *Epichloë* endophytes vary widely with respect to the presence and absence of each group of alkaloids (Schardl et al., 2013a; 2013b).

In my thesis, I studied *Epichloë* endophytes with different chemical profiles. *Epichloë festucae* var. *lolii* produces high concentrations of vertebrate toxic lolitrem B and often ergot alkaloids, and peramine in their host grass *L. perenne* (Schardl et al., 2013a). *Epichloë uncinata* and *E. siegelii* are hybrids, possibly sharing a common ancestor, and mainly produce the insect deterring/toxic loline alkaloids in the association with *F. pratensis* (Craven et al., 2001; Saikkonen et al., 2016). In chapter II, I mainly focused on the quantification of lolitrem B in *E. festucae* var. *lolii*-infected ryegrass to predict the intoxication risk of livestock. Effects on insect pest species due to invertebrate toxic/deterring loline alkaloids, were predicted for *Epichloë* endophyte-infected meadow fescue in chapter III.

I.3 Managing the problems - *Epichloë* endophytes in an agronomic environment

Grasslands provide various habitats for a large number of species of different organisms, but also are among the most important land-use types in central Europe, and thus are highly affected by anthropogenic land use (Bluethgen et al., 2012). Land use started to increase with increasing the number of livestock and grazing periods, the application of chemical fertilizer and frequent mowing events, resulting in the transition of less fertile grasslands into high productive meadows and pastures (Vickery et al., 2001). The intensification of land use and simplification of agronomic landscapes are major drivers of drastic declines in species diversity (Klimek et al., 2007; Tscharntke et al., 2005). Fertilization and grazing influence the vegetation structure and the availability of nutrients to the plants respectively, and have been shown to alter individual fungal abundances, species richness and structure of microbial communities (Donnison et al., 2000; Parrent et al., 2006; Soliveres et al., 2016; Valyi et al., 2015), as well as the interactions of *Epichloë* endophytes, their host grasses and other interacting organisms.

In managed grasslands and especially in highly grazed areas, the fitness of *Epichloë* endophyte-infected grasses was shown to be enhanced due to a higher reproductive success and increased dispersal compared to un-infected grasses (Clay et al., 2005), resulting in an increased occurrence of *Epichloë* endophyte infections in pastures recorded by several studies (Gwinn et al., 1998; Jensen and Roulund, 2004). Similar, the concentrations of *Epichloë* endophyte-derived alkaloids increased after grazing was simulated by clipping the plant, and indicate that the alkaloid production of *Epichloë* endophytes might be induced by grazing herbivores such as livestock (Bultman et al., 2004; Fuchs et al., 2017b). The application of fertilizer in experimental studies also resulted in increased alkaloid concentrations (Krauss et al., 2007; Lane et al., 1997), but decreased in another laboratory study (Rasmussen et al., 2007). In natural ecosystems the *Epichloë* endophyte-grass associations were unaffected by fertilization (Bylin et al., 2014; Repussard et al., 2014). Thus, alkaloid production might be low and/or *Epichloë* endophyte infection might be lost or decrease in frequency, when herbivory pressure is low or the host grasses grow under optimal conditions (e.g. specific environmental conditions or when fertilized; Clay, 2014; Rasmussen et al., 2007).

Besides the potential to harm grazing livestock, *Epichloë* endophytes can also alter species composition of grassland ecosystems by providing

various fitness advantages and increased competitive ability to their hosts compared to un-infected grasses and other plants (Hoveland, 1993; Rudgers et al., 2010). With the naturalization of the in Europe originated grass species and until the discovery of animal health problems associated with *Epichloë* endophytes, the systemic endophytes were unknowingly spread in the United States (Bacon et al., 1977), and also dominate todays improved pastures in Australia and New Zealand (Hoveland, 1993; Hume et al., 2016). In my thesis, I focused on the two grass species, perennial ryegrass (*L. perenne*) and meadow fescue (F. *pratensis*; Figure I.5). Due to their high feeding value and sward persistence, both grass species are of high agronomic importance (Malinowski and Belesky, 2006). European-bred cultivars of such commonly used forage and pasture grasses can occasionally be *Epichloë* endophytes-infected (Saari et al., 2009; Saari et al., 2010a), and thus the additionally application of cultivars can enhance the dispersal of *Epichloë* endophytes and their effects on vegetation structure, even in native habitats such as European grasslands.

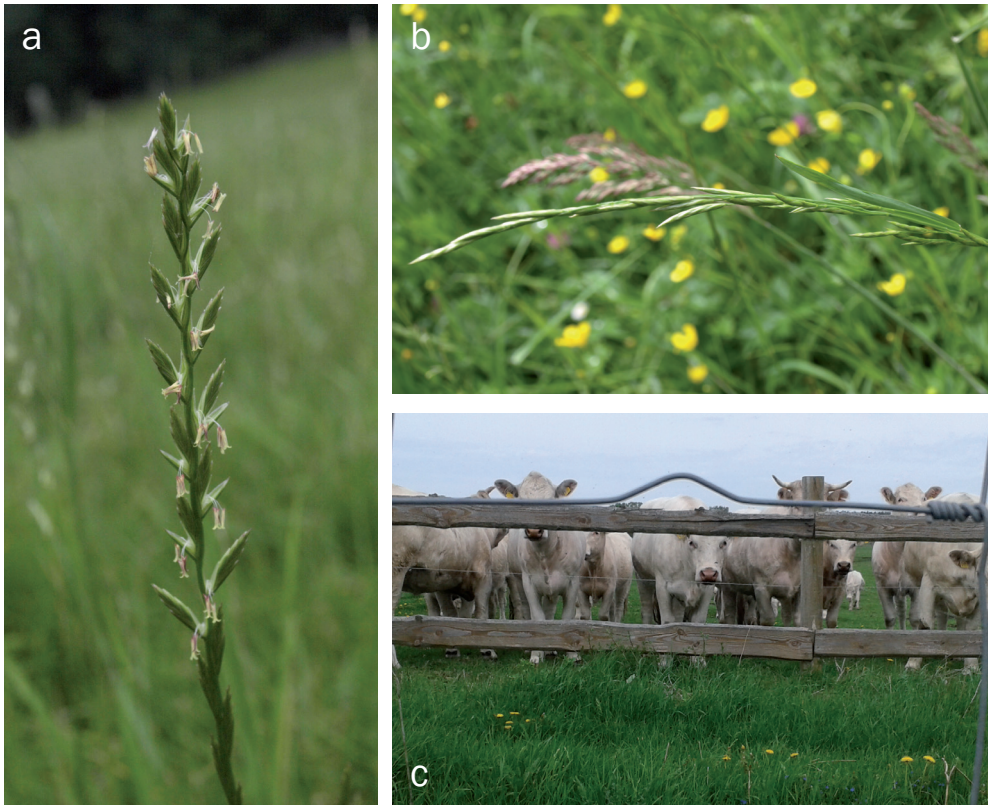

Figure I.5 (a) Inflorescence of perennial ryegrass, *Lolium perenne*; (b) inflorescence of meadow fescue, *Festuca pratensis*; (c) pasture site, grazed mainly by cattle, containing both grass species.

Several studies indicated that the *Epichloë* endophytes may benefit from land-use intensification which can largely affect the economic value of forage production in managed grassland ecosystems, providing increased

sward persistence and resistance to several insect pest species. However, the association with *Epichloë* endophytes can also have tremendous consequences at community and agroecosystem levels, including (1) increased intoxication risk for vertebrate livestock, (2) interactions with other microorganisms and plants, and (3) possible fitness disadvantages of higher trophic levels (e.g. beneficial insects) by alkaloids cascading up insect food chains (Fuchs et al., 2013). For that reasons, I studied if land-use intensity, including the common management practices grazing, fertilization and mowing, as well as the application of additional grass seeds, change the distribution and alkaloid content, and thus possibly influence the effects on interacting organisms (Figure I.6), of the *Epichloë* endophytes of the two host grasses *L. perenne* (chapter II) and *F. pratensis* (chapter III).

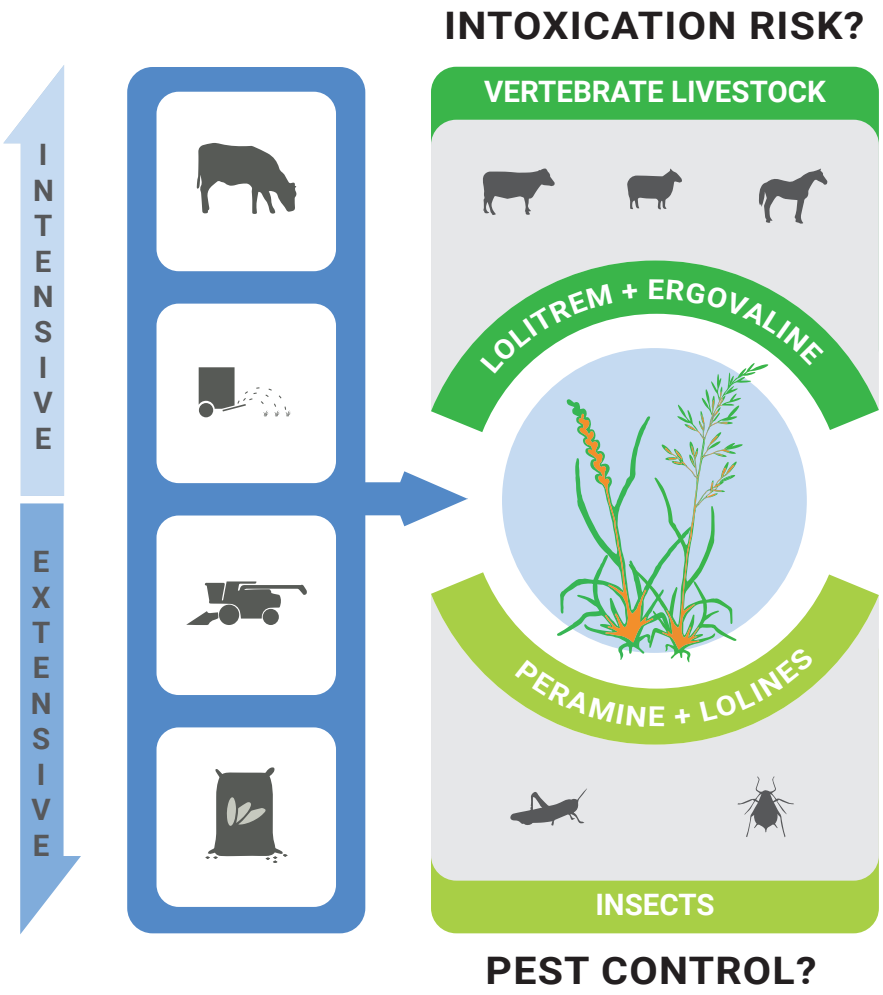

Figure I.6 Land use increase *Epichloë* endophyte infection rates and the production of alkloids (e.g. Gwinn et al., 1998; Krauss et al., 2007), but which impact has the intensity of land use on the infection rates and alkloids of *Epichloë* endophytes of *L. perenne* and *F. pratensis* in German grasslands?

I.4 Heat it up – *Epichloë* endophytes under the impact of seasonal changes

In agroecosystems, grasses and their associated *Epichloë* endophytes are exposed to several environmental predictors which can be abiotic factors such as temperature, nutrition and water supply, or biotic factors like symbionts, competitors and enemies (Mitchell et al., 2006).

In the association of grasses and *Epichloë* endophytes, the distribution of *Epichloë* endophyte-derived alkaloids, as well as the mycelia, is closely linked to the growing phase of the symbiotic host grass and varies in plant tissue (Justus et al., 1997; Spiering et al., 2005a). Although, the distribution of the endophyte mycelia did not play a major role in the distribution of alkaloids in the plant, endophyte mycelia and alkaloids mainly accumulate in the basal leaf sheaths of the *Epichloë* endophyte-infected grasses (Spiering et al., 2005a). The highest concentrations of endophyte-derived alkaloids can be mainly detect at the fully ripe stage of mature grasses (Guerre, 2016; Justus et al., 1997).

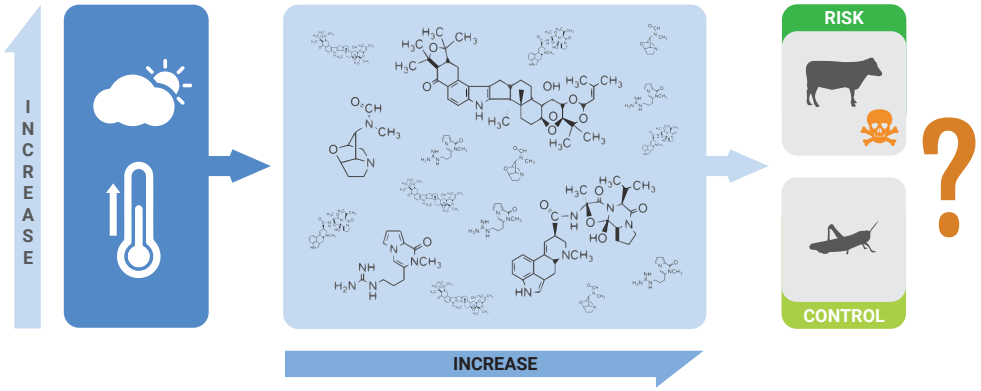

Figure I.7 The accumulation of *Epichloë* endophyte-derived alkaloids change with season and increasing temperatures (e.g. Repussard et al., 2014) and thus, may increase (a) the intoxication risk for grazing livestock and (b) the potential to control insect pest species in German grasslands.

However, the production of alkaloids is strongly determined by climatic conditions (Ryan et al., 2015) and were found to accumulate in dry summer periods (Repussard et al., 2014). Alkaloid concentrations showed a seasonal rhythm indicating a strong impact of temperature on the alkaloid production of *Epichloë* endophytes (Fuchs et al., 2017c; Justus et al., 1997; Repussard et al., 2014; Tong et al., 2006). Further, alkaloid accumulation possibly correlates with outbreaks of livestock intoxications which

occurred especially in years with rainy spring and dry-hot summers (Reed et al., 2011; Figure I.7). Thus, in my thesis, I analysed how concentrations especially of the alkaloids lolitrem B and peramine in *Epichloë* endophyte-infected *L. perenne* (chapter II), and loline alkaloids in *Epichloë* endophyte-infected *F. pratensis* (chapter III), change with season, which can influence their potential to affect interacting organisms such as grazing livestock or insect herbivores.

I.5 The game is on – *Epichloë* endophytes in a fungal community

The biotic predictors which interact with the *Epichloë* endophytes range from herbivorous insects and grazing livestock to other plant microbes such as mycorrhiza fungi or fungal endophytes. Many studies demonstrate performance effects on insect species mediated by *Epichloë* endophytes (e.g. Barker et al., 2015c; Krauss et al., 2007), but studies analysing effects of *Epichloë* endophytes on the community of other plant-associated fungi are rare.

However, the genus *Epichloë* only represent a small fraction of a diverse fungal endophyte community of grasses (Neubert et al., 2006; Rodriguez et al., 2009). Most of all grass species are infected with a large number of fungal endophytes and one host often harbours hundreds of species colonizing roots, stems and leaves (Sánchez Márquez et al., 2007). In contrast to the *Epichloë* endophytes, most other endophytic species are limited in their capability to colonize tissue of the host grasses systemically (Rodriguez et al., 2009). Their frequencies and patterns of colonization are very unequal, but a few dominant genera, such as *Alternaria* Nees, *Acremonium* Link, *Cladosporium* Link and *Penicillium* Link, occur in multiple grass species (Sánchez Márquez et al., 2012). These endophytes appear to be non-systemic and not transmitted vertically, involved in a wide spectrum of interaction types from being latent pathogens to latent or facultative saprotrophs (Sánchez Márquez et al., 2012).

Fungal endophytes ability to colonize, persist and disperse are largely influenced by abiotic and biotic factors (Rodriguez et al., 2009). The composition of the mycobiome of grasses varies among grass species, individual plants, plant organs and organ age (Rodriguez et al., 2009; Stone et al., 2004) and frequencies of endophyte species change with spatial distance and season (Sánchez Márquez et al., 2012).

The interactions among endophytes are little understood and may include competition for plant resources such as space and nutrients, or direct/

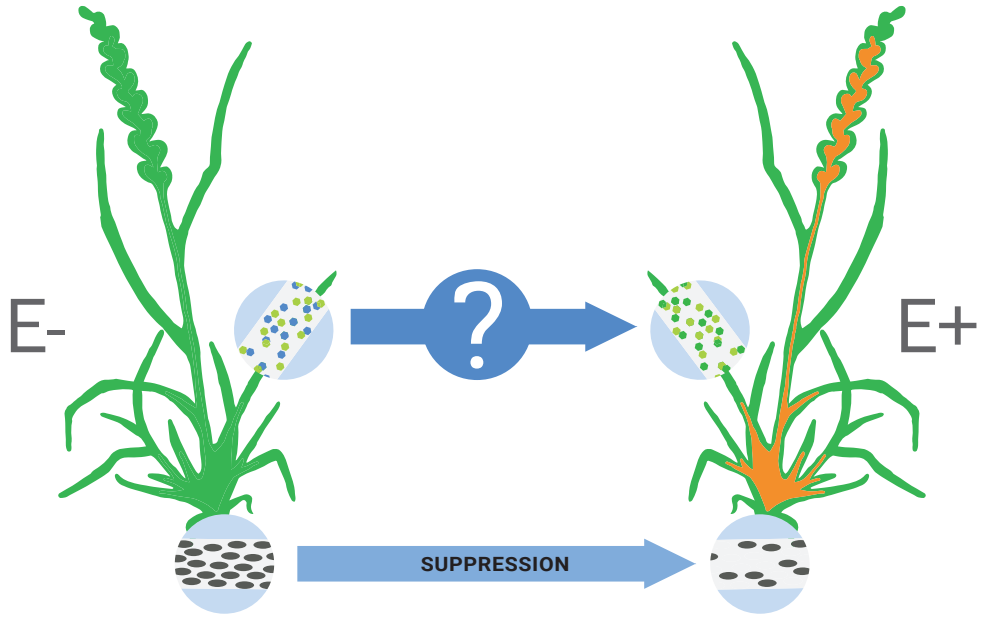

Figure I.8 The infection with *Epichloë* endophytes (E+) suppress mycorrhizal colonization of the grass roots (e.g. Vandegrift et al., 2015), but do they also have modifying effects on the fungal assemblages of *L. perenne* leaves?

indirect counteractions against fungal pathogens (Saunders et al., 2010; Suryanarayanan, 2013). *Epichloë* endophytes have been associated with the production of specific chemicals in culture which inhibit the growth of pathogenic fungi (Koshino et al., 1988; 1989; Yoshihara et al., 1985; Yue et al., 2000). However, *in planta*, effects are not completely correlated with the *in vitro* studies. *Epichloë typhina* (Pers) Tul. & C. Tul. was the first *Epichloë* endophyte which was observed to confer resistance to its host grass timothy (*Phleum pratense* L.) against leaf spot (*Cladosporium phlei* (C.T. Greg.) G.A. de Vries; Shimanuki and Sato, 1983). *Epichloë coenphialum*-infected tall fescue was enhanced in resistance to dollar spot disease (*Sclerotinia homoeocarpa* F. T. Benn.; Clarke et al., 2006) and crown rust (*Puccinia coronate* Corda) was reduced compared to *Epichloë* endophyte-free plants (West et al., 1989), but no effect was found on stem rust (*Puccinia graminis* subsp. *graminicola* Urban; Welty et al., 1991). Moreover, it is suggested that *Epichloë* endophytes generate major shifts in below ground subsystems and suppress root colonization of mycorrhiza fungi (Mack and Rudgers, 2008; Omacini et al., 2012; Vandegrift et al., 2015). Thus, species composition of non-systemic fungal endophytes in the grass leaves might also change due to *Epichloë* endophytes (Figure I.8). Therefore, as part of my thesis, I studied if *E. festucae* var. *lolii* have modifying effects on the fungal assemblages of *L. perenne* leaves in dependence of land-use intensity and season (chapter IV).

I.6 Fitting partners - The Biodiversity Exploratories

Recent studies on *Epichloë* endophytes are laboratory based or have been conducted mainly as common garden experiments and the impact of land use was analysed by focusing on only one management type, such as grazing or fertilization (e.g. Bylin et al., 2014; Fuchs et al., 2017b; Krauss et al., 2007; Saari et al., 2010b). However, the effects of different abiotic conditions in environments of distinct regions or different management intensities may change the impact of *Epichloë* endophytes.

My thesis was conducted as the project "DEFENSE" (KR 3559/3-1) under the framework of the German large-scale and long-term project "Biodiversity Exploratories" (www.biodiversity-exploratories.de), a joint project funded by the German Research Foundation (Deutsche Forschungsgesellschaft, DFG Priority Program 1374) which serves a research platform addressing critical questions on the feedback between land use, biodiversity and ecosystem processes in real-world ecosystems (Fischer et al., 2010b).

The exploratories were established in three regions: The UNESCO Biosphere Reserve Schorfheide-Chorin (Brandenburg, NE Germany); the National Park Hainich (Thuringia, C Germany); and the UNESCO Biosphere Reserve Schwäbische Alb (Baden-Württemberg, SW Germany). While the north-eastern Schorfheide-Chorin (10-140 m ASL) is defined by marshy grasslands and glacially formed landscapes, the central located uplands of National Park Hainich (200-400 ASL) and the low mountain ranges of south-western Schwäbische Alb (720-850 ASL) consist of calcareous bedrock (Fischer et al., 2010a). The regions span a latitudinal gradient of 800 km from south to north Germany and represent different landscape types and reflect a climatic gradient of increasing precipitation, rising altitude and slightly decreasing annual mean temperatures from north eastern to south western Germany (Figure I.9). Thus, the project Biodiversity Exploratories gave me the possibility to study *Epichloë* endophytes under environmental conditions in three different German regions as native habitats and on real-world grassland ecosystems.

In each region, 100 study sites, 50 in grassland and 50 in forests, were selected for experimental manipulations and biodiversity monitoring. Out of the 50 experimental plots (EP), 9 study sites (the so called Very-Intensive-Plots (VIP)) in grassland and forests respectively, were chosen for detailed and labour-intensive investigations (Fischer et al., 2010a; Figure I.9). In my thesis, I exclusively focused on grassland study sites, of which some (25 %) were once or had been sown with seeds of commercial cultivars in-

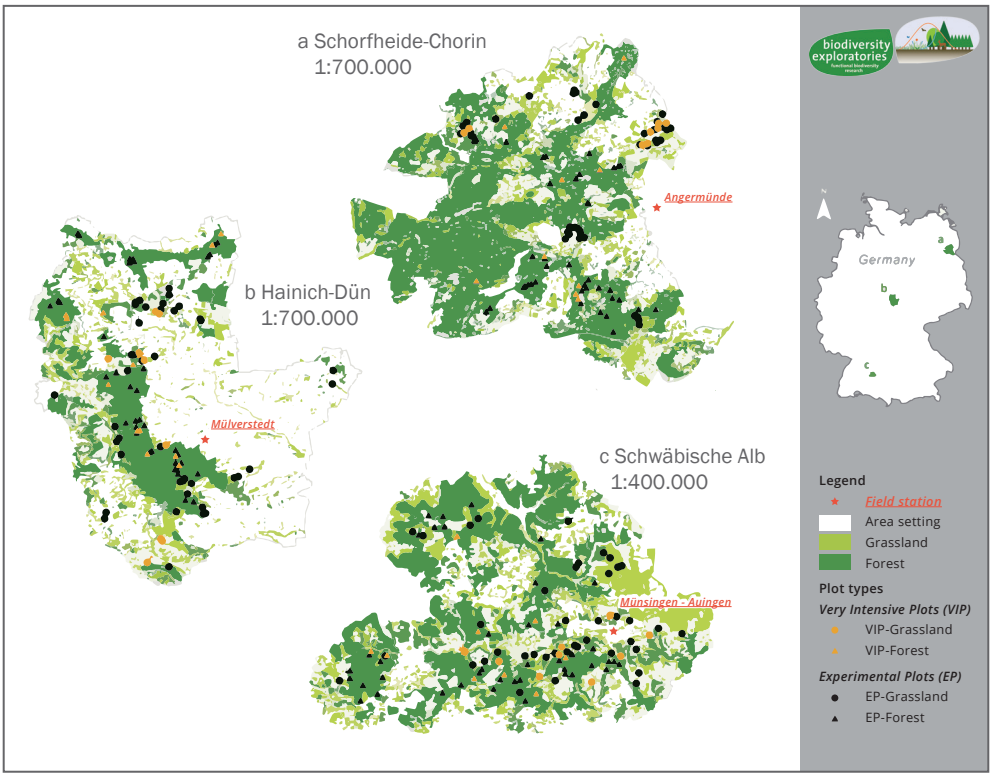

Figure I.9 Study regions of the German project „Biodiversity Exploratories", (a) north-eastern UNESCO Biosphere Reserve Schorfheide-Chorin; (b) Nationalpark Hainich-Dün in central Germany; (c) south-western UNESCO Biosphere Reserve Schwäbische Alb (Fischer et al., 2010a).

cluding grass seeds within the last ten years. However, the most common management practices for grasslands of the Biodiversity Exploratories include fertilization, mowing and grazing.

The intensities of those management types, are integrated into one index (land-use intensity index, LUI; Bluethgen et al., 2012; Figure I.10). This integrated index allows the analysis of *Epichloë* endophytes and their

$$Li = \frac{Fi}{FR} + \frac{Mi}{MR} + \frac{Gi}{GR}$$

Figure I.10 The land-use intensity index (LUI) adds fertilization plus mowing plus grazing intensities. For each grassland site *i*, land-use intensity *Li* is defined by fertilization level (kg nitrogen/ha*year) *Fi*, the frequency of mowing per year *Mi,* the density of grazing livestock (livestock units days of grazing/ha*year) *Gi* and *FR, MR and GR* their respective mean within its region for a given year (Bluethgen et al., 2012).

production of potentially toxic/deterring alkaloids along a land-use intensity gradient, including extensively managed grasslands (e.g. semi-natural grasslands such as protected calcareous grasslands and wetlands), as well as intensively managed and high productive meadows and pastures (Figure I.11).

Figure I.11 The established grassland sites within the Biodiversity Exploratories comprise different grassland and management types, including meadows, mown pastures and pastures (Fischer et al., 2010a).

In this thesis, I analysed the distribution of *Epichloë* endophytes and their content of bioprotective alkaloids produced under the impact of land use and under natural conditions on real-world grasslands. In order to predict effects of *Epichloë* endophytes on interacting species, such as herbivores and other fungi, approaches considered *Epichloë* species with different chemical profiles, in dependence of different management practices and along a land-use intensity gradient in three German regions and for two season.

In **Chapter II** of this thesis, I studied the effects of land-use intensity using LUI on the infection rates of *E. festucea* var. *lolii* in *L. perenne* and concentrations of the *Epichloë* endophyte-derived alkaloids lolitrem B, ergovaline and peramine. Further, I tested if single components of the LUI, grazing (no/yes) and fertilization (no/yes), as well as additional sowing, affect the rate of infections and the alkaloid concentrations, and I analysed seasonal effects on the accumulation of alkaloids. My main hypotheses were:

- Endophyte infection rates and alkaloid concentrations increase with increasing management intensity
- Concentrations of alkaloids are higher in summer than in spring

In **Chapter III** of this thesis, I analysed if land-use intensity using LUI has an impact on the infection rates of *Epichloë* endophytes in *F. pratensis* and concentrations of the *Epichloë* endophyte-derived loline alkaloids. Further, I tested the effects of the LUI components grazing (no/yes) and fertilization (no/yes) on the rate of infections and the alkaloid concentrations. Additionally, I studied if the accumulation of loline alkaloids is seasonal dependent. My predictions on this topic were:

- Endophyte infection rates and loline alkaloid concentrations depend on study region and alkaloid concentrations are higher in summer compared to spring
- Endophyte infection rates and loline alkaloid concentrations increase with increasing land-use intensity (including fertilized vs. not fertilized and grazed vs. not grazed grasslands)
- Loline concentrations are above toxicity threshold for insects and vertebrate toxic endophyte-derived alkaloids are low or missing

In **Chapter IV** of this thesis, I analysed species composition of fungal assemblages isolated from *L. perenne* leaves. I tested if an infection with *E. festucae* var. *lolii* determine foliar fungal assemblages in dependence of land use and season. Further, I studied whether land-use intensity using LUI or seasonal changes influence species richness, species evenness and Shannon diversity of the foliar fungal communities.

In this chapter I asked, whether or not the presence of systemic grass endophytes of the genus *Epichloë* changes the species composition of foliar fungal assemblages in a host grass along a land-use intensity gradient in two seasons (spring and summer) and in three geographic regions.

Chapter II

Infection rates and alkaloid concentrations of *Epichloë festucae* var. *lolii* in *Lolium perenne* along a land-use gradient in Germany

The common forage grass *Lolium perenne* has evolved with the systemic fungal endophyte *Epichloë festucae* var. *lolii*. The endophyte provides herbivore resistance to the grass due to defensive alkaloids, some of which are toxic to grazing livestock. In this field study, we determine whether distribution of the endophyte-grass association change along a land-use intensity gradient on 87 managed grasslands in three German regions. Endophyte infections were detected in 66 % of the studied sites and infection rates within infected sites ranged from 1 % to 95 %. Alkaloid concentrations of lolitrem B (vertebrate toxin) exceeded the toxicity thresholds in 50 (14 %) of 351 infected plants and of peramine (invertebrate deterrent/ toxin) in 12 (3 %) of 351 plants. Infection rates and alkaloid concentrations were not significantly affected by land-use intensity and region, but alkaloid concentrations were higher in summer compared to spring. We conclude that risks for livestock intoxication are currently low, as (1) average alkaloid concentrations per grassland were always below toxicity thresholds and as (2) none of the grasslands was dominated by *L. perenne*. We suggest avoidance of grass monocultures in Europe to keep intoxication risks for livestock low; we also recommend regular examination of seeds and grasslands, as seed producers might accidentally distribute infected seeds, and as climate warming might further enhance the distribution of *Epichloë* endophytes in European grasslands.

II.1 Introduction

In central Europe, grasslands are among the most important land-use types in agricultural systems and are highly affected by anthropogenic land-use (Bluethgen et al., 2012). Over centuries, the simplification of landscapes and transformation of less fertile grasslands into high productivity meadows and pastures has led to a drastic decline in species and community diversity. This in turn has influenced species interactions such as symbioses between plants and bacteria or fungi (Klimek et al., 2007; Tscharntke et al., 2005).

Many common forage grasses of the Poaceae family, e.g. *Lolium* (ryegrass) and *Festuca* (fescue), have evolved as symbiotic associates with asexual, systemic fungal endophytes of the genus *Epichloë* (Leuchtmann, 1992). These grass endophytes live their entire life cycle asymptomatically within the intercellular spaces of aerial parts of the host grass and are transmitted vertically via grass seeds (Schardl et al., 2004). The endophyte improves the fitness and persistence of the grass by producing bioprotective alkaloids against herbivores (Schardl et al., 2013a). Some of these alkaloids have detrimental effects on grazing livestock. The alkaloids toxic to vertebrates induce different forms of intoxication such as “ryegrass staggers”, “fescue foot” and lameness in cattle, sheep and horses (Douthit et al., 2012; Porter, 1995; Rowan, 1993). These endophyte-mediated poisonous effects have economic costs of up to two billion dollars per year in the United States, Australia and New Zealand (Aiken and Strickland, 2013).

The endophytes and their grass associates originated in Europe (Lewis, 1997; Malinowski and Belesky, 2006). Endophyte infections of different host grass species have been observed frequently in wild grass populations from the Mediterranean to Scandinavia (Do Valle Ribeiro et al., 1996; Dobrindt et al., 2013; Jensen and Roulund, 2004; Leyronas and Raynal, 2001; Oldenburg, 1997; Saikkonen et al., 2000; Zabalgogeazcoa et al., 2003; Zurek et al., 2012). Reports of livestock intoxication in Europe are rare, but outbreaks have been shown to correlate with increased infection rates and alkaloid concentrations in host grasses (Zabalgogeazcoa and Bony, 2005). Several studies have demonstrated increased endophyte infections under intensive management (e.g. grazing and fertilization), which indicates that *Epichloë* endophytes may benefit from land-use intensification (Gwinn et al., 1998; Jensen and Roulund, 2004). Alkaloid concentrations are strongly determined by climatic conditions (Fuchs et al., 2017a). Increased alkaloid concentrations under rising temperatures indicate that the effects of endophytes are season-dependent (Repussard et al., 2014; Ryan et al., 2015).

Recent studies on the effect of land use on infection rates and alkaloid production of grass endophytes have been conducted mainly as laboratory or common garden experiments, or have manipulated only one management type, such as grazing or fertilization (Fuchs et al., 2017b; Krauss et al., 2007). Studies on real-world grasslands (semi-natural to intensively used) under natural environmental conditions are missing. In this field study, we examined the infection rates of *Epichloë festucae* var. *lolii* in *Lolium perenne* in managed grasslands of three German regions. We describe the impact of land use on infection rates using a land-use intensity gradient which integrates the most important management practices. In order to analyse the risk of endophyte-mediated intoxication of livestock or the benefit of invertebrate toxification, we quantified the alkaloids lolitrem B, ergovaline and peramine. Our predictions are:

- Endophyte infection rates and alkaloid concentrations increase with increasing management intensity
- Concentrations of alkaloids are higher in summer than in spring

II.2 Methods and Materials

II.2.1 Study species

The perennial ryegrass *Lolium perenne* was selected as the study species, as (1) it is native to Europe, (2) it is of high agronomic importance as a forage supply for grazing livestock, and (3) it is often associated with the vertically transmitted endophyte *Epichloë festucae* var. *lolii* (formerly *Neotyphodium lolii*; Klapp and Opitz von Boberfeld, 2013; Leuchtmann et al., 2014). The endophyte produces three groups of herbivore toxic metabolites; lolitrems, ergot alkaloids, and peramine (Schardl et al., 2013a). Concentrations above 2 µg/g of lolitrem B and 0.3 µg/g of ergovaline (ergot alkaloid) are vertebrate toxins and concentrations above 3 µg/g of peramine are invertebrate deterrents/toxins (Hovermale and Craig, 2001; Siegel and Bush, 1996). All alkaloids cascade up insect food chains and may be responsible for fitness disadvantages of higher trophic levels (Fuchs et al., 2013).

II.2.2 Study sites

We selected 150 grasslands with different land-use intensities as study sites to detect endophyte infection rates and alkaloid concentrations of

L. perenne populations. The study sites are part of the German Biodiversity Exploratory (www.biodiversity-exploratories.de) regions. Study sites span a latitudinal gradient of 800 km from south to north Germany and experience different climatic conditions, soil types, and different intensities of land use (Fischer et al., 2010a). The UNESCO Biosphere Reserve Schwäbische Alb (Baden-Württemberg, ALB) is located in the low mountain ranges of south-western Germany, while the National Park Hainich (Thuringia, HAI) is located in central Germany. Both regions are defined by calcareous bedrock. The UNESCO Biosphere Reserve Schorfheide-Chorin, located in the northeast (Brandenburg, SCH), is defined by marshy grasslands and glacially formed landscapes (Fischer et al., 2010a).

All selected study sites are real-world grasslands with owners and farmers exploiting the grasslands for their needs following public laws. Due to the heterogeneous management of the grasslands, all sites have been classified along a land-use intensity gradient (LUI) integrating into one index the most common practices, such as mowing, grazing, and fertilization (Bluethgen et al., 2012). Intensively managed and highly productive grasslands are treated with fertilizers, mown repeatedly and/or grazed mainly by cattle several times during the year. In contrast, extensively managed grasslands, e.g. semi-natural grasslands such as protected calcareous grasslands and wetlands, are not fertilized, mown only once and/or are grazed only for a short time, mainly by sheep (Bluethgen et al., 2012).

We used the LUI to test whether infection rates and alkaloid concentrations are dependent on land-use intensity, but we also tested two main components of the LUI separately, as fertilization and grazing can have independent effects on endophyte-grass symbiosis (Gwinn et al., 1998; Krauss et al., 2007). Some of our study sites (25 %) were once or had repeatedly been sown with seeds of commercial cultivars within the last ten years. As European-bred cultivars can occasionally be *Epichloë* endophyte-infected due to a lack of regular controls (Saari et al., 2009; Saari et al., 2010a), we tested whether infection rates and alkaloid concentrations at the study sites depended on whether or not the farmer had sown grass seeds within the last ten years. We received the data on sowing events from grassland farmers and grassland owners.

II.2.3 Plant sampling

We sampled individual plants in three surveys. In the first survey, from June to July 2014, we visited all 150 study sites in the three regions; ALB, HAI and SCH, to record the occurrence of the host grass *L. perenne*. All

studied sites were mixed grasslands containing a minimum of nine vascular plant species in a 16 m^2 plot in the centre of the grasslands (Fischer et al., 2010a). On average, the grasslands contained between 16 and 35 vascular plant species (in 16 m^2) depending on management (Socher et al., 2013). In none of the studied grasslands was *L. perenne* the dominant species (personal observations Julia König).

Lolium perenne populations were observed and sampled on 87 study sites. On these 87 sites, plant sampling was repeated in the second survey from April to June 2015, and in the third survey from July to August 2015. The two surveys in 2015 were used to test seasonal variations in alkaloid concentrations, as previous publications have shown higher alkaloid concentrations in summer compared to spring for the same endophyte-grass symbiosis (Fuchs et al., 2017a; Repussard et al., 2014).

In each sampling interval, plants were sampled at different locations on each study site. Depending on the population size of the host grass *L. perenne* and on recent mowing or grazing events, the number of sampled *L. perenne* plants differed. As we mainly focus on detecting infection rates of each study site, we sampled a minimum of ten to a maximum of 56 plants per site, resulting in a total of 2786 plants. To reduce the probability of sampling the same plant twice, we kept a minimum distance of 1 m between sampled plants. As marking sampled plants was not possible due to mowing events and grazing herbivores on many sites, we cannot exclude the possibility that the same plant was sampled in more than one survey.

Approximately 3 cm of one grass tiller per each individual plant were cut near the ground, as the endophyte mycelia and alkaloids accumulate mainly in basal leaf sheaths of endophyte-infected grasses (Spiering et al., 2005a; Figure II.1 a). In less than 1 %, we sampled additionally one to two tillers when plants were small to achieve enough material for alkaloid analyses. In these cases, infection states were congruent for the tillers of the same plant. Samples were stored in 2.5 ml Eppendorf tubes. All samples were cooled with dry ice during the field survey and stored at -20°C afterwards to prevent degradation of the alkaloids in endophyte-infected grasses.

II.2.4 Endophyte detection and alkaloid analysis

For endophyte detection of the 2786 plant samples, we used a commercially available kit for immunoblot assays (www.agrinostics.com), following the protocol of Agrinostics. An additional staining with phenolic blue and

microscopic investigation of 629 (23 %) of the plants showed a congruence of 91 % to the results of immunoblot assays. As immunoblot assays are less time consuming compared to staining, we exclusively considered the immunoblot assays for endophyte detection.

In total, 351 plants were detected as endophyte infected. For these 351 immuno-positive and, in addition as a control, for ten immuno-negative plants, alkaloid content was quantified by using Ultra High Performance Liquid Chromatography-Tandem-Mass Spectrometry (UPLC-Tandem-MS).

To quantify the alkaloids we followed the protocol published by Fuchs et al. (2013). Until the preparation for UPLC-Tandem-MS, grass samples were kept frozen at -20°C. Samples were ground in liquid nitrogen and alkaloids were extracted with methanol and dichloromethane. Concentrations of lolitrem B, peramine and ergovaline were determined by reference to the internal standard compounds homoperamine and ergotamine. The limit of detection was 5 ng for all alkaloids (Fuchs et al., 2013).

Alkaloid concentrations correlate with the presence of the endophyte mycelia (Spiering et al., 2005a). Thus, a successful quantification of alkaloid content indicates an infection with *Epichloë* endophytes. No alkaloid content was detected in the ten immuno-negative plants. However, 108 immuno-positive plants did also not contain alkaloids. In such cases, the *Epichloë* endophyte might lack the genes for alkaloid production (Schardl et al., 2013b) or alkaloid concentrations could be below the detection limit of the UPLC-Tandem-MS. Only the 243 plants where at least one of the alkaloids lolitrem B, ergovaline or peramine was detected, were used for the analyses of the alkaloid concentrations, while all 351 immuno-positive plants were used for analyses of the infection rates.

II.2.5 Statistical analysis

All statistical analyses were conducted using the software R version 3.1.1 (R Development Core Team, 2014). We first tested the effect of region, land-use intensity (LUI) and their interaction on endophyte infection rate using generalized least square models (GLS, nlme package; Pinheiro et al., 2016). Infection rates were logit-transformed to improve normality of residuals. To deal with heteroscedasticity between regions, we incorporated a 'varIdent' variance structure in the models (form = ~1|Regions; GLS, nlme package; Pinheiro et al., 2016).

In a second model, we tested the effect of season, region, LUI and their interaction on alkaloid concentrations (lolitrem B and peramine) using linear

mixed effect models (LME, nlme package; Pinheiro et al., 2016). Study site ID was included in the model as random effect (random intercept models). Ergovaline was detected in only 32 (9 %) of 351 endophyte-infected grass samples, and was therefore not statistically analysed. Alkaloid concentrations were square root transformed to improve normality and homoscedasticity of residuals.

In a separate analysis, the continuous explanatory variable LUI was replaced in both models by one of the three categorical explanatory variables; fertilization (no/yes), grazing (no/yes), or sowing (no/yes).

II.3 Results

II.3.1 Infection rates of *Epichloë festucae* var. *lolii* in *Lolium perenne*

The grass species *L. perenne* was recorded in 2014 on 87 (58 %) of the 150 surveyed grassland study sites. The semi-natural grasslands such as calcareous meadows in the regions ALB and HAI and wetlands in the region SCH did not contain *L. perenne* populations. On 57 (66 %) of the 87 sampled study sites we detected an infection of the grass with the fungal endophyte *E. festucae* var. *lolii*. In total, 351 (13 %) of the 2786 plant samples were infected with the endophyte. The study sites with endophyte infections had infection rates between 1 % and 95 %.

Study sites in the most southern region ALB (mean: 10 %, range: 3 %–95 %), the sites in the regions HAI (mean: 13 %, range: 2 %–60 %) and SCH (mean: 15 %, range: 2 %–58 %) had similar infection rates (N ALB = 33, N HAI = 27, N SCH = 27; $F_{2,81} = 0.09$, $p = 0.910$; Figure II.1 b). Considering only those 243 samples with detectable alkaloid content, region had a significant effect on infection rates ($F_{2,81} = 4.81$, $p = 0.011$) with lowest infection rate in the region ALB (mean: 3 %) compared to HAI (mean: 12 %) and SCH (mean: 11 %; Supplementary Figure II.S1). Neither land-use intensity ($F_{1,81} = 0.03$, $p = 0.862$), fertilization (N fertilized = 40, N not fertilized = 47; $F_{1,81} = 0.14$, $p = 0.713$) nor grazing (N grazed = 62, N not grazed = 25; $F_{1,81} = 0.97$, $p = 0.327$) had a significant effect on infection rates. Similarly, the infection rates were independent from additional sowing (N sown = 30, N not sown = 57; $F_{1,81} = 0.10$, $p = 0.756$).

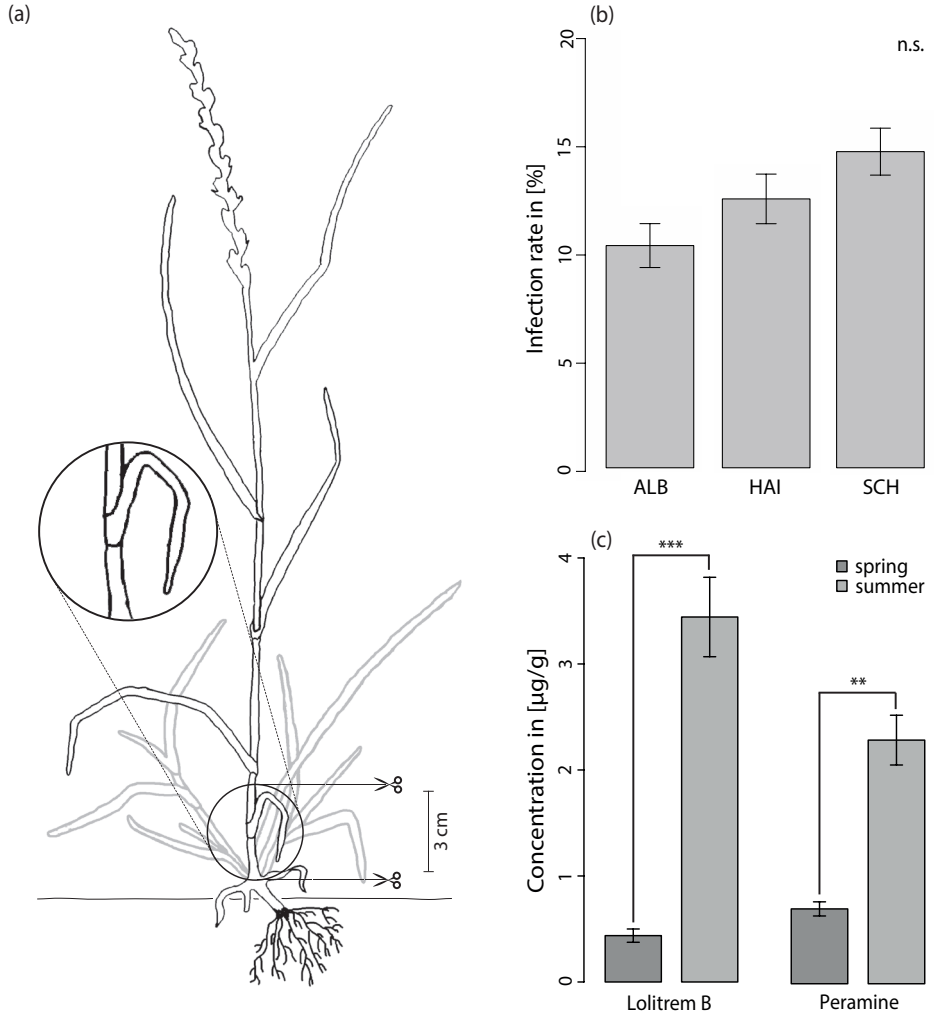

Figure II.1 (a) Analysed parts of grass samples of *Lolium perenne* include basal stems, leaves, and leaf sheaths of each collected tiller; (b) infection rates of *Epichloë festucae* var. *lolii* in *L. perenne,* by study region; (c) mean concentrations of lolitrem B and peramine of *E. festucae* var. *lolii* infected samples depending on the season spring (dark grey) and summer (light grey). Error bars represent mean standard error. Significant levels: n.s. = not significant; ** $p < 0.01$; ***$p < 0.0001$.

II.3.2 Alkaloid concentrations

Alkaloid content was detected in 243 of the 351 immuno-positive plants and contained peak concentrations of 17.26 µg/g lolitrem B, 0.77 µg/g ergovaline and 11.50 µg/g peramine (Table II.1). Peak concentrations of lolitrem B and peramine were detected on two study sites which are owned by one farmer and additionally seeded with a seed mixture including *L. perenne*. Of the endophyte-infected plants, 21 % contained alkaloid concentrations above the toxicity threshold, with more than 2 µg/g of the ver-

Table II.1 Concentrations of alkaloids lolitrem B, ergovaline and peramine in (a) *Epichloë festucae* var. *lolii*-infected individual grass samples of *Lolium perenne*, and (b) means of *E. festucae* var. *lolii* infected and un-infected grass samples of *L. perenne* per study site (population), where at least one individual was infected. Concentrations above toxicity thresholds and peak values are highlighted in bold.

	(a) Individual concentrations						(b) Mean concentrations per study site					
Conc.	Lolitrem B		Ergovaline		Peramine		Lolitrem B		Ergovaline		Peramine	
[µg/g]	IND	[%]	IND	[%]	IND	[%]	SITE	[%]	SITE	[%]	SITE	[%]
0	14	5.8	211	86.8	0	0	1	2.7	25	67.6	0	0
0 – 0.3	100	41.2	30	12.3	55	2.1	27	72.9	12	32.4	30	81.1
0.3 – 1	50	20.6	**2**	**0.8**	89	36.6	9	24.3	0	0	7	18.9
1 – 2	29	11.9	0	0	67	27.6	0	0	0	0	0	0
2 – 3	**22**	**9.1**	0	0	17	7.0	0	0	0	0	0	0
3 – 5	**16**	**6.6**	0	0	**9**	**3.7**	0	0	0	0	0	0
5 – 10	**10**	**4.1**	0	0	**5**	**0.8**	0	0	0	0	0	0
>10	**2**	**0.8**	0	0	**1**	**0.4**	0	0	0	0	0	0
Maximum	**17.26 µg/g**		**0.767 µg/g**		**11.50 µg/g**		**0.836 µg/g**		**0.017 µg/g**		**0.992 µg/g**	

IND number of plant samples; SITE number of study sites.

tebrate toxin lolitrem B. Only 1 % of the plants exceeded toxicity thresholds of the vertebrate toxin ergovaline, with more than 0.30 µg/g, and 5 % of plants with more than 3 µg/g of the invertebrate deterrent/toxin peramine (Table II.1a). Mean concentrations per study site of lolitrem B, ergovaline and peramine, including endophyte-infected and endophyte-free *L. perenne* plants (the population level), on average never exceeded toxicity thresholds (Table II.1b).

Mean alkaloid concentrations of study sites in spring were lower (N = 30; lolitrem B: 0.44 ± 0.1 µg/g, peramine: 0.70 ± 0.1 µg/g) than concentrations in summer (N = 15; lolitrem B: 3.44 ± 0.4 µg/g, peramine: 2.28 ± 0.2 µg/g; Figure II.1c). Region and land-use intensity had no significant effect on alkaloid concentrations of lolitrem B and peramine (Table II.2). Fertilization (N fertilized = 18, N not fertilized = 27; lolitrem B: $F_{1,35} = 0.96$, $p = 0.34$, peramine: $F_{1,35} = 0.20$, $p = 0.66$), grazing (N grazed = 37, N not grazed = 8; lolitrem B: $F_{1,39} = 0.63$, $p = 0.44$, peramine: $F_{1,39} = 0.39$, $p = 0.54$) and additional sowing (N sown = 12, N not sown = 33; lolitrem B: $F_{1,35} = 0.34$, $p = 0.57$, peramine: $F_{1,35} = 0.84$, $p = 0.37$) had also no significant effect on the alkaloid concentrations.

II.4 Discussion

This study shows that *E. festucae* var. *lolii* is widespread in *L. perenne* populations of managed grasslands across Germany. However, endophyte

Table II.2 Effects of season, study region, and land-use intensity (LUI) on alkaloid concentrations of the vertebrate toxin lolitrem B and the invertebrate toxin peramine. Note: For this analysis only the spring and summer surveys of 2015 were taken into account. Significant p-values are highlighted in bold.

		LOLITREM B		PERAMINE	
	df	**F**	**p**	**F**	**p**
SEASON	1,35	45.49	**<0.001**	20.90	**0.002**
REGION	2,35	2.62	0.091	1.19	0.321
LUI	1,35	0.05	0.827	0.09	0.768
SEASON x REGION	2,35	0.89	0.449	2.60	0.135
SEASON x LUI	1,35	4.17	0.076	0.01	0.919
REGION x LUI	2,35	0.26	0.772	0.91	0.415

Data were analysed by a linear mixed-effect model with study site ID as random effect.

infection rates vary on a local scale. It also indicated seasonal variations in the accumulation of the alkaloids lolitrem B and peramine.

Our results suggest that the infection rates and alkaloid concentrations of *E. festucae* var. *lolii* in *L. perenne* are not significantly driven by land-use intensity. In two previous studies, grazing increased endophyte infection rates. In one study, moderate to high grazing pressure increased endophyte infection rates on experimental tall fescue pastures in the USA (Gwinn et al., 1998); in the second study, infection rates of *E. festucae* var. *lolii* in *L. perenne* were higher on grazed than on ungrazed semi-natural permanent grasslands in Denmark (Jensen and Roulund, 2004). An experimental approach, in which grazing was simulated by clipping, showed increased lolitrem B concentrations (Fuchs et al., 2017b). In our study, neither endophyte infection rates nor alkaloid concentrations were affected by grazing. This may be explained by differences in grazing intensities by different grazing species and races, and by low grazing pressure on *L. perenne* in species rich grasslands. In a common garden experiment, 200 kg/ha NPK fertilizer increased alkaloid concentrations in endophyte-infected *L. perenne* (Krauss et al., 2007), while in a laboratory study decreasing alkaloid concentrations were observed when fertilized (Rasmussen et al., 2007). Another field study showed no effects of additional fertilizer on lolitrem B concentrations (Repussard et al., 2014). Our study also detected no effect of fertilization on infection rates or on alkaloid concentrations. We assume that fertilization may significantly change alkaloid concentrations only under specific environmental conditions. For example, fertilization may enhance alkaloid concentrations under drought stress and low nitrogen availability for the host plant.

In several countries, highly *Epichloë* endophyte-infected seeds of *L. perenne* were sown as monocultures and resulted in intoxication of large numbers of livestock (Hume et al., 2016). On average, 34 % of our studied sites had been additionally sown within the last ten years, but infection rates of *E. festucae* var. *lolii* in *L. perenne* were not significantly different from grasslands which were not sown. A possible explanation is that farmers used un-infected grass seeds or have sown seed mixtures (e.g. different species of grasses, clover and other plants) on the grassland sites rather than seeds of one grass species exclusively. Species richness of vascular plants range in average between 16 to 35 species (in 16 m^2) depending on management (Socher et al., 2013). However, on two study sites owned by one farmer who sowed a seed mixture including *L. perenne* in the year prior to our sampling, we detected peak concentrations of lolitrem B and peramine up to eight times higher than the toxicity thresholds. As such high concentrations occurred only in single plants, we assume no overall negative effects for grazing livestock on the studied grasslands.

We also found a seasonal dependence of lolitrem B and peramine concentrations and an increase in alkaloid concentrations during summer, similar to results reported from France and Germany (Fuchs et al., 2017a; Repussard et al., 2014). Our results confirm that the endophyte-grass association depends on environmental conditions (Börschig et al., 2014) and also indicates that infection rates are highly variable on local scale. Overall, the mean infection rates of all tested plants within the three study regions ALB (10 % of 896 plants), HAI (13 % of 829 plants) and SCH (15 % of 1061 plants), are similar to the mean infection rates of other locations in Germany which varied from 6 % in Lower Saxony to 28 % in Mecklenburg-West Pomerania (Dobrindt et al., 2013; Oldenburg, 1997).

We conclude that land-use intensity is not a major driver of *Epichloë* endophyte infection rates in *L. perenne* and alkaloid production in Germany. Moreover, the accumulation of alkaloids and thus the risk of livestock intoxication are likely promoted by rising temperatures and possibly influenced by the changing climate (Fuchs et al., 2017b; Ryan et al., 2015). The endophyte-grass association might profit, for example, from climate change due to enhanced drought tolerance of infected grass (Hesse et al., 2003). We found highly toxic alkaloid concentrations in plants on German grasslands, but at the population level concentrations were below toxicity thresholds. Furthermore, agricultural practices in Germany and Europe rarely include grassland monocultures or species poor seed mixtures (Klaus et al., 2013; Malinowski and Belesky, 2006; Socher et al., 2012). Therefore, we assume that there is, at least for the studied sites, currently no intoxication risk for grazing livestock. The application of endophyte-in-

fected seed material accidently or knowingly can also promote an increase in distribution of *Epichloë* endophyte-infected grasses (Saari et al., 2009; Saari et al., 2010a). If these endophytes are "novel" endophyte strains with low vertebrate toxic alkaloid concentrations it would cause minimal or no health problems to livestock (Woodfield and Easton, 2004). We recommend frequent examinations of seed material and grasslands for *Epichloë* endophyte infections and the alkaloid content to prevent future mass intoxication of livestock, as has been reported from the United States, Australia and New Zealand (Aiken and Strickland, 2013).

II.5 Supplementary Figure

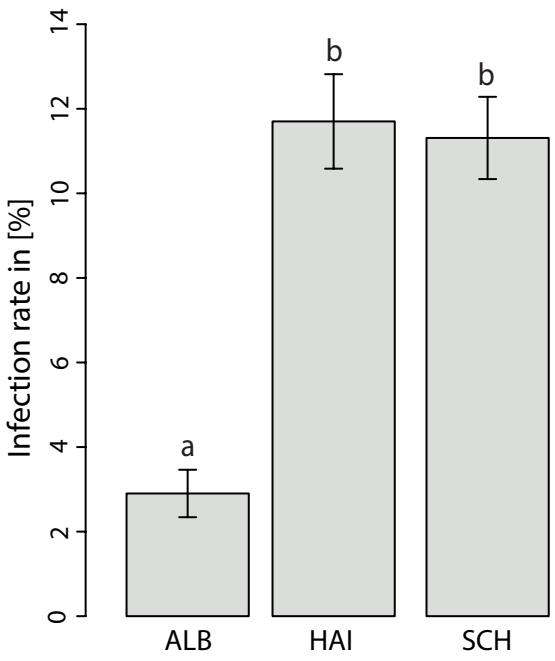

Figure II.S1 Infection rates of *E. festucae* var. *lolii* in *L. perenne,* by study region ($F_{2,81}$ = 4.81, p = 0.011), only considering plants with detectable alkaloid content. Significant levels: * p > 0.05.

Chapter III

Predicting the potential of *Epichloë* endophytes in protecting meadow fescue from herbivory on managed grasslands

The endophytic fungi *Epichloë uncinata* and *E. siegelii* are associates of the cool season grass species *Festuca pratensis* and produce insect toxic/deterring loline alkaloids. However, infection rates in managed European grasslands has been rarely studied and alkaloid concentrations and toxicity against insect herbivores are unknown. We identified the *Epichloë* infections and quantified concentrations of four endophyte-derived alkaloids with UPLC-Tandem-MS of 74 grasslands in three German regions and in two seasons. Our results showed, that endophyte infection rates are high (81 % of 1495 sampled plants) and that infection rates decreased with increasing land-use intensity and fertilization. Infection rates and seasonal accumulation of loline alkaloids differed between regions. Infected plants did not produce endophyte derived alkaloids like lolitrem B, ergovaline and peramine, but produce N-formyl- and N-acetyl-loline. Thereby, loline concentrations were substantially lower (<14 µg/g) than *in planta* levels and toxicity thresholds reported from other studies. We conclude, that intensively managed grasslands can be a disadvantage for endophyte-grass symbioses and that in German grasslands loline concentrations are too low to be effective herbivore repellents.

III.1 Introduction

Grasslands are the most important land-use types in agricultural systems in Europe providing habitats for various species (Bluethgen et al., 2012). In the last decades, intensification of land use changed less fertile grasslands into high productive meadows and pastures, which led to a loss of species diversity and changing species interactions, including symbiotic relationships between plants and fungi or bacteria (Klimek et al., 2007; Tscharntke et al., 2005).

Some of the best known plant-fungus associations are those of cool season grass species, e.g. *Lolium* (ryegrass) and *Festuca* (fescue), with systemic endophytes of the genus *Epichloë*. These endophytes asymptomatically colonize the internal tissue of the above ground plant material and are transmitted horizontally (sexual) by symbiotic flies (*Botanophila*) or vertically (asexual) by the grass seeds (Schardl et al., 2004). The host grass can benefit from an *Epichloë* endophyte infection by an increased stress tolerance and the production of herbivore-defensive alkaloids (Schardl et al., 2013a). Major produced alkaloids are cyclic indole-isoprenoid lolitrems, ergot alkaloids, the pyrrolopyrazine alkaloid peramine and pyrrolizidine lolines. While lolitrems and ergot alkaloids can have detrimental effects on grazing vertebrates, peramine and lolines are deterring or toxic to several insect species (Schardl et al., 2013a).

Lolines occur almost exclusively in grasses, which are associated with *Epichloë* endophytes, e.g. *E. uncinatum*, *E. siegelii*, *E. coenophiala* and *E. festucae*, but were also isolated from few non-grass species, e.g. Convolvulaceae (Craven et al., 2001; Siegel et al., 1990; Steiner et al., 2006; Tofern et al., 1999; Wilkinson et al., 2000). *Epichloë* endophytes produce different derivatives of lolines in above and below ground plant tissue, which are known for their broad spectrum of deterrence and insecticidal activity against different insect species (Bush et al., 1997; Schardl et al., 2007), including several aphid species (Siegel et al., 1990; Wilkinson et al., 2000), root aphids (Schmidt and Guy, 1997), fall armyworm and European corn borer (Riedell et al., 1991), Japanese beetle (Patterson et al., 1991), black beetle and crickets (Barker et al., 2015a; b), as well as Argentine stem weevil (Jensen et al., 2009) and grass grub (Schmidt and Guy, 1997). *Epichloë* endophyte species which produce exclusively loline alkaloids are of economic interest as they protect the plant without possible intoxication of grazing livestock. Several studies have indicated that *Epichloë* endophytes may benefit from land use and have shown increased infection rates and enhanced alkaloid production under intensive management, such as grazing and fertilization (Fuchs et al., 2017b; Gwinn et al.,

1998; Jensen and Roulund, 2004; Krauss et al., 2007), but for *F. pratensis*, *Epichloë* infection rates were also shown to be lower in grazed grassland sites (Saari et al., 2010b). Alkaloid concentrations can also increase with rising temperatures indicating a season-dependence (Fuchs et al., 2017c; Justus et al., 1997; König et al., in press; Repussard et al., 2014; Tong et al., 2006). Abiotic differences between different regions and sites might also affect infection rates and alkaloid concentrations (Müller and Krauss, 2005). Most studies on *F. pratensis* infected with *Epichloë* endophytes are laboratory based (Barker et al., 2015a; Bylin et al., 2014; Dirihan et al., 2015). Other studies considering the effects of land use focused on one management type only (Ahlholm et al., 2002; Saari et al., 2010b). Studies considering loline concentrations of *Epichloë*-infected meadow fescues in natural environments and on real-world grassland ecosystems are rare (Leuchtmann et al., 2000).

The presence of *Epichloë* endophytes in host grass is often verified by immunoblot assays (Koh et al., 2006) or histological staining (Saha et al., 1988), beside uncertainties to detect an *Epichloë* endophyte infection correctly (Dombrowski et al., 2006; Jensen et al., 2011). We therefore used immunoblot assays, histological staining and alkaloid detections combined to compare the methods and to enhance the chance to detect all *Epichloë*-infected samples in this study.

In this field study, we tested the *Epichloë* endophyte infection rates in *Festuca pratensis* in 74 real-world managed grasslands in three German regions. Further, we quantified the concentrations of all common herbivore toxic/deterring alkaloids including lolines *in planta* to predict the toxicity and the potential for pest control. Our predictions are:

- Endophyte infection rates and loline alkaloid concentrations depend on study region and alkaloid concentrations are higher in summer compared to spring
- Endophyte infection rates and loline alkaloid concentrations increase with increasing land-use intensity (including fertilized vs. not fertilized and grazed vs. not grazed grasslands)
- Loline concentrations are above toxicity threshold for insects and vertebrate toxic endophyte-derived alkaloids are low or missing

III.2 Methods and Materials

III.2.1 Study species

We chose meadow fescue, *Festuca pratensis*, as study species, as it is one of the most important forage grasses in mixed grasslands in Europe (Malinowski and Belesky, 2006; Pfannmöller et al., 1997). *Festuca pratensis* was shown to be often highly infected with the vertically transmitted endophyte *Epichloë uncinata* (formerly *Neotyphodium uncinatum*; Leuchtmann et al., 2014), but can also be associated with *Epichloë siegelii* (formerly *Neotyphodium siegelii*; Craven et al., 2001; Leuchtmann et al., 2014). *Epichloë siegelii* was firstly isolated and described from *F. pratensis* plants from a German plant introduction centre, but did not appear in plants grown out from seeds collected at various locations across Europe (Craven et al., 2001). Thus, it is suggested that *E. uncinata* is the predominant endophyte infecting *F. pratensis*. However, both endophytic strains are hybrids possibly sharing a common ancestor (Craven et al., 2001; Saikkonen et al., 2016) and mainly produce the insect toxic/deterring lolines in concentrations up to 1000 times higher compared to other endophyte-derived alkaloids (Schardl et al., 2013a; Siegel and Bush, 1996; Spiering et al., 2005b; Zhang et al., 2009). N-formyl- and N-acetyl-loline accumulate in highest concentrations, often between 1000 µg/g to 4000 µg/g in vegetative shoots and their insecticidal activities have been proven to be comparable to nicotine (Leuchtmann et al., 2000; Riedell et al., 1991; Siegel and Bush, 1996). Additionally, a recent study from Poland indicated the production of the vertebrate toxic alkaloid ergovaline in the association of *Epichloë* endophytes and *F. pratensis* (Zurek et al., 2017).

III.2.2 Study sites

Within the framework of the Biodiversity Exploratories (www.biodiversity-exploratories.de), the study was conducted on 150 grassland sites. They cover three different regions across Germany and represent different landscape types and reflect a climatic gradient of increasing precipitation, rising altitude and slightly decreasing annual mean temperatures from north eastern to south western Germany: The UNESCO Biosphere Reserve Schorfheide-Chorin (Brandenburg, NE Germany, SCH), defined by marshy grasslands and glacially formed landscapes (10-140 m ASL); the National Park Hainich (Thuringia, C Germany, HAI), consisting of calcareous uplands (200-400 m ASL); and the UNESCO Biosphere Reserve Schwäbische Alb (Baden-Württemberg, SW Germany, ALB), a calcareous low mountain range (720-850 m ASL; Fischer et al., 2010a).

All selected study sites are permanent agriculturally used grasslands and represent the full range of land use practices and intensities typical for Germany, from extensively and rarely managed grasslands (e.g. protected calcareous grasslands and wetlands) to highly fertilized and intensively used meadows and pastures. All sites have been classified along a land-use intensity gradient (LUI) integrating into one index the most common practices, such as mowing, grazing, and fertilization (Bluethgen et al., 2012). For testing whether infection rates and alkaloid concentrations depend on land-use intensity we used this index. As fertilization and grazing, can also have independent effects on endophyte-grass symbioses (e.g. Gwinn et al., 1998; Krauss et al., 2007), we also tested these two components separately. European-bred grass cultivars can occasionally contain endophyte-infected seeds (Saari et al., 2009; Saikkonen et al., 2000). However, the studied grassland sites in the regions ALB, HAI and SCH were seldom sown with additional seeds and none of the used seed mixtures contained *F. pratensis* (data received from grassland farmers and owners).

III.2.3 Plant sampling

We sampled individual plants of *F. pratensis* in two field surveys from April to June (spring) and July to August in 2015 (summer), as previous studies have shown variable loline concentrations between seasons (Tong et al., 2006) and higher alkaloid concentrations in summer compared to spring were recorded for the *Lolium perenne* and *Epichloë festucae* var. *lolii* association (Fuchs et al., 2017c; König et al., in press; Repussard et al., 2014). We found 74 study sites of the 150 grasslands with *F. pratensis* populations and randomly collected *F. pratensis* plants at different locations on the sites in each survey. A minimum distance of 1 m between sampled plants was kept, to reduce the probability of sampling the same plant twice. In total, 1612 individual *F. pratensis* plants were sampled, with a minimum of ten and maximum of 41 individuals per study site. The number of sampled plants differed due to recent mowing or grazing events and variable population sizes of the host grass *F. pratensis* on the sampled study sites.

As mycelia and alkaloids of the *Epichloë* endophytes mainly accumulate in basal leaf sheaths of the grasses (Justus et al., 1997; Spiering et al., 2005a), we cut approximately 3 cm of three grass tillers per individual plant, including basal stems and leaf sheaths, near the ground (König et al., in press). For ~5 % of the sampled plants, we collected less than three tillers, as plants were just starting to grow after e.g. a mowing/grazing event or largely withered at the time of sampling.

The tillers of each sample were stored in 2.5 ml reaction tubes and were cooled during field survey with dry ice. Afterwards samples were stored at -20°C, to prevent degradation of alkaloids in endophyte-infected plants.

III.2.4 Endophyte detection and alkaloid analysis

For endophyte detection three tillers of each plant sample were stained with lacto-phenolic blue and identified by microscopic examination (Clark et al., 1983). One tiller per plant was randomly chosen to be additionally screened for endophytes by using a commercially available kit for immunoblot assays (www.agrinostics.com), following the manufacturer's protocol.

We quantified alkaloid content of the most commonly distributed alkaloids in endophyte-grass symbioses. These alkaloids were the vertebrate toxic compounds lolitrem B and ergovaline, as well as the invertebrate toxic/deterring compounds peramine, N-formyl- and N-acetyl-loline (Schardl et al., 2013a). The three tillers of each plant were pooled into one sample for the alkaloid detection. Plants were ground in liquid nitrogen and alkaloids were extracted with methanol and dichloromethane.

To detect the alkaloids, we used Ultra High Performance Liquid Chromatography-Tandem-Mass Spectrometry (UPLC-Tandem-MS), following the protocol published by Fuchs et al. (2013). *Epichloë* endophytes in meadow fescues mainly produce loline alkaloids (Schardl et al., 2013a). As we had not analysed these alkaloids before, we expanded and changed our UPLC-Tandem-MS protocol in few points.

UPLC-Tandem-MS analysis was carried out using a Waters Acquity BEH HILIC column (2.1 x 50 mm, 1.7 µm particle size, with a 5 x 2.1 mm guard column) and a column temperature of 40°C. The injection volume was 2.5 µl per sample and the alkaloids were separated using a mobile phase consisting of 0.1 % formic acid (solvent A) and acetonitrile at a flow rate of 0.2 ml/min. A gradient elution was performed starting from 1 % A to 60 % solvent A within 5 min. Alkaloids were detected by multiple reaction monitoring (MRM), instrument parameters for ionization and collision induced dissociation (CID) with argon as collision gas were determined by flow injection analysis of N-formyl- and N-acetyl-loline. The ESI source was operated in the positive electrospray mode at a temperature of 120°C, a capillary voltage of 3.0 kV and a cone voltage of 30 V. Desolvation of the mobile phase was carried out using 800 l/h nitrogen at a temperature of 350°C and 25 l/h as cone gas. For each compound, two specific fragments were monitored at a collision energy of 20 eV and a dwell time of

25 ms per MRM transition (N-formyl-loline: m/z 183 > 112, m/z 183 > 155; N-acetyl-loline: m/z 197 > 112, m/z 197 > 155). Semi-quantitative amounts of endophyte-derived alkaloids were calculated using Homoperamine and Ergotamine as internal standards.

For 117 of the 1612 *F. pratensis* plants, the collected plant material was not adequate to process the three detection methods, resulting in 1495 plants which were analysed by histological staining, immunoblot assays and UPLC-Tandem-MS.

III.2.5 Statistical analysis

All statistical analyses were conducted using the software R version 3.1.1 (R Development Core Team, 2014). We first tested the effect of region, land-use intensity (LUI) and their interaction on endophyte infection rate using generalized linear models (GLM) with binomial distribution and logit link function (Crawley, 2013). To correct for over dispersion (residuals/degree of freedom > 1) quasi GLM was used. Model selection was performed backward by using F-test.

In a second model, we tested the effect of season, region, LUI and their interactions on alkaloid concentrations (N-formyl- and N-acetyl-loline) using linear mixed effect models (LME, nlme package; Pinheiro et al., 2016). Alkaloid concentrations were log10 transformed to improve normality and homoscedasticity of residuals. In separate analyses, the continuous explanatory variable LUI was replaced in both models by one of the two categorical explanatory variables; fertilization (no/yes) or grazing (no/yes). Study site ID was included in all models as random effect (random intercept models).

To compare infection rates and alkaloid concentrations between regions, fertilized/not fertilized sites and season, we performed Tukey‘s HSD comparisons of groups in mixed effects models (GLHT, multcomp package; Hothorn et al., 2008). Mean ± SE are represented throughout the manuscript, if not otherwise specified.

III.3 Results

III.3.1 *Epichloë* endophyte detection methods

Epichloë infection rates differed depending on the used detection method. The lowest infection rates were found for immunoblot assays of one tiller with 51 % (762 samples), followed by staining of three tillers with 60 %

Table III.1 *Epichloë* endophyte infections of *Festuca pratensis* comparing three different detection methods.

Staining	Immunoblot assays	UPLC-Tandem-MS	No. of samples	No. of samples [%]
3/3 tiller 60 % infected	1/3 tiller 51 % infected	3/3 tiller 79 % infected	All 1495	Total 81 % infected
1	1	1	551	36.9%
1	1	0	8	0.5%
0	1	0	7	0.5%
0	0	0	284	19.0%
0	0	1	112	7.5%
1	0	1	320	21.4%
1	0	0	17	1.1%
0	1	1	196	13.1%

First three columns 0 not detected with the method, 1 detected with the method.

(896 samples). The UPLC-Tandem-MS recorded specific alkaloids (lolines) from the *Epichloë* endophyte-grass associations in 79 % (1179 samples). Only in 36.9 % of the 1495 samples all three methods detected an endophyte infection in parallel. A further 34.5 % were detected with UPLC-Tandem-MS and one further method. In 19.0 % none of the three methods showed an endophyte infection (Table III.1). Assuming no false positive detections of *Epichloë* endophytes with the three methods, we considered all plants as endophyte-infected with one positive infection (81 %) and all as endophyte-free which showed no infection with all three methods (19 %).

III.3.2 Infection rates of *Epichloë* endophytes in *Festuca pratensis*

On 74 (49 %) of the 150 grassland study sites, the grass species *F. pratensis* was recorded in 2015. Populations of *F. pratensis* were not observed on semi-natural grasslands such as calcareous meadows in the regions ALB and HAI and wetlands in the region SCH. Infections of the *F. pratensis* population with the *Epichloë* endophytes were detected on 70 (96 %) of the 74 sampled grassland study sites combining all methods. In total, 1211 (81 %) of the 1495 individual grass samples were infected with *Epichloë* endophytes. Infection rates of the grassland sites with an endophyte infection ranged from 5 % to 100 %.

Epichloë infection rates in *F. pratensis* were different between the regions (Table III.2), with highest infection rates in the most southern region ALB (mean: 95 %, range: 10 %–100 %, N = 29), intermediate infection rates in

the north-eastern region SCH (mean: 77 %, range: 29 %–100 %, N = 10) and lowest in the region HAI (mean: 69 %, range: 5 %–100 %, N = 35; Figure III.1 a).

Table III.2 Effects of study region and (a) land-use intensity (LUI) or (b) fertilization (no/yes) or (c) grazing (no/yes) on infection rates of *Festuca pratensis* with *Epichloë* endophytes. Significant p-values highlighted in bold.

	Infections		
	df	**F**	**p**
REGION	2, 71	8.12	**0.001**
LUI	1, 70	14.80	**< 0.001**
REGION x LUI	2, 68	0.77	0.466
REGION	2, 71	8.17	**< 0.001**
FERTILIZATION	1, 70	14.40	**< 0.001**
REGION x FERTILIZATION	1, 69	0.01	0.915
REGION	2, 71	6.81	**< 0.001**
GRAZING	1, 70	0.00	0.954
REGION x GRAZING	2, 68	3.94	**0.024**

Data were analysed using generalized linear model.

In contrast to our hypothesis, *Epichloë* infection rates decreased with increasing land-use intensity (Table III.2, Figure III.1 b). *Epichloë* infection rates were higher in not fertilized (mean: 86 %, N = 48) compared to fertilized grassland sites (mean: 70 %, N = 26; Figure III.1 c). The interaction term between region and grazing was significant (Table III.2). Thereby infection rates in region ALB follows our hypothesis and is higher on grazed (mean: 99 %, N = 17) compared to not grazed grassland sites (mean: 87 %, N = 12). In the region SCH, infection rates are higher on not grazed (mean: 95 %, N = 5) compared to grazed grassland sites (mean: 61 %, N = 5), while infection rates in HAI were not different (grazed mean: 69 %, N = 30; not grazed mean: 69 %, N = 5; Table III.2).

III.3.3 Concentrations of N-formyl- and N-acetyl-loline

Alkaloid contents were detected in 1179 of the 1211 endophyte-infected samples. The alkaloids lolitrem B, ergovaline and peramine were not detected in any of the analysed *F. pratensis* plants. N-formyl-loline concentra-

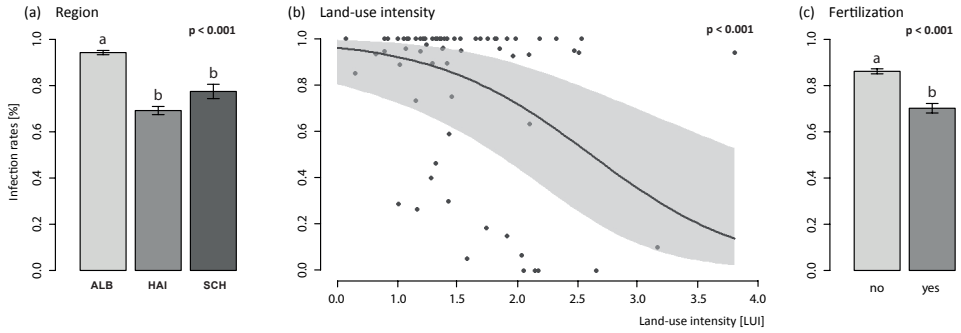

Figure III.1 Infection rates of *Epichloë* endophytes in *Festuca pratensis* depending on (a) study region (ALB, HAI, SCH), (b) land-use intensity (LUI) and (c) fertilization (no/yes). Mean ± SE are shown for bar plots, grey area in b is the 95 % confidence interval. Different letters above bars show significant difference with Tukey HSD.

tions were up to 13.88 µg/g and N-acetyl-loline concentrations up to 9.70 µg/g, mean concentrations were 0.57 ± 0.04 µg/g for N-formyl-loline and 0.39 ± 0.03 µg/g for N-acetyl-loline. Mean loline concentrations per study site, including also the endophyte-free *F. pratensis* plants (the population level), further decreased the average concentrations (N-formyl-loline: 0.46 ± 0.03 µg/g; N-acetyl-loline: 0.31 ± 0.02 µg/g) and ranged from 0.03 ±

Table III.3 Effects of season and study region and (a) land-use intensity (LUI) or (b) fertilization (no/yes) or (c) grazing (no/yes) on alkaloid concentrations of N-formyl- and N-acetyl-lolines of *Epichloë* endophytes in *Festuca pratensis*.

		N-Formyl-Loline		N-Acetyl-Loline	
	df	**F**	**p**	**F**	**p**
SEASON	1, 20	2.46	0.132	0.51	0.483
REGION	2, 65	0.28	0.757	3.29	**0.044**
LUI	1, 65	1.78	0.187	2.17	0.146
SEASON x REGION	2, 20	33.00	**< 0.001**	27.89	**< 0.001**
SEASON	1, 20	2.48	0.131	0.52	0.479
REGION	2, 65	0.28	0.755	3.37	**0.041**
FERTILIZATION	1, 65	2.78	0.101	4.32	**0.042**
SEASON x REGION	2, 20	33.14	**< 0.001**	28.17	**<0.001**
SEASON	1, 20	2.49	0.130	0.52	0.478
REGION	2, 65	0.28	0.754	3.39	**0.040**
GRAZING	1, 65	0.37	0.544	1.25	0.268
SEASON x REGION	2, 20	34.62	**< 0.001**	30.15	**< 0.001**

Data were analysed using linear mixed-effect model with study site ID as random effect.

0.03 to 3.17 ± 0.83 µg/g N-formyl-loline and 0.01 ± 0.01 to 2.89 ± 0.67 µg/g N-acetyl-loline.

Neither land-use intensity, nor grazing had a significant effect on concentrations of loline alkaloids (Table III.3). Loline concentrations were lower on fertilized (N-formyl-loline: 0.33 ± 0.03 µg/g, N-acetyl-loline: 0.24 ± 0.02 µg/g, N = 29) than on not fertilized grassland sites (N-formyl-loline: 0.67 ± 0.05 µg/g, N-acetyl-loline: 0.45 ± 0.03 µg/g, N = 54) and differed between the regions ALB (N-formyl-loline: 0.38 ± 0.03 µg/g, N-acetyl-loline: 0.31 ± 0.02 µg/g, N = 36), HAI (N-formyl-loline: 0.83 ± 0.08 µg/g, N-acetyl-loline: 0.53 ± 0.06 µg/g, N = 36) and SCH (N-formyl-loline: 0.50 ± 0.04 µg/g, N-acetyl-loline: 0.25 ± 0.03 µg/g, N = 11; Table III.3). The interaction term between season and region is significant for both lolines. Thereby loline concentrations in region HAI follows our hypothesis and is higher in summer (N-formyl-loline: 2.31 ± 0.23 µg/g, N-acetyl-loline: 1.46 ± 0.17 µg/g, N = 10) compared to spring (N-formyl-loline: 0.28 ± 0.05 µg/g, N-acetyl-loline: 0.19 ± 0.03 µg/g, N = 26). However, in ALB and SCH, loline concentrations are higher in spring (ALB N-formyl-loline: 0.67 ± 0.06 µg/g,

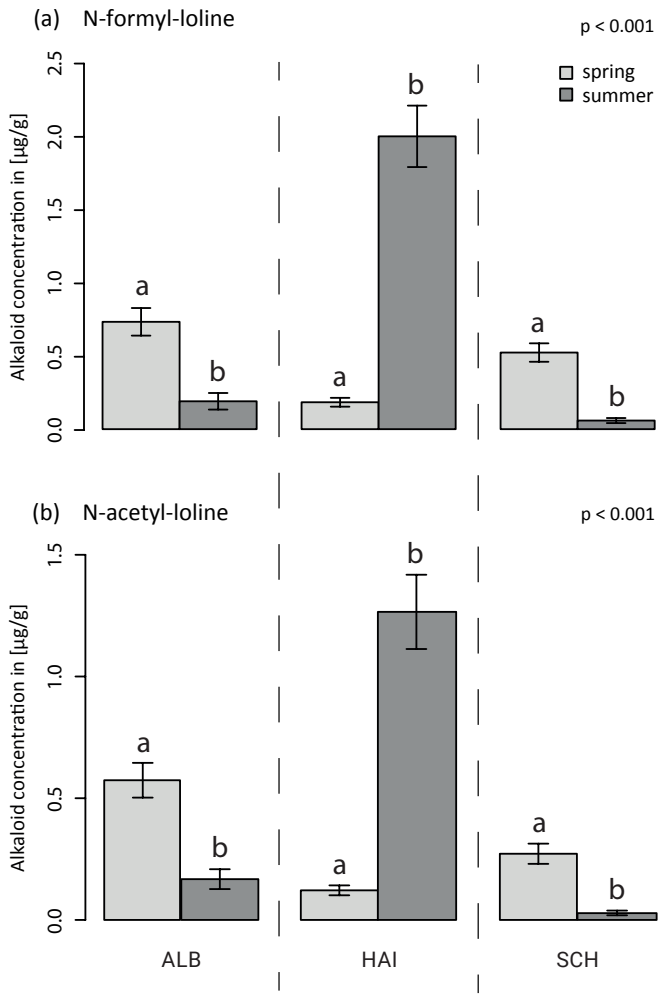

Figure III.2 Alkaloid concentrations of (a) N-formyl-loline and (b) N-acetyl-loline, in dependence of region ALB, HAI and SCH, and season spring (light grey) and summer (dark grey). Mean ± SE are shown. Different letters above bars show significant difference with Tukey HSD.

N-acetyl-loline: 0.51 ± 0.04 µg/g, N = 16; SCH N-formyl-loline: 0.58 ± 0.05 µg/g, N-acetyl-loline: 0.30 ± 0.03 µg/g, N = 6) compared to summer (ALB N-formyl-loline: 0.14 ± 0.01 µg/g, N-acetyl-loline: 0.13 ± 0.01 µg/g, N = 20; SCH N-formyl-loline: 0.09 ± 0.02 µg/g, N-acetyl-loline: 0.04 ± 0.01 µg/g, N = 5; Table III.3, Figure III.2).

III.4 Discussion

Our study shows that *Festuca pratensis* population of managed grasslands in Germany are often highly infected with *Epichloë* endophytes. Thereby infection rates vary on a regional scale and in contrast to our hypothesis decrease with land use. Our results also show contradictory results for seasonal loline concentrations across the study regions.

III.4.1 Comparison of *Epichloë* endophyte detection methods

Epichloë infection rates of *F. pratensis* depend on the method of endophyte detection. In general, alkaloid concentrations correlate with the presence of the endophyte mycelia (Spiering et al., 2005a). Moreover, loline alkaloids can occur in grass species, as far as we know, exclusively if an *Epichloë* endophyte is associated (Wilkinson et al., 2000) and thus, the detection of alkaloids by UPLC-Tandem-MS was the best method to verify the *Epichloë* occurrence in our study. Only 4 % showed a positive *Epichloë* endophyte infection with immunoblots or histological staining, but not with the UPLC-Tandem-MS detection of the alkaloids. This can be explained by the lack in the genes for alkaloid production (Schardl et al., 2013b) or that alkaloid concentrations were below the detection limit of the UPLC-Tandem-MS. A lower detection probability of *Epichloë* endophytes using immunoblot assays can be explained by our method, as we only used one of the tree sampled tiller for the immunoblots, reducing the chance to find the infection as hyphal growth of *Epichloë* endophytes can vary in plant tissue and may not always occur in all tillers of a plant (Spiering et al., 2005a). *Epichloë* infection rates increased up to 15 % when three instead of one tiller were tested with immunoblot assays in *Epichloë-F. pratensis* associations (own, unpublished data). Even though we stained all three tillers, the mycelia of *Epichloë* endophytes can have low abundances which can be easily overlooked by microscopic examination (Dombrowski et al., 2006; Groppe and Boller, 1997). We decided in this study to consider all positive detections as *Epichloë* endophyte-infected, but false positives are occasionally also described and criticised, e.g. for immunoblot assays

(Jensen et al., 2011), or wrong identifications after histological staining (Dombrowski et al., 2006; Takenaka, 1995) or even unknown producers of lolines.

III.4.2 Effects of region and season

In comparison to *Epichloë* infection rates in *L. perenne* (13 %) from the same grassland sites (König et al., in press), *Epichloë* infection rates in *F. pratensis* (81 %) were higher and are similar to infection rates of seeds and plants of wild and cultivated meadow fescue from other European countries (Craven et al., 2001; Leyronas and Raynal, 2001), e.g. from different populations of Finland (range: 30-73 %; Saikkonen et al., 2000), seeds collected in Italy (mean: 72 %; Latch et al., 1987), and plants from different provinces in Poland (range: 8-90 %; Panka, 2011; Panka et al., 2013). Infections rates, as well as loline concentrations, differed between the studied regions and our results confirm that *Epichloë*-grass associations depend on environmental conditions (Börschig et al., 2014; König et al., in press). Moreover, loline alkaloids were affected by season, but in contrast to our hypothesis this was also region dependent. Concentrations of both loline alkaloids were higher in summer compared to spring in one region, while they were *vice versa* in the other two regions. Lolines have been shown to increase from spring to the maturation and again in vegetative growth in late summer (Justus et al., 1997). Additionally, foliage damage due to herbivores, can induce strong anti-herbivory response especially in younger tissue lacking of physical defence in *Epichloë*-grass associations (Saari et al., 2010b). Thus, loline production and concentration of N-formyl- and N-acetyl-loline may vary with time of sampling in our study and might be influenced by regional/local environmental conditions as well as herbivore pressure.

III.4.3 Effects of land-use intensity, fertilization and grazing

In contrast to our hypothesis and several other studies (e.g. Gwinn et al., 1998), *Epichloë* endophytes in *F. pratensis* did not benefit from land use and our results showed decreasing infection rates with increasing land-use intensity. As the used land-use intensity index (LUI) integrates common management practices including fertilization, the effects of land-use intensity also correlate with the effects of fertilization in our analysis. *Epichloë* infection rates, as well as loline concentrations, were higher on not fertilized grassland sites in the studied regions. Under high nitrogen supply, similar decreases were shown for endophyte and alkaloid concentrations in *L. perenne* under laboratory conditions (Rasmussen et al., 2007). In

contrast, alkaloid concentrations increased with intensive fertilization in two other studies (Krauss et al., 2007; Lane et al., 1997). Alkaloids of *Epichloë-L. perenne* associations were not affected by fertilization in field studies from France and Germany (König et al., in press; Repussard et al., 2014). Grass species tolerate *Epichloë* endophytes, when benefits, e.g. nutrient uptake, outweigh costs (Saikkonen et al., 2006). Thus, the benefit of an infection with *Epichloë* endophytes might be low when the host grass grows under optimal conditions, e.g. when fertilized (Rasmussen et al., 2007). This could explain low infection rates and lower alkaloid concentrations in our study.

Effects of grazing on *Epichloë* infection rates depended on the studied regions. Infection rates in the region ALB showed higher rates on grazed grassland sites. This result correlates with increased infection rates in tall fescue (*F. arundinaceae*) pastures in USA due to moderate to high grazing pressure (Gwinn et al., 1998) and with higher endophyte infection rates in *L. perenne* plants in grazed compared to not grazed permanent grasslands in Denmark (Jensen and Roulund, 2004). In contrast and similar to a study on *Epichloë*-infected *F. pratensis* in successional pastures in Finland (Saari et al., 2010b), *Epichloë* infection rates were lower under grazing pressure in the region SCH. In parallel to a study on the association of *E. festucae* var. *lolii* and *L. perenne* in the same study regions (König et al., in press), grazing had no effect on infection rates of *Epichloë* endophytes in *F. pratensis* in the region HAI. We conclude, that *Epichloë* infection rates strongly depend on the regional and local environment and probably differ depending on the *Epichloë*-grass symbiosis.

Loline concentrations were not significantly affected by grazing in our study. There is evidence that wounding (e.g. by clipping) of the grass can induce alkaloid production in *Epichloë*-grass associations (Fuchs et al., 2017b; Schardl et al., 2007). Loline concentrations of *Epichloë*-infected *F. arundinaceae* and *F. pratensis*, were increased in regrown plant tissue, increasing the herbivore resistance compared to un-infected plants (Bultman et al., 2004; Craven et al., 2001). Thus, defensive response of *Epichloë*-grass associations can enhance the production of insect toxic/deterring alkaloids, such as lolines, under herbivore pressure. Decreased loline concentrations were caused by high concentrations of the aphid induced phytohormone salicylic acid (Bastias et al., 2018) and indicate that herbivores may also induce the plants immune response which interacts with biology of the *Epichloë* endophytes (Bastias et al., 2017). Loline concentrations are often higher compared to concentrations of other *Epichloë* endophyte-derived alkaloids such as lolitrem B, peramine and ergovaline (Siegel and Bush, 1996). The highest concentrations of N-formyl- and N-acetyl-loline in our

study were comparably low and below toxicity levels (50 µg/g) for larvae of a rice leaf bug in a feeding experiment (Shiba and Sugawara, 2009). Similarly, loline concentrations above 100 µg/g are necessary to observe toxicity in Argentine stem weevil (Jensen et al., 2009). *In planta,* higher loline concentrations are common with concentrations up to 5500 µg/g detected in cultivars and *Epichloë*-infected breeding lines of *F. pratensis* from European studies (Bylin et al., 2014; Leuchtmann et al., 2000). We assume, that herbivory pressure can play a key role in loline alkaloid accumulation, but in general might be below levels which induce alkaloid production in *Epichloë*-*F. pratensis* associations on the studied grassland sites, explaining the rather low loline concentrations in our study.

III.5 Conclusion

We conclude, that (1) *Epichloë* endophyte detection methods need to be improved. One possibility is to detect some of the *Epichloë* endophyte-derived alkaloids. (2) The *Epichloë* endophyte-*F. pratensis* symbioses from German grasslands produce loline derivates, but not the alkaloids lolitrem B, ergovaline and peramine. Thereby the concentrations of N-formyl- and N-acetyl-loline are surprisingly low (< 14 µg/g) and have not the potential to be effective herbivore repellents. (3) *Epichloë* infection rates were 81 % of all sampled *F. pratensis* plants. In contrast to our hypothesis, increasing land-use intensity and fertilization decreased *Epichloë* infection rates, which might be explained by low herbivory rates on German grasslands and costs of *Epichloë* endophyte infection for the host grass. (4) Study region played an important role for infection rates and alkaloid accumulations between seasons. We suggest a regular monitoring of *Epichloë* infections and alkaloid concentrations in European grasslands, to detect changes due to climate change and shifts in grassland communities, perhaps due to changes of management practices.

Due to contamination of the loline standards the quantification of the loline contents is not reliable. This information was available after the manuscript and thesis was finalised. Further studies with new and clean loline standards are needed to verify the results. At the current stage the uncertainties are too high to trust any measured loline concentrations mentioned in the text. The results and interpretation of loline quantifications therefore must remain preliminary. Nonetheless, the occurrence of lolines could be detected with high probability.

Chapter IV

The effects of *Epichloë* endophytes on foliar fungal assemblages in perennial ryegrass in dependence of season and land-use intensity

Epichloë endophytes associated with cool season grass species can protect their hosts from herbivory and can suppress mycorrhizal colonization of the hosts' roots. However, little is known about whether or not *Epichloë* endophyte infection can also change the foliar fungal assemblages of the host. We tested 52 grassland study sites along a land-use intensity gradient in three study regions over two seasons (spring vs. summer) to determine whether *Epichloë* infection of the host grass *Lolium perenne* changes the fungal community structure in leaves. Foliar fungal communities were assessed by Next Generation Sequencing of the ITS rRNA gene region. Fungal community structure was strongly affected by study region and season in our study, while land-use intensity and infection with *Epichloë* endophytes had no significant effects. We conclude that effects on non-systemic endophytes resulting from land use practices and *Epichloë* infection reported in other studies were masked by local and seasonal variability in this study's grassland sites.

IV.1 Introduction

Fungi include ubiquitous and highly diverse microbial symbionts associated with a large number of plant species in all terrestrial ecosystems (Dix and Webster, 1995). Such associations can have profound effects on ecosystems (Bundrett, 2006; Clay and Holah, 1999; Omacini et al., 2001; van der Heijden et al., 1998). Besides mycorrhizae, some of the best known plant-fungus interactions are the symbioses of endophytes of the genus *Epichloë* (Ascomycota, Clavicipitaceae) with cool-season grass species in the family Poaceae (Schardl et al., 2004; Tanaka et al., 2012). *Epichloë* endophytes systemically colonize above ground tissues of the host grass. Asexual *Epichloë* species are vertically transmitted through the seeds and provide several benefits to their hosts, like herbivore resistance and enhanced fitness (Schardl et al., 2004). Sexual species of *Epichloë* endophytes produce spores which are transmitted horizontally by symbiotic flies of the genus *Botanophila* and suppress the hosts' seed development (Schardl et al., 2004). Depending on abiotic and biotic conditions, the asexual *Epichloë* endophyte-grass association can shift from a mutualistic symbiosis to an antagonistic symbiosis, e.g when herbivore pressure is low and nitrogen availability for the host is limited (Müller and Krauss, 2005; Saikkonen et al., 1998).

All grass species are associated with a large number of different fungal endophytes and often harbour more than one hundred species which colonize roots, stems and leaves of the plants (Sánchez Márquez et al., 2007). Therefore the systemic *Epichloë* endophytes represent only a small fraction of a diverse fungal community in grass species (Neubert et al., 2006). In contrast to *Epichloë* endophytes, most other endophytic fungi have a limited capacity to systemically colonize the plant organs or seeds (Rodriguez et al., 2009). In addition, the diverse endophytic fungi have unequal colonization frequencies with a few dominant genera, such as *Alternaria, Acremonium, Cladosporium* and *Penicillium*, which occur in multiple grass species, as well as in non-grass hosts (Sánchez Márquez et al., 2012).

Diverse foliar fungal endophytes of plants are influenced by several abiotic and biotic factors which may compromise the host species' ability to colonize, persist and disperse (Rodriguez et al., 2009). The fungal assemblages of grass leaves and frequencies of endophyte species change with spatial distance and season (Sánchez Márquez et al., 2012). As these fungal species vary in their dispersal ability, dissimilarities between fungal assemblages increase with distance (David et al., 2016). Depending on the prevailing microclimate, variability can be high at small spatial scales, e.g. between individual leaves of a single tree, as well as between individual

plants or different plant species (Cordier et al., 2012; Peršoh, 2015; Rodriguez et al., 2009; Scholtysik et al., 2013; Stone et al., 2004).

Several grass species, such as *Lolium perenne*, are of high agronomic importance and are part of a regular food supply for livestock (Malinowski and Belesky, 2006). Such grass species and their interacting symbionts can be influenced by management of grasslands. For example, fertilization and grazing can influence the availability of nutrients for host plants and vegetation structure respectively, and have been shown to change individual fungal abundances, species richness and the microbial community structure of fungal communities in soil (Donnison et al., 2000; Parrent et al., 2006; Soliveres et al., 2016; Valyi et al., 2015). Thus, land-use intensity may also determine foliar fungal assemblages of meadow or pasture grasses.

The interactions between species within fungal communities are little understood and may include direct and/or indirect competition for plant resources (Saunders et al., 2010; Suryanarayanan, 2013). The systemic *Epichloë* endophytes produce chemical compounds which inhibit growth of pathogenic fungi and generate shifts in below ground subsystems by suppressing the root colonization of mycorrhizal fungi (Clarke et al., 2006; Kumar and Kaushik, 2012; Mack and Rudgers, 2008; Omacini et al., 2012; Siegel and Latch, 1991; Vandegrift et al., 2015; Yue et al., 2000). Thus, *Epichloë* endophytes may change the species composition of non-systemic fungal endophytes in grass leaves as well. In this field study, we ask whether or not the presence of systemic grass endophytes of the genus *Epichloë* changes the species composition of foliar fungal assemblages in a host grass along a land-use intensity gradient in two seasons (spring and summer) and in three geographic regions.

IV.2 Methods and Materials

IV.2.1 Study sites

The study was conducted on 150 grassland sites within the framework of the Biodiversity Exploratories (www.biodiversity-exploratories.de), which includes three distinct regions across Germany. The three study regions; Schwäbische Alb (south-west Germany, ALB), Hainich-Dün (central Germany, HAI) and Schorfheide-Chorin (north-east Germany, SCH), represent different climatic conditions, soil types, landscapes and land-use types, as well as different management intensities (Fischer et al., 2010a). All se-

lected study sites are real-world grasslands and are not experimental plots (Fischer et al., 2010a). Rather, they are grasslands used by the owners or farmers to meet their needs without artificial changes by researchers (Fischer et al., 2010a). Some owners' management strategies have included sowing grasslands with commercial seed mixtures within the last ten years. Such real-world study systems are necessary to show how ecosystems work, but bear the risk of lower replicability compared to controlled laboratory experiments. The grasslands are classified along a land-use intensity gradient (LUI), which integrates the most common practices such as mowing, grazing, and fertilization, into one index, comprising values from zero (extensive) to four (intensive; Bluethgen et al., 2012). Intensively managed grasslands are fertilized, grazed by livestock several times during the year and/or mown repeatedly. Extensively managed grasslands, such as semi-natural grasslands, including protected calcareous grasslands and wetlands, are not fertilized and are mown only once and/or grazed for only a short time (Bluethgen et al., 2012). For this study we used the LUI calculated for the management in 2014, one year before our sampling in 2015.

Field work permits were issued by the responsible state environmental offices of Baden-Württemberg, Thüringen, and Brandenburg (according to § 72 BbgNatSchG).

IV.2.2 Plant sampling

The perennial ryegrass *Lolium perenne* was selected as the study species, as it is an important forage grass which is commonly associated with the vertically transmitted endophyte *Epichloë festucae* var. *lolii* (formerly *Neotyphodium lolii*; Klapp and Opitz von Boberfeld, 2013; Leuchtmann et al., 2014). Samples of *L. perenne* were collected in all three study regions in spring and summer surveys in 2015. In total, 80 sites within the 150 grasslands contained *L. perenne* populations and were sampled. In each survey, we sampled up to 20 *L. perenne* plants randomly at different locations at each study site, with a minimum distance of 1 m between sampled plants to reduce the probability of sampling the same plant twice. The number of sampled plants per study site differed depending on the population size of *L. perenne* and on recent mowing and/or grazing events. Overall, 2147 plants were sampled.

Approximately 3 cm of one grass tiller from each plant was collected, and included basal stem, leaf sheaths, and basal leaf blades (König et al., in press). The mycelia of *Epichloë* endophytes accumulate mainly in basal leaf sheaths of the grasses (Spiering et al., 2005a). The collected basal stem and leaf sheaths of the tillers were therefore used to detect *Epichloë* infections using immunob-

lot assays (Figure IV.1 a). As leaf blades contain a high diversity of fungal endophytes (Sánchez Márquez et al., 2007; 2008; 2010), foliar fungal assemblages of one *Epichloë*-infected and one *Epichloë*-free grass individual per study site and season were assessed in the basal leaf blades (Figure IV.1 b). The sampled plant material was stored separately for each individual in 2.5 ml Eppendorf reaction tubes. During the field survey, all samples were immediately cooled with dry ice. To prevent degradation of the fungal DNA, plant samples were stored afterwards at -20°C (Millberg et al., 2015; Peay et al., 2013).

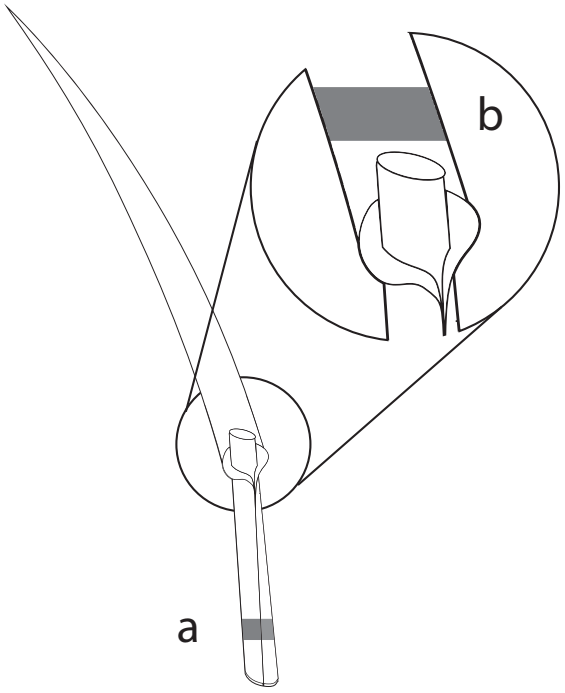

Figure IV.1 Material from the same grass tiller of each sampled *L. perenne* plant was used for (a) detection of *Epichloë* endophyte by immunoblot assays, using parts of the basal stem and leaf sheaths; and (b) analyses of the foliar fungal assemblages by NGS, using parts of the basal leaf blades.

IV.2.3 *Epichloë* endophyte detection

To detect *Epichloë* endophytes in the basal stem and leaf sheaths, a commercially available kit for immunoblot assays was used, following the manufacturer's protocol (www.agrinostics.com). In total, 270 (12.6 %) of 2147 sampled *L. perenne* plants were infected with an *Epichloë* endophyte. To compare fungal assemblages of *Epichloë*-infected and *Epichloë*-free samples, 52 sites with *Lolium perenne* which contained both infected and un-infected individuals were chosen from the 80 grassland sites. Depending on recent mowing or grazing events, 21 of the 52 grasslands were sampled exclusively in spring and 15 grasslands exclusively in summer, while 16 grasslands could be sampled in both seasons. As 43 % of grasslands contained less than three *Epichloë*-infected plant individuals, one immuno-positive (*Epichloë*-infected, E+) and one immuno-negative (*Epichloë*-free, E-) plant sample for each grassland site and season was randomly selected, resulting in a total of 68 E+ and 68 E- samples.

IV.2.4 Foliar fungal assemblages

For analyses of the foliar fungal assemblages by Next Generation Sequencing (NGS), 68 E+ and 68 E- leaf blades were used. In order to detect the complete foliar (i.e. epi- and endophytic) fungal assemblage on the grass leaves the leaf blades were not surface sterilized.

IV.2.4.1 DNA extraction

The ChargeSwitch® gDNA Plant Kit (Invitrogen™) was used to extract DNA as recommended by the manufacturer, but with volumes scaled down to 10 %. Cell disruption was achieved using a FastPrep®-24 Instrument (MP Biomedicals) as detailed by Guerreiro et al. (2018).

IV.2.4.2 Library preparation and sequencing

The fungal barcoding region, i.e., the ITS rRNA gene region, was amplified as detailed by Guerreiro et al. (2018). Briefly, library preparation comprised two sequential amplification steps. In the first PCR, the fungus specific primers ITS1F and ITS4 were used and modified at the 5'-ends to include sample-specific TAG sequences. In the second PCR, the sequencing primers, indices, and the P5 and P7 adapters for the Illumina sequencing were appended. Libraries were processed by the sequencing service of the Faculty of Biology at LMU Munich, and sequenced using an Illumina MiSeq® sequencer (Illumina, Inc., San Diego, CA, USA) with 2 × 250 bp paired end sequencing (MiSeq Reagent Kit v3 Chemistry, Illumina, Inc., San Diego, CA, USA).

IV.2.4.3 Processing of sequencing data

The obtained sequence reads were processed as detailed by Guerreiro et al. (2018). Briefly, the sequences were demultiplexed using QIIME version 1.9.0 (Caporaso et al., 2010). Using the FastX toolkit (http:/hannonlab.cshl.edu/fastx_toolkit), reads were trimmed at the 5'-end to comprise only the final 11 bp of the SSU rRNA gene region. These pre-processed sequence data were deposited in the European Nucleotide Archive database (http://www.ebi.ac.uk/ena/data/view/PRJEB23523). CD-HIT-OTU for Illumina reads. Version 0.0.1 (http://weizhongli-lab.org/cd-hit-otu; Li et al., 2012) was selected for clustering reads into Operational Taxonomic Units (OTUs) at a similarity threshold of 97 %, according to a previous comparison of

the performance of clustering algorithms (Röhl et al., 2017). A matrix (OTU table) listing the read count per OTU and sample was generated, which was used for statistical analyses and was deposited in BExIS database (ID 22188). The taxonomic affiliation of each OTU was assigned using the UNITE database version 7.1 (Kõljalg et al., 2005) as reference.

Samples with less than 15,000 reads were discarded and the OTU read counts were standardized per sample by the total number of reads. Sequence processing revealed 247 fungal OTUs, represented by 4,907,006 quality-filtered ITS1 sequence reads. As eight samples had to be discarded after the ITS sequencing, we ended up with a sample size of 128 (63 E+ and 65 E-) from (a) 19 sites in the region ALB, together 46 samples (spring 24, summer 22); (b) 14 sites in the region HAI, together 30 samples (spring 22, summer 8); and (c) 19 sites in the region SCH, together 52 samples (spring 23, summer 29).

IV.2.5 Statistical analysis

For statistical analyses, the software R version 3.1.1 (R Development Core Team, 2014) was used. The effects of the following explanatory variables were tested using linear mixed effect models (LME, nlme package; Pinheiro et al., 2016): (1) presence of *Epichloë* endophytes, (2) season, (3) region, (4) land-use intensity on the response variables "species richness" (number of OTUs), "species evenness" and "Shannon diversity" of the OTUs. Study site ID was included as random intercept. Unequal sample sizes for region and season lead to an unbalanced sampling design, but E+ and E- samples were almost equal (one E+, one E-) for each studied grassland. To compare species richness, species evenness, and Shannon diversity between season and region, Tukey's HSD comparison of groups in mixed effects models was used (GLHT, multcomp package; Hothorn et al., 2008).

Differences between the composition of foliar fungal assemblages in dependence of region, season, land-use intensity and *Epichloë* endophyte infection were tested with a PERMANOVA (9999 permutations; ADONIS, package vegan; Oksanen et al., 2017). It fits a linear model to a distance matrix and tests hypotheses by permutations, thus not assuming normality of the data (Anderson, 2001). To characterize compositional differences between foliar fungal assemblages, non-metric multidimensional scaling (NMDS, vegan package; Oksanen et al., 2017) based on Bray–Curtis dissimilarities was used. Mean ± SE are used throughout the manuscript unless otherwise specified.

IV.3 Results

We identified 247 fungal OTUs associated with 128 *L. perenne* leaves (63 E+, 65 E-) and identified 33 genera. In total, 59 % of the OTUs were assigned to Ascomycota, 33 % to Basidiomycota and less than 1 % to Chytridiomycota (Table IV.1). Approximately 8 % of the OTUs could be not identified at the phylum level (Table IV.1), which is below the proportion of unassignable fungi in NGS surveys of different habitats such as tree leaves (40 %; Yang et al., 2016), submerged litter (36 %; Röhl et al., 2017), or dead wood (16 %; Peršoh and Borken, 2017). The orders contributing the most species to the foliar fungal assemblages of *L. perenne* were the Pleosporales (15 % of OTUs), Heliotiales (8 % of OTUs) and Hypocreales (5 % of OTUs; Table IV.1). The dominant genera contributing to the foliar fungal assemblages of *L. perenne* were *Cryptococcus* (25 % of the sequencing reads), a genus of Basidiomycota, and *Mycosphaerella* (11 % of the sequencing reads; Table IV.2), a genus of Ascomycota.

The sample sizes of analysed *L. perenne* plants differed slightly between the studied regions (ALB = 46, HAI = 30, SCH = 52) and between seasons (spring = 69, summer = 59). Nonetheless, separate species (OTU) accumulation curves for the studied regions (ALB = 216 OTUs, HAI = 205 OTUs, SCH = 220 OTUs) and seasons (spring = 233 OTUs, summer = 215 OTUs; Figure IV.2 a, b) were close to saturation.

The foliar fungal assemblages of *L. perenne* were significantly different among the regions (Table IV.3), with lowest species richness and highest species evenness in the southernmost and coolest region, ALB (number of OTUs = 51 ± 3, evenness = 0.23 ± 0.004), compared to HAI (number of OTUs = 63 ± 3, evenness = 0.20 ± 0.003) and SCH (number of OTUs = 62 ± 3, evenness = 0.21 ± 0.004), but with no differences in Shannon diversity (ALB = 2.55 ± 0.10, HAI = 2.52 ± 0.10, SCH = 2.63 ± 0.10; Figure IV.3). The assemblages in ALB and SCH were distributed in a loosely scattered pattern, while fungal assemblages in the HAI region were more similar to one another (Figure IV.4 a).

Season significantly affected species richness and composition of the assemblages (Table IV.3). Species richness and Shannon diversity were higher and compositions of the foliar fungal assemblages were more similar in summer (number of OTUs = 69 ± 3, Shannon = 2.66 ± 0.10) compared to spring (number of OTUs = 49 ± 2, Shannon = 2.50 ± 0.10). Species evenness, however, peaked in spring (spring: evenness = 0.23 ± 0.004, summer: evenness = 0.21 ± 0.003; Figure IV.3; IV.4 b).

Table IV.1 Fungal taxa identified in 128 *L. perenne* leaves from 52 grasslands in three German study regions by Next Generation Sequencing of the ITS rRNA gene region. Shown are the number of OTUs of each order, as well as their percent relative abundance and their proportions of the sequencing reads.

Phylum	Class	Order	OTUs	% of OTUs	% of reads
Ascomycota			145	58.7	42.8
	Dothideomycetes		60	24.3	26.1
		Capnodiales	13	5.3	14.8
		Dothideales	1	0.4	0.1
		Incertae sedis	5	2.0	0.3
		Pleosporales	36	14.6	10.3
		unidentified	5	2.0	0.6
	Eurotiomycetes		9	3.6	0.2
		Chaetothyriales	6	2.4	0.1
		Eurotiales	3	1.2	0.1
	Lecanoromycetes		2	0.8	0.0
		Lecanorales	2	0.8	0.0
	Leotiomycetes		24	9.7	7.6
		Erysiphales	3	1.2	0.1
		Helotiales	21	8.5	7.6
	Pezizomycetes		1	0.4	0.1
		Pezizales	1	0.4	0.1
	Pezizomycotina		4	1.6	1.1
		Incertae sedis	4	1.6	1.1
	Saccharomycetes		2	0.8	0.0
		Saccharomycetales	2	0.8	0.0
	Sordariomycetes		27	10.9	5.2
		Hypocreales	13	5.3	2.2
		Sordariales	4	1.6	0.2
		Incertae sedis	1	0.4	0.2
		Xylariales	9	3.6	2.6
	Taphrinomycetes		2	0.8	0.1
		Taphrinales	2	0.8	0.1
	unidentified		14	5.7	2.4
Basidiomycota			82	33.2	53.9
	Agaricomycetes		7	2.8	0.2
		Agaricales	5	2.0	0.1
		Polyporales	1	0.4	0.0
		Trechisporales	1	0.4	0.0
	Agaricostilbomycetes		5	2.0	0.2
		Agaricostilbales	5	2.0	0.2
	Cystobasidiomycetes		3	1.2	0.3
		Incertae sedis	2	0.8	0.1
		unidentified	1	0.4	0.2
	Exobasidiomycetes		3	1.2	0.1
		Entylomatales	1	0.4	0.0
		unidentified	2	0.8	0.1

	Microbotryomycetes		19	7.7	6.4
		Leucosporidiales	9	3.6	1.9
		Microbotryales	2	0.8	0.2
		Incertae sedis	1	0.4	0.0
		Sporidiobolales	7	2.8	4.2
	Pucciniomycetes		1	0.4	0.0
		Pucciniales	1	0.4	0.0
	Tremellomycetes		38	15.4	43.5
		Cystofilobasidiales	6	2.4	2.9
		Filobasidiales	6	2.4	4.4
		Tremellales	24	9.7	34.9
		unidentified	2	0.8	1.2
	Ustilaginomycotina		3	1.2	0.1
		Malasseziales	3	1.2	0.1
	Wallemiomycetes		1	0.4	0.1
		Wallemiales	1	0.4	0.1
	unidentified		2	0.8	3.1
Chytridiomycota			1	0.4	0.1
	Chytridiomycetes		1	0.4	0.1
		Rhizophlyctidales	1	0.4	0.1
unidentified			19	7.7	3.3

Table IV.2 Dominant fungal genera identified in 128 *L. perenne* leaves from 52 grasslands in three German study regions by Next Generation Sequencing of the ITS rRNA gene region. Shown is the proportion of sequencing reads for each genus. Table includes only genera which account for a minimum of 1 % of the sequencing reads and does not include undefined OTUs.

Phylum	Class	Order	Family	Genus	% of reads
Basidiomycota	Tremellomycetes	Tremellales	Incertae sedis	Cryptococcus	24.9
Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Mycosphaerella	11.1
Basidiomycota	Tremellomycetes	Tremellales	Incertae sedis	Bullera	4.9
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	Filobasidium	4.4
Basidiomycota	Tremellomycetes	Tremellales	Incertae sedis	Dioszegia	4.2
Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Neoascochyta	4.1
Basidiomycota	Microbotryomycetes	Sporidiobolales	Incertae sedis	Sporobolomyces	4.1
Ascomycota	Leotiomycetes	Helotiales	Incertae sedis	Helgardia	3.1
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Articulospora	2.6
Ascomycota	Sordariomycetes	Xylariales	Incertae sedis	Monographella	2.4
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	Itersonilia	1.1
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae	Leucosporidium	1.1
Ascomycota	Dothideomycetes	Pleosporales	Incertae sedis	Boeremia	1.0
Ascomycota	Pezizomycotina_cls_Incertae_sedis	Incertae sedis	Incertae sedis	Volucrispora	1.0
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Incertae sedis	Mrakiella	1.0

Neither land-use intensity nor infection with *Epichloë* endophytes had a significant effect on species richness (number of OTUs: E+ = 56 ± 3, E- = 60 ± 3) or composition of fungal assemblages of *L. perenne* leaves (Table IV.3, Figures IV.4 c, d).

From all 63 immunoblot positive (E+) samples of basal stems and leaf sheaths, only 17 % (11 samples) indicated the occurrence of *Epichloë festucae* var. *lolii* in lower leaf blades using the NGS method. In 5 % (7 of all 128 analysed *L. perenne* samples), NGS detected *Epichloë uncinata*.

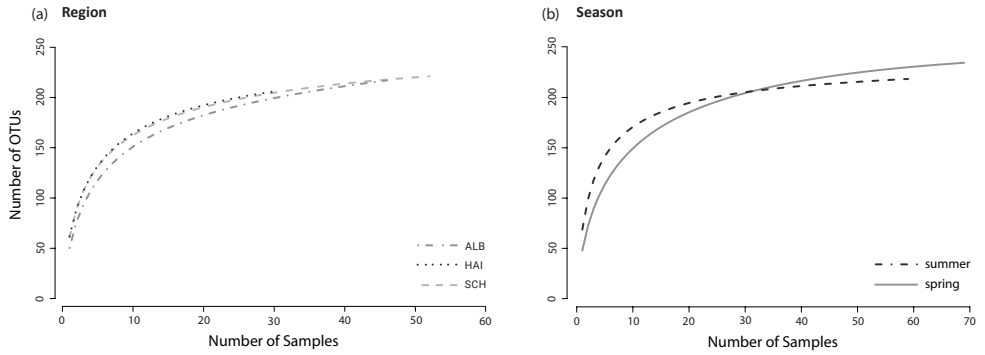

Figure IV.2 Species accumulation curves of fungal OTUs found in lower leaf blades of *L. perenne* indicate species saturations (a) for each study region: ALB, HAI, and SCH and (b) for both seasons: spring, summer. All fungal OTUs were included, resulting in asymptotic curves.

IV.4 Discussion

Many of the dominant and ubiquitous ascomycetes detected by NGS in our study, including several taxa such as *Acremonium, Alternaria, Cladosporium, Epicoccum* and *Penicillium*, have previously been recorded in other grass species (Sánchez Márquez et al., 2007; 2010) and in *L. perenne* (Thomas and Shattock, 1986) with direct isolation methods. In contrast to these studies, the fungal genus that dominated in our study belonged to the Basidiomycota (*Cryptococcus*). With direct isolation methods, only cultivable fungi can be detected, while indirect methods such as NGS can also detect fungi which cannot be cultured *in vitro*. Such differences in detection probabilities may have resulted the observed differences between our study and those of others. However, the presence of numerous fungal species seems to be characteristic of the mycobiome of grasses, leading to large compositional similarities in comparisons of fungal assemblages from different grass species (Neubert et al., 2006; Sánchez Márquez et al., 2007; 2008; 2010; White and Backhouse, 2007).

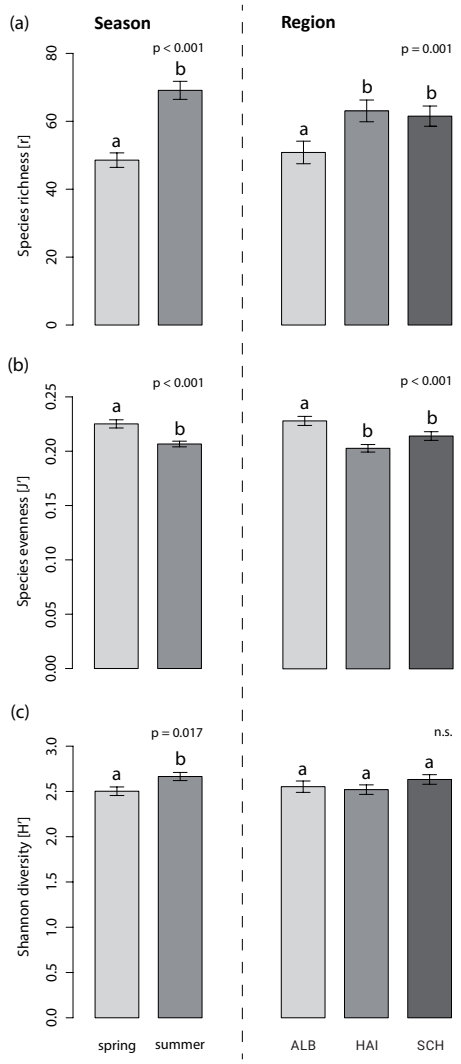

Figure IV.3 The (a) species richness (number of OTUs), (b) evenness and (c) diversity of the foliar fungal assemblages in the grass *L. perenne* depending on season (left) and study region (right). Means ± SE are shown. Different letters above bars indicate significantly different groups at $p < 0.05$, corrected for multiple comparisons.

IV.4.1 Effects of region, season and land-use intensity

The three study regions; ALB, HAI and SCH, span a latitudinal gradient from south to north across Germany, including different grassland types with variable vegetation structures (Fischer et al., 2010a). The total number of OTUs was similar between regions, but the fungal assemblages, including mean number of OTUs, differed strongly between the regions in our study. Similarly, other studies have also found differences between regions for fungal assemblages of other grass species (Neubert et al., 2006; Sánchez Márquez et al., 2008; Wilberforce et al., 2003; Wirsel et al., 2001). The environmental context of study sites, including soil, vegetation, surrounding landscape, weather, and climate may contribute to differences in the foliar fungal assemblages among the three regions in our study.

Apart from study region, species richness and species composition changed between spring and summer. Similar seasonal changes have been observed in fungal assemblages of different tree species (Peršoh, 2015). This seasonal pattern may be due to the accumulation of aerial and rain-dispersed fungal spores over time (Sánchez Márquez et al., 2012). As leaves grow older, susceptibility to infections by horizontally transmitted fungal endophytes increases (Balazs et al., 1973; Iwasa et al., 1996); and leaves of temperate grasses tend to die with summer drought (Sánchez Márquez et al., 2012). This could explain the significantly higher species richness and Shannon diversity but lower species evenness in summer as compared to spring.

In contrast to region and season, land-use intensity had a minor impact on foliar fungal assemblages of *L. perenne* in our study. A recent study found that taxonomic richness of different endophytic fungi, including mycorrhizal fungi in roots, decreased with increasing mowing intensity on the same study sites in the three regions (Simons et al., 2017). Another study found that both species richness and diversity of below ground fungi were negatively affected by increased N mineralization rates, but effects on abundances of different taxa varied (Parrent et al., 2006). We assume that different management practices, such as mowing or fertilization, essentially change the likelihood of the occurrence of any single species, but that the overall effect on fungal assemblages remains rather low.

IV.4.2 Effects of *Epichloë* endophytes

While infections with *Epichloë* endophytes has been shown to affect mycorrhizal colonization of grasses (Mack and Rudgers, 2008; Omacini et

al., 2012; Vandegrift et al., 2015), it had no significant effect on the foliar fungal assemblages of *L. perenne* in our study. Similar to a recent study on *Festuca rubra* and *Epichloë festucae* (Zabalgogeazcoa et al., 2013), neither species richness nor the composition of fungal communities in leaves changed between *Epichloë*-infected and *Epichloë*-free samples. We analysed fungi from the surfaces and internal tissues of *L. perenne* leaves. In addition to endophytic species, fungal epiphytes and spores on the outer surface of the grass leaves were detected in our study. The presence of these epiphytes and spores may have confounded our results somewhat, as we assume that *Epichloë* endophytes have a stronger effect on fungi which had invaded the leaves.

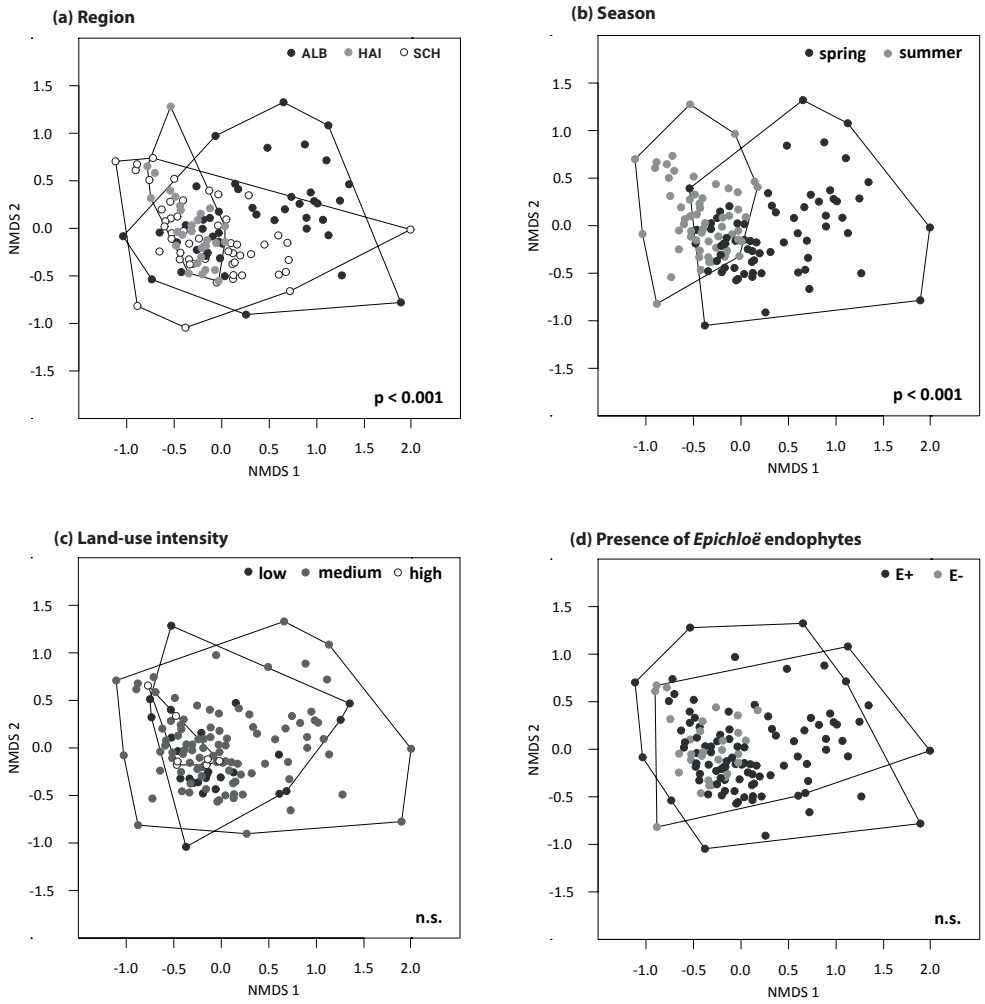

Figure IV.4 NMDS ordination (stress = 0.20) of foliar fungal community composition. Relationships with (a) study region, (b) season, (c) land-use intensity and (d) the presence of *Epichloë* infection are highlighted. Dots represent different foliar fungal assemblages of *L. perenne*. Polygons indicate clustering of fungal compositions based on the analysed variables.

Interestingly, in only 17 % of the plants where an infection with *E. festucae* var. *lolii* was detected in the basal stems and leaf sheaths by immunoblot assays (E+), the endophyte species was also detected by NGS in the lower leaf blades. Infection rates of *Epichloë* may differ among plant parts (Spiering et al., 2005a) and the limited specificity of immunoblot assays may result in false positive results (Jensen et al., 2011). *Epichloë* fungal DNA in the host plants increase with plant age (Fuchs et al., 2017c) and therefore younger basal stems have a lower detection probability of the fungi compared to older plants. The reason why we achieved such low overlap between the two methods needs further study, as a causal connection could not be established with our study design and sampling methods, using different plant material for the immunoblot assays and NGS.

Furthermore, in some *L. perenne* plants (5 %), independent of the *Epichloë* infection detected by immunoblot assays, NGS detected *Epichloë uncinata*, a species not found in *L. perenne* (Leuchtmann et al., 2014). Since we sampled *L. perenne* plants at the vegetative stage in heterogeneous and species rich grasslands, we may have sometimes inadvertently sampled the hybrid Festulolium or young meadow fescue tillers (*Festuca pratensis*). Festulolium can be visually difficult to distinguish from *L. perenne* and farmers have seeded this hybrid; it is frequently included in seed mixtures used by managers (personal communication with grassland farmers and owners). Both Festulolium and *F. pratensis* species can serve as hosts of *E. uncinata*. *Epichloë uncinata* itself is a hybrid of the species *Epichloë bromicola* and the *E. typhina* complex, and *E. typhina* has been recorded in *L. perenne* (Leuchtmann et al., 2014). This may explain, at least in part, the detection of *E. uncinata* in our samples.

IV.5 Conclusion

Our results demonstrated that, in all regions, the leaves of the grass *L. perenne* contain more than 200 taxa of fungal endo- and/or epiphytes. The number of OTUs ranges from 50 to 70 fungal taxa per study site depending on region and season. We therefore conclude that the fungal community composition of the leaves depends on study region and season, while land-use intensity of the grasslands and the occurrence of *Epichloë* endophytes in the grass has a minor influence on the foliar endo- and epiphytes in our study. However, land-use intensity has been shown to drive communities of endophytes (Parrent et al., 2006; Simons et al., 2017; Soliveres et al., 2016; Valyi et al., 2015) and the occurrence of *Epichloë* endophytes changes the expression of over one third of the host genes (Dupont et al.,

2015) and can also increase resistance to pathogenic fungi (Bonos et al., 2005; Clarke et al., 2006; Vignale et al., 2013; Xia et al., 2015). Further studies are needed to exclude the epiphytes and spores on the leaves (e.g. by surface sterilisation), and to detect effects of land use and *Epichloë* endophytes on the fungal diversity of host plants, with a focus on specific and perhaps competing groups of endophytic fungi in the host.

Chapter V

General Discussion

Epichloë endophytes can largely affect grassland ecosystems. *Epichloë* endophytes of the studied host grass species *L. perenne* and *F. pratensis* frequently appeared in the grasslands of the Biodiversity Exploratories. Depending on the endophyte-host genotypes, effects of intensive land use were different, but never beneficial. Responses of *Epichloë* endophytes are strongly determined by environmental factors such as seasonal changes and local abiotic and biotic conditions, and possibly benefit from climate warming. The effects of the two studied associations were diluted in the diverse vegetation of mixed grasslands in Germany as native habitats. However, the introduction of *Epichloë* endophyte-infected seeds knowingly or accidentally, can shift relations of the endophyte, their host grasses and other interacting species, and may change their role within grassland ecosystems. Furthermore, still undiscovered mechanisms have to be clarified to understand *Epichloë* endophytes, their consequences at community level and the role of endophyte-derived alkaloids.

The discovery of *Epichloë* endophytes changed the knowledge of the ecology of grasses and factors which determine the fitness of the grass individuals. The *Epichloë* endophyte-grass associations can largely affect the economic value of forage production in managed grassland ecosystems, but consequences of *Epichloë* infection at community and agroecosystem levels has been rarely studied (Garcia Parisi et al., 2014; Omacini et al., 2012; Saikkonen et al., 2004; 2006). Although, the *Epichloë* endophytes and their host grass species originated in Europe, most of the studies were undertaken in countries where the grass species became naturalized (Faeth, 2002; Malinowski and Belesky, 2006; Saikkonen et al., 2004). The project Biodiversity Exploratories gave me the possibility to study *Epichloë* endophytes under environmental conditions in three different regions across Germany as native habitats and on real-world grassland ecosystems which are managed by different land-use practices and intensities (Fischer et al., 2010a; 2010b).

In my PhD thesis, I mainly focused on how the associations of *Epichloë* endophytes with the common forage grass species perennial ryegrass, *Lolium perenne*, and meadow fescue, *Festuca pratensis*, and their production of bioprotective alkaloids (chapter II, III) are influenced by land use and whether they are affected by seasonal changes. Further, I predicted the possible consequences of *Epichloë* infections and concentrations of endophyte-derived alkaloids on vertebrate and insect herbivores (chapter II, III), and investigated the impact on the species composition in foliar fungal assemblages of *L. perenne* by the presence of *Epichloë festucae* var. *lolii* (chapter IV).

V.1 *Epichloë* endophytes in managed grasslands of Germany

At present, studies of *Epichloë* endophytes predominantly based on two model grass species: (1) perennial ryegrass, *L. perenne*, containing the endophyte *E. festucae* var. *lolii*; and (2) tall fescue, *F. arundinaceae*, containing the endophyte *E. coenophiala* (Faeth, 2002; Saikkonen et al., 2006). These *Epichloë* endophytes have been proven for their positive effects on the host grass fitness and persistence, but also confer negative consequences on animal health by the production of vertebrate toxic alkaloids (Clay and Schardl, 2002). Thus, *Epichloë* endophytes, which produces mainly alkaloids against insect herbivores, such as *Epichloë* endophytes associated with meadow fescue, *F. pratensis*, can have agronomic advantages without the intoxication of grazing livestock (Schardl et al., 2013a).

In order to address my research questions to *Epichloë* endophyte species with different chemical profiles (Schardl et al., 2013a), for my PhD thesis I chose two host grass species *L. perenne* and *F. pratensis*, which are associated with different *Epichloë* endophytes (Leuchtmann et al., 2014).

Lolium perenne and *F. pratensis* both frequently appeared within the mixed grassland sites in all three regions of the Biodiversity Exploratories (chapter II, III). The presence of various *Epichloë* endophyte-infected grass species has been documented for seeds and plants of agricultural and natural grasslands from several European countries (Craven et al., 2001; Do Valle Ribeiro et al., 1996; Jensen and Roulund, 2004; Lewis et al., 1997; Leyronas and Raynal, 2001; Saikkonen et al., 2000; Zabalgogeazcoa et al., 2003; Zurek et al., 2012). I showed in chapter II, that infection rates of *E. festucae* var. *lolii* in *L. perenne* are consistent to infections rates observed from other German regions (Dobrindt et al., 2013; Oldenburg, 1997) and in chapter III, that *Epichloë* infection rates of *F. pratensis* are similar to infection rates from plants of different populations of Finland and Poland, as well as seeds collected from Italy (Latch et al., 1987; Panka, 2011; 2013; Saikkonen et al., 2000).

When we compare our results of chapter II and III, we found lower infection rates of *E. festucae* var. *lolii* in *L. perenne* with 13 %, compared to the 81 % of *Epichloë* infection rates in *F. pratensis*, which may depend on the *Epichloë* endophyte–grass symbiosis and the genetic combination of the associated *Epichloë* endophyte and host grass species (Hume et al., 2016). *Epichloë* infection rates in *L. perenne*, varied in a wide range on local sites, but overall and in contrast to *Epichloë* infection rates of *F. pratensis*, they did not differ between the studied regions. However, variable environmental conditions on local/regional scale can influence *Epichloë* endophytes (Börschig et al., 2014; Hume et al., 2016), and thus differences in e.g. soil texture, the surrounding vegetation and landscape, can explain the variability of *Epichloë* infection rates between the studied regions and grassland sites.

V.2 Effects of land-use intensity and land-use types

Measured in terms of sward persistence and livestock performance, *Lolium* and *Festuca* are of high agronomic importance. As they are host grass species of *Epichloë* endophytes, they have been the subject of extensive breeding programs and subsequently introduced to managed grasslands worldwide as highly productive forage (Hoveland, 1993; Hume et al., 2016). Meadows and pastures are important elements of the agronomic

landscapes of Germany (Bluethgen et al., 2012). Anthropogenic land use is well known to affect grassland biodiversity (Klimek et al., 2007; Tscharntke et al., 2005), but also influence the relations between organisms, such as *Epichloë* endophytes, their host grasses and other interacting species.

Several studies suggest that intensive land use, such as grazing and fertilization, can have beneficial effects on the *Epichloë* endophytes, e.g. increased infection rates or higher alkaloid production (Gwinn et al., 1998; Jensen and Roulund, 2004; Krauss et al., 2007). In contrast to those studies and against my expectations, both *Epichloë* endophyte species did not benefit from land-use intensity or the type of management in my studies (chapter II, III). While land use had no significant effect on the association of *E. festucae* var. *lolii* and *L. perenne*, infection rates, as well as loline alkaloid concentrations of *Epichloë* endophytes in *F. pratensis,* decreased with an increasing land-use intensity.

The effects of land-use intensity on *Epichloë* infection rates and alkaloid concentrations also correlate with the effects of fertilization in my study, as the used land-use intensity index (LUI) integrates common management practices including fertilization. Thus, while *E. festucae* var. *lolii* in *L. perenne* was unaffected (chapter II; König et al., in press), *Epichloë* infection rates and concentrations of loline alkaloids in *F. pratensis* were higher on unfertilized grassland sites in the studied regions (chapter III). However, the impact of fertilization on *Epichloë* endophytes was often analysed in experimental and laboratory studies, resulted in either increased (Krauss et al., 2007; Lane et al., 1997) or decreased (Rasmussen et al., 2007) alkaloid concentrations. In field studies and in natural ecosystems, the *Epichloë* endophyte-grass associations were unaffected by the use of fertilizer (Bylin et al., 2014; Repussard et al., 2014). It is suggested, when benefits, e.g. nutrient uptake, outweigh costs, the grass species tolerate *Epichloë* endophytes (Saikkonen et al., 2006). Thus, when the host grasses grow under optimal conditions (e.g. specific environmental conditions or when fertilized), the benefit of an *Epichloë* infection might be low (Rasmussen et al., 2007), which explain that there was no increase in *Epichloë* infection rates and alkaloid concentrations in both host grass species *L. perenne* (chapter II) and *F. pratensis* (chapter III) in the studied regions of the Biodiversity Exploratories.

Grazing did not affect the association of *E. festucae* var. *lolii* and *L. perenne* (chapter II; König et al., in press) and the production of loline alkaloids in *Epichloë* endophyte-infected *F. pratensis* (chapter III). In contrast, the effect of grazing on the *Epichloë* infection rates in *F. pratensis* differed between the studied regions, with no effect of grazing in the Nationalpark Hainich, lower infection rates under grazing pressure in the Schorfhei-

de-Chorin, and higher infection rates on grazed sites in Schwäbische Alb (chapter III). Higher infection rates were mainly recorded from highly grazed grasslands in non-native habitats (Gwinn et al., 1998). As many *Lolium* and *Festuca* species evolved in Europe, grazing pressure possibly was modest in the Biodiversity Exploratories and my studies (chapter II), compared to those encountered in the United States, Australia and New Zealand (Malinowski and Belesky, 2006). However, my results also indicate that *Epichloë* endophyte associations strongly depend on their regional and local environment, and probably effects of grazing differ between *Epichloë* endophyte and host grass species forming the symbiosis (chapter III).

Pastoral agriculture in countries like the United States, Australia or New Zealand largely consists of cultivated *Lolium* and *Festuca*, and *Epichloë* endophyte-infected plants dominate improved pastures, but increased infection rates often resulted in frequent intoxication of livestock (Aiken and Strickland, 2013; Hoveland, 1993; Hume et al., 2016; Rowan et al., 1990). Some grasslands of the Biodiversity Exploratories were frequently sown with additionally grass seeds (König et al., in press). Although, European-bred cultivars can be infected with *Epichloë* endophytes (Saari et al., 2009; Saari et al., 2010a), the infection rates of *E. festucae* var. *lolii* in *L. perenne* and their production of alkaloids were not affected (chapter II). The sown seeds possibly were un-infected and were exclusively sown as seed mixtures on a small scale, rather than comprehensively sown seeds of one grass species (König et al., in press). Interestingly, peak concentrations of the vertebrate toxic lolitrem B and insect toxic/deterring peramine in *L. perenne* were detected on two study sites which were sown with a seed mixture containing *L. perenne* in the year before my sampling campaign. However, as concentrations above toxicity thresholds only occurred in single plants, the overall negative effects on grazing animals were rather low (chapter II; König et al., in press). Additionally, in comparison to pastures and meadows in the United States, Australia and New Zealand, grasslands of the studied regions in the Biodiversity Exploratories are botanically diverse (Klaus et al., 2013; Socher et al., 2012) and *Epichloë* endophyte infections and alkaloid content can be diluted in the total sward of the studied grasslands, and thus also in the diet of grazing livestock (Hume et al., 2016; Malinowski and Belesky, 2006).

Compared to lolitrem B and peramine, loline alkaloids normally exist in higher concentrations in *Epichloë* endophyte-infected grasses (Bush et al., 1997; Siegel and Bush, 1996). *In planta* levels of up to 5500 µg/g are common in *Epichloë* endophyte-infected *F. pratensis* from European studies (Bylin et al., 2014; Leuchtmann et al., 2000), but the highest concentrations of loline alkaloids were substantially lower (< 14 µg/g) in my analyses (chapter III). Although, loline concentrations were not affected by

grazing in my study (chapter III), wounding of the grass is suggested to induce the production of *Epichloë* endophyte-derived alkaloids (Fuchs et al., 2017b; Schardl et al., 2007). Loline concentrations in regrown plant tissue of *Festuca* plants increased after clipping (plant damage), leading to an increased herbivore resistance (Bultman et al., 2004; Craven et al., 2001). Thus, linking the enhanced production of invertebrate toxic/deterring alkaloids with the response of *Epichloë* endophyte-grass associations under herbivore pressure, insect herbivore rate in the studied regions of the Biodiversity Exploratories might be below levels which induce loline production as a defensive response of the association of *Epichloë* endophytes and *F. pratensis* (chapter III).

Although, the overall effect on foliar fungal assemblages in *L. perenne* was rather low (chapter IV), the results of my thesis indicate, that land use can be a determining factor of *Epichloë* endophytes (chapter III). Based on the *Epichloë* endophyte and grass species, benefits of the associations are not symmetric under natural conditions (chapter II, III) and effects also depend on the prevailing habitat on local sites (König et al., in press). However, besides regional and local variations in soil types and nutrient availability, also the vegetation structure and diversity might play an important role in determining the effects of *Epichloë* endophyte-grass associations (chapter II). Furthermore, I showed that herbivore resistance by an enhanced production of *Epichloë* endophyte-derived alkaloids can also vary among *Epichloë* endophyte-grass associations and possibly depend on insect herbivore pressure (chapter III).

V.3 Temporal effects by seasonal changes

In agroecosystems, grasses and their associated endophytes are exposed to climatic conditions which change over time. Seasonal changes can be conducive (e.g. drought temporarily) to the production of high concentrations of *Epichloë* endophyte-derived alkaloids (Fuchs et al., 2017a; Repussard et al., 2014; Ryan et al., 2015). In chapter II, I demonstrated that the alkaloids lolitrem B and peramine of the association of *E. festucae* var. *lolii* and *L. perenne*, mainly accumulated during summer (König et al., in press). In contrast to alkaloid concentrations in *L. perenne* and against my hypothesis, loline concentrations in *Epichloë* endophyte-infected *F. pratensis* were higher in spring or higher in summer depending on the studied region (chapter III).

Rising temperatures likely promote the risk of livestock intoxication (chapter II; König et al., in press) and the potential of *Epichloë* endophytes bee-

ing effective repellents against insect pests (chapter III). However, alkaloid concentrations also vary among the plants‘ growing phase, with highest accumulation during the maturation of the grasses (Guerre, 2016; Justus et al., 1997), and foliage damage by herbivores can induce anti-herbivory responses due to an enhanced alkaloid production (Saari et al., 2010b). My results indicate that alkaloid concentrations can vary at time of harvest and are additionally influenced by several environmental factors, and possibly correlates with herbivore pressure (chapter III).

V.4 Effects on foliar fungal assemblages

In fungal communities of grasses, the systemic *Epichloë* endophytes represent only a small part of a diverse mycobiome, while most of the other endophytic species are limited in their capability of a systemic colonization of the plant tissue (Neubert et al., 2006; Rodriguez et al., 2009). However, interactions among the fungal species within a community are inevitable and the systemic *Epichloë* endophytes have been associated with shifts in fungal colonization of plants‘ root system (Mack and Rudgers, 2008; Omacini et al., 2012; Vandegrift et al., 2015; Yue et al., 2000).

In chapter IV, I found no visible effects of *E. festucae* var. *lolii* on the composition of foliar fungal assemblages of *L. perenne.* The fungal assemblages were mainly affected by region and season. As other studies have also found regional differences for fungal assemblages of other grass species (Neubert et al., 2006; Sánchez Márquez et al., 2008; Wilberforce et al., 2003; Wirsel et al., 2001), variable environmental conditions can be also responsible for regional differences in fungal assemblages in *L. perenne* leaves in my study (chapter IV). However, the fungal community composition of *L. perenne* leaves was largely driven by seasonal changes. I detected highest species richness in summer, which is known from fungal communities of different tree species (Peršoh, 2015) and might be explained by the increased exposure to aerial fungal spores over time (Sánchez Márquez et al., 2012). Temperate grasses tend to die with increasing drought (Sánchez Márquez et al., 2012), which leads to infections with a multitude of horizontally transmitted fungal endophytes (Balazs et al., 1973; Iwasa et al., 1996). Thus, higher temperatures during summer influence the composition of foliar fungal assemblages and may also change interactions between fungal species within a community (chapter IV).

V.5 Conclusion

The outcomes of *Epichloë* endophyte-grass associations have consequences not only for the symbionts themselves, but also for the whole ecosystem that comprises many insect and vertebrate herbivores, as well as other plant-associated microorganisms such as fungi. The effects of *Epichloë* endophytes on host plants are ranging from strongly positive to neutral and sometimes strongly negative, which may suggest, that *Epichloë* endophytes and their ecological functions are highly influenced by the genetic background of endophyte and grass species (Hume et al., 2016). However, effects at population and community level are also dependent on their abiotic and biotic environments.

In the studies of my thesis, *Epichloë* endophyte infections and alkaloid concentrations are possibly diluted in a diverse vegetation in German grasslands as native habitats with low insect herbivore rates. Intoxication risk for grazing livestock are rather low (chapter II) and *Epichloë* endophytes have not the potential to be effective herbivore repellents (chapter III). The results also indicate that rising temperatures can likely promote an increase in alkaloid concentrations and thus, increase the risk for *Epichloë* endophyte-mediated diseases in livestock. Additionally, alkaloid content, as well as infection rates, are also strongly influenced by regional and local dependent environmental conditions (chapter II, III).

These outcomes suggest that biotic factors possibly have a greater impact on the *Epichloë* endophyte-grass associations than land-use intensity and management type on grasslands in Germany (chapter II, III). Moreover, the *Epichloë* endophyte-grass association might profit from climate change due to enhanced drought tolerance and competitive ability of *Epichloë* endophyte-infected grasses compared to un-infected grasses and other plant species (Clay and Schardl, 2002; Hesse et al., 2003). The accidently application of *Epichloë* endophyte-infected seed material, or the consciously introduction of “novel” *Epichloë* endophyte strains or only loline-producing *Epichloë* endophyte-grass associations, can possibly alter species composition of natural or semi-natural grasslands (Hoveland, 1993; Rudgers et al., 2010). Indeed, agricultural practices in Germany and Europe rarely include grassland monocultures or species poor seed mixtures (Klaus et al., 2013; Malinowski and Belesky, 2006; Socher et al., 2012), but due to the lack of regular controls, European-bred grass cultivars can contain *Epichloë* endophytes (Saari et al., 2009; 2010a). To prevent future mass intoxication of livestock due to the predominance of *Epichloë* endophyte-infected grasses on pastures and meadows as it has been reported from the United States, Australia and New Zealand, frequent examinations of seed material and grasslands in their native European habitats are required (Aiken and Strickland, 2013).

The interactions within fungal communities are highly complex and the mechanisms behind are still unclear (Kumar and Kaushik, 2012; Saunders et al., 2010; Suryanarayanan, 2013), and possibly also change under certain conditions. Although *Epichloë* endophytes had no modifying effect on the foliar fungal assemblages in *L. perenne* of the studied German regions (chapter IV), the *Epichloë* endophytes can influence fungal species and the expression of their host genes (Bonos et al., 2005; Clarke et al., 2006; Dupont et al., 2015; Vignale et al., 2013; Xia et al., 2015) and thus, further studies are needed.

However, the association of *Epichloë* endophytes and temperate grasses represents a complex symbiosis which affects diverse biological components of agroecosystems and address questions to different scientific fields and applied research. The outcomes and results of my thesis contribute only a small part to understand the ecological role of the *Epichloë* endophytes in grassland ecosystems, but there are still undiscovered mechanisms which have to be clarified, to predict and explain their possible consequences at community levels in natural environments and the role of different *Epichloë* endophyte-produced alkaloids in protecting plants against herbivorous pests.

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