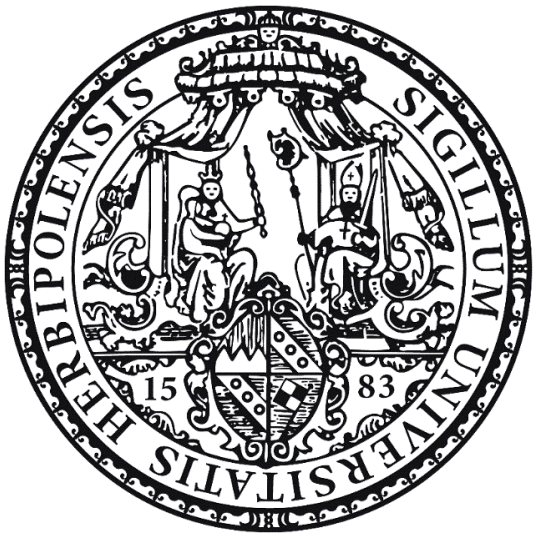
Epichloë endophyte-grass symbioses in Germany – Infection rates, alkaloid concentrations and possible intoxication risks

Epichloë Endophyt-Gras Symbiosen in Deutschland – Infektionsraten, Alkaloidkonzentrationen und mögliche Vergiftungsrisiken



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Veronika Vikuk

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Lolium perenne plant infected with *Epichloë* spp. ©Veronika Vikuk

Erratum

In Chapter I, on page 16, Figure I1 the following information needs to be added in the caption: "Reprinted by permission from Springer, Fungal Diversity, The exploitation of epichloae endophytes for agricultural benefit, Linda J. Johnson, Anouck C. M. de Bonth, Lyn R. Briggs, John R. Caradus, Sarah C. Finch, Damien J. Fleetwood, Lester R. Fletcher, David E. Hume, Richard D. Johnson, Alison J. Popay, Brian A. Tapper, Wayne R. Simpson, Christine R. Voisey & Stuart D. Card, Copyright (2013)".

In Chapter I, on page 21, Figure I4 the following information needs to be added in the caption: "Reprinted from Current Opinion in Plant Biology, Vol. 16, Christopher L Schardl, Simona Florea, Juan Pan, Padmaja Nagabhyru, Sladana Bec and Patrick J Calie, The *Epichloë* alkaloid diversity and roles in symbiosis with grasses, 480-488, Copyright (2013), with permission from Elsevier." Additionally, the figure source should be changed from (Schardl et al., 2013a) to (Schardl et al., 2013c) and the following source should be added in the reference list: Schardl, C.L., Florea, S., Pan, J., Nagabhyru, P., Bec, S., and Calie, P.J. (2013c). The *Epichloë* alkaloid diversity and roles in symbiosis with grasses. Current Opinion in Plant Biology 16, 480–488.

Table of Contents

Affidavit

I hereby declare that my thesis entitled „***Epichloë* endophyte-grass symbioses in Germany – Infection rates, alkaloid concentrations and possible intoxication risks**" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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Summary

Chapter I – General Introduction

Endophytes live in partial symbiosis inside a plant and have been detected in all tested plants. They belong to the group of fungi or bacteria and their ecological function is mostly unknown. The fungal endophytes of the genus *Epichloë* belong to a special group of endophytes. *Epichloë* endophytes live symbiotically inside cool season grass species and some of them are able to produce alkaloids toxic to vertebrates and insects. Their symbiosis is seen as mutualistic for the following reasons: the fungus provides the plant herbivore resistance by producing alkaloids, and it increases the plant's drought tolerance as well as its biomass production. In return, the grass provides the fungus shelter, nutrients and dispersal. *Epichloë* endophytes are host specific and the ability to produce alkaloids differs between species. In order to estimate intoxication risks in grasslands, it is necessary to detect infection rates of different grass species with *Epichloë* endophytes, and to determine the genotypes and chemotypes of the *Epichloë* species as well as the produced alkaloid concentrations. Factors like land-use intensity or season may have an influence on infection rates and alkaloid concentrations. Also, different methodological approaches may lead to different results. In this doctoral thesis my general aim was to evaluate intoxication risks in German grasslands caused by *Epichloë* endophytes. For that I investigated infection rates of different grass species and the genotypes and chemotypes of their *Epichloë* endophytes in German grasslands (Chapter II). Furthermore, I compared alkaloid concentrations detected with dry and fresh plant weight and different analytical methods. I also detected possible changes on the influence of season or land-use intensity (Chapter III). Additionally, I examined infections with *Epichloë* endophytes and alkaloid concentrations in commercially available grass seed mixtures and determined how that influences the intoxication risk of grazing animals in Europe (Chapter IV).

Chapter II – Infection rates and alkaloid patterns of different grass species with systemic *Epichloë* endophytes

Most studies focus on agriculturally important grass species like *Festuca arundinacea* or *Lolium perenne*, because *Epichloë* endophytes infecting these grass species may produce toxic alkaloids responsible for severe intoxication events in New Zealand, Australia or the USA. Little is known about infection rates and alkaloid contents in Germany. In the first study I investigated infection rates of 13 different cool season grass species with *Epichloë* spp. in three different regions in Germany. Then I detected pathway alkaloid genes and produced alkaloids in the infected grass species. Infections in five different grass species were found: *Festuca pratensis* (81 %), *Festuca ovina* agg. (73 %), *Lolium perenne* (15 %), *Festuca rubra* (15 %) and *Dactylis glomerata* (8 %). *Epichloë uncinata* in *F. pratensis* produced insect toxic loline compounds, whereas *Epichloë festucae* var. *lolii* in *L. perenne* produced insect deterring peramine and vertebrate toxic lolitrem B, but no ergovaline. I showed that the ergotalkaloid starting gene *dmaW* was missing, thus ergovaline was not produced. In *Festuca ovina* agg. samples we detected different precursors of the indole-diterpene pathway, which potentially have toxic effects on vertebrates. I concluded that the ability of the *Epichloë* endophyte to produce alkaloids should be asessed to estimate intoxication risks. Studying infection rates without these parameters is not sufficient.

Chapter III – Alkaloid concentrations of *Lolium perenne* infected with *Epichloë festucae* var. *lolii* with different detection methods – a re-evaluation of intoxication risk in Germany?

Intoxication risks for animals posed by alkaloids produced by the fungus *Epichloë festucae* var. *lolii* in *Lolium perenne* is evaluated using infection rates but also the quantitation of alkaloid concentrations. Hence, an exact quantitation of alkaloid concentrations is crucial to determine, whether concentrations exceed toxicity thresholds for livestock or insect pests. Different alkaloid detection methods are used to quantify alkaloid concentrations, which can complicate the comparability of results. Aim of this study was to confirm results or detect changes in infection rates and alkaloid concentrations compared to a previous study on the same study

sites. Additionally, I analyzed the effect of season on alkaloid concentrations on five study sites in Germany, which previously showed high alkaloid levels. Furthermore, I analyzed the effect of season on alkaloid concentrations in a common garden study. Then, I examined if the seasonal trend changed when dry instead of fresh plant weight was used and if alkaloid concentrations differed between two alkaloid detection methods. Alkaloid concentrations of individual plants on a wider selection of grasslands in three German regions exceeded toxicity thresholds, but not on population level. Alkaloid concentrations on five German grasslands peaked in summer but were also always below toxicity thresholds on population level. Additionally, I showed, that alkaloid concentrations follow the same seasonal trend, regardless if dry or fresh plant weight was used for the quantitation. Nevertheless, alkaloid concentrations detected with dry plant weight were about three times higher than with fresh plant weight. Also, alkaloid concentrations can be biased by the use of different alkaloid detection methods. I concluded that toxicity risks should be analyzed using dry plant weight instead of fresh plant weight. Still, seasonal trends stay the same with both methods.

Chapter IV – *Epichloë* endophyte infection rates and alkaloid content in commercially available grass seed mixtures in Europe

Asexual forms of *Epichloë* endophytes are distributed by infected grass seeds, hence contaminated commercially available grass seed mixtures can be a distributor of *Epichloë* infections. Additionally, benefits for the plant like drouhgt resistance and increased biomass are used in agriculture and plants are infected intentionally with vertebrate safe *Epichloë* endophytes, which produce no toxic alkaloids. In the third study I tested 24 commercially available grass seed mixtures for infections with *Epichloë* endophytes and alkaloid contents. I detected infections in six of the seed mixtures and additional alkaloid contents in four of the seed mixtures. Two of the alkaloids containing seed mixtures were forage grass seed mixtures. Since *Epichloë* infections can harm livestock, when infected grass dominates grasslands, seed breeders should test and communicate *Epichloë* infection status in seed mixtures and should avoid *Epichloë* infected seed mixtures if possible.

Chapter V – General Discussion

It is of agricultural interest to estimate intoxication risks for grazing livestock on German grasslands due to *Epichloë* infected grass species. Therefore, it is important to investigate which grasses are infected with the *Epichloë* endophyte, if the endophytes have the ability to produce vertebrate and invertebrate toxic alkaloids and if the alkaloids are indeed produced. I showed that *Epichloë festucae* var. *lolii* infecting agriculturally important *Lolium perenne* lacked the starting gene for ergovaline biosynthesis. Hence, vertebrate toxic ergovaline was not detected in the majority of the collected *L. perenne* plants. The detection of alkaloid concentrations is an important tool to estimate intoxication risk for vertebrates, but also invertebrates. My studies showed that the usage of dry plant material is crucial to quantify the correct alkaloid concentrations, and that alkaloid concentrations can vary depending on the detection method. Hence, the usage of validated, similar detection methods is important to be able to compare alkaloid concentrations from different studies. Nevertheless, the trends of seasonal changes and the influence of land-use intensity stayed the same, regardless if dry or fresh plant weight was used. Also, alkaloid concentrations were below toxicity thresholds on population level, regardless of the method used. Two commercially available forage grass and two commercially available turf grass seed mixtures were infected with *Epichloë* endopyhtes and alkaloids were detected. This might contribute to the spreading of *Epichloë* endopyhtes in Germany, therefore seed mixtures should be tested for *Epichloë* infections. My results indicate that the intoxication risk is generally low in Germany at the moment, although that might change due to climate change, an increase of monocultural land-use, or the seeding of *Epichloë* infected grass seeds.

Zusammenfassung

Kapitel I – Allgemeine Einleitung

Endophyten leben, zumindest zeitweise, symbiontisch in Pflanzen und sind bisher in allen untersuchten Pflanzen nachgewiesen worden. Es handelt sich dabei um Pilze oder Bakterien und ihre ökologische Funktion ist meistens unbekannt. Eine spezielle Gruppe der Endophyten sind Pilzendophyten der Gattung *Epichloë*. Diese leben symbiontisch innerhalb von kaltgemäßigten Grasarten und einige sind in der Lage vertebraten- und/oder insektentoxische Alkaloide herzustellen. Die Symbiose wird meist als mutualistisch bezeichnet, weil der Pilz der Pflanze einen Herbivorenschutz durch die Produktion der Alkaloide und eine gesteigerte Trockenresistenz und Biomassesteigerung bietet. Das Gras hingegen bietet dem Pilz Unterkunft, Nährstoffe und Verbreitung. *Epichloë* Endophyten sind wirtsspezifisch und die Fähigkeit Alkaloide zu produzieren schwankt zwischen den Arten. Um das Vergiftungsrisiko im Grünland einzuschätzen, ist es nötig Infektionsraten verschiedener Grasarten mit *Epichloë* Endophyten, die Geno- und Chemotypen der *Epichloë* Arten, und die produzierten Alkaloidkonzentrationen zu bestimmen. Faktoren wie Landnutzungsintensität oder die Jahreszeit können Infektionsraten und Alkaloidkonzentrationen beeinflussen. Ebenso können Alkaloidkonzentrationen von methodischen Faktoren abhängen. In dieser Doktorarbeit habe ich Infektionsraten verschiedener Grasarten in Deutschland und die Geno- und Chemotypen ihrer *Epichloë* Endophyten untersucht (Kapitel II). Außerdem habe ich Alkaloidkonzentrationen mit Frisch- bzw. Trockengewicht gemessen und mit verschiedenen analytischen Methoden verglichen, um mögliche Änderungen beim Einfluss von Jahreszeiten oder der Landnutzungsintensität zu detektieren. Des Weiteren habe ich das Vergiftungsrisiko auf deutschen Grasflächen abgeschätzt (Kapitel III). Zusätzlich habe ich kommerziell erhältliche Grassaatgutmischungen auf *Epichloë* Infektionen und Alkaloidgehalt untersucht und habe versucht einzuschätzen, wie sich das auf das Vergiftungsrisiko von Weidevieh in Europa auswirkt (Kapitel IV).

Kapitel II – Infektionsraten und Alkaloidmuster verschiedener Grasarten mit systemischen *Epichloë* Endophyten

Die meisten Studien fokussieren sich auf landwirtschaftlich wichtige Grasarten, wie *Festuca arundinacea* und *Lolium perenne*, weil *Epichloë* Endophyten, die diese Gräser infizieren giftige Alkaloide produzieren können, die für schwere Vergiftungsereignisse in Neuseeland, Australien und den USA verantwortlich sind. In Deutschland ist über Infektionsraten der Gräser und ihren Alkaloidgehalt nur wenig bekannt. In dieser ersten Studie habe ich Infektionsraten von 13 verschiedenen Grasarten mit *Epichloë* spp. in drei Regionen in Deutschland untersucht und anschließend Pfadalkaloidgene der *Epichloë* Pilze, sowie produzierte Alkaloide detektiert. In den folgenden fünf Grasarten habe ich Infektionen nachweisen können: *Festuca pratensis* (81 %), *Festuca ovina* agg. (73 %), *Lolium perenne* (15 %), *Festuca rubra* (15 %) und *Dactylis glomerata* (8 %). *Epichloë uncinata* in *F. pratensis* produzierte insektentoxische Lolin-Alkaloide, wohingegen *Epichloë festucae* var. *lolii* in *L. perenne* insektenabwehrendes Peramin und vertebratentoxisches Lolitrem B, aber kein Ergovalin produzierte. Ich konnte zeigen, dass das Startgen des Ergotalkaloidsyntheseweges *dmaW* gefehlt hat, weshalb Ergovalin nicht produziert werden konnte. In den *Festuca ovina* agg. Proben konnte ich verschiedene Vorstufen des Indolditerpensyntheseweges nachweisen, die auch potenziell vertebratentoxisch sein können. Ich konnte zeigen, dass Infektionsraten allein nicht ausreichen, um ein Vergiftungsrisiko einzuschätzen, da auch andere Eigenschaften, wie die Fähigkeit der *Epichloë*-Pilze Alkaloide produzieren zu können, berücksichtigt werden sollten.

Kapitel III – Die Toxizität von *Epichloë* infizierten *Lolium perenne* Pflanzen in Grassländern in Abhängigkeit von unterschiedlichen analytischen Methoden – eine Reevaluierung des Vergiftungsrisikos in Deutschland?

Das Vergiftungsrisiko für Tiere auf Grund von Alkaloiden, die vom endophytischen Pilz *Epichloë festucae* var. *lolii* in *Lolium perenne* produziert werden, wird mit Hilfe der Detektion von Infektionsraten, aber auch durch die Quantifizierung von Alkaloidkonzentrationen bestimmt. Eine genaue

Quantifizierung der Alkaloidkonzentrationen ist ausschlaggebend, um abschätzen zu können, ob die Konzentrationen eine Toxizitätsschwelle für Weidevieh oder Insektenschädlinge übersteigt. Verschiedene Alkaloid-Detektionsmethoden werden genutzt, um die Alkaloidkonzentrationen zu quantifizieren, was die Vergleichbarkeit der Ergebnisse erschwert. Ziel dieser Studie war es den Einfluss von Landnutzungsintensität auf Infektionsraten und Alkaloidkonzentrationen auf Graslandflächen in drei Regionen in Deutschland im Vergleich zu einer vorherigen Studie zu bestätigen bzw. Änderungen festzustellen. Außerdem wurden jahreszeitliche Effekte auf die Alkaloidkonzentrationen auf fünf Versuchsflächen in Deutschland untersucht, auf denen zuvor hohe Alkaloidkonzentrationen nachgewiesen wurden. Zusätzlich habe ich jahreszeitliche Effekte auf die Alkaloidkonzentrationen in einer Gartenstudie analysiert und Alkaloidkonzentrationen verglichen, die mit Trocken- bzw. Frischgewicht der Pflanze detektiert wurden. Die Alkaloidkonzentrationen einzelner Pflanzen auf Graslandflächen in drei Regionen Deutschlands lagen zwar über der Toxizitätsschwelle, aber nicht auf Populationsebene. Die Alkaloidkonzentrationen auf den fünf deutschen Graslandflächen haben ihren Höhepunkt im Sommer erreicht, allerdings lagen die Werte auch hier unterhalb der Toxizitätsschwellen auf Populationsebene. Zusätzlich konnte ich zeigen, dass Alkaloidkonzentrationen im Jahresverlauf dem gleichen Trend folgen, egal ob Trocken- oder Frischgewicht der Pflanze für die Quantifizierung verwendet wurde. Trotzdem waren die Alkaloidkonzentrationen, die mit Trockengewicht der Pflanze detektiert wurden, ca. dreimal höher, als die mit Frischgewicht gemessenen. Außerdem können Alkaloidkonzentrationen durch die Nutzung verschiedener Alkaloid-Detektionsmethoden beeinflusst werden. Ich konnte zeigen, dass das Vergiftungsrisiko mittels Trockengewicht der Pflanze ermittelt werden sollte, anstatt mit Frischpflanzengewicht. Allerdings bleiben die Trends der Alkaloidkonzentrationen im Jahresverlauf gleich, egal ob Frisch- oder Trockenpflanzengewicht verwendet wurde.

Kapitel IV –Infektionen mit *Epichloë* Endophyten und Alkaloidgehälter in kommerziell erwerblichen Grassamenmischungen in Europa

Asexuelle Formen von *Epichloë* Endophyten werden über infizierte Grassamen verbreitet. Deshalb können *Epichloë* Infektionen auch durch kontaminiertes Saatgut verbreitet werden. Außerdem werden die Vorteile der Pflanze durch die Symbiose mit dem *Epichloë*-Pilz, wie Trockenresistenz und gesteigerte Biomasse, gerne landwirtschaftlich genutzt und Pflanzen werden absichtlich mit vertebratensicheren *Epichloë* Endophyten infiziert, die keine giftigen Alkaloide produzieren. In der dritten Studie habe ich 24 kommerziell erwerbliche Grassamenmischungen auf Infektionen mit *Epichloë* Endophyten und ihren Alkaloidgehalt untersucht. Ich konnte in sechs der Saatgutmischungen Infektionen nachweisen und in vier zusätzlich auch Alkaloide. Zwei der alkaloidhaltigen Saatgutmischungen waren Futtergrasmischungen. Da *Epichloë*-Infektionen Weidevieh schaden können, wenn infizierte Gräser die Wiesen dominieren, empfehle ich Saatgutfirmen ihre Samen auf *Epichloë* Infektionen zu testen, diese Informationen öffentlich zu machen und *Epichloë* infiziertes Saatgut zu vermeiden.

Kapitel V – Allgemeine Diskussion

Die Einschätzung von Vergiftungsrisiken für Weidevieh aufgrund von *Epichloë* infizierten Grasarten auf deutschen Graslandflächen ist von landwirtschaftlichem Interesse. Deshalb ist es wichtig zu untersuchen, welche Grasarten mit *Epichloë* Endophyten infiziert sind, ob der Endophyt in der Lage ist vertebraten- oder insektentoxische Alkaloide zu produzieren und ob diese tatsächlich produziert werden. Ich konnte zeigen, dass *Epichloë festucae* var. *lolii*, welches das landwirtschaflich wichtige *Lolium perenne* infiziert, das Startgen für die Ergovalinbiosynthese fehlt. Deshalb wurde das vertebraten-toxische Ergovalin in der Mehrheit der gesammelten *L. perenne* Pflanzen nicht nachgewiesen. Die Detektion von Alkaloidkonzentrationen ist ein wichtiges Werkzeug, um das Vergiftungsrisiko für Vertebraten aber auch Invertebraten einschätzen zu können. Ich konnte zeigen, dass die Verwendung von trockenem Pflanzenmaterial essenziell

ist, um korrekte Alkaloidkonzentrationen zu quantifizieren und dass Alkaloidkonzentrationen in Abhängigkeit von der Detektionsmethode schwanken können. Deshalb ist die Verwendung von validierten, ähnlichen Detektionsmethoden wichtig, um die Alkaloidkonzentrationen von verschiedenen Studien vergleichen zu können. Dennoch blieben die jahreszeitlichen Trends und der Einfluss von Landnutzungsintensität gleich, egal ob Trocken- oder Frischgewicht der Pflanze verwendet wurde und Alkaloidkonzentrationen lagen unter der Toxizitätsschwelle auf Populationsebene. Ich konnte außerdem zeigen, dass zwei kommerziell erwerbliche Futtergrasmischungen, sowie zwei Rasengrasmischungen mit *Epichloë* Endophyten infiziert waren und auch Alkaloide detektiert werden konnten. Das könnte zu einer weiteren Ausbreitung von *Epichloë*-Endophyten in Deutschland beitragen, weshalb Saatgutmischungen auf *Epichloë* Infektionen getestet werden sollten. Meine Ergebnisse zeigen, dass das Vergiftungsrisiko in Deutschland im Moment generell eher niedrig ist. Allerdings kann sich das auf Grund von Klimawandel, zunehmenden Monokulturen in der Landnutzung, aber auch der Aussaat von *Epichloë* infiziertem Saatgut ändern.

Chapter I

General Introduction

Stromata (yellow fruit bodies) forming *Epichloë uncinata* in *Dactylis glomerata* on a grassland close to Salamanca, Spain. Photo © Veronika Vikuk

Chapter I

General Introduction

Epichloë endophytes are agronomically important fungal grass endophytes, which improve drought resistance and plant biomass and are able to produce alkaloids, which can be toxic for vertebrates and invertebrates. Intoxication events of vertebrates are often reported from New Zealand, Australia and the USA, but hardly from Germany. In this thesis I investigated *Epichloë* diversity in cool-season grass species in Germany (Chapter II) as well as seasonal changes in alkaloid concentrations (Chapter III). I also estimated intoxication risks of livestock in Germany with field studies, a common garden experiment (Chapter III) and by investigation of grass seed mixtures (Chapter IV). Additionally, I identified methodological differences in the detection and quantitation of alkaloids (Chapter III).

I.1. Endophytes

Endophytes are bacterial or fungal symbionts, which live, at least part of their lifecycle, inside a plant (Wilson, 1995), without any obvious pathogen effect on the host plant (Hirsch and Braun, 1992; Stone et al., 2004). Endophytes have been detected in all presently examined plants and are multidivers, but their ecological function is mostly unknown (Arnold et al., 2003; Clay, 2004). Studies indicate that endophytes might be part of the plant immune system and protect plants against fungal pathogenes (Arnold et al., 2003). Arnold et al. (2003) showed that cacao tree leaves inoculated with endophytes showed a better defense against fungal pathogenes than leaves without endophytes. Better studied than the multidivers, horizontally transmitted woody angiosperm endophytes are the relatively species poor horizontally transmitted *Epichloë* endophytes in cool season grasse species (Arnold et al., 2003) on which I will focus in this thesis. It is assumed that *Epichloë* endophytes are among the most common microorganisms after mycorrhizal root fungi and nitrogen fixing bacteria (Hume et al., 2016).

I.2. *Epichloë* endophytes in cool season grass species

Cool season grass species can be infected with endophytic fungi of the genus *Epichloë* (Sampson, 1933). Endophytic *Epichloë* fungi live asymptomatically inside the grass plant (Sampson, 1933). The fungus increases plant biomass and drought resistance of the plant (Bourguignon et al., 2015; Gundel et al., 2020), whereas the plant provides shelter, nutrition and dispersal for the fungus (Schardl, 1996). Moreover, *Epichloë* spp. are able to produce alkaloids, which can be toxic for invertebrates and vertebrates and thus contribute to the herbivore resistance of the plant (Schardl, 1996). It has been suggested, that *Epichloë* infection could also be part of an indirect plant defense by altering volatile organic compounds of the plants, which then attract insect herbivore predators (Fuchs and Krauss, 2019). It is known that *Epichloë* infection can change expression of up to one third of host plant genes and thus enhances the host plant immune system for example (Bastias et al., 2017; Dupont et al., 2015).

Epichloë endophytes have different lifecycles. Asexual forms, formerly known as *Neotyphodium*, are transmitted vertically via grass seeds, whereas sexual forms produce fruit bodies, so called stromata, and are horizontally transmitted via flies of the genus *Botanophila* (formerly known as *Phorbia*) (Bultman et al., 1998; Pagel et al., 2019) (Figure I1). *Botanophila* flies are attracted by volatiles of the fungi (Steinebrunner et al., 2008), feed on perithecial tissue containing spermatial spores and lay their eggs on the stromata (Pagel et al., 2019). They cross-fertilize the *Epichloë* fungi, which is self-incompatible (Bultman et al., 1998).

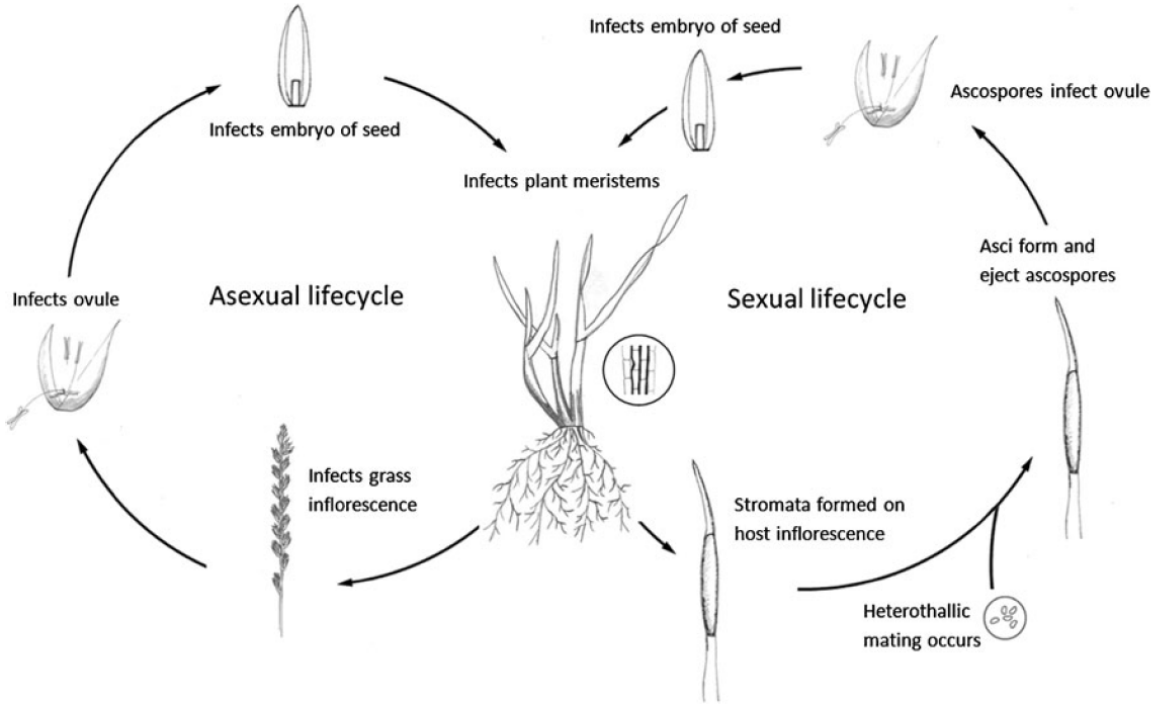


Figure I1: Asexual and sexual lifecycle of *Epichloë* fungi. In the asexual lifecycle (**left**) the infection spreads from the plant meristeme to the inflorescence. The fungus infects the ovule and is dispersed via the infected seed. In the sexual lifecycle (**right**) the fungus forms stromata on the host inflorescence, heterothallic mating occurs via *Botanophila* flies, asci form and ejects ascospores, which infect ovules and seed embryo of a new grass plant. Figure from (Johnson et al., 2013)

The *Epichloë* fungi lives between plant cells, but never inside plant cells (Johnson et al., 2013). It infects plant meristeme and in the asexual lifecycle also the inflorescences, the ovule and the seed embryo. Afterwards it is transmitted via the

grass seeds and infects the new plant meristem (Figure I1) (Johnson et al., 2013; Schardl, 1996). In the sexual lifecycle the fungus grows also intercellularly in the leaf sheath without causing symptoms, but then forms a stroma on the host inflorescence. After the quasi-pollination interaction with *Botanophila* flies, heterothallic mating occurs, asci form and eject ascospores, which then infect the ovule and as a result the seed embryo of a new plant, which grows for a new *Epichloë* infected grass plant (Figure I1) (Johnson et al., 2013; Pagel et al., 2019; Schardl, 1996). Horizontally transmitted *Epichloë* fungi possess haploid genomes, whereas most vertically transmitted *Epichloë* fungi are interspecific hybrids with heteroploid genomes (Moon et al., 2004). The species *Epichloë coenophiala*, infecting *Festuca arundinacea*, for example, is an asexual hybrid, whereas *Epichloë festucae* var. *lolii*, infecting *Lolium perenne*, is a non-hybrid, evolved from sexual ancestors, which lost their stromata-forming ability (Johnson et al., 2013; Kuldau et al., 1999).

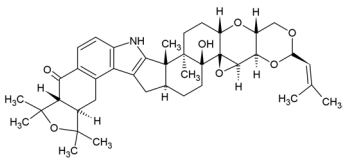
The symbiosis of cool season grass species and *Epichloë* fungi is mostly seen as mutualistic, but an antagonistic effect is also discussed (Müller and Krauss, 2005; Saikkonen et al., 1998; Schardl, 1996). In a mutualistic symbiosis both parties benefit from each other, whereas in an antagonistic symbiosis, one partner has negative effects on the symbiosis. Whether the symbiosis between the grass and the *Epichloë* endophyte is mutualistic or antagonistic depends on genetic interactions between grass and endophyte, different abiotic factors like nutrients, water or light, and different biotic interactions, like herbivores, pathogens or competitors (Müller and Krauss, 2005). Therefore, Müller and Krauss (2005) postulated a mutualism-parasitism continuum. Sexually transmitted *Epichloë* fungi cause choke disease, and suppress flower and seed production of the host (Figure I2) (Müller and Krauss, 2005). Hence, this symbiosis is rather seen as antagonistic , in contrast to the more mutualistic symbiosis with the asexual *Epichloë* form (Müller and Krauss, 2005). Sexually distributed *Epichloë* can also produce toxic alkaloids, but normally they produce less alkaloids and lower concentrations (Hume et al., 2016; Leuchtmann et al., 2000; Schardl et al., 2012). A recent study showed that 10 % of the plant genes and hundreds of fungal genes were expressed differently in the stroma tissue of sexual *Epichloë festucae* infecting *Festuca rubra* subsp. *rubra* compared to not infected tissue (Wang et al., 2019). The authors showed that plant-stress-related genes in particular were up-regulated, which supports the hypothesis of a more antagonistic

relationship (Wang et al., 2019). Sexual forms are less dependent on the host plant fitness, whereas asexual *Epichloë* forms depend on the fitness of their host plant for distribution and survival. Hence, the production of defensive alkaloids contributes to the mutualism, since both parties benefit: The grass is protected from herbivores, which also secures the survival of the *Epichloë* fungi (Bush et al., 1997; Hume et al., 2016; Schardl et al., 2012).

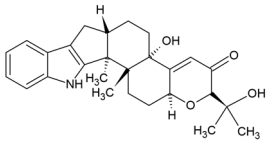
Figure I2: Stromata (yellow fruit bodies) forming *Epichloë uncinata* in *Dactylis glomerata* on a grassland close to Salamanca, Spain. Photo: © Veronika Vikuk

I.3. Alkaloids produced by *Epichloë* endophytes and intoxications

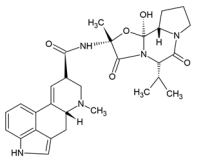
Up to four different alkaloid classes can be produced by *Epichloë* spp. (Schardl, 1996; Schardl et al., 2012, 2013a) (Figure I3). Loline alkaloids and peramine have insect toxic and insect deterring properties, respectively, whereas ergotalkaloids and indole-diterpenes are vertebrate toxic (Schardl, 1996; Schardl et al., 2012, 2013a). It was shown that *Epichloë* endophytes are host specific (Schirrmann et al., 2018) and the ability to produce alkaloids depends on the *Epichloë* species in the host grass (Schardl et al., 2012) (Table I1). Endophyte diversity and thus also alkaloid diversity can be high in a single grass species (Charlton et al., 2014; Oberhofer and Leuchtmann, 2012; Shymanovich et al., 2017).



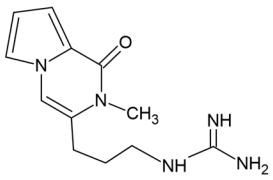
lolitrem B (vt)



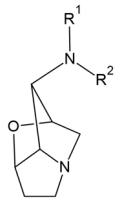
paxilline (vt)



ergovaline (vt)



peramine (id)



lolines (it)
N-acetyl-lolin: R_1= $COCH_2$, R_2= CH_3
N-formyl-lolin: R_1= COH, R_2= CH_3

Figure I3: Most common alkaloids which can be produced by *Epichloë* spp. in cool season grass species. Letters in brackets indicate if alkaloid is mainly vertebrate toxic (vt), insect toxic (it) or insect deterring (id).

Lolines (LOL), ergot alkaloids (EAS) and indole-diterpenes (IDT) are encoded by several biosynthesis steps (Schardl et al., 2013a, 2014), whereas peramine is encoded by a single gene, *perA* (Figure I4) (Tanaka et al., 2005). Insect toxic lolines are, for example, typically produced by *Epichloë uncinata* in *Festuca pratensis*, whereas *Epichloë festucae* var. *lolii* in *Lolium perenne*, for example, produces

insect deterring peramine, but also vertebrate toxic lolitrem B and ergovaline (Table I1, Figure I3) (Schardl et al., 2012; Spiering et al., 2005a; van Zijll de Jong et al., 2008).

Lolitrem B and some of its precursors, like paxilline, induce tremors in vertebrates (Figure I3+I4) (Imlach et al., 2008; Menna et al., 2012). Intoxications caused by lolitrem B from *L. perenne* plants infected with *E. festucae* var. *lolii* are also known as ryegrass staggers. The alkaloid is affecting the nervous system of the grazing animal (Blythe et al., 2007; Canty et al., 2014; Gallagher and Hawkes, 1986; Menna et al., 2012). It is characterized by falling hypersensitivity, disturbed coordination and functional disturbance of nervous tissue function (Canty et al., 2014). Death occurs due to accidents and starvation as consequence to the tremors, however intoxications are reversible, when animals are moved to an endophyte free pasture (Menna et al., 2012).

Ergovaline, an ergotalkaloid, is, for example, produced by *Epichloë coenophiala* in *Festuca arundinace* or *E. festucae* var. *lolii* in *L. perenne* (Table I1, Figure I3). It can cause fescue toxicosis, which is characterized by low weight gain and milk yield, hypersalivation and the seeking for shade of the animals (Canty et al., 2014). But also fescue foot, the loss of limbs or tails due to local vascular constrictions, and prolonged pregnancies can be caused by ergovaline (Canty et al., 2014).

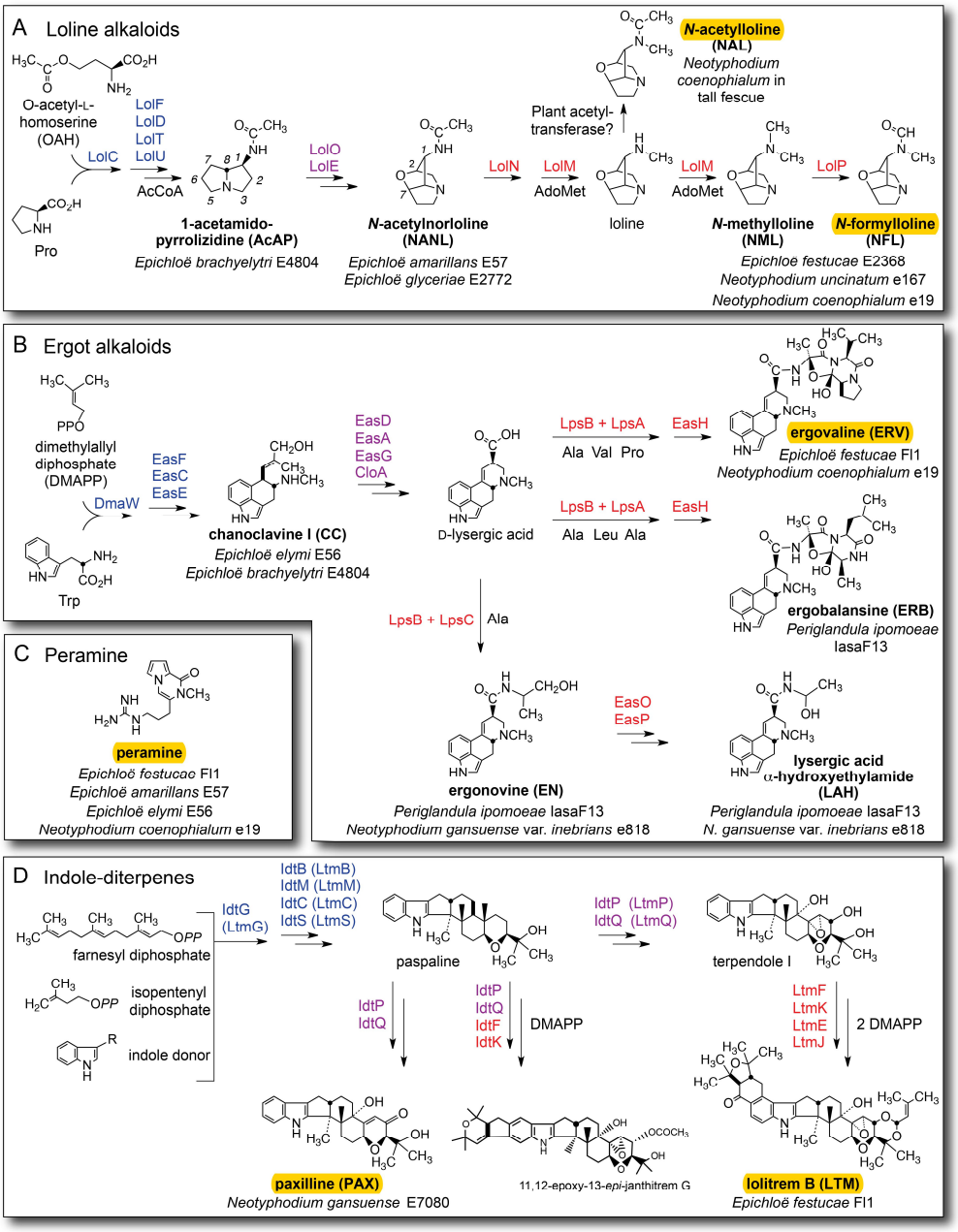


Figure I4: Proposed biosynthesis pathway structures of alkaloids in *Epichloë* spp and *Periglandula*. **a**: pathway for lolines, **b**: pathway for ergot alkaloids, **c**: structure of peramine, **d**: pathway for indole-diterpenes, **blue**: early pathway genes, **purple:** intermediate pathway genes, **red**: late pathway genes, **bold**: endproducts. Below the endproducts the species and strains that produce them are written. Figure from (Schardl et al., 2013a). *Neotyphodium* is an old genus name for asexual *Epichloë* spp., but is now renamed to *Epichloë* (Leuchtmann et al., 2014). Yellow highlighted names are the most common toxic alkaloids produced by *Epichloë* spp.

Table I1: *Epichloë* species, corresponding host grass species, produced alkaloids and type of transmission[a]. Selection of grass species due to presence in German grasslands. Empty cells indicate that no data is available yet

***Epichloë* species (H = Hybrid)**	**Host species**	**Ergotalkaloides (vertebrate toxic)**	**Indole-diterpenes (vertebrate toxic)**	**Lolines (insect toxic)**	**Peramine (insect deterring)**	**transmission**
***E. coenophiala* (H)**	*Festuca arundinacea*	ergovaline, chanoclavine	Maybe terpendoles (not tested)	N-formylloline, N-acetylnorloline, N-methylloline, N-acetylloline	Peramine	vertical
E. festucae	*Festuca rubra, Festuca trachyphylla, Lolium giganteum* *Festuca gigantea* *Festuca ovina* (only erogovaline and peramine)	ergovaline, chanoclavine (both not detected in *F. rubra, L. giganteum*)	lolitrem B (not detected in *F. rubra, L. giganteum*)	N-Formylloline, N-Methylloline, N-acetylnorololine N-acetylloline	Peramine	horizontal/vertical
E. festucae var. lolii	*Lolium perenne*	ergovaline	lolitrem B terpendole janthitrems	no	Peramine	vertical
E. festucae* var. *lolii* x *E. typhina (H)	*Lolium perenne*	ergovaline chanoclavine	terpendole	-	-	horizontal
E. siegelii	*Festuca pratensis*	no	Paxiline (not tested)	N-Formylloline, N-Methylloline, N-acetylnorololine N-acetylloline	Peramine	vertical
E. typhina	*Lolium perenne, Dactylis glomerata, Anthoxanthum odoratum, Poa nemoralis, Poa pratensis, Poa sylvicola, Poa trivialis, Puccinellia distans, Brachypodium pinnatum, Phleum pratense* *Brachypodium pinnatum*	no	no	no	Peramine Just in *Poa trivialis* and *Lolium perenne*	horizontal (in some vertical, in some horizontal) stroma forming is obligatory
E. typhina ssp. clarkii	*Holcus lanatus*	-	-	-	Peramine	horizontal
E. uncinatum (H)	*Festuca pratensis*	no	Not tested	N-Formylloline, N-Methylloline, N-acetylnorololine N-acetylloline	no	vertical
E. baconii	*Agrostic tenuis* *Agrostis stolonifera*	no	no	no	no	-
E. bromicola	*Bromus erectus, Broumus benekenii, Bromus ramosus*	no	no	no	Peramine	horizontal
E. sylvatica	*Brachypodium sylvaticum*	no	no	no	no	-

a: Compiled from (Leuchtmann et al., 2000, 2014; Schardl et al., 2012, 2013a, 2013b; Young et al., 2015)

The appearance of clinical symptoms of intoxications depends on the alkaloid concentration in the grass. Toxicity thresholds have been estimated, which indicate the threshold, above which clinical symptoms appear (Craig et al., 2014). Toxicity thresholds can differ between animals. An ergovaline concentration above 0.3 μg/g dry weight, for example, is considered to be toxic for horses and cattle, but for sheep the toxicity threshold is 0.5 μg/g (Craig et al., 2014). Toxicity thresholds are determined with feeding experiments on sheep or cattle (Blythe et al., 2007; Tor-Agbidye et al., 2001) or mice (Finch et al., 2007). The toxicity threshold for lolitrem B is considered as 1.8 – 2 μg/g dry weight (Craig et al., 2014). Lolines are considered to be toxic above concentrations of 50 – 100 μg/g dry weight (Jensen et al., 2009; Shiba and Sugawara, 2009) and peramine is considered to be insect deterring above concentrations of 2 μg/g dry weight, determined with Argentine stem weevil larvae feeding on artificial diets (Rowan et al., 1990; Siegel and Bush, 1996). Another study states a strong deterring effect of peramine against Argentine stem weevil adults from concentrations above 15 μg/g in planta (Popay and Wyatt, 1995). The latter toxicity threshold is used in agricultural contexts in New Zealand to determine a protection against Argentine stem weevil (Hewitt et al., 2020). Peramine is hydrophilic and therefore evenly distributed throughout the plant (Ball et al., 1997a), whereas ergovaline concentrations are higher in the very basal plant tissues (Spiering et al., 2005b) and the inflorescences (Repussard et al., 2014a, 2014b). Lolitrem B, a hydrophobic compound, tends to be higher in older leaves, but seems to be highest in the seeds, respectively the inflorescences (Ball et al., 1997b; Repussard et al., 2014b; Spiering et al., 2005b). *Epichloë festucae* var. *lolii* tends to accumulate in the lowest part of *Lolium perenne* (Spiering et al., 2005b), whereas *Epichloë coenophiala* in *Festuca arundinacea* is distributed in the whole plant (Takach et al., 2012). It is important to be aware of the distribution of the alkaloids in the plant, when sampling plant material for the determination of alkaloids.

I.4. Intoxication events

Intoxication events due to alkaloids produced by *Epichloë* spp. are mostly known from New Zealand, Australia and the USA (Hume et al., 2016; Young et al., 2013). In these countries single grass species are dominating the grasslands (Johnson et al., 2013). In the late 19th century *Lolium perenne* (perennial ryegrass) arrived with the European settlers in New Zealand (Johnson et al., 2013). Most *Epichloë* spp. infect grasses of the Northern Hemisphere, but there are also three native grasses in New Zealand, which are infected with *Epichloë* (Hume et al., 2020). However, mass intoxication events are predominantly caused by introduced *Epichloë* infected grass species. In the 1980s the alkaloids responsible for biological activity produced by the endophytic *Epichloë* species, were first identified (Johnson et al., 2013). The idea of not using *Epichloë* infected grasses was abandoned, because another non-native species, the Argentine stem weevil (*Listronotus bonariensis*), was accidentially introduced in New Zealand and destroyed many grasslands. Due to the production of the insect deterring alkaloid peramine by the *Epichloë* fungi, only *Epichloë* infected *L. perenne* grasslands persisted against the herbivore. Hence, the widespread use of *L. perenne* in New Zealand and the introduction of the Argentine stem weevil led to severe intoxication events in grazing animals in New Zealand (Hume et al., 2016; Johnson et al., 2013). Agriculture in New Zealand was conflicted between the benefits of insect deterrence against the Argentine stem weevil and the intoxication risks for vertebrates. Farmers seeded infected grass seeds, due to the financial losses caused by the Argentine stem weevil. Hence, many grasslands became highly toxic for vertebrates in New Zealand (Johnson et al., 2013). Nowadays, vertebrate safe *Epichloë* endophyte strains, like AR1, are developed, which do not produce vertebrate toxic alkaloids, but the insect deterring alkaloid peramine (Johnson et al., 2013; Moate et al., 2012; Young et al., 2013) (Table I2). Those new strains contribute about $200 million per annum to the New Zealand economy (Johnson et al., 2013).

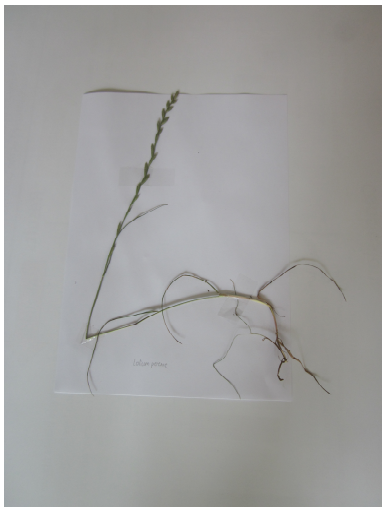
Table I2: Agriculturally used *Epichloë* species/strains, produced alkaloids, key traits and region of use, adapted from: (Johnson et al., 2013)

Common or commercial name	*Epichloë* species	alkaloids produced	Traits	Region of use
Common-toxic (wild-type)	*Epichloë festucae* var. *lolii*	Lolitrem B Peramine Ergovaline	-Ryegrass staggers +Argentine stem weevil and black beetle resistance	Ryegrass pastures and turf New Zealand, Australia, South America
Common-toxic (wild-type)	*Epichloë coenophiala*	Peramine Ergovaline Lolines	-Fescue toxicosis +broad insect restistance	Tall fescue pastures and turf USA
Common-toxic (wild-type)	*Epichloë uncinata*	Lolines	+broad insect resistance	Meadow fescue pastures USA, Europe
Endosafe	*Epichloë festucae* var. *lolii*	Peramine Ergovaline	+no ryegrass staggers, good argentine stem weevil resistance	Ryegrass pastures New Zealand
AR1	*Epichloë festucae* var. *lolii*	Peramine	+no ryegrass staggers, good Argentine stem weevil resistance	Ryegrass pastures New Zealand, Australia, South America
Endo5	*Epichloë festucae* var. *lolii*	Peramine Ergovaline	+no ryegrass staggers, good argentine stem weevil and black beetle resistance	Ryegrass pastures Australia
NEA2	Mix of *Epichloë festucae* var. *lolii* strains	Lolitrems Ergovaline Peramine	+good black beetle resistance	Ryegrass pastures New Zealand, Australia
AR37	*Epichloë* spp.	Epoxy-janthitrems	-some ryegrass staggers +Broad insect resistance, excellent animal performance	Ryegrass pastures New Zealand, Australia
MaxQ	*Epichloë coenophiala* strain AR542 and AR584 (MaxQII)	Lolines Peramine	+no fescue toxicosis, broad insect resistance	Tall fescue pastures, USA
Max P	*Epichloë coenophiala* strain AR542 and AR584	Lolines Peramine	+no fescue toxicosis, broad insect resistance	Tall fescue pastures New Zealand, USA
Avanex	*Epichloë coenophiala* strain AR601	Ergovaline Lolines Peramine	Bird and wildlife deterrent	Tall fescue pastures Airports
Avanex	*Epichloë coenophiala* strain AR94/95	Ergovaline Lolitrem B (only AR95)	Bird and wildlife deterrent	Ryegrass sport fields, recreational parks

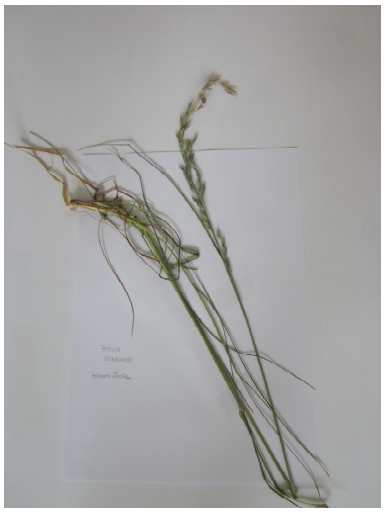
Intoxication events in the USA are predominantly caused by the *Epichloë* cultivar Kentucky 31 in *Festuca arundinacea* (tall fescue) (Hume et al., 2016; Johnson et al., 2013) (Table I2) and the beef cattle industry had financial losses of US$ 1.0 - 1.5 billion per year due to *Epichloë* infected tall fescue (Aiken and Strickland, 2013; Hume et al., 2016).

Therefore, *Epichloë* infections in *L. perenne* and *F. arundinacea* (Figure I5) in these countries are well studied, whereas studies of other grass species are rare (Saikkonen et al., 2006). The estimated number of possibly infected species is

approximately 900 and for more than 100 grass species infections with *Epichloë* endophytes have been shown (Hume et al., 2016; Leuchtmann, 1993). Additionally, infection rates and alkaloid concentrations in Germany are not well studied, although the origin of the symbiosis is in the Northern Hemisphere (Hume et al., 2020). For the evaluation of intoxication risks for vertebrates but also invertebrates it is important to investigate infection rates, but also the genotypes and chemotypes of the *Epichloë* endophytes. Alkaloid pathway genes are detected with polymerase-chain-reactions to detect the genotype and this provides the opportunity to predict the possibly produced alkaloids. Analytical measurements like high pressure liquid chromatography are used to quantify alkaloid concentrations and determine the chemotype of the *Epichloë* species, meaning the produced alkaloids. Hence, I investigated infection rates, genotypes as well as chemotypes of *Epichloë* endophytes in 13 German grass species in my doctoral thesis (Chapter II) (Figure I6). In chapter II and III, I evaluated the intoxication risk of vertebrates and invertebrates due to *E. festucae* var. *lolii* infected *L. perenne* in Germany.



Lolium perenne



Festuca arundinacea

Figure I5: *Lolium perenne* (perennial ryegrass) and *Festuca arundinacea* (tall fescue). Photos © Veronika Vikuk

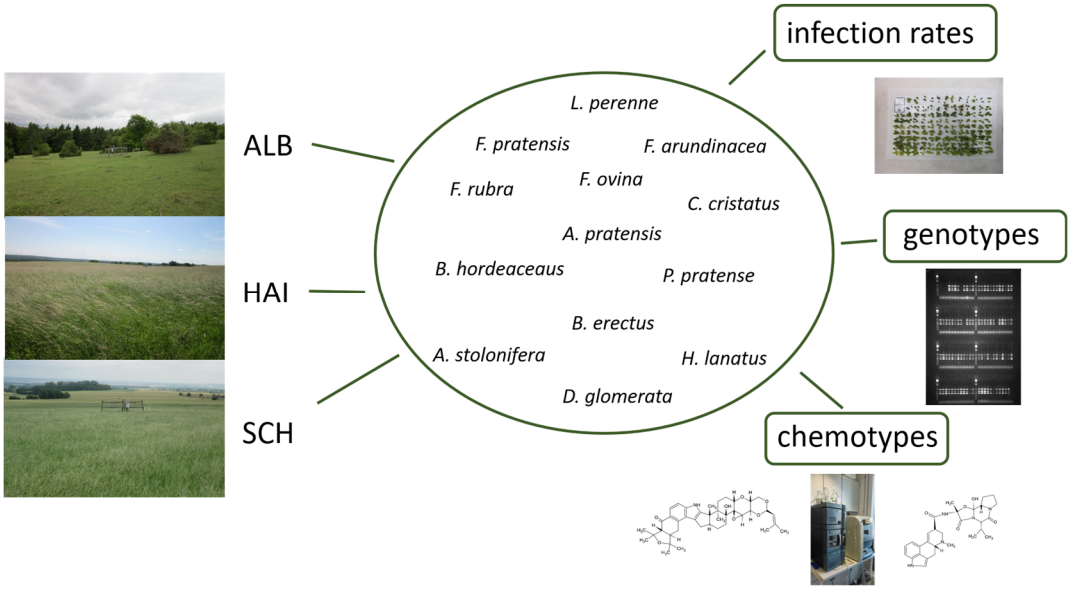


Figure I6: I sampled 13 grass species (*Lolium perenne, Festuca pratensis, Festuca arundinacea, Festuca rubra, Festuca ovina, Cynosurus cristatus, Alopecurus pratensis, Bromus hordeaceus, Phleum pretense, Bromus erectus, Agrostis stolonifera, Holcus lanatus, Dactylis glomerate*) in three different regions in Germany: ALB: Schwäbische Alb, HAI: National park Hainich, SCH: Schorfheide and detected infection rates of the grass species with *Epichloë* endophytes and determined genotypes and chemotypes of the *Epichloë* species. © Veronika Vikuk

As already mentioned, in order to estimate intoxication risks it is necessary to know infection rates, but it is even more important to know about alkaloid concentrations in the grasses. However, measurements for alkaloid concentrations differ between different laboratories in their detection methods, but also in the used plant material. High performance liquid chromatography is often used with different detection methods like fluorescence (Craig et al., 2014; Finch et al., 2012, 2013; Gallagher et al., 1985; Murty et al., 2018; Repussard et al., 2014c) tandem mass spectrometry (Fuchs et al., 2013; König et al., 2018; Rudolph et al., 2018), or mass spectrometry with electrospray ionisation (Shelby et al., 1997). Enzyme immunoassays are used as well (Bauer et al., 2017). For the detection of lolines, often produced by *Epichloë uncinata* in *Festuca pratensis* (Schardl et al., 2012; Spiering et al., 2005a), gas chromatography with flame ionisation (Justus et al., 1997; Yates et al., 1990) or mass spectrometry with electron ionization (Blankenship et al., 2001; Schardl et al., 2007) are used. However, liquid chromatography with mass spectrometry is also used for loline detection (Adhikari et al., 2016; Rudolph et al., 2018). *Epichloë* alkaloids have been extracted from fresh (Fuchs et al., 2017a; König

et al., 2018) or freeze dried plant material (Rottinghaus et al., 1991), straw (Craig et al., 2014; Repussard et al., 2014c), horse serum (Rudolph et al., 2018) or excretion of cattle (Murty et al., 2018). Since alkaloid concentrations may differ between methods, I investigated the difference of alkaloid concentrations detected with fresh or dry plant weight in Chapter III and compared two different analytical methods.

I.5. Different study designs: The Biodiversity Exploratories and a common garden experiment

I used the study design of the German Biodiversity Exploratories project (www.biodiversity-exploratories.de) to sample grass species on 150 study sites in three regions in Germany for experiments I described in Chapter II and III (Figure I7). The three regions cover different landscape types and span a latitude of 800 km from north to south Germany (Fischer et al., 2010). The UNESCO Biosphere region Schwäbische Alb (ALB), located in southwest Germany (Baden-Württemberg) and the Hainich National Park (HAI), located in central Germany (Thüringen) are both characterized by calcareous bedrock (Fischer et al., 2010). The UNESCO Biosphere region Schorfheide-Chorin (SCH), located in northeastern Germany (Brandenburg), is characterized by glacially formed landscapes (Fischer et al., 2010) (Figure I7). The study sites in each region have been selected along a land-use intensity gradient. Study sites are fertilized and unfertilized and can be divided into pastures, mown pastures and meadows (Figure I8). Sites with low intensity were for example wetlands and calcareous grasslands. Sites with highest intensity had high stocking rates (70 livestock units/ha; cattle, sheep, horse or goat), four to five cuts per year and a large amount of fertilizer was applied (400 kg/ha/year) (Socher et al., 2013).

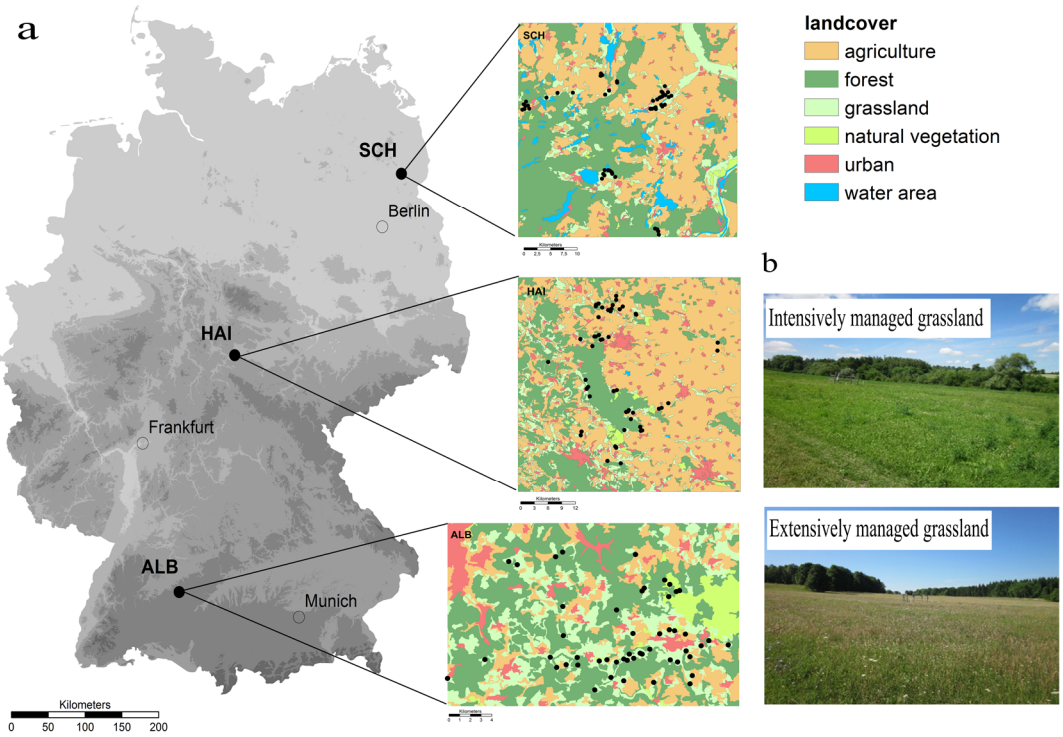


Figure I7: Left: Three regions of the Biodiversity Exploratories: Schorfheide-Chorin (SCH), Hainich National Park (HAI) and Schwäbische Alb (ALB). **Center:** Grass species were sampled on 150 grassland study sites in the three regions. **Right:** A land-use intensity gradient is spanning across the regions and study sited from intensively managed grassland to extensively managed grassland (right). Figure from (Vikuk et al., 2019).

Land-use intensity was calculated with the so called land-use intensity index (LUI) with the following formula (Blüthgen et al., 2012):

$$L_i = \frac{F_i}{F_R} + \frac{M_i}{M_R} + \frac{G_i}{G_R}$$

With L_i representing land-use intensity for the study site i, F_i the fertilization level (kg nitrogen ha^{-1} $year^{-1}$), M_i the frequency of mowing per year and G_i the grazing intensity reflected by the density of livestock (livestock units days of grazing ha^{-1} $year^{-1}$). F_R, M_R and G_R represent the respective mean within the region R for one year. L_i is dimensionless (Blüthgen et al., 2012).

Figure I8: Example study sites in the Biodiversity Exploratories including intensively and extensively managed grasslands as well as meadows, pastures and mown pastures. © Veronika Vikuk

Another part of the experiments in Chapter III was conducted in a common garden in Würzburg (Figure I9). *L. perenne* seeds infected with *E. festucae* var. *lolii* and non-infected seeds were sown in the greenhouse and translocated to the common garden of the University of Würzburg after six weeks (Figure I9).

Figure I9: *Lolium perenne* plants grown in the greenhouse (**left**). After six weeks plants were translocated to a common garden at the University of Würzburg (**right**). Signs indicate if grasses are infected with *Epichloë festucae* var. *lolii* (red) or not (light blue). © Veronika Vikuk

I.6. Influence of different factors on infection rates and alkaloid concentrations

Alkaloid concentrations from *Epichloë* spp. in their host grasses depend on different abiotic factors. Temperature, respectively season (Bourguignon et al., 2015; Fuchs et al., 2017a; Hennessy et al., 2016; König et al., 2018; McCulley et al., 2014), but also herbivory (Fuchs et al., 2017b) can induce alkaloid concentrations. It is also suggested that herbivory might increase the vertical transmission of the endophyte and thus increases the distribution of the endophyte in the population (Gundel et al., 2020). Furthermore, land-use intensity in general can have an influence on *Epichloë* infections (Gwinn et al., 1998; Jensen and Roulund, 2004). It has been shown, that alkaloid contents increase in summer and decrease again in winter in common garden experiments (Fuchs et al., 2017a), in sown field cultures (Jensen, 2005; Repussard et al., 2014b) and in managed grasslands (König et al., 2018). Temperature and precipitation seem to be the main driver for this seasonal change. Higher temperatures for example resulted in increased epoxyjanthitrem concentrations in *Epichloë festucae* var. *lolii* (AR37) infected perennial and Italian ryegrass in New Zealand (Hennessy et al., 2016). Warming also increased ergovaline

concentrations in *Epichloë coenophiala* infected tall fescue plants (Bourguignon et al., 2015; McCulley et al., 2014) and in combination with elevated precipitation loline concentrations increased as well (McCulley et al., 2014). Ryegrass staggers often occur in autumn, after hot and dry summers (Hume et al., 2016), which could be explained by increased alkaloid concentrations associated with warm temperatures. Elevated temperature also increased tillering and biomass of tall fescue (Bourguignon et al., 2015), a benefit of the *Epichloë* grass symbioses used in agriculture.

Moreover, alkaloid concentrations increase with plant age (Fuchs et al., 2017a; Hewitt et al., 2020). A recent study showed that alkaloid production in seedlings started around six days after sowing, but initially peaked around 8 to 10 day old seedlings, then decreased until 43 days post sowing, when concentrations increased again (Hewitt et al., 2020). Furthermore, it was shown that alkaloid contents accumulate over years (Fuchs et al., 2017a). Another study showed that grazing cattle, simulated by clipping, induced lolitrem B, whereas locusts increased peramine concentration (Fuchs et al., 2017b). Hence, the specific alkaloid is induced, depending on the herbivore, whereas intensive grazing or public usage can increase infection rates (Gwinn et al., 1998; Jensen and Roulund, 2004) and fertilization can increase peramine concentration (Krauss et al., 2007).

Drought resistance is often mentioned as a benefit of *Epichloë* spp. infections in grasses (Hume et al., 2016; Saikkonen et al., 2006). But the effect seems to depend on genotypes of the host-endophyte combinations and environmental conditions (Hume et al., 2016). The drought adaptation, for example, depends on the origin of the plant genotype (Annicchiarico et al., 2011). Hence, *Epichloë* infections can have a positive effect on the number of tillers and biomass under drought conditions in grasses from areas, where drought is known to occur e.g. in Italy, Tunisia, Morocco and Turkey (Kane, 2011). But there are also reports that *Epichloë* infections had no influence on drought resistance of the plants (Vázquez-de-Aldana et al., 2013) or that biomass was induced with daily watering instead of drought in a growth chamber experiment with *Elymus virginicus*, a native grass species in the US, infected with *Epichloë elymi* (Rudgers and Swafford, 2009).

In order to improve the understanding of the occurrence of *Epichloë* infections in grass species in Germany and to prevent possible intoxication events, I investigated the influence of land-use intensity on infection rates and alkaloid contents in *L. perenne* in Germany as well as seasonal changes in alkaloid concentrations in *L. perenne* in Schorfheide-Chorin. Additionally, I investigated seasonal changes in a common garden experiment depending on the usage of different analytical methods and the use of fresh or dry plant weight (Chapter III) (Figure I10). Experiments, which studied the influence of land-use intensity and season in the Biodiversity Exploratories (König et al., 2018), as well as the influence of season in a common garden experiment (Fuchs et al., 2017a) in *L. perenne* infected with *E. festucae* var. *lolii* have already been conducted. Since these previous studies detected alkaloid concentrations with fresh plant weight and used another analytical method, I repeated these studies in order to detect changes, depending on whether alkaloid concentrations are detected with dry plant weight, or if another analytical method is used (Chapter III). I also investigated, if intoxication risks in German grasslands need to be re-evaluated, depending on whether alkaloid concentrations are detected with dry plant weight or with different analytical methods (Chapter III) (Figure I10).

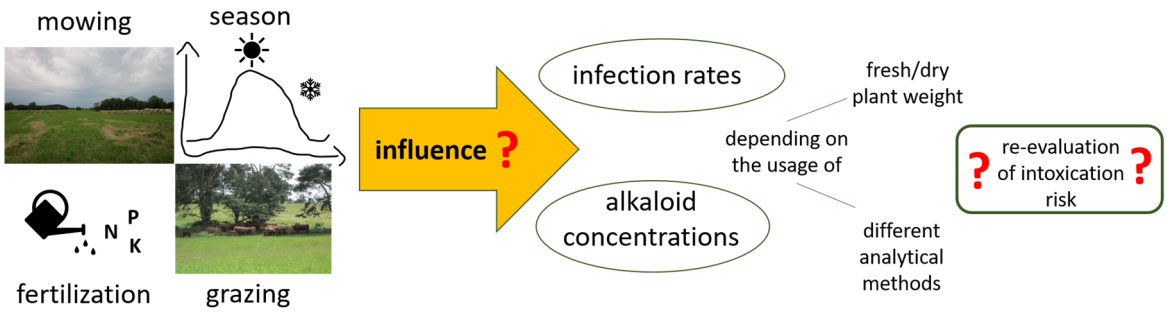


Figure I10: I investigated if mowing, fertilization, grazing or season had an influence on infection rates or alkaloid concentrations depending on whether fresh/dry plant weight or different methods were used and if that leads to a re-evaluation of intoxication risks in German grasslands (Chapter III). ©Veronika Vikuk

I.7. Seed breeding and different *Epichloë* cultivars

Since it is known that some of the alkaloids produced by *Epichloë* spp. can harm livestock, vertebrate safe *Epichloë* sp. were developed for example in New Zealand (Hume et al., 2020) (Table I2). These *Epichloë* endophytes produce less or no vertebrate toxic compounds, but still insect toxic or deterring alkaloids and keep other positive effects on the host grass, like drought resistance and biomass increase (Hume et al., 2020; Johnson et al., 2013; Karpyn Esqueda et al., 2017). Vertebrate safe endophytes are widely used in New Zealand, Australia and the USA, countries which had problems with intoxication events in the past (Hume et al., 2020; Johnson et al., 2013). In Europe, or particularly Germany, the usage of infected grass seeds with vertebrate safe or natural *Epichloë* endophytes is not common, thus *Epichloë* infected seed mixtures are hardly studied (Jensen, 2005; Saikkonen et al., 2000). But since *Epichloë* infected grasses can be advantegous, considering e.g. climate change, it is possible that seed breeders might use *Epichloë* infected grass seeds in future (Humphreys et al., 2006; Karpyn Esqueda et al., 2017). It is not known so far, if commercially available seed mixtures (Figure I11) already contain *Epichloë* infected grass seeds, because it is not mandatory to claim the usage of *Epichloë* endophytes in Germany. *Epichloë* infected seeds are less predated by insects (Popay et al., 2000) and birds (Madej and Clay, 1991), and can contain higher alkaloid concentrations than in the mature plants (Ball et al., 1997a, 1997b; Hewitt et al., 2020). Hence, I investigated infection rates and alkaloid contents in 24 commercially available seed mixtures in Chapter IV.

Figure I11: Examples for tested commercially available seed mixtures. © Veronika Vikuk

I.8. Research questions

In this doctoral thesis, I focus on the following questions:

1. Which *Epichloë*-grass symbioses are present on German grasslands and how are they characterized (genotypically and chemotypically)? (Chapter II)
2. Do different methods, e.g. the usage of dried or fresh plant weight or different analytical methods, have an influence on alkaloid concentrations measured in *L. perenne* infected with *E. festucae* var. *lolii*, and thus on seasonal changes and the influence of land-use intensity? If yes, does the intoxication risk in Germany need to be re-evaluated? (Chapter III)
3. Are commercially available grassseed mixtures in Europe infected with *Epichloë* endopyhtes and can alkaloids be detected? Does this influence the intoxication risks of grazing animals in Europe? (Chapter IV)

Chapter II

Infection rates and alkaloid patterns of different grass species with systemic *Epichloë* endophytes

Grassland in Germany. Photo: © Veronika Vikuk

"We should preserve every scrap of biodiversity as priceless while we learn to use it and come to understand what it means to humanity."

E. O. Wilson, The Diversity of Life

Chapter II

Infection rates and alkaloid patterns of different grass species with systemic *Epichloë* endophytes

Symbiotic *Epichloë* species are fungal endophytes of cool-season grasses that can produce alkaloids with toxicity to vertebrates and/or invertebrates. Monitoring infections and presence of alkaloid in grasses infected with *Epichloë* species can provide an estimate of possible intoxication risks for livestock. We sampled 3046 individuals of 13 different grass species in three regions on 150 study sites in Germany. We determined infection rates and used PCR to identify *Epichloë* species diversity based on the presence of different alkaloid biosynthesis genes then confirmed the possible chemotypes with HPLC/UPLC-MS and GC-MS measurements. Infections of *Epichloë* spp. were found in *Festuca pratensis* Huds. (81%), *Festuca ovina* L. agg. (73%), *Lolium perenne* L. (15%), *Festuca rubra* L. (15%) and *Dactylis glomerata* L. (8%). The other eight grass species did not appear to be infected. For the majority of *Epichloë*-infected *L. perenne* samples (98%) the alkaloids lolitrem B and peramine were present, but ergovaline was not detected, which was consistent with the genetic evaluation as *dmaW* the gene encoding the first step of the ergot alkaloid biosynthesis was absent. *Epichloë uncinata* in *F. pratensis* produced anti-insect loline compounds. The *Epichloë* sp. observed in the *F. ovina* agg. samples showed the greatest level of diversity and different intermediates of the indole-diterpene pathway could be detected. *Epichloë* infection rates alone are insufficient to estimate intoxication risks for livestock, as other factors like the ability of the endophyte to produce the alkaloids also need to be assessed.

Severe problems of livestock intoxication from *Epichloë* infected forage grasses have been reported from New Zealand, Australia and the USA, but much less from Europe, or particularly Germany. Nevertheless, it is important to monitor infection rates and alkaloids of grasses with *Epichloë* fungi, to estimate possible intoxication risks. Most studies focus on agricultural grass species like *Lolium perenne* and *Festuca arundinacea*, but other cool season grass species can also be infected. We show that in Germany, infection rates and alkaloids differ between grass species

and that some of the alkaloids can be toxic to livestock. Changes in grassland management due to changing climate, especially with a shift towards grasslands dominated with *Epichloë* infected species such as *Lolium perenne*, may result in greater numbers of intoxicated livestock in the near future. We therefore suggest regular monitoring of grass species for infections and alkaloids and call for maintaining heterogenous grasslands for livestock.

II.1. **Introduction**

Grasslands are some of the most species rich ecosystems in central Europe (Socher et al., 2013). In these grasslands more than 100 grass species are naturally infected with fungal endophytes from the genus *Epichloë* and the estimated number of possibly infected grass species is approximately 900 (Hume et al., 2016; Leuchtmann, 1993). Endophytic *Epichloë* species live as systemic, asymptomatic symbionts inside the plant (Sampson, 1933). The endophyte-plant interaction can range from a mutualistic to an antagonistic symbiosis (Saikkonen et al., 1998; Schardl, 1996). In the often mutualistic symbiosis, the grass provides the endophyte nutrients, shelter and dispersal, while the fungus can provide drought and herbivore resistance for the grass (Schardl, 1996). The herbivore resistance is associated with the production of bioactive alkaloids produced by the endophytic fungi, which can be toxic to vertebrates and invertebrates. Additionally, *Epichloë* spp. have been shown to also enhance host plant immune response, as infection with the fungus can change expression of up to one third of host plant genes (Bastias et al., 2017; Dupont et al., 2015).

Epichloë spp. can produce up to four different alkaloid classes (Schardl, 1996; Schardl et al., 2013a). The 1-aminopyrrolizidines (including lolines) and peramine are toxic or deterrent to insects, whereas the indole-diterpenes and ergot alkaloids are known to cause toxicity to vertebrates in addition to possessing anti-insect properties (Schardl, 1996; Schardl et al., 2013a). The production of alkaloids depends on the symbiotic *Epichloë* species in the host grass. Several studies screening for *Epichloë* species in specific grass species showed that endophyte diversity and, thus also alkaloid diversity, can be high within a single grass species (Charlton et al.,

2014; Oberhofer and Leuchtmann, 2012; Shymanovich et al., 2017; Yi et al., 2018). The genes encoding each class of alkaloid have been identified and for most genes, the relative step they encode within each biosynthetic pathway is known (Saikia et al., 2012; Schardl et al., 2013a; Young et al., 2015). Peramine synthesis is encoded by the single gene, *perA* (Tanaka et al., 2005), whereas the ergot alkaloids (EAS), indole-diterpenes (IDT) and lolines (LOL) each have multiple genes associated in gene clusters (Schardl et al., 2013a, 2014). Much of the alkaloid diversity present in the *Epichloë* is due to presence or absence of the alkaloid biosynthesis genes, which makes it possible to predict synthesized alkaloids based on detection of genes encoding key pathway steps (Charlton et al., 2014; Chen et al., 2015; Shi et al., 2017; Shymanovich et al., 2017; Yi et al., 2018).

Lolitrem B, an indole-diterpene, is produced by *Epichloë festucae* var. *lolii* in *Lolium perenne* (perennial ryegrass) plants, as well as by *Epichloë* sp. FaTG-2 present in *Festuca arundinacea* (tall fescue) (Christensen et al., 1993; Takach et al., 2012). Lolitrem B is known to cause ryegrass staggers, which is characterized by tremors in grazing livestock (Menna et al., 2012). Most notably, New Zealand and Australia have experienced outbreaks of ryegrass staggers, which are responsible for severe economic loss of livestock (Hume et al., 2016). These outbreaks typically occur in summer and autumn due to endophyte growing and producing alkaloids in association with the plant in response to high temperatures and peaking in late summer (Fuchs et al., 2017a; König et al., 2018). Other indole-diterpenes such as paxilline, a related indole-diterpene, can also have tremorgenic effects on livestock and should be considered in the evaluation of toxicity (Imlach et al., 2008). The ergot alkaloid ergovaline can be produced by *E. festucae* var. *lolii* and by *Epichloë coenophiala* in *Festuca arundinacea* (Guerre, 2015), where high ergovaline concentrations are responsible for fescue toxicosis, which results in poor livestock performance, and is often seen in regions where tall fescue is dominant in the USA (Hoveland, 1993).

Most studies worldwide focus on the agriculturally important grass species *L. perenne* and *F. arundinacea* as model systems, because most reports of intoxication are in livestock grazing these two species (Saikkonen et al., 2006). Endophyte infection rates for *L. perenne* appear low in Germany (8-28%) (Dobrindt et al., 2013;

König et al., 2018; Oldenburg, 1997) in contrast to New Zealand and Australia (70%) (Easton and Tapper, 2008). For German grasslands, only infection rates of *L. perenne* (Dobrindt et al., 2013) and concentrations of alkaloids have been documented (Bauer et al., 2018; König et al., 2018; Oldenburg, 1997).

The aim of this study is to provide an overview on infection rates of *Epichloë* ssp. in different regionally common grass species in German grasslands. We collected 13 grass species in three regions in Germany along a land-use intensity gradient. Our focus was on the agronomically important grasses *L. perenne*, *F. pratensis*, *F. arundinacea* and *D. glomerata*, which have been widely reported to be infected with *Epichloë* (Clay and Schardl, 2002). We also sampled less examined wild species, which are reported to possibly be infected with *Epichloë*, for example *Festuca ovina* agg. and *Holcus lanatus* (Leyronas and Raynal, 2001). We determined infection rates and linked the endophyte genetic diversity based on presence or absence of alkaloid pathway genes to the actual alkaloid chemotype. Finally, based on our observations, we speculate on the risks for grazing livestock on the studied grasslands.

II.2. Materials and Methods

II.2.1. Plant material and sampling.

In 2015 and 2017 tillers of 13 grass species were collected in three geographically separated regions in Germany. In 2017 *Lolium perenne* L., *Festuca pratensis* Huds. (syn. *Schedonorus pratensis* (Huds.) P. Beauv.), *Festuca ovina* L. aggregate (agg.), *Festuca arundinacea* Schreb. (syn. *Schedonorus arundinaceus* Roem. & Schult.), *Festuca rubra* L., *Phleum pratense* L., *Bromus erectus* Huds., *Bromus hordeaceus* L. and *Agrostis stolonifera* L. were collected. We collected the grass species in the northern region Schorfheide-Chorin (SCH) in June 2017, in the middle region Hainich National Park (HAI) in June and July 2017 and in the southern region Schwäbische Alb (ALB) in July 2017. Additionally, samples from a collection in 2015 from *Dacytlis glomerata* L., *Alopecurus pratensis* L., *Holcus lanatus* L. and *Cynosurus cristatus* L. were used. These samples were collected in the Hainich National Park (HAI) and

the Schwäbische Alb (ALB) in June 2015 and in the Schorfheide-Chorin in July 2015. The grass species have been selected due to their abundance in the sampling regions and these species have been previously reported as *Epichloë* -infected (Leyronas and Raynal, 2001).

We sampled 150 different study sites (Figure II1), 50 per region, which are part of the German Biodiversity Exploratories project (www.biodiversity-exploratories.de). The three regions span a latitude of 800 km from north to south Germany and cover different landscape types (Fischer et al., 2010). The UNESCO Biosphere region Schorfheide-Chorin (SCH) is located in the state of Brandenburg, in northeast Germany, and is defined by glacially formed landscapes (Fischer et al., 2010). Hainich National Park, located in central Germany (Thüringen, HAI), and the UNESCO Biosphere region Schwäbische Alb (ALB) located in southwest Germany (Baden-Württemberg), are both characterized by calcerous bedrock (Fischer et al., 2010) (Figure II1). Study sites were located inside and in the surrounding areas of the protected zones.

All study sites are part of real-life grasslands, which are conventionally managed by farmers. The study sites can be divided into meadows, pastures and mown pastures with both fertilized and unfertilized study sites (Fischer et al., 2010). The 50 grassland study sites in each region have been selected along a land-use intensity gradient, but land-use intensity was not used as an explanatory variable in this study (Fischer et al., 2010). The sites with the highest intensity had four to five cuts per year, high stocking rates (70 livestock units/ha, cattle, horse, sheep or goat) and a high amount of fertilizer was applied (up to 400 kg/ha/year) (Socher et al., 2013). The lowest intensity sites were species-rich semi-natural calcareous grasslands and wetlands. For each specific study site a 50 m x 50 m plot within the grasslands was selected (Fischer et al., 2010).

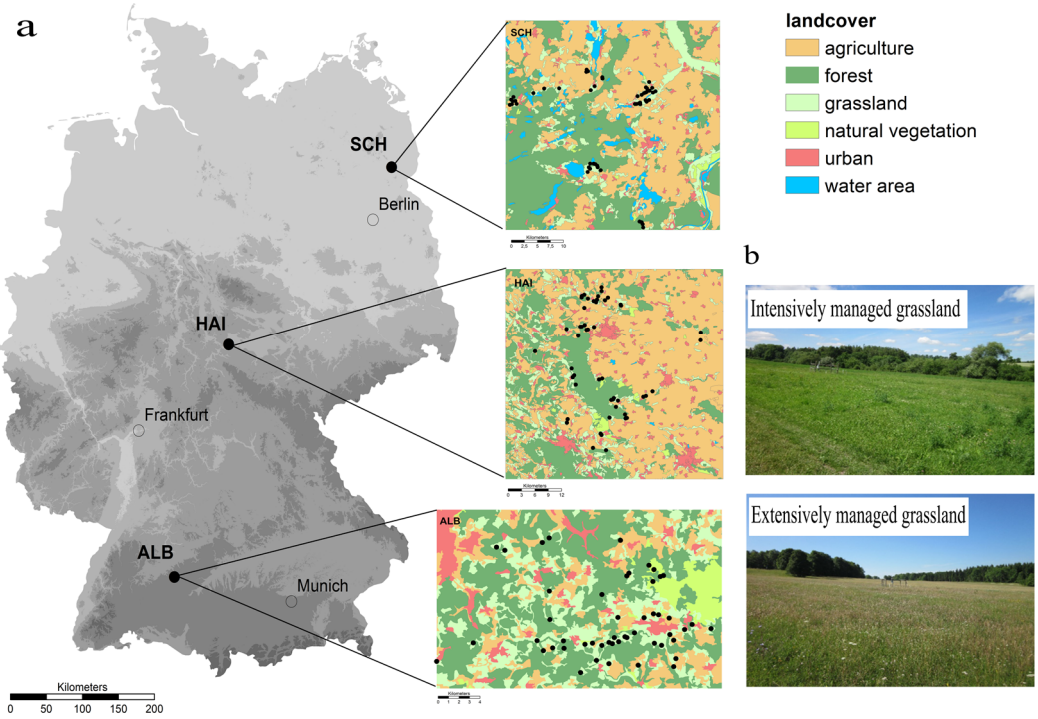


Figure II1: Study site locations **a.** Topographic map of Germany with the three study regions Schorfheide-Chorin (SCH) in the north, Hainich National Park (HAI) in central Germany and the Schwäbische Alb (ALB) in the south. Each region is depicted enlarged with corresponding land cover. Black dots indicate the 50 grassland study sites per region. **b.** Pictures of one intensively and one extensively managed grassland in SCH taken in June 2017. Intensively managed grassland have three to six cuts or more per year and are heavily fertilized (up to 400kg N/ha) and extensively managed grasslands are one to three-cut meadows, pastures with low stocking densities and low fertilization (Socher et al., 2013)

In each study site all selected grass species were collected when present. From each individual plant, three tillers of approximately 3 cm height were collected (König et al., 2018). We sampled the lowest part of the grass tiller between the ground and the first leaf, because this region is where the highest concentrations of alkaloids and the *Epichloë* hyphae accumulate (König et al., 2018; Spiering et al., 2005b). For each tiller we only selected tissue that was still green. We sampled 20 different individuals, but on some sites, this was reduced to 10 due to small populations of these species. Individual plants growing at least 3 m distance apart were sampled to avoid representing the same individual twice. The samples were

collected in 2 ml Eppendorf tubes, kept on dry ice in the field and stored at -20°C before further processing.

II.2.2. Endophyte detection by multiplex PCR.

A multiplex polymerase-chain-reaction (PCR) method for all collected species was performed to detect the endophyte and determine the presence of alkaloid biosynthesis genes (Charlton et al., 2014). With this method the endophyte infection rates for all collected grass species were determined. Initially, multiplex PCR (M1) with *Epichloë* specific primers was performed to determine if the samples were infected (primers: Table II1). Further PCR analyses were only performed for infected samples. The number of plant samples examined per species were: 1109 *L. perenne* samples, 1154 *F. pratensis*, 164 *F. ovina* agg., 176 *F. arundinacea*, 133 *D. glomerata*, 40 *F. rubra*, 24 *A. pratensis*, 23 *C. cristatus*, 45 *P. pratense*, 24 *H. lanatus*, 50 *B. erectus*, 50 *B. hordeaceus* and 45 *A. stolonifera*.

For *Epichloë* infected samples, five multiplex PCR (M1- M5) with different primer combinations (Table II1) were performed to predict the genotypic diversity of the different *Epichloë* species. All alkaloid biosynthesis primers were designed to bind to conserved gene regions using available information from *Epichloë* fungal genome and gene sequences (Charlton et al., 2014; Takach et al., 2012). We isolated total genomic DNA from the freeze-dried and ground grass tillers with the MagAttract 96 DNA plant core Kit (QIAGEN inc., Valencia, CA). PCR reactions were performed with a volume of 25 µl in total containing 3 µl DNA (1 ng/µl), 5 µl 5X Green GoTaq™ Reaction Buffer containing 1.5 mM $MgCl_2$, 0.5 µl of dNTPs (10 mM, Promega corp., Madison, WI), 0.2 µl 1.0 U Go Taq™ DNA Polymerase (Promega corp., Madison, WI) and 0.5 µl of each target specific primer (10 µM) (Table II1). The PCR parameters were 2 min initial denaturation at 94°C, 30 cycles of 15 s 94°C, 30 s 56°C, and 1 min 72°C followed by 10 min at 72°C. For samples with faint PCR products in the first multiplex PCR (M1) the number of cycles was increased to 40, which was necessary for all *L. perenne* samples and some *F. ovina* agg. samples. For multiplex 5 (M5) the annealing temperature step during the cycles was changed from 56°C to 60°C for 30 s and only 30 cycles were applied, because

40 cycles decreased the quality of the amplicons. PCR products were visualized by gel electrophoresis with 1.5% agarose gel in 1 x Tris-Boric-EDTA (TBE) buffer and following ethidium bromide staining and UV transillumination.

II.2.3. Phylogenetic analyses.

Samples were selected for phylogenetic placement using the sequences of the mating type genes as we were able to obtain better amplification for the polyploid species we evaluated. We selected five samples of *L. perenne*, six *F. pratensis*, nine *F. ovina* agg. samples and four *D. glomerata* samples. The mating type genes *mtBA* and *mtAC* (primers in Table II1) were sequenced for these 24 representatives. The PCR reactions were performed in 50 µl containing 8 µl DNA (1 ng/µl), 10 µl 5X Green GoTaq™ Reaction Buffer, 0.4 µl 1.0 U Go Taq™ DNA Polymerase, 1 µl of dNTPs (10 mM) and 1 µl of the target specific primers (10 µM). The same PCR parameters as for the multiplex PCR were used but the extension time was elongated to 2 min to account for the longer amplicon sizes. PCR amplicons were visualized as described previously. Only samples showing a single PCR product were selected for purification. PCR products were purified with the QIAquick PCR Purification Kit (250) (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. Big Dye Terminator Chemistry (version 3.1, Applied Biosystems, Foster City, CA) was used for sequencing. Due to low DNA concentration of the template we could only generate sequence data for the mating type genes. Geneious™ 11.1.5 (Bioweights Ltd, Auckland, New Zealand) was used for alignment and sequence editing. Forward and reverse reads were joined and trimmed and the final consensus sequences were then aligned with reference sequences from NCBI (Table SII1) using MUSCLE (version 3.7) and default settings. RaxML 8.2.11 (Stamatakis, 2014) was used to infer a phylogenetic tree with 1000 fast bootstraps. Accession numbers of the 24 partial mating type gene sequences are MK992983 to MK993006.

Table II1: Primers used in PCR to genotype endophytes

Multiplex primer combinations	Locus	Gene	Primer name	Forward primer (5´-> 3`)	Primer name	Reverse primer (5´-> 3´)	Size (bp)
M 1	*house-keeping*	*tefA*	tef1-exon1d-1	GGGTAAGGACGAAAAGACTCA	tef1-exon6u-1	CGGCAGCGATAATCAGGATAG	860
	PER	*perA*	perA-T2-F	TCTTCAGGCATCGCAGGAAC	perA-T2-R	TCGGCCACCTCCAGCCTGATG	600
	LOL	*lolC*	lolC-3a	GGTCTAGTATTACGTTGCCAGGG	lolC-5b	TCTAAACTTGACGCAGTTCGGC	442
	EAS	*dmaW*	dmaW-F4	GTGTACTTTACTGTGTTCGGCATG	dmaW-6R	GTGGAGATACACACTTAAATATGGC	282
	IDT/LTM	*idtG*	idtG-F	GAGCTTGAGAAGCTTACGAATCC	idtG-R	GGGCAATGGAGCGATTCTCTC	113
M 2	*MT*	*mtAC*	mtAC-F	CAATGGTGGTCACCTGAGAAG	mtAC-R	CGGTCTCATTCTTCCAGAGAGAGG	785
	PER	*perA-R*	perA-red F2	GAGATCAGTTCGCAGTTGTCAG	perA-red R	CTAGCCTCCAGATCTTGTGAAAG	447-589
	MT	*mtBA*	mtBA-F2	ATCAGTTGAGGGCGATTTGG	mtBA-R2	AGGCTTGCTTGACTCTATCCGC	213
M 3	*EAS*	*dmaW* alt[a]	dmaW818 (311+21)d	AACCCATCAACGGAGCAACTG	dmaW818 (1068-24)u	GCCAAACACTGTGAAATACACCTG	758
	EAS	*lpsB*	p12-F	CCGTCTTCCCGTATACCGAA	p12-R	TACCCACTGCCTCGAACTTG	598
	IDT/LTM	*idtQ*	ltmQ-313	CTACCAGGACAGGCGTGACGTCC	ltmQ-282	CAGAGGTTTAACCCTCTTGACGC	334
	LOL	*lolA*	lolA-F1	GAGACACTAGAGAAATGGCAGCTGC	lolA-R1	GGCATCCATGGTGGCGAAGATGTG	270
M 4	*LOL*	*lolO*	lolO-F1	GTGAACTGGCAGTAGTCCGTATG	lolO-R1	AATCCATGCCAGTGTCGGGAATG	492-719
	EAS	*easA*	easA-F	GCGGTTGCATTGAGAATCGCTC	easA-R	ATCTACCACAAGCTTGGCGGAC	350
	EAS	*easC*	easC-F2	CTGGAGCATATGGAGAGTTTG	easC-R2	AATGTTCAGGCAAACCCAGTC	278
M 5	*IDT/LTM*	*ltmE*	ltmE-356		ltmE-341		687
	LOL	*lolP*	lolP-F1	GTTCTAAACATCGTGACTGGGC	lolP-R1	GGTAGGTCAGCATCTTGTCAACG	566
	EAS	*cloA*	cloA-MP-F2	CGCACAACGCTCCATTGATGGC	cloA-MP-R2	AAGCTCGTGCCGGGAATTAGGC	434
	PER	*perA*	perA-5`F3	ATGACGAGCTCGGAGCGAGTTG	perA-5`R	TCGCAGCTGCAAGTCGAGCAC	309
	IDT/LTM	*idtF*	ltmF-359	GAATTATGTTACTCTTGGGG	idtF-MP-R	GTCTTCACCTGAGGGAAGTC	217
Amplicon sequencing primers	*EAS*	*dmaW*[b]	dmaW-F10	CCAACAATGACCAGAGGCTATG	dmaW-R10	TATAACAAGTTRAATCYCGCCG	1450
	LOL	*lolC*	lolC-F1	ATGACAGTAGATACGATTACTTCG	lolC-R1	TCATCTGTGACGCCAGCCTCAG	1630
	IDT/LTM	*idtM*[b]	idtM-F	GCGACTTCAAGGTAATAATCGTGG	idtM-R	CATCCTACAAAGCTTGGTCTATT	1551
	housekeeping	*tefA*[c]	tef1-exon1d-1	GGGTAAGGACGAAAAGACTCA	tef1-exon6u-1	CGGCAGCGATAATCAGGATAG	860
	housekeeping	*tubB*[d]	tubB F1	CTCTGTTTGTCTTGGGGACC	T1.2	CTGGTCAACCAGCTCAGCAC	660
	MT	*mtAC*[b]	mtAC-F	CAATGGTGGTCACCTGAGAAG	mtAC-R	CGGTCTCATTCTTCCAGAGAGAGG	785
	MT	*mtBA*[a,b]	mtBA-F2	ATCAGTTGAGGGCGATTTGG	mtBA-R2	AGGCTTGCTTGACTCTATCCGC	620
	EAS	*cloA*	cloA-F2	GGTTCTATTGGAACTGGATACGAG	cloA-R2	GTCCTTCACGATAGTGACCTC	1900
Fragment analysis primers	*B10*[e]	Peak color: green, label: VIC	B10.1	CGCTCAGGGCTACATACACCATGG	B10.2	CTCATCGAGTAACGCAGGCGACG	

Primers used for endophyte detection, alkaloid gene profile, mating type, phylogeny analysis and fragment analysis and expected product sizes (modified from (Charlton et al., 2014)), a: (Shymanovich et al., 2015), b: (Charlton et al., 2012), c: (Craven et al., 2001), d: (Young et al., 2005), e: (Moon et al., 1999)

II.2.4. Analysis of simple sequence repeats (SSR) in *Epichloë uncinata*.

As the genetic profiles the *F. pratensis* infected samples were all similar we used SSR primer pairs B10 and B11 (Moon et al., 1999) on a subset of 96 samples to determine if endophyte diversity could be detected in these samples. Total genomic DNA (1 ng/µl) was diluted 10x and 4 µl diluted DNA was used in the PCR reaction. The total reaction volume was 10 µl containing 1 µl 10x PCR buffer (without $MgCl_2$) (Invitrogen Life Technologies), 0.3 µl $MgCl_2$ (50 mM), 0.2 µl of dNTPs (5 mM), 0.2 µl of each primer (10 µM) and 0.15 µl Platinum Taq (Invitrogen, 5U/µl). PCR products from B10 and B11 were labeled with VIC and NED fluorescent dyes, respectively (Takach and Young, 2014). The PCR parameters were 4 min at 94°C, 35 cycles of 30 s at 94°C, 30 s 60 °C, and 30 s 72°C, followed 7 min at 72°C. Samples were kept at 4°C overnight. PCR products were diluted 10 times and an aliquot of 1.5 µl PCR product, 9.9 µl formamide and 0.1 µl 500 LIZ size standard (Applied Biosystems/Invitrogen Life Technologies) were added per reaction (Takach and Young, 2014). PCR products were denaturated at 96°C for 5 min and immediately placed on ice for 20 min. Applied Biosystems 3730 DNA Analyzer was used to seperate the PCR fragments and Peak Scanner v1.0 (Applied Biosystems) used to visualize fragment sizes. Only the B10 data was informative for determining ecotypes based on published data (Clayton et al., 2017).

II.2.5. Alkaloid analyses.

Alkaloids of a representative subset of 10 *L. perenne*, 24 *F. pratensis* and 35 *F. ovina* agg. samples were analyzed. Quantitation of ergovaline, peramine and lolitrem B in frozen *L. perenne* samples (fresh plant weight) was performed by ultra high performance liquid chromatography-tandem-mass spectrometry (UPLC-MS) using a published protocol (Fuchs et al., 2013). We prepared the samples as described in König et al. (2018). Homoperamine and ergotamine were used as internal standards. For a subset of 35 freeze-dried *F. ovina* agg. samples we analyzed indole-diterpene (IDT) alkaloids by HPLC/MS using a published protocol (Lee et al., 2017). Endophyte-infected, freeze-dried *F. pratensis* samples were analyzed for loline alkaloids. For each *F. pratensis* sample, loline alkaloids from 75 mg, lypholized tissue

was extracted and prepared for GC/MS analysis using a Varian CP-3800 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA,) coupled to a Varian Saturn 2200 mass spectrometer (Agilent Technologies) (Faulkner et al., 2006; Pan et al., 2014). The standard calibration curve was performed using *N*-formylloline (NFL). The concentrations of other loline alkaloids were calculated based on the NFL standard curve and thus, concentrations are considered relative to NFL concentrations.

Due to insufficient sample amount for *F. pratensis* and *F. ovina* agg. we pooled samples that originated from the same region and that appeared genetically similar based on the marker analyses. The pooling strategy resulted in analyses for *F. pratensis* of seven ecotype 1 samples, one ecotype 4 and 16 ecotype 3 samples. Additionally, we compared differences in loline alkaloid production of ecotypes 1 and 3 with Mann-Whitney U-Test for total amount of lolines and with a generalized mixed model with bionomial errors for presence or absence of the different loline alkaloids (tested individually). Influence of presence and absence of different loline alkaloid combinations on the ecotype and the amount of lolines was tested with a permanova with 999 runs and a Bray-Curtis similarity matrix (Oksanen et al., 2018). Data was analyzed with R version 3.5.2.

II.3. Results

II.3.1. Endophyte infection rates

In total 1147 *F. pratensis*, 1109 *L. perenne*, 164 *F. ovina* agg. and 133 *D. glomerata* plants were sampled and tested from all three regions. *Festuca arundinacea* (176 plants) did not occur in the ALB location and were only sampled from HAI and SCH regions. For the other plant species, a subset of the sampled plants from each region was tested (Table II2).

Using PCR, we determined the infection rates of *F. pratensis*, *L. perenne*, *F. ovina* agg., *D. glomerata* and *F. rubra.* as 81%, 15%, 73%, 8% and 15%, respectively. It should be noted that the amplification of the L. perenne and F. ovina agg. samples resulted in faint bands and the percentage of infected samples may be higher than

we have reported. The eight other grass species did not show *Epichloë* infections. In total, of the populations tested 92.3% of the *F. pratensis*, 42.4% *L. perenne*, 100% *F. ovina* agg., 26.3% *D. glomerata* and 75% *F. rubra* were infected with *Epichloë* (Table II2). Infection rates differed slightly between regions.

Table II2: *Epichloë* infection rates, determined by PCR, of plants collected across all study sites. Empty cells indicate that no samples were collected.

Grass species	Endophyte species	Infection rates [%][b]	Infection ALB [%]	Infection HAI [%]	Infection SCH [%]	Study sites sampled	Study sites infected
Festuca pratensis	*E. uncinatum*[a]	80.7 (926/1147)	94.2 (437/464)	62.0 (375/499)	75.2 (114/184)	78	72
Lolium perenne	*E. festucae* var. *lolii*[a]	14.5 (163/1109)	1.2 (4/335)	17.1 (55/322)	23.0 (104/452)	66	28
Festuca ovina agg.[c]	*E. festucae*[a]	73.2 (120/164)	60.0 (24/40)	86.8 (92/106)	22.2 (4/18)	11	11
Dactylis glomerata	*E. typhina*[a]	8.3 (11/133)	9.0 (8/89)	0.0 (0/16)	10.7 (3/28)	19	5
Festuca rubra	*E. festucae*	15.0 (6/40)			15.0 (6/40)	4	3
Festuca arundinacea	*E. coenophiala*	0.0 (0/176)		0.0 (0/46)	0.0 (0/130)	11	0
Alopecurus pratensis	*E. typhina*	0.0 (0/24)		0.0 (0/24)		5	0
Cynosurus cristatus	unknown	0.0 (0/23)	0.0 (0/23)			5	0
Phleum pratense	*E. typhina*	0.0 (0/45)	0.0 (0/15)	0.0 (0/15)	0.0 (0/15)	20	0
Holcus lanatus	*E. typhina ssp. clarkii*	0.0 (0/24)			0.0 (0/24)	5	0
Bromus erectus	*E. bromicola*	0.0 (0/50)	0.0 (0/25)	0.0 (0/25)		22	0
Bromus hordeaceus	unknown	0.0 (0/50)		0.0 (0/25)	0.0 (0/25)	21	0
Agrostis stolonifera	*E. baconii*	0.0 (0/45)	0.0 (0/12)	0.0 (0/18)	0.0 (0/15)	14	0

[a] *Epichloë* species confirmed by sequencing using the mating type genes were from samples of *L. perenne*, *F. pratensis*, *F. ovina* agg. and *D. glomerata.* Other host endophyte interactions are according (Leuchtmann et al., 2014).

[b] Percentage infection rate followed by number of *Epichloë* infected individuals and the total number of individuals screened are presented in brackets.

[c] The actual endophyte infection rates could be higher but the samples were difficult to analyze due to low template concentration, since the tillers are so fine.

II.3.2. Endophyte genotypic diversity

All 926 infected *F. pratensis* samples were consistent with respect to the markers, which were expected for infection with *Epichloë uncinata*. Both mating types were present (mtAC, mtBA), and all PER markers (per A5, perA-T2, perA-R) and LOL markers (lolC, lolA, lolO, lolP) were present (Table II3). EAS and IDT genes were not detected.

Two genetic profiles were observed for *Epichloë* in *L. perenne* plants (Table II3). Both genetic profiles were identical with respect to the presence of mating type B (*mtBA*), *perA* gene for peramine biosynthesis, and *idtG*, l*tmQ* and *ltmE* of the indole-diterpene pathway (IDT), but differed with respect to the ergot alkaloid locus (EAS). In 98% of the samples (161) the *dmaW* marker, used to detect the gene encoding the first step in the EAS pathway, was not present, but the other EAS genes were detected. Only three samples (2%) contained *dmaW* and likely contained a functional EAS pathway as the other EAS genes were present (Table II3). To verify that the lack of *dmaW* was not due to mismatched primers, another primer (dmaW10, Table II1) for this gene was tested, but also did not result in amplification of a PCR product.

F. ovina agg. samples showed high variability with the alkaloid gene profiles of the endophyte (Table II3 and II4). Both mating types were present within locations, but more mating type B samples were observed. The majority of samples contained all the *perA* markers, but for some samples (23%) the region encoding the *perA* reductase domain was not detected. The *EAS* genes were present in 88% of samples. *IDT* genes were present in 27% of samples but not all of these contained *idtG* that encodes the first step of the *IDT* pathway (Table II3 and II4). In total, nine different combinations of alkaloid biosynthesis genes could be observed in the endophyte-infected *F. ovina* agg. samples (Table II4). The most common samples (58%) contained all the *perA* and *EAS* markers and were predicted to produce peramine and ergot alkaloids. We predicted that 22% of the samples would be able to produce early pathway indole-diterpenes, from paspaline through to terpendole C, but due to the low template concentration, we could not confirm the presence of additional *IDT* genes.

We found two different genotypes in the six *F. rubra* samples. Both genotypes were mating type A, lacked the region encoding the *perA* reductase domain, and contained all the *EAS* markers, but they differed with respect to the *IDT* markers. Five of the samples lacked *IDT* genes, and one sample contained *idtG* and *ltmQ* (Table II3).

Three different genetic profiles were present in the 15 *D. glomerata* samples (Table II3). Both mating types were present, and 10 were mating type A, five were mating type B. Variation was associated with the presence (seven samples) or absence (three samples) of the region encoding the *perA* reducatase domain.

Alkaloid gene profiles can be also used for identification of *Epichloë* and their host grass species. In total, 15 of 1109 samples (1.4%) *L. perenne* were likely misidentified as the endophyte gene profiles were consistent with the *F. pratensis* samples. Other possible misidentified samples were, seven *F. pratensis* samples (0.6%), five *F. ovina* agg. (3.0%) and one *D. glomerata* sample (0.7%). Misidentification of samples are possible as a large proportion of the grass samples were collected vegetatively, occasionally after a mowing event or on strongly grazed study sites. We used the complete sampled plant material for the analyses of genotypes and chemotypes of the *Epichloë* fungi. Therefore, the identification of the plant species with plant marker genes is not possible. For the calculation of infection rates, possible misidentified samples were excluded.

Table II3: Genotypes of different *Epichloë* in the host grass species. `+` show presence, `-` absence of gene. ´+/-´ some samples contain the markers some not. Housekeeping gene *tefA* was used to confirm the infection with the endophyte. Mating type genes and selected pathway genes of different alkaloids were tested for presence or absence. Numbers of samples with the determined genotype are also shown. Predicted chemical profile (PER: peramine, IDT: indole-diterpenes, EAS: ergot alkaloids, LOL: lolines)

		Housekeeping gene	**Mating type**		**Peramine**			**Lolines (LOL)**				**Ergot alkaloids (EAS)**					**Indole diterpenes (IDT)**				
Grass species	***Epichloë* species**	**tefA**	**mtAC**	**mtBA**	**perA 5**	**perA-T2**	**perA -ΔR**	**lolC**	**lolA**	**lolO**	**lolP**	**dmaW**	**easC**	**easA**	**cloA**	**lpsB**	**idtG**	**idtQ**	**idtE**	Sample size	Predicted chemical profile
F. pratensis	*E. uncinata*	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	926	PER,LOL
L. perenne	*E. festucae* var. *lolii*	+	-	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	161	PER, IDT
		+	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	3	PER, EAS, IDT
F. ovina agg.[a]	*E. festucae*	+	-	+	+	+	+/-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	84	PER, EAS, IDT
		+	+	-	+	+	+/-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	36	PER, EAS, IDT
D. glomerata	*E. typhina*	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	3	PER
		+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	7	-
		+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-
F. rubra	*E. festucae*	+	+	-	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	5	EAS
		+	+	-	+	+	-	-	-	-	-	+	+	+	+	+	+	+	-	1	EAS, IDT

[a] further explanation of variants in Table II4

Table II4: Alkaloid biosynthesis gene diversity present in *F. ovina* agg. samples. `+` presence, `-` absence of gene. Predicted chemical profile (PER: peramine, IDT: indole-diterpenes, EAS: ergot alkaloids) and detected IDTs.

Peramine (PER)			**Ergot alkaloids (EAS)**					**Indole diterpenes (IDT)**						
***perA* 5**	***perA*-T2**	***perA*-ΔR**	***dmaW***	***easC***	***easA***	***cloA***	***lpsB***	***idtG***	***idtQ***	***idtE***	**No. samples**	**% of total**	**Predicted chemical profile**	**Detected IDTs (SZ) [a]**
+	+	+	+	+	+	+	+	+	+	-	8	7%	PER, EAS, IDT	Paxilline isomer, Paspaline, Terpendole C, Terpendole E, Terpendole I isomer (2)
+	+	-	+	+	+	+	+	+	+	-	3	3%	EAS, IDT	Paspaline (1)
+	+	+	-	-	-	-	-	+	+	-	9	8%	PER, IDT	Paspaline, 13-Desoxypaxilline isomer, Emindole SB (3)
+	+	-	-	-	-	-	-	+	+	-	6	5%	IDT	
+	+	+	+	+	+	+	+	-	-	-	69	58%	PER, EAS	
+	+	-	+	+	+	+	+	-	-	-	16	13%	EAS	
+	+	+	+	+	+	+	+	+	-	-	2	2%	PER, EAS, IDT?	
+	+	+	+	+	+	+	+	-	+	-	5	4%	PER, EAS	Paxilline isomer, Paspaline, Terpendole C (1)
+	+	-	+	+	+	+	+	-	+	-	2	2%	EAS	

[a]IDTs detected with HPLC-MS. Order of the substances indicates the abundancy of the peak. Detections only for a subset of samples, sample size (SZ) in brackets. Retention times of isomers: Paxilline isomer: 29.7 min, Terpendole I isomer: 29.6 min, 13-Desoxypaxilline isomer: 26.8 min. Empty cells indicate that measurement of IDTs was not possible due to limited sample amount.

II.3.3. Determining phylogenetic placement by evaluating mating type genes

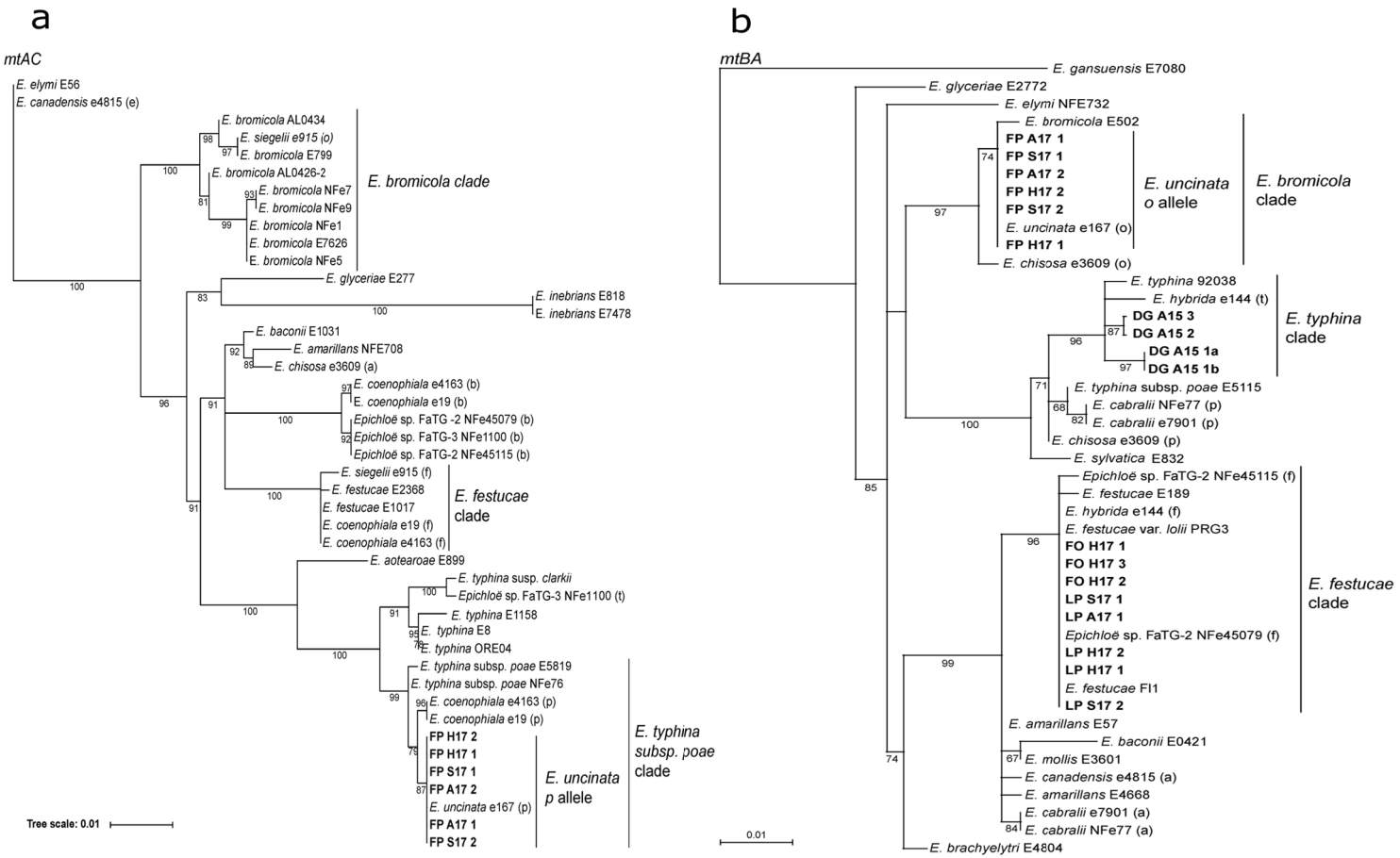
Mating type genes (*mtBA*, *mtAC*) were chosen for phylogenetic analyses as the template quantity for each sample was limited and these fragments amplified well.

The *F. pratensis* endophytes had both mating type genes, *mtAC* and *mtBA*, and the sequence grouped with the expected *E. uncinta* reference sequences in either the *E. typhina* subsp. *poae* clade (*mtAC*) or the *E. bromicola* clade (*mtBA*). Hence, the *F. pratensis* endophytes were identified as *Epichloë uncinata* (Figure II2). Interestingly, all *E. uncinata mtAC* genes, including the reference sequence for this species, are considered pseudogenes as a sequence transition results in a premature stop codon.

All sequenced *L. perenne* samples grouped in the *mtBA Epichloë festucae* clade along with *F. ovina* agg. (Figure II2), which is consistent with expected *E. festucae* var. *lolii* for *L. perenne*. Although it should be noted that there is no current way of distinguishing *E. festucae* from *E. festucae* var. *lolii* based on sequence data.

The *D. glomerata* samples grouped in the *mtBA E. typhina* clade, but the samples segregated into two branches. The *D. glomerata* samples segregated based on the presence or absence of the region encoding the *perA* reductase domain (Figure II2).

Figure II2: Phylogenetic analyses of mating type genes of *Epichloe* isolates from selected plant samples (bold). a. Phylogenetic tree derived from maximum likelihood analysis of mtAC introns, including *Epichloë* isolates from *Festuca pratensis* (FP_A17_1, 2, FP_H17_1, 2, FP_S27_1, 2). b. Phylogenetic tree derived from maximum likelihood analysis of mtBA introns including *Epichloë* isolates from *Dactylis glomerata* (DG_A15_1a, b, 2, 3), *Festuca pratensis* (FP_A17_1, 2, FP_H17_1, 2, FP_S27_1, 2), *Festuca ovina* agg. (FO_H17_1, 2, 3) and *Lolium perenne* (LP_H17_1, 2, LP_A17_1, LP_S27_1, 2). Trees are rooted at the left edges according to other published housekeeping gene trees (Schardl et al., 2014b). The first two letters for each isolate designation signify the grass species: DG = *Dactylis glomerata*, FP = *Festuca pratensis*, FO = *Festuca ovina* agg., LP = *Lolium perenne*; H = Hainich National Park, A = Swabian Alb, S = Schorfheide-Chorin. 17: sampled in 2017, 15: sampled in 2015; 1, 2, 3: sample number. The samples were clustered with reference gene sequences from NCBI. Sequences which are from hybrids are marked with a single letter abbreviation required for the allele: a = *E. amarillans*, o = *E. bromicola*, p = *E. typhina subsp. poae*, f = *E. festucae*, b = *E. baconii* and LAE clade.



II.3.4. Analysis of simple sequence repeats (SSR) of *E. uncinata*

Since all the *E. uncinata* samples from *F. pratensis* had the same genetic profile based on presence or absence of alkaloid and mating type genes, further genetic diversity was investigated with SSR primers. We selected and analyzed 90 *F. pratensis* samples (7.85% of the infected samples) representing all regions of the study. B10 was the most informative marker, which resulted in three different patterns associated with previously documented variation (Table II5, SII2) (Clayton et al., 2017). In total, 30 samples were classified as ecotype 1, 56 as ecotype 3 and four as ecotype 4. For the analyzed subset of samples ecotype 4 was only identified in HAI, whereas ecotype 3 occured in all three regions and ecotype 1 in HAI and SCH. Ecotypes 3 and 4 have been previously reported for samples found in Germany (Table II5) (Clayton et al., 2017).

Table II5: Classification of *Epichloë uncinata* from *Festuca pratensis* into ecotypes based on typical DNA fragments of the primer B10 and their distribution in Europe. *E. uncinata* samples from the three regions (ALB: Swabian Alb, HAI: Hainich National Park and SCH: Schorfheide-Chorin).

***E. uncinata* ecotype**[1]	**B10 (bp)**	**Distribution**[1]	**ALB**	**HAI**	**SCH**	**Total**
1	159, 194	Norway	0	16	14	30
2	159, 177	Bulgaria	0	0	0	0
3	171, 191	Germany	31	8	17	56
4	159, 196	Germany	0	4	0	4

[1] Ecotype data from Clayton et al., 2017

II.3.5. Chemical diversity

Based on the genetic profile of *E. uncinata* in *F. pratensis* we predicted that the *E. uncinata*-infected plants would produce the lolines *N*-formylloline (NFL) and *N*-acetylloline (NAL). GC/MS analyses confirmed that all 24 tested samples contained NFL and all but one contained NAL (Table SII3). Other metabolites in the loline pathway were also detected, such as *N*-acetylnorloline (NANL), loline and *N*-methylloline (NML), but 1-acetamidopyrrolizidine (AcAP) was not detected. The total concentrations of lolines varied from 18 to 3515 μg/g. Significant differences were not found between ecotype 1 and 3 for total concentrations of lolines ($p=0.92$; $W=54$) or the presence or absence of different lolines (tested

individually: generalized mixed effect model: NANL: p=0.54, Z=0.61, Df: 1,22; Loline: p=0.75, Z=0.32, Df: 1,22; NAL: p=0.10, Z=0.003, Df: 1,22; NML: p=0.41, Z=-0.83, Df: 1,22; NFL: p=1, Z=0, Df: 1,22, ACAP: p=1, Z=0, Df: 1,22; combination tested: permanova: p=0.66; Df: 1,22; F=0.42). There was a significant positive correlation of the presence of different loline combinations and the total concentration of lolines (permanova: p=0.01 ***; Df: 1,22; F=15.16).

The genetic profile of *E. festucae* var. *lolii* in *L. perenne* indicated that peramine and indole-diterpenes could be produced. The samples (2%) that contained *dmaW* were predicted to produce ergovaline, which was confirmed by UPLC-MS analyses. Of the 10 samples tested for ergovaline, the two samples with *dmaW* produced ergovaline and peramine, and one also produced lolitrem B, whereas the eight samples without *dmaW* only produced peramine and lolitrem B. Ergot alkaloids were not produced in the majority of the samples as they lacked a functional copy of the *dmaW* gene, which endcodes the first step of the ergot alkaloid biosynthesis pathway. These data provided evidence that the PCR screen is effective for identifying endophyte-infected samples that differ with respect to potential alkaloid production.

We examined 33 *F. ovina* agg. plants from all three regions, of which we could detect at least one *IDT* gene, either *idtG* or *idtP* in 17 samples. Different indole-diterpene patterns could be detected in samples that contained *IDT* genes, but this was not always consistent with what we were expecting for the sample, and may be the result of low amount of sample. For the samples that produced IDTs, we detected emindole SB and paspaline, both early pathway alkaloids, but also a paxilline isomer, terpendole C, terpendole E, a terpendole I isomer and a 13-desoxypaxilline isomer (Table II4). We detected different patterns of alkaloid production of which some could be explained by the presence of *IDT* genes, however not all.

II.4. Discussion

In this study, we determined the level of endophyte-infection of 13 grass species across a land-use intensity gradient. By utilizing PCR with *Epichloë* specific primers we were able to determine the infection rates of each host species and obtain information on the alkaloid potential for each endophyte-infected plant. We were able to confirm the *Epichloë* species found in four host species and linked genotypic diversity of alkaloid pathway genes present in the endophyte to alkaloid production. *L. perenne*, *F. arundinacea* and *F. pratensis* are agriculturally important forage grasses known to harbor *Epichloë* species (Hume et al., 2016). However, endophyte-infected *L. perenne* and *F. arundinacea* have also caused significant livestock production losses due to the production of the alkaloids, lolitrem B and ergovaline that are toxic to vertebrates (Hume et al., 2016). The cases of livestock toxicity has been more limited to Australia, New Zealand and the United States where these grasses are more heavily cultivated and dominated by a single species (Young et al., 2013). Livestock intoxication associated with European pastures have been rarely reported, likely due to greater plant diversity and lower infection rates compared to other continents. Reports of ryegrass staggers from the United Kingdoms, France, Germany and Netherlands, lay back to 1990th (Dapprich et al., 1996; Hume et al., 2016; Lewis, 1997; Zabalgogeazcoa and Bony, 2008) with only one report from France in 2004 (Benkhelil et al., 2004; Zabalgogeazcoa and Bony, 2008). No other livestock intoxications have been reported in the international scientific literature, although informal reports about animals showing "strange behavior" exist (Zabalgogeazcoa and Bony, 2008).

II.4.1. Infection rates varied across host species

We showed that five of 13 grass species, *L. perenne*, *F. pratensis*, *F. ovina* agg., *F. rubra* and *D. glomerata*, were infected with *Epichloë*. These grass species are all used as forage or lawn grasses. No endophyte infection was found in *F. arundinacea*, *A. stolonifera*, *A. pratensis*, and *P. pratense* or in the wild grasses *C. cristatus*, *H. lanatus*, *B. erectus* and *B. hordeaceus*. In our study, the average infection rate for *L. perenne* (15%) corresponded with other studies in Germany with between 8-28% (Dobrindt et al.,

2013; König et al., 2018; Oldenburg, 1997), whereas infection rates in New Zealand and Australia for *L. perenne* are higher (70%) (Easton and Tapper, 2008). We found higher endophyte infection rates of *F. pratensis* (81%) and *F. ovina* agg. (73%) than reported in other European countries with *F. pratensis* ranging between 42-74% and *F. ovina agg.* between 24-29%. Interestingly, our *F. arundinacea* samples were not infected, whereas in other European countries infections have varied between 32-98%. The infection rate of *D. glomerata* (8%) in our study was consistent with that of other European countries (0 – 17%) (Saikkonen et al., 2000; Zurek et al., 2017). Typically, *Epichloë* infected *D. glomerata* will produce stromata, fruiting bodies of sexually transmitted *Epichloë* fungi, that are observed during flowering (Leyronas and Raynal, 2001; Sampson, 1933). However, as we often sampled prior to flowering we did not observe stromata on any *D. glomerata* samples in the field. The endophyte infection rate for *F. rubra* was 15% in German grasslands, which is lower than reported infection rates in Finland with 32 % (Saikkonen et al., 2000) or in Poland with 41% (Zurek et al., 2017). Infection rates can vary in different regions or countries due to differences in environmental conditions. Abiotic conditions like temperature, precipitation and geology affect infection frequencies with *Epichloë* species (Dobrindt et al., 2013; Zurek et al., 2017). Grasses infected with *Epichloë* are also better adapted under drought (Ju et al., 2006; Rudgers and Swafford, 2009).

II.4.2. Genotypes and chemical diversity of different grass endophytes

Many studies have used endophyte infection but only focused on pathway end products such as production of ergovaline, lolitrem B, peramine and *N*-formylloline (Christensen et al., 1993; Clay and Schardl, 2002; König et al., 2018). Yet in undisturbed native environments, more alkaloid diversity can be observed among and within populations of endophyte-infected grasses (Charlton et al., 2014; Shymanovich et al., 2015, 2017; Yi et al., 2018), even for *F. arundinacea* and *L. perenne* (Christensen et al., 1993; Takach and Young, 2014; Takach et al., 2012). In some grass-endophyte associations alkaloids can be induced, for example by grazing animals as defense reaction (Fuchs et al., 2017b; Rudgers et al., 2016; Sullivan et al., 2007). In addition, environmental conditions and plant genotype can influence

production of alkaloids (Faeth and Fagan, 2002). Recently, more emphasis has been placed on evaluating the genetic capability of *Epichloë* species to produce alkaloids by determining which alkaloid biosynthesis genes are present within the endophyte (Schardl et al., 2012, 2013a; Young et al., 2009, 2015). Understanding which alkaloid biosynthesis genes are present can affirm the alkaloid potential of any given isolate, as much of the alkaloid diversity observed in *Epichloë* species is due to gene presence/absence polymorphisms that can be easily observed by PCR, providing an effective tool to rapidly evaluate samples from large population sizes (Charlton et al., 2014; Shi et al., 2017; Shymanovich et al., 2017; Yi et al., 2018). Alkaloid chemotypes that do not match the predictions based on the PCR have been shown to have altered gene expression (little or no expression) that silences the pathway, or a gene can lack functionality due to the presence of nonsense mutations that would result in a different pathway end product (Berry et al., 2015; Charlton et al., 2014; Shi et al., 2017; Young et al., 2009, 2015).

F. pratensis is an agriculturally important grass species that was widely distributed across the collection sites. All infected *F. pratensis* samples, apart from 0.6% of samples that may have been misidentified at sampling, were infected with *E. uncinata* and the genetic profile of mating type and alkaloid biosysnthesis genes was consistent with that described for occurence in meadow fescue (Berry et al., 2015; Schardl et al., 2012, 2013b; Takach and Young, 2014). *Epichloë uncinata* is known to produce NFL (Spiering et al., 2005a), which was consistent with our samples. Total loline concentrations up to 5500 μg/g (dry weight) are reported in literature (Leuchtmann et al., 2000) and the toxicity threshold of lolines for invertebrates vary from 50 μg/g (dry weight) (Shiba and Sugawara, 2009) up to 100 μg/g (dry weight) (Jensen et al., 2009). Most samples showed concentrations above 100 μg/g (dry weight) with a maximum of 3515 μg/g (dry weight) (Table SII3). Although *E. uncinata* appears to have the *perA* gene, it has been previously described as nonfunctional due to independent frameshift mutations in both alleles (Berry et al., 2015). Despite the fact that we did not find any difference in the alkaloid gene profile of *E. uncinata* infected *F. pratensis* samples, we were able to distinguish additional diversity using the previously published SSR marker B10 found in different ecotypes from Europe (Clayton et al., 2017; Moon et al., 1999). Regional differences were observed between the three sampling regions. The occurence of

different ecotype distribution might be explained by unknown environmental factors.

The endophytes found in *L. perenne* are known to range in alkaloid production varying in their capability of producing peramine, lolitrem B, and ergovaline (van Zijll de Jong et al., 2008). Perhaps the most documented strains are those that have caused livestock toxicity through the production of lolitrem B, a neurotoxin that causes ryegrass staggers (Gallagher et al., 1985). However, there are also commercial cultivars released with safe endophytes, such as AR1 , which do not produce vertebrate toxic alkaloids (Johnson et al., 2013; Moate et al., 2012; Young et al., 2013). *Lolium perenne* was well represented in our collections and the majority of the endophyte-infected samples were capable of producing lolitrem B and peramine. Interestingly, these samples also contained many of the genes required for ergot alkaloid biosynthesis, but lacked *dmaW* that encodes 4-y,y-dimethylallyltryptophan synthase, the determinant step in ergot alkaloid biosynthesis (Wang et al., 2004). As expected, we were unable to detect ergovaline in these samples, therefore, there should be no risk of ergovaline intoxication for grazing animals on these study sites. However, since lolitrem B was detected in the *L. perenne* samples, intoxication of animals grazing on those pastures may be possible.

Festuca ovina agg. is a plant with high genetic variation and large gene flows (Prentice et al., 1995). The species is variable and contains many subspecies, which are hard to distinguish (Wilkinson and Stace, 1991). We detected high genotypic diversity of *E. festucae* in *F. ovina* agg. We also detected both mating types within the populations and we conclude that these may represent sexually active populations. Populations that are recombining could also result in greater alkaloid gene diversity. The greatest diversity within the endophyte-infected *F. ovina* agg. samples were associated with variation of *IDT* genes. *Epichloë festucae* is known to differ in the presence of *IDT* alkaloid pathway genes (Schardl et al., 2013a; Young et al., 2009) and we detected different pathway intermediates of the IDT alkaloid pathway in our samples. In addition, we only detected IDTs in half of the IDT gene-positive samples. It is possible that the gene-positive samples in which IDTs were not detected do contain IDTs but at concentrations that were below the limits of

detection. As alkaloid production is known to be induced by herbivory (Fuchs et al., 2017b; Rudgers et al., 2016), we assume, the genes were not upregulated or possibly could be nonfunctional. Naturally occurring pathway variation can result from a combination of altered gene expression and/or sequence variations, including sequence polymorphisms within a gene resulting in non-synonymous changes or the complete absence of the gene (Young et al., 2009). Terpendole C, as well as paxilline, are known to be tremorgenic in mice (Gardner et al., 2018). We detected a paxilline isomer, however, it is unkown if this isomer is tremorgenic, as relatively small changes in the structure of the molecule can change its toxicity (Munday-Finch et al., 1996). The grass *F. ovina* agg. often occurs on nutrient poor grassland which can be found in the regions of HAI and ALB, where most of the samples were collected. *F. ovina* agg. shows good drought resistance, but due to its poor growth it is only used as lawn grass, and not as forage for livestock (Bundessortenamt, 2019). Therefore, the intoxication risk for grazing animals by infected *F. ovina* agg. is low.

Variation was also present with respect to the peramine biosynthesis gene in the *F. ovina* agg., *F. rubra* and *D. glomerata* endophyte-infected samples. The region encoding the peramine reductase domain has been previously shown to be required for permaine biosynthesis (Berry et al., 2015). Interestingly, *Epichloë typhina* in *D. glomerata* is not known to produce alkaloids (Leuchtmann et al., 2000), which was confirmed recently with *D. glomerata* infected with *Epichloë typhina* from Oregon, USA where the the region encoding the *perA* reductase domain was not detected by PCR (Bushman et al., 2018). We observed variation with the *perA* markers in our endophyte-infected *D. glomerata* samples, but since choke in *D. glomerata* in the USA has only been reported since 1996 (Pfender and Alderman, 1999), genetic variation of *E. uncinata* in *D. glomerata* in European grasslands may be greater.

II.5. Conclusion

We have evaluated grasses represented in three diverse locations in Germany. Our study has looked beyond endophyte infection rates and used a combination of genetic and chemotypic evaluation to determine endophyte diversity. A significant finding of the study was that although the endophyte infection rates for some host species were higher than other regions in Europe not all of these samples could be considered toxic to vertebrates. The host with the highest endophyte infection rates was *F. pratensis*, but it has no known livestock toxicity, although insect toxic properties can be of advantage for farmers. *L. perenne* samples were able to produce the vertebrate toxin lolitrem B, however, ergovaline was not of concern, because the gene encoding the determinant step for ergot alkaloid biosynthesis was absent in most samples. Interestingly, none of the *F. arundinacea* samples were infected, whereas, infected *F. ovina* agg. samples contained various alkaloid biosynthesis precursors for which the toxicity potential is not clear. Further studies are necessary to estimate the influence of abiotic and biotic effects on infections, alkaloid gene expression and alkaloid production on managed grasslands. Previous studies showed no influence of land-use intensity on infection rates or alkaloid production in *Epichloë* infected *L. perenne* plants (König et al., 2018). Other studies showed that abiotic factors like soil type or soil moisture can alter infection rates of grasses infected with *Epichloë* species (Dobrindt et al., 2013; Rudgers et al., 2016). We conclude that some grasses in Germany are infected with endophytes that could be toxic to livestock and these should be monitored to make sure they will not become dominant grasses in the pastures. We showed that PCR screens provide a robust way of testing the endophyte status of pasture grasses and could be a helpful tool for future monitoring. It is necessary to regularly record *Epichloë* infection rates as well as alkaloid profiles and concentrations for a large number of cool season grass species in natural and agricultural grasslands, to predict the risks and chances of grass endophytes in a changing climate and with new breeds of *Epichloë*-grass varieties by international companies.

II.5. Supplemental Material

Table SII1: Reference sequences for phylogeny

Species name	Strain	Ploidy	Accession number	Mating type	Species name	Strain	Ploidy	Accession number	Mating type
E. amarillans	E4668	1x	MK992923	B	*E. festucae*	E189	1x	MK992932	B
E. amarillans	E57	1x	MK992924	B	*E. festucae*	E2368	1x	HQ680587	A
E. amarillans	NFE708	1x	MK992972	A	*E. festucae*	Fl1	1x	HQ680590	B
E. aotearoae	E899	1x	MK992964	A	*E. festucae* var. *lolii*	PRG-3	1x	MK992929	B
E. baconii	E421	1x	MK992933	B	*E. inebrians*	E818	1x	MK992953	A
E. baconii	E1031	1x	MK992973	A	*E. gansuensis*	E7080	1x	MK992925	B
E. brachyelytri	E4804	1x	MK992936	B	*E. glyceriae*	E277	1x	MK992954	A
E. bromicola	AL0426/2	1x	MK992962	A	*E. glyceriae*	E2772	1x	MK992930	B
E. bromicola	AL0434	1x	MK992961	A	*E. hybrida*	e144	2x	MF464368, MF464367	BB
E. bromicola	E502	1x	MK992927	B	*E. mollis*	E3601	1x	MK992935	B
E. bromicola	E799	1x	MK992963	A	*E. siegelii*	e915	2x	MK992941 MK992942	AA
E. bromicola	E7626	1x	MK992960	A	*E. sylvatica*	E832	1x	MK992934	B
E. bromicola	NFe1	1x	MK992956	A	*E. typhina*	92038	1x	MK992928	B
E. bromicola	NFe5	1x	MK992957	A	*E. typhina*	E1158	1x	MK992967	A
E. bromicola	NFe7	1x	MK992958	A	*E. typhina*	E8	1x	MK992965	A
E. bromicola	NFe9	1x	MK992959	A	*E. typhina*	ORE04	1x	MK992966	A
E. cabralii	NFe77	2x	MK992947 MK992948	BB	*E. typhina* subsp. *clarkii*	Holcus3	1x	MK992968	A
E. cabralii	e7091	2x	MK992949 MK992950	BB	*E. typhina* subsp. *poae*	E5115	1x	MK992931	B
E. canadensis	e4815	2x	MK992951 MK992952	AB	*E. typhina* subsp. *poae*	E5819	1x	MK992969	A

E. chisosa	e3609	3x	MK992980 MK992981 MK992982	ABB	*E. uncinata*	e167	2x	MK992938 MK992937	AB
E. coeno-phiala	e19	3x	MK992974 MK992975 MK992976	AAA	*Epichloë* sp. FaTG-2	NFe45079	2x	MK992943 MK992944	AB
E. coeno-phiala	e4163	3x	MK992977 MK992978 MK992979	AAA	*Epichloë* sp. FaTG-2	NFe45115	2x	MK992945 MK992946	AB
E. elymi	NFE732	1x	MK992926	B	*Epichloë* sp. FaTG-3	NFe1100	2x	MK992939 MK992940	AA
E. elymi	E56	1x	MK992955	A	*E. typhina* subsp. *poae*	NFe76	1X	MK992970	A
E. festucae	E1017	1x	MK992971	A					

Table SII2: Analysis of simple sequence repeats

Sample Name	LUI	landuse	ecotype	B10	B11
A17_FP_17_2	1,56	meadow	3	**171, 191**	118
A17_FP_17_3	1,56	meadow	3	**171, 191**	118
A17_FP_17_4	1,56	meadow	3	**171, 191**, 162, 180	113, 118
A17_FP_17_5	1,56	meadow	3	**171, 191**, 162, 180	118, 113
A17_FP_23_1	1,55	meadow	3	**171, 191**	118
A17_FP_23_2	1,55	meadow	3	**171, 191**	113, 118
A17_FP_23_3	1,55	meadow	3	**171, 191**, 180, 186, 160	113, 118
A17_FP_23_4	1,55	meadow	3	**171, 191**, 178	118
A17_FP_32_3	0,73	pasture	3	**171, 191**	118
A17_FP_32_4	0,73	pasture	3	**171, 191**, 160	118, 113
A17_FP_32_5	0,73	pasture	3	**171, 191**, 180, 162	113, 118
A17_FP_33_1	0,68	pasture	3	**171, 191**	113, 118
A17_FP_33_2	0,68	pasture	3	**171, 191**, 181	118
A17_FP_33_3	0,68	pasture	3	**171, 191**, 180, 162	113, 118
A17_FP_33_4	0,68	pasture	3	**171, 191**, 162	113, 118
A17_FP_36_24	2,34	meadow	3	**171, 191**	118
A17_FP_36_25	3,34	meadow	3	**171, 191**	113, 118
A17_FP_36_26	4,34	meadow	3	**171, 191**, 162	113, 119
A17_FP_36_27	5,34	meadow	3	**171,191,** 180	113, 118
A17_FP_42_42	2,02	meadow	3	**171, 191**	118, 113
A17_FP_42_43	2,02	meadow	3	**171, 191**	118
A17_FP_42_44	2,02	meadow	3	**171, 191**	118, 113

A17_FP_42_45	2,02	meadow	3	**171, 191**	118, 113
A17_FP_44_10	1,43	pasture	3	**171, 191**, 180	113, 118
A17_FP_44_11	1,43	pasture	3	**171, 191**	118
A17_FP_44_12	1,43	pasture	3	**171, 191**, 180	118
A17_FP_44_9	1,43	pasture	3	**171, 191,** 161, 180	118, 113
A17_FP_46_25	1,57	pasture	3	**171, 191,** 162, 180	118, 113
A17_FP_46_26	1,57	pasture	3	**171, 191**, 180, 160	113, 118
A17_FP_46_27	1,57	pasture	3	**171, 191**	118
A17_FP_46_28	1,57	pasture	3	**171, 191,** 162, 180	113, 118
H17_FP_14_1	1,87	meadow	1	**159, 194,** 151	113, 118
H17_FP_14_2	1,87	meadow	1	**159, 194**, 220	113, 118
H17_FP_14_4	1,87	meadow	1	**159, 194,** 180, 188, 304	78, 118, 113
H17_FP_18_24	1,07	mown pasture	1	**159, 194**, 151,	118, 288
H17_FP_18_26	1,07	mown pasture	1	**159, 194**, 180, 188,	113, 118
H17_FP_18_27	1,07	mown pasture	1	**159, 194,** 188	113, 118
H17_FP_26_27	0,96	meadow	3	**171, 191**, 221	118
H17_FP_26_28	0,96	meadow	3	**171, 191**, 221	118
H17_FP_26_29	0,96	meadow	4	**159, 196**, 221, 304	118
H17_FP_26_30	0,96	meadow	3	**171, 191**	118
H17_FP_38_5	1,74	pasture	4	**159, 196,** 220, 304, 151	118, 113
H17_FP_38_6	1,74	pasture	4	**159, 196**, 150	118, 113
H17_FP_38_7	1,74	pasture	1	**159, 194**, 220, 304, 151	113, 118
H17_FP_38_8	1,74	pasture	1	**159, 194,** 212, 220	113, 118
H17_FP_40_42	1,82	pasture	3	**171, 191**	118
H17_FP_40_43	1,82	pasture	3	**171, 191**	118
H17_FP_40_44	1,82	pasture	3	**171, 191**	113, 118
H17_FP_40_45	1,82	pasture	3	**171, 191**	118
H17_FP_42_1	0,74	pasture	1	**159, 194**	113, 118
H17_FP_42_2	0,74	pasture	1	**159, 194**, 150	118
H17_FP_42_3	0,74	pasture	1	**159, 194**, 134	118
H17_FP_42_4	0,74	pasture	3	**171, 191**, 221	118
H17_FP_43_2	0,64	pasture	1	**159, 194**	113, 118
H17_FP_43_3	0,64	pasture	1	**159, 194,** 151, 188	113, 118
H17_FP_43_4	0,64	pasture	4	**159, 196**	118, 164, 229, 455

H17_FP_43_5	0,64	pasture	1	**159, 194,** 304	118, 337
H17_FP_49_3	1,57	meadow	1	**159, 194**	120, 180
H17_FP_49_4	1,57	meadow	1	**159, 194,** 221, 304	118
S17_FP_1_4	1,44	mown pasture	3	**171, 191**, 180	118
S17_FP_1_5	1,44	mown pasture	3	**171, 191**	118
S17_FP_1_6	1,44	mown pasture	1	**159, 194**	118, 113
S17_FP_1_8	1,44	mown pasture	3	**171, 191**	118
S17_FP_12_22	1,44	mown pasture	1	**159, 194,** 180, 186	118
S17_FP_12_23	1,44	mown pasture	1	**159, 194**	113, 118
S17_FP_12_24	1,44	mown pasture	1	**159, 194**, 189, 187	118
S17_FP_12_25	1,44	mown pasture	1	**159, 194**, 187	118
S17_FP_14_1	1,39	mown pasture	3	**171, 191**, 210, 220	118
S17_FP_14_2	1,39	mown pasture	3	**171, 191**	118
S17_FP_14_3	1,39	mown pasture	3	**171, 191**	118
S17_FP_14_4	1,39	mown pasture	3	**171, 191**	118
S17_FP_16_1	0,46	pasture	1	**159, 194**	118
S17_FP_16_2	0,46	pasture	3	**171, 191,** 97, 220, 304	118
S17_FP_16_3	0,46	pasture	1	**159, 194**	118
S17_FP_16_4	0,46	pasture	1	**159, 194**	118
S17_FP_26_4	1,36	meadow	3	**171, 191**	118, 135
S17_FP_26_5	1,36	meadow	3	**171, 191**	118
S17_FP_26_6	1,36	meadow	1	**159, 194,** 134,	118
S17_FP_26_7	1,36	meadow	3	**171, 191**	118
S17_FP_29_2	0,89	pasture	3	**171, 191**, 220	118
S17_FP_29_3	0,89	pasture	3	**171, 191,** 162	113, 118
S17_FP_29_4	0,89	pasture	3	**171, 191,** 162	113, 118
S17_FP_31_4	0,96	meadow	3	**171, 191**	118
S17_FP_31_5	0,96	meadow	1	**159, 194**	118, 180
S17_FP_31_6	0,96	meadow	3	**171, 191**	118, 113
S17_FP_31_7	0,96	meadow	3	**171, 191**, 158	118
S17_FP_39_22	1,57	mown pasture	1	**159, 194,** 220	113, 118
S17_FP_39_23	1,57	mown pasture	1	**159, 194**	118
S17_FP_39_24	1,57	mown pasture	1	**159, 194**, 221	118
S17_FP_39_25	1,57	mown pasture	1	**159, 194,** 188	118

Sample name (H = Hainich National Park, A = Swabian Alb, S = Schorfheide-Chorin. 17: sampled in 2017; FP: *Festuca pratensis*; 39_6: plot 39, sample number 6), land-use intensity factor (LUI), landuse, ecotype and DNA fragment sizes for the primers B10 and B11

Table SII3: Loline alkaloids

sample	ecotype	lolines [µg/g dry weight]	AcAP	NANL	Loline	NAL	NML	NFL	alkaloids
H17_FP_38_eco1	eco1	2166	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
H17_FP_14_eco1	eco1	2084	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
H17_FP_18_eco1a	eco1	18	-	-	-	-	-	+	NFL
H17_FP_18_eco1b	eco1	793	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
S17_FP_39_eco1	eco1	482	-	+	-	+	+	+	NANL, NAL, NML, NFL
S17_FP_16_eco1	eco1	500	-	+	-	+	+	+	NANL, NAL, NML, NFL
S17_FP_12_eco1	eco1	545	-	+	-	+	+	+	NANL, NAL, NML, NFL
A17_FP_36_eco3	eco3	1620	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
A17_FP_23_eco3a	eco3	3515	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
A17_FP_23_eco3b	eco3	1723	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
A17_FP_17_eco3a	eco3	1769	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
A17_FP_17_eco3b	eco3	1906	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
A17_FP_46_eco3	eco3	2303	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
A17_FP_44_eco3	eco3	1502	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
A17_FP_33_eco3a	eco3	1011	-	+	-	+	+	+	NANL, NAL, NML, NFL
A17_FP_33_eco3b	eco3	533	-	+	-	+	+	+	NANL, NAL, NML, NFL
A17_FP_32_eco3	eco3	1411	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL
H17_FP_40_eco3a	eco3	21	-	-	-	+	-	+	NAL, NFL
H17_FP_40_eco3b	eco3	26	-	+	-	+	-	+	NANL, NAL, NFL
H17_FP_26_eco3	eco3	38	-	+	-	+	-	+	NANL, NAL, NFL
S17_FP_1_eco3	eco3	346	-	+	-	+	+	+	NANL, NAL, NML, NFL
S17_FP_26_eco3	eco3	218	-	+	-	+	-	+	NANL, NAL, NFL
S17_FP_29_eco3	eco3	138	-	+	-	+	-	+	NANL, NAL, NFL
eco4	eco4	1874	-	+	+	+	+	+	NANL, Loline, NAL, NML, NFL

Detected loline alkaloids in a subset of 24 *F. pratensis* samples with different ecotypes. Total amount of lolines [µg/g dry weight], presence (+) or absence (-) of different lolines (AcAP: 1-acetamido-pyrrolizidine, NANL: N-acetylnorloline, NAL: N-acetylloline, NML: N-methylloline, NFL: N-formylloline) and chemotypic profile of the samples. (H = Hainich National Park, A = Swabian Alb, S = Schorfheide-Chorin. 17: sampled in 2017; FP: *Festuca pratensis* number: plot number

Chapter III

Alkaloid concentrations of *Lolium perenne* infected with *Epichloë festucae* var. *lolii* with different detection methods – a re-evaluation of intoxication risk in Germany?

Cattle on a grassland in Germany. Photo: ©Veronika Vikuk

"Poison is in everything, and no thing is without poison.

The dosage makes it either a poison or a remedy."

Paracelsus

Chapter III

Alkaloid concentrations of *Lolium perenne* infected with *Epichloë festucae* var. *lolii* with different detection methods – a re-evaluation of intoxication risk in Germany?

Mycotoxins in agriculturally used plants can cause intoxications in animals and can lead to severe financial losses for farmers. The endophytic fungus *Epichloë festucae* var. *lolii* living symbiotically within the cool season grass species *Lolium perenne* can produce vertebrate and invertebrate toxic alkaloids. Hence, an exact quantitation of alkaloid concentrations is essential to determine intoxication risk for animals. Many studies use different methods to detect alkaloid concentrations, which complicates the comparability. In this study, we showed that alkaloid concentrations of individual plants exceeded toxicity thresholds on real world grasslands in Germany, but not on population level. Alkaloid concentrations on five German grasslands with high alkaloid levels peaked in summer but were also below toxicity thresholds on population level. Furthermore, we showed that alkaloid concentrations follow the same seasonal trend, regardless if fresh or dry plant weight was used, in the field and in a common garden study. However, alkaloid concentrations were around three times higher when detected with dry weight. Finally, we showed that alkaloid concentrations can additionally be biased by different alkaloid detection methods. We highlight that toxicity risks should be analyzed using dry plant weight, but concentration trends of fresh weight are reliable.

III.1. Introduction

Mycotoxins are secondary metabolites produced by fungi, which can cause disease and death in humans and other vertebrates, but also in invertebrates, plants and microorganisms (Bennett and Klich, 2003; Pitt, 2000). Fungal endophytes of the genus *Epichloë* live symbiotically and asymptomatically inside cool season grass species (Sampson, 1933). They can produce different alkaloids, which can be toxic for vertebrates or invertebrates and provide protection from herbivores for the plant (Müller and Krauss, 2005; Schardl, 1996). The plant provides shelter, nutrition and dispersal for the fungus (Schardl, 1996), whereas the fungus increases plant fitness, biomass and drought resistance (Bourguignon et al., 2015; Gundel et al., 2020).

One economically important grass endophyte is *Epichloë festucae* var. *lolii* infecting *Lolium perenne* L. (perennial ryegrass) (Hume et al., 2016). This fungus is able to produce the pyrrolopyrazine peramine, an insect deterring alkaloid, as well as the vertebrate toxic alkaloids lolitrem B, an indole-diterpene, its precursor paxilline and the ergot alkaloid ergovaline (Hume et al., 2016; Imlach et al., 2008; Menna et al., 2012; Reddy et al., 2019). Biologically active concentrations for peramine vary between 2 μg/g dry weight (DW), when Argentine stem weevil larvae fed on artificial diets (Rowan et al., 1990; Siegel and Bush, 1996) and 15 μg/g in planta for a strong resistance against Argentine stem weevil (Hewitt et al., 2020; Popay and Wyatt, 1995). Lolitrem B and ergovaline showed signs of intoxication in livestock from 1.8 μg/g (DW) and 0.3 μg/g (DW), respectively, based on dry plant weight (Craig et al., 2014; Hovermale and Craig, 2001). Lolitrem B causes intoxications of livestock, known as ryegrass staggers (Hume et al., 2016; Menna et al., 2012; Saikkonen et al., 2006). Severe outbreaks of ryegrass staggers are known from New Zealand and Australia (Hume et al., 2016). In Australia, approximately 100,000 animals died in 2002 due to ryegrass staggers caused by lolitrem B produced by *E. festucae* var. *lolii* infecting *L. perenne* (Hume et al. 2016). Financial losses for farmers caused by *Epichloë* endophyte intoxication events are estimated worldwide at around 2 billion dollars per year (Hume et al. 2016). Perennial ryegrass (*L. perenne*) is a commonly used forage grass species due to its fast initial growth and persistence

(Spangenberg et al., 2012). Hence, an evaluation of intoxication risks caused by *Epichloë* infections is important for farmers.

In Europe, intoxication events caused by *Epichloë* endophytes are scarce and intoxication risk in Germany is considered to be low (König et al., 2018; Vikuk et al., 2019), explained by low infection rates (15 %) (Vikuk et al., 2019) compared to New Zealand (70 %) (Easton and Tapper, 2008) and more heterogenous grasslands (Kauppinen et al., 2016; Vikuk et al., 2019). Abiotic factors like drought and elevated temperature can influence infection rates of grasses with *Epichloë* endophytes and alkaloid concentrations (Bourguignon et al., 2015; Malinowski and Belesky, 2000) as well as land-use intensity (Gwinn et al., 1998; Jensen and Roulund, 2004). However, a previous study conducted on German grasslands in 2015 showed no influence of land-use intensity on infection rates or alkaloid concentrations (König et al., 2018).

The main method to estimate intoxication risks is the quantitation of alkaloids in grasses with analytical chemistry methods. However, methodologies differ substantially between studies. Some use high-performance liquid chromatography with different detection methods like mass spectrometry (Shelby et al., 1997), tandem mass spectrometry (Krauss et al., 2020; Rudolph et al., 2018; Vikuk et al., 2019), or fluorescence detection (Craig et al., 2014; Finch et al., 2012, 2013; Gallagher et al., 1985; Repussard et al., 2014c) for the quantitation of lolitrem B, paxilline, ergovaline and peramine. Other studies use enzyme immunoassays (Bauer et al., 2017). Furthermore, analytical reference compounds for exact quantitation of alkaloids are rarely commercially available. For the validation of analytical methods the respective substance is needed (e.g. lolitrem B, peramine or ergovaline), whereas for the quantitation the same substance is used as external standard (e.g. lolitrem B in (Finch et al., 2012, 2013)) or a structurally similar substance is used as internal standard (e.g. homoperamine for peramine (Fuchs et al., 2013; König et al., 2018; Krauss et al., 2020; Vikuk et al., 2019), or stable isotope labelled compounds(Rudolph et al., 2018)). But also fluorometric response curves (Gallagher et al., 1985) and fluorescence detectors are used (Helander et al., 2016). Homoperamine has also been used as internal standard for quantitation of lolitrem B, although for a non-validated quantitation due to the structural differences (Fuchs

et al., 2013; König et al., 2018; Krauss et al., 2020; Vikuk et al., 2019), whereas ergovaline is often quantified with ergotamine as internal standard compound (Fuchs et al., 2013; König et al., 2018; Krauss et al., 2020; Rottinghaus et al., 1991; Shelby and Flieger, 1997; Spiering et al., 2002; Vikuk et al., 2019), as well as with lysergic acid diethylamide-d3 (LSD-d3) (Rudolph et al., 2018). However, standard calibration curves in combination with HPLC-fluorescence analyses are used as well for the quantitation of ergovaline (Hovermale and Craig, 2001; Shelby and Flieger, 1997). The original plant material used for extraction is also important for the extraction of alkaloids. Few studies used fresh plant weight to analyze alkaloid concentrations (Fuchs et al., 2013; König et al., 2018), whereas the use of dry plant weight is the standard to detect toxicity of compounds (Craig et al., 2014; Repussard et al., 2014c; Rottinghaus et al., 1991). Sometimes fresh plant weight is used for analytical detection, if the target substance degrades or chemically changes (e.g. some phytohormones) while drying the plant samples in the oven (Baldwin, 1988; Glauser et al., 2009) or if the amount of plant material is limited (König et al., 2018). However, using fresh plant weight complicates comparison of alkaloid contents between different studies. Hence, plant samples are often freeze-dried, because this method protects susceptible samples and solves the problems of varying amount of water. However, there are also studies, which used straw for alkaloid analyses (Craig et al., 2014; Repussard et al., 2014c). Many of the published toxicity thresholds for peramine, lolitrem B and ergovaline are based on analyses of dry plant weight. In best practice, new methods need to be validated, compared to other licensed methods and tested for their robustness (Rudolph et al., 2018; Vassiliadis et al., 2019), before they can be licensed for the quantitation of alkaloids. However, there are also differences in methods, which lead to differently detected alkaloid concentrations. Bauer et al. (2018) showed for example that paxilline concentrations detected with enzyme immunoassays differed compared to measurements with HPLC-MS/MS analysis.

In this study, we conducted a study following König et al. (2018) using dry weight instead of fresh weight to (i) confirm or detect changes of infection rates and alkaloid concentrations in German grasslands. Additionally, we (ii) analyzed seasonal changes in alkaloid concentrations of *L. perenne* on five selected grasslands in northern Germany for ten months during the growing period. Finally, we

conducted a common garden experiment with *L. perenne* plants infected with *E. festucae* var. *lolii*, to (iii) detect seasonal changes in alkaloid concentrations and (iv) compare changes in alkaloid concentrations detected with fresh and dry plant weight over six months. Besides using fresh and dry plant weight, we applied two different UPLC methods (Fuchs et al., 2013; Krauss et al., 2020) to (v) show changes in alkaloid concentrations depending on the detection method.

III.2. Materials and Methods

III.2.1. Dataset 1: Field study in Germany

Our field study covered 150 study sites in three regions, which are part of a nation-wide long-term study in Germany, called Biodiversity Exploratories (www.biodiversity-exploratories.de). We conducted our field study in three regions: UNESCO biosphere region Schwäbische Alb (south-west Germany), national park Hainich (central Germany) and UNESCO biosphere region Schorfheide (north-west Germany) (Vikuk et al., 2019). The sampled regions cover different landscape types and land-use intensity gradients, further spanning a latitudinal area of 800 km from north to south within Germany (Fischer et al., 2010). Each study site is 50 m x 50 m in size within grassland areas (Fischer et al., 2010). Land-use intensity (LUI) was calculated with a formula, developed for the Biodiversity Exploratories integrating fertilization, mowing and grazing (Blüthgen et al., 2012). The influence of LUI was tested on infection rates of *L. perenne* with *Epichloë festucae* var. *lolii* sampled in 2017 and the produced alkaloids. Since fertilization, mowing and grazing can also have separate influence on infection rates and alkaloid concentrations (König et al., 2018; Krauss et al., 2007), we also analysed them separately. We monitored all study sites from May until August 2017 for the occurrence of *Lolium perenne*, and sampled 20 individual plants per study site, where sufficient numbers were found. We sampled individuals with a minimum distance of three meters to avoid sampling the same individual twice. In 2017 we sampled the same study sites as in König et al. (2018) in 2015 to compare the effects of land-use intensity and region on infection rates and alkaloid concentrations. One tiller per plant individual was sampled in a previous survey in 2015 (König et al., 2018) three were sampled

in (2017). Each sample was taken of approximately 3 cm from the lowest part of the grass tiller, because alkaloids and *Epichloë* endophyte accumulate there (König et al., 2018; Spiering et al., 2005b). We collected the samples in 2 ml Eppendorf tubes, kept them on dried ice in the field and stored them at – 20 °C before further processing. Infection rates of *L. perenne* with *Epichloë festucae* var. *lolii* were detected with specific primers by a polymerase chain reaction (PCR) method in all collected samples from 2017 as described in (Vikuk et al., 2019). After PCR, only positive samples were tested for the presence of alkaloids. Alkaloid concentrations were calculated on population level (infected and not infected samples per study site) and individual level.

III.2.2. Dataset 2: Alkaloid concentrations throughout the year in a field study

We sampled *L. perenne* plants on five different study sites in the region Schorfheide-Chorin, northern Germany in 2018, which were previously confirmed with high alkaloid concentrations of *Epichloë* infected *L. perenne* individuals in 2015 (König et al., 2018) (SIII1) in order to detect seasonal changes in the alkaloid production. We sampled three tillers of 10-20 plants per study site every two months, at the beginning of April and June and at the end of July, September, November 2018 and January 2019. Infection rates were checked with a commercially available kit for immunoblot assays (www.agrinostics.com) and only infected grass samples were tested for alkaloids. Alkaloid concentrations, quantified with dry weight, were calculated on population level (mean alkaloid concentration of infected and not infected samples per study site) and for infected samples only.

III.2.3. Dataset 3: Common garden study

In 2019 we conducted a common garden experiment with sown *Epichloë* infected *L. perenne* plants to detect seasonal changes in alkaloid production in six months during the main growing season in central Europe (April until September). We used the same experimental setup as described in Fuchs et al. (2017b) with some

minor changes: On 9th of January 2019, we planted 216 endophyte-infected *Lolium perenne* seeds (A26558, cv. Grasslands Samson 'common toxic' (Johnson et al., 2013)) in 4 trays, each tray with 54 pots (diameter 5 cm, height 5 cm) and one tray with 54 endophyte-free seeds of the same plant cultivar (A11104, Grassland Samson Nil endo). Seeds were provided by AgResearch, New Zealand. Substrate was potting compost (Plantiflor Pro Natur Bio Quality) and pots were positioned in a green house (day: 5 am – 9 pm, temperature: 20 °C, RH: 50 %; night: 9 pm – 5 am, temperature: 15 °C, RH: 75 %) at the University of Würzburg, Germany. After six weeks of growth, plants were repotted in common garden soil (Einheitserde classic CLED73, Profi Substrat) in single pots (11x11x11cm). After 14 weeks in the greenhouse, the plants were again repotted in bigger pots (17x17x17 cm) and placed randomly in a common garden setup at the University of Würzburg, Germany for the entire experimental period.

We sampled biweekly ten random endophyte-infected plants from 9th of April to 25th of September 2019. Sampling was conducted by cutting randomly 10 complete grass tillers from each pot, which were immediately frozen and kept at -20 °C until further preparation for chemical analyses. For alkaloid detection the plant material of each sample was ground and freeze-dried for 72 hours. Sampled plants were marked to avoid sampling the same plant twice. The first plant samples were taken after 13 weeks of growth in the greenhouse, before the plants were located in the common garden, in order to be able to compare alkaloid concentrations in the greenhouse with the common garden. For ten sampling points (April – September), samples were divided after homogenization and half of the sample was analyzed with fresh plant weight, whereas the other half was freeze-dried. Thereby, we were able to directly compare alkaloid concentrations in fresh and dried plant weight. Alkaloid detections were performed with approximately 40 mg fresh plant weight and approximately 20 mg freeze-dried plant weight.

III.2.4. Alkaloid detection methods

Alkaloid concentrations for datasets (1) and (2), as well as for the comparison of fresh and dry weight concentrations in dataset (3) were detected by using the following method (method 1) (SIII2): Quantitation of peramine, lolitrem B, ergovaline and paxilline was performed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) using a Waters Acquity UPLC combined with a Quattro Premier triple quadrupole mass spectrometer. After grinding and freeze drying for 72h, grass samples were prepared as described in a previous publication (Krauss et al., 2020). We used approximately 20 mg of freeze-dried plant material. After extraction with 250 µl dichloromethane/methanol 1:1 (v/v) containing the internal standards ergotamine (500 ng), lysergic acid diethylamide-d3 (LSD-d3) (125 ng) and homoperamine (500 ng) and a centrifugation (14000 rpm, 10 min), the supernatant was recovered, and the pellet was reextracted with 250 µl of dichlormethane/methanol 1:1 (v/v). The organic phases were combined, and two aliquots of the supernatants were evaporated under reduced pressure. One sample was reconstituted in water/acetonitrile/formic acid 50:50:0.1 (v/v/v) for the analysis of lolitrem B and paxilline, and the other in water/acetonitrile/formic acid 80:20:0.1 (v/v/v) for the analysis of peramine and ergovaline. The solubility and the chromatographic separation of lolitrem B and paxilline was better in water/acetonitrile/formic acid 50:50:0.1 (v/v/v), whereas the chromatographic separation of peramine and ergovaline needed a higher water content (water/acetonitrile/formic acid 80:20:0.1 (v/v/v)).

Chromatographic separation was conducted according to a published protocol with slight modifications (Rudolph et al., 2018). An Aquitiy UPLC column (100x2.1 mm; 1.7 µm; Waters GmbH, Eschborn, Germany) was used. 0.1 % formic acid dissolved in water (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B) was used for the following gradient elution: 0–1.0 min 98% solvent A, 1.0–3.0 min to 90% solvent A, 3.0–5.0 min to 85% solvent A, 5.0–7.5 min to 80% solvent A, 7.5–10.0 min to 75% solvent A, 10.0–11.5 min to 70% solvent A, 11.5–13.0 min to 65% solvent A, 13.0–14.5 min to 50% solvent A, 14.5–16.0 min to solvent 40% A, 16.0–19.0 min to 0% solvent A, 19.0–22.0 hold 0% solvent A, 22.0–

23.0 back to 98% solvent A and hold for another 2 min (total run time 25 min). Injection volume was 10 µl for the analyses of peramine and ergovaline and 5 µl for the analyses of lolitrem B and paxilline. Parameters for alkaloid detection were set as in Fuchs et al. (2013), using multiple reaction monitoring (MRM). Additionally, LSD-d3 was used as internal standard with the following specific transitions: m/z 327.1→208.1 and 327.1→226.1. Paxilline was detected using the following transitions: m/z 436.3 → 130.1 and 436.3→182.1. Following the protocol of (Rudolph et al., 2018), we tested LSD-d3 as internal standard for the quantitation of ergovaline, but quantitation with ergotamine as internal standard gave more reliable results due to a better similarity concerning the physicochemical properties of both compounds (retention time, ionisation efficiency and fragmentation of ergotamine were more similar to ergovaline). In method 1, the limit of quantitation (LOQ) for paxilline and lolitrem B was 0.5 ng on column and 0.1 ng on column for ergovaline and peramine, respectively.

In dataset (3), we additionally compared alkaloid concentrations by using dry plant material determined with method 1, as described in this paper and an UPLC-MS/MS method based on parameters published in (Fuchs et al., 2013) (method 2) (SIII2). Method 1 was optimized to detect a higher number of endophyte derived alkaloids like lipophilic alkaloids such as paxilline and other *Epichloë* alkaloids, like lolines, in one analysis. We used dried plant material from week 29 (cut number 8) and detected the same ten samples with both methods in order to evaluate if methods produce comparable results and can be used interchangeably. Methodological differences in method 2 were as following: Samples were diluted in 80 % methanol before HPLC measurements to allow analysis of lolitrem B, peramine and ergovaline from one sample. Data for the detection of paxilline was not aquired within this method. Another column (Acquitiy BEH column (50x2.1 mm; 1.7 µm; Waters GmbH, Eschborn, Germany)) was used for separation at a flow rate of 0.3 mL/min, with the same solvents (A+B) as method 1, but with a different gradient elution: from 5% to 25% solvent B in 5 min, followed by 25% to 75% solvent B in 0.5 min, then 75% to 100 % solvent B in 2.5 min (total run time 10 min).

III.2.5. Statistical analyses

Statistical analyses were conducted using the statistical software R (version 3.5.2). We tested the effects of land-use intensity (LUI) and region on infection rates, followed by additional models replacing LUI by either mowing, grazing or fertilization using a generalized linear model (glmer, package: lme4) (Bates et al., 2014) with study site as a random effect. The model was followed by a Wald chi-square test (Anova: type II, Wald Chisquare (X^2) tests, package: Car) (Fox and Weisberg, 2019). Difference between regions were tested using a Tukey posthoc test.

The response variables peramine and lolitrem B concentrations were analysed with the same explanatory variables using a linearized mixed effect models (lmer, package: lmerTest (Kuznetsova et al., 2017)) with study site as random effect. We only tested samples statistically, where alkaloid concentrations were detected by UPLC-MS/MS. Samples which showed no alkaloids were excluded. The response variable alkaloid concentration was log transformed (natural log) to improve homoscedasticity and normality of residuals. In the *L. perenne* samples from 2017 ergovaline was detected in only three samples, because of that the variable ergovaline was not analysed statistically.

Differences between alkaloid concentrations detected with two analytical methods (method 1 and method 2) were compared with a paired t-test.

III.2.6. Data availability

The related raw data were deposited on the BExIS database of the Biodiversity Exploratories (www.bexis.uni-jena.de) with the following data set IDs: 26046 and 26047.

III.3. Results

III.3.1. Dataset 1: Field study in Germany

In 2017 we collected and analysed 1122 *L. perenne* individuals on 66 grassland study sites in the three study regions. Neither infection rates nor alkaloid concentration were significantly affected by land-use intensity, mowing, grazing or fertilization (Table III1). In the regions Schorfheide-Chorin (SCH) and Hainich (HAI) occurred significantly more infections compared to the Schwäbische Alb (ALB), but alkaloid concentrations were not significantly different ($p > 0.05$) (Table III1).

Table III1: Generalized linear models (infection rates) and linear mixed effect models (peramine and lolitrem B). For the alkaloid concentrations (peramine and lolitrem B) only infected samples were used for analyses.

	Infection rates			**Peramine**			**Lolitrem B**		
	χ^2 dF	χ^2	p	dF	F	p	dF	F	p
Region	**2**	**17.45**	**<0.001*** **	2,107	1.84	0.19	2,75	2.77	0.11
LUI	1	0.53	0.47	1,107	3.21	0.10	1,75	0.24	0.64
Mowing (y/n)	1	0.09	0.76	1,107	0.004	0.95	1,75	0.01	0.94
Mowing tot	1	0.09	0.76	1,107	0.10	0.75	1,75	0.001	0.98
Grazing (y/n)	1	0.81	0.37	1,107	0.08	0.79	1,75	0.02	0.90
Grazing tot	1	0.12	0.73	1,107	0.22	0.64	1,75	0.01	0.91
Fertilization (y/n)	1	1.70	0.19	1,107	0.33	0.58	1,75	0.15	0.70
Fertilization tot	1	0.36	0.55	1,107	4.14	0.08	1,75	0.09	0.78

In the Schwäbische Alb (ALB) we detected concentrations of 3.38 ± 1.90 μg/g (DW) of peramine (mean ± SE) and 1.51 ± 1.17 μg/g (DW) of lolitrem B, but due to low samples size (n=3) we did not analyze it statistically. In the Nationalpark Hainich (HAI) a mean peramine concentration of 2.23 ± 0.36 μg/g (DW) and a mean lolitrem B concentration of 0.61 ± 0.10 μg/g (DW) were

detected. In Schorfheide-Chorin region mean concentrations of peramine were 1.25 ± 0.12 µg/g (DW) and for lolitrem B 0.34 ± 0.06 µg/g (DW). Although alkaloid concentrations were extracted from dry weight, alkaloid concentrations on population level were, in accordance with König et al. (2018), below toxicity thresholds for insect and vertebrates. At an individual level, 23 *L. perenne* samples showed peramine concentrations above toxicity threshold for insects and seven vertebrate-toxic lolitrem B concentrations exceeded the threshold (Table III2).

Table III2: Number of individuals (IND, only infected) or study sites (SITE, infected and not infected) within alkaloid concentration range [µg/g DW] of peramine, ergovaline, lolitrem B and paxilline and their relative proportion of all individuals, or all study sites. Max: maximum of alkaloid concentrations. Individuals or study sites which show concentrations above toxicity thresholds are marked in bold. Individuals: n=126, study sites: n=66.

	Individual concentrations								**Mean concentrations per study site**							
	Peramine		Ergovaline		Lolitrem B		Paxilline		Peramine		Ergovaline		Lolitrem B		Paxilline	
Conc. (µg/g)	IND	%	IND	%	IND	%	IND	%	SITE	%	SITE	%	SITE	%	SITE	%
0.0	4	3.2	125	99.2	50	39.7	125	99.2	38	57.6	65	98.5	42	63.6	65	98.5
0-0.3	12	9.5	1	0.8	28	22.2	1	0.8	15	22.7	1	1.5	23	34.9	1	1.5
0.3-1	51	40.5	**0**	**0.0**	31	24.6	0	0.0	9	13.6	**0**	**0.0**	1	1.5	0	0.0
1.0-2.0	36	28.6	**0**	**0.0**	10	7.9	0	0.0	4	6.1	**0**	**0.0**	0	0.0	0	0.0
2.0-3.0	**9**	**7.1**	**0**	**0.0**	**4**	**3.2**	0	0.0	**0**	**0.0**	**0**	**0.0**	**0**	**0.0**	0	0.0
3.0-5.0	**8**	**6.4**	**0**	**0.0**	**3**	**2.4**	0	0.0	**0**	**0.0**	**0**	**0.0**	**0**	**0.0**	0	0.0
5.0-10.0	**4**	**3.2**	**0**	**0.0**	**0**	**0.0**	0	0.0	**0**	**0.0**	**0**	**0.0**	**0**	**0.0**	0	0.0
>10.0	**2**	**1.6**	**0**	**0.0**	**0**	**0.0**	0	0.0	**0**	**0.0**	**0**	**0.0**	**0**	**0.0**	0	0.0
Max (µg/g)	12.0		0.17		4.2		0.15		1.7		0.0		0.5		0.01	

III.3.2. Dataset 2: Alkaloid concentrations throughout the year in a field study

In 2018 we sampled 575 *L. perenne* plants on five study sites throughout the year, as high alkaloid concentrations have been detected in 2015 (SIII1).

133 out of 575 samples showed infections with the immunoblot assay and were analyzed for their alkaloid concentrations, as well as 12 not infected samples as negative controls. Alkaloid concentrations could not be analyzed for 10 infected samples, due to limited amount of plant material and were excluded from the following analyses. None of the UPLC-MS/MS- analyzed immunoblot-negative samples showed detectable alkaloid concentrations. In eight immunoblot-positive samples we did not detect alkaloids. In the remaining 116 infected samples we detected peramine with concentrations ranging between 0.04 and 23.38 μg/g (DW). Peramine concentrations above the toxicity threshold of 2 μg/g (DW) were detected in 65 of the samples, 27 were collected in September, 15 in July, 12 in June, 7 in November and 5 in January. Mean concentration per month on population level for September was with 3.23 ± 0.61 μg/g (DW) (mean ± SE) above the toxicity threshold (Figure III1, SIII3), in three of the study sites SEG43 (5.11 ± 1.63 μg/g (DW)), SEG44 (2.66 ± 1.46 μg/g (DW)) and SEG46 (7.34 ± 1.43 μg/g (DW)) (SIII3). In 76 of the endophyte-infected samples we detected lolitrem B with concentrations ranging between 0.07 and 23.81 μg/g (DW), of which 31 contained concentrations above the toxicity threshold of 1.8 μg/g (DW). All samples with concentrations above toxicity threshold for lolitrem B were sampled during the summer months (June, July, September). But on population level all lolitrem B concentrations, per month (Figure 1) and per study site, were below toxicity threshold. Paxilline was not detected in the samples and ergovaline was only detected in four samples and therefore not statistically analyzed. Ergovaline concentrations in all four samples were all below the specific toxicity threshold of 0.3 – 0.4 μg/g (DW). Only considering infected samples (Figure III1, filled dots), peramine concentrations exceeded the toxicity threshold in July, September and November and lolitrem B concentrations exceeded the threshold in September, highlighting that *Epichloë* infected monocultures could be toxic for livestock in late summer.

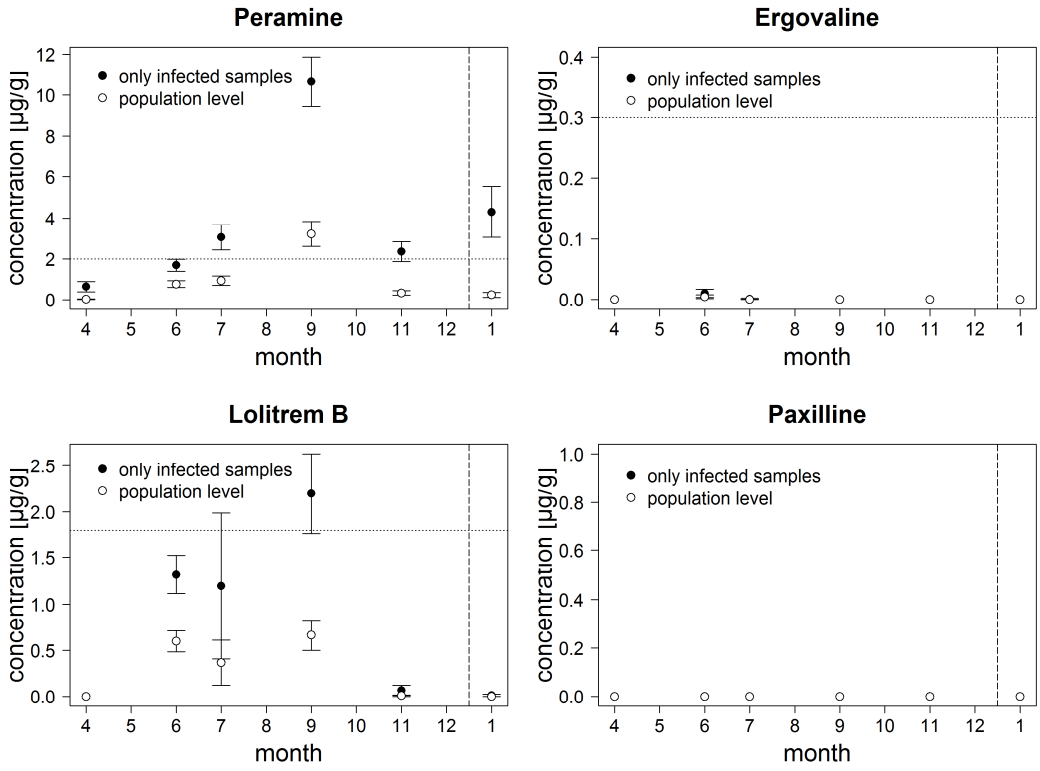


Figure III1: Concentrations of peramine and lolitrem B in one year in 2018 and the beginning of 2019 on five study sites in the Schorfheide-Chorin, Germany on population level (unfilled dots: µg/g dry weight (DW), mean ± SE, n=575). Filled dots show only infected samples (mean ± SE, n=133). Dotted horizontal line indicates toxicity threshold of the alkaloid based on dry weight and dashed vertical line indicates the turn of the year.

III.3.3. Dataset 3: Common garden study

Alkaloid concentrations in *L. perenne* seeds infected with *E. festucae* var. *lolii* from New Zealand, varied monthly during the study period (April-September) in our common garden experiment (Figure III2). In all samples (fresh and dry weight), concentrations of peramine exceeded the estimated toxicity threshold of 2 µg/g (DW), except in calendar week 21. The first samples were taken during plant rearing in the green house, before plants were placed to the common garden (dashed vertical line) starting with a mean concentration of 26.21 ± 2.97 µg/g dry weight (DW) (mean ± SE). Concentration decreased until calendar week 21 to 1.02 ± 0.10 µg/g (DW) peramine, increased again until calendar week 33 (August) to 35.63 ± 3.10 µg/g (DW) and decreased again to 16.32 ± 2.30 µg/g (DW) at the end of

September (Figure III2, SIII4). The concentrations analyzed from fresh weight showed a similar trend as dry plant weight but were only 24 – 37 % of the concentrations determined in dry weight (averaged approx. 3.12 times less).

Ergovaline concentration reached the peak in calendar week 25 (June) with 1.33 ± 0.30 μg/g (DW) and this level maintained until end of September when concentrations decreased to 0.81 ± 0.18 μg/g (DW) (Figure III2, SIII4). Concentrations analyzed based on dry plant weight were all above toxicity threshold of 0.3 μg/g (DW), except for calendar week 21 (May, 0.03 ± 0.01 μg/g (DW)). Concentrations analyzed with fresh plant weight followed the same trend but were only 20-49 % of the concentrations determined in dry plant weight (averaged approx. 3.55 times less).

Lolitrem B concentrations exceeded the toxicity threshold of 1.8 μg/g (DW) only in calendar week 29 (July, 2.00 ± 0.32 μg/g (DW) and 33 (August, 6.57 ± 1.09 μg/g (DW)) (Figure III2, SIII4). Concentration changed with season and peaked in calendar week 33 (in August) for both fresh and dry weight. (Figure III2). Concentrations analyzed with fresh plant weight were only 13-40 % of the concentrations determined in dry plant weight (averaged approx. 3.65 times less).

Paxilline concentration decreased after plant transfer to the common garden to concentrations bellow detection limit but increased from calendar week 31 until calendar week 37 (September) up to 0.56 ± 0.28 μg/g (DW) (Figure III2, SIII4). Concentrations analyzed with fresh weight were below detection limit.

Detected differences between alkaloid concentrations analyzed from fresh and dry plant weight were higher in summer than in spring or autumn (Figure III2).

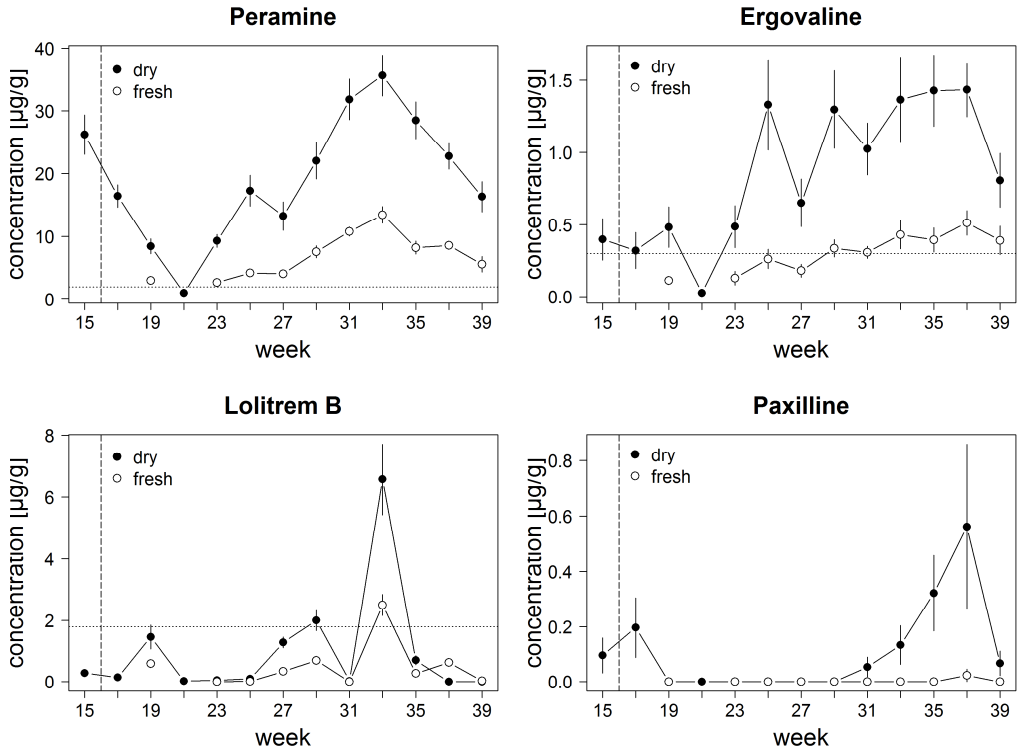


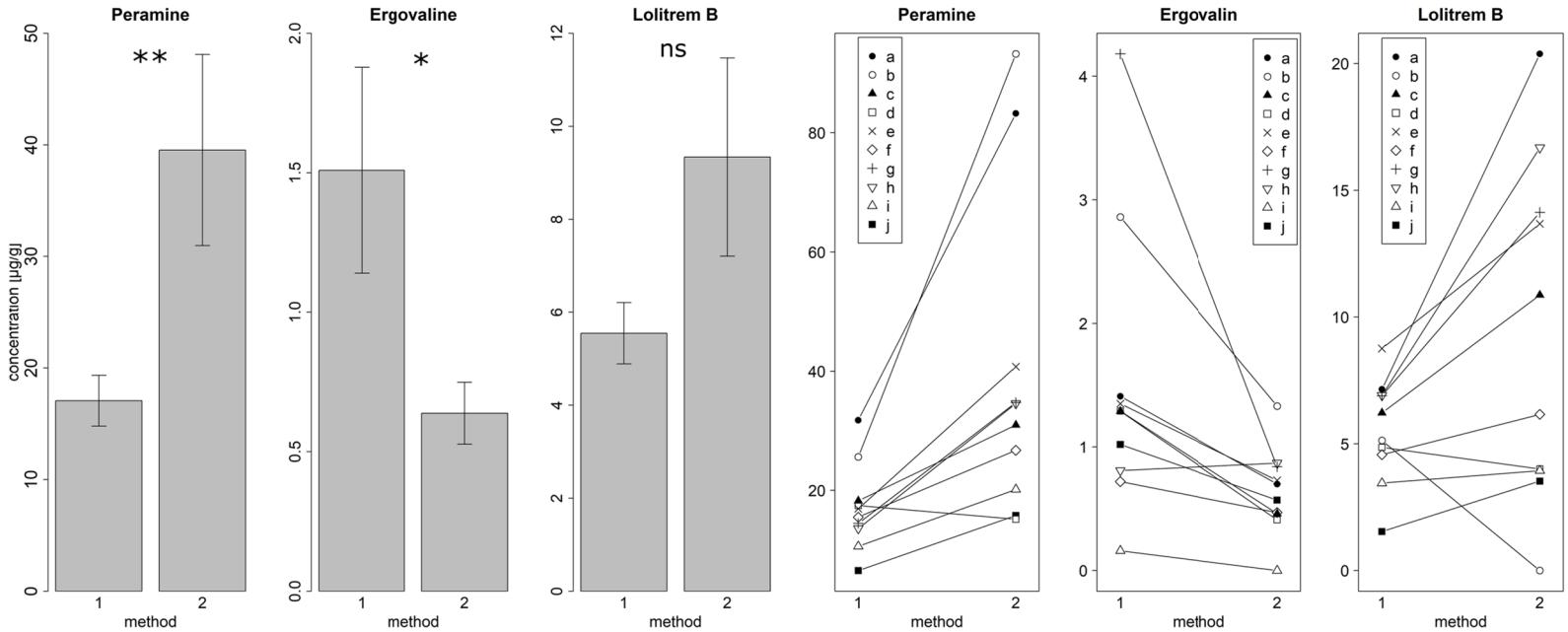
Figure III2: Alkaloid concentrations in [µg/g] (mean ± SE) in *L. perenne* plants infected with *Epichloë festucae* var. *lolii* during several weeks (calendar weeks) in a common garden experiment analyzed with dry (filled circles) and fresh (empty circles) plant weight. Plants were sampled every two weeks from the beginning of April until the end of September. The first sampling (calendar week 15) was performed in the greenhouse, afterwards the plants were placed outside in a common garden (marked with a vertical dashed line). Toxicity thresholds based on dry weight are marked with a horizontal dotted line.

III.3.4. Comparison of two alkaloid detection methods

Concentrations (DW) based on the method from (Fuchs et al., 2013) (method 2) were significantly higher for peramine (averaged approx. 2.2 times) compared to the method described in this study (Method 1) (paired t-test (n=20): t = -3.34, df = 9, p = 0.008**) (Figure III3). In contrast, ergovaline concentrations (DW) were significantly lower (averaged approx. 0.42 times) when detected with method 2 compared to method 1 (paired t-test (n=19): t = -2.82, df = 8, p = 0.02*) (Figure III3). Lolitrem B concentrations (DW) were marginally higher (averaged approx. 1.7 times) when detected with method 2 compared to method 1 (paired t-test (n=19): t = 2.23, df = 9, p = 0.052) (Figure III3).

Comparing each sample with the two methods showed that some samples showed an opposite trend (peramine: sample *d*, ergovaline: sample *j*, lolitrem B: sample *b*, *d*) (Figure 4). Peramine, ergovaline and lolitrem B were found in each sample when analyzed with method 1. There was no detection of ergovaline in sample *f* and no detection of lolitrem B in sample *b* when using method 2.

Figure III3: a: Comparison of alkaloid concentrations [µg/g DW] (peramine, ergovaline and lolitrem B, mean ± SE) detected with the UPLC method from this study (method 1) and the UPLC method from (Fuchs et al., 2013) (method 2). Peramine: n=10, p = 0.008**, ergovaline: n=9, p = 0.02* and lolitrem B: n=10, p = 0.052, ns=not significant). **b:** Comparison of alkaloid concentrations [µg/g] (peramine, ergovaline and lolitrem B) of single samples (*a-j*) detected with the UPLC method from this study (method 1) and the UPLC method from (Fuchs et al., 2013) (method 2).



III.4. Discussion

III.4.1. Field study in Germany

Our results are in accordance with the results found in König et al. (2018) that land-use intensity, mowing, grazing or fertilization had no influence on infection rates and alkaloid concentrations of *L. perenne* infected with *E. festucae* var. *lolii* in three regions in Germany, even though our results are now based on dry plant weight (i). Although other studies showed influence of grazing (Faeth and Fagan, 2002; Fuchs et al., 2017b; Gwinn et al., 1998; Jensen and Roulund, 2004) or fertilization (Faeth and Fagan, 2002; Krauss et al., 2007) on alkaloid concentrations, we detected no significant effects in our field study sites. However, in most of the studies, results are based on functional treatments from common garden experiments, whereas our study represents results from real world agricultural fields, highlighting the importance of authentic field studies. Infection rates differed significantly between the three regions in this study and in König et al. (2018), but this could not be explained by land-use intensity, mowing, grazing or fertilization. König et al. (2018) also showed that sowing had no influence on infection rates or alkaloid concentrations. However, there are hints, that commercially available seed mixtures can contain *Epichloë* infected seeds in Germany (Krauss et al., 2020), which might lead to increasing *Epichloë* infections. In contrast to König et al (2018), we extracted alkaloids from dry plant weight with an altered quantitation method (Krauss et al., 2020). We showed that intoxication risk on population level on German grasslands is similarly low using dry plant weight compared to fresh weight (König et al., 2018). Single samples exceeded toxicity thresholds in both studies, but not on population level. We assume that the difference in alkaloid concentrations often dilutes on population level in Germany like in other regions, e.g Spain (Soto-Barajas et al., 2019).

III.4.2. Alkaloid concentrations throughout the year in a field study

We monitored alkaloid concentrations in *Lolium perenne* infected with *Epichloë festucae* var. *lolii* over ten months and showed an increase in alkaloid concentrations detected in dried plant material in summer (ii). Other studies also indicated, that alkaloid concentrations peak in late summer (Fuchs et al., 2017a; Jensen, 2005; König et al., 2018; Repussard et al., 2014b). Our results represent the course of alkaloid concentrations in a real world field without additional watering, compared to other studies, where observations of alkaloid concentrations over several months are often highly controlled e.g. performed on grasslands sown with *Epichloë* endophyte infected seeds (Jensen, 2005), grasses from the field, but selected for *Epichloë* infections and potted (Repussard et al., 2014b), or potted *Epichloë* infected grasses (Fuchs et al., 2017a).

On field sites, we only detected lolitrem B and peramine, but not ergovaline, which is consistent with the results of a previous study, showing that the starting gene for the ergovaline biosynthesis pathway *dmaW* is missing in *E. festucae* var. *lolii* in *L. perenne* in the same German grasslands (Vikuk et al., 2019). Another study showed varying ergovaline contents in wild *Festuca rubra* plant infected with *Epichloë festucae* depending on geographic origin and transplanting sites, indicating phenotypic plasticity (Vázquez de Aldana et al., 2020). We also did not detect paxilline, a precursor of lolitrem B. That indicates that the amount of paxilline was below the detection limit and the precursor was probably immediately converted to lolitrem B. Paxilline is currently mostly neglected in studies concerning endophyte-mediated toxicity. Due to its tremorgenic effects on vertebrates, paxilline should be considered in future intoxication studies (Imlach et al., 2008; Vikuk et al., 2019). We calculated alkaloid concentrations on the population level (mean of infected and not infected *L. perenne* samples per study site) and of only infected samples (mean of infected individual plants per study site), which would reflect alkaloid concentrations in a monoculture with only infected *L. perenne* plants. Concentrations of insect deterring peramine on the population level exceeded the toxicity threshold of 2 µg/g (DW) (Rowan et al., 1990) only in September. However, mean alkaloid concentrations per study sites for infected plants showed exceeding concentrations throughout the entire summer, which can be a benefit for

farmers due to herbivore protection. Considering the deterring threshold of 15 µg/g (DW) as postulated for the deterrence of Argentine stem weevil (Hewitt et al., 2020; Popay and Wyatt, 1995), none of the tested samples exceeded this threshold.

Lolitrem B concentrations exceeding toxicity thresholds of 1.8 µg/g (DW) can lead to intoxication events in grazing livestock by causing ryegrass staggers (Craig et al., 2014). On the population level, lolitrem B never exceeded the toxicity threshold, whereas it did in September, when only considering infected samples for the calculation of mean alkaloid concentrations per study site. Therefore, intoxication of livestock due to lolitrem B might be possible in late summer on *L. perenne* monocultures, with a high frequency of *Epichloë* infection.

III.4.3. Common garden study

We conducted a common garden study, monitoring alkaloid concentrations over a ten months period following the setup of (Fuchs et al., 2017a) to compare alkaloid concentrations between samples extracted from fresh and dried plant material. In this experiment, we used *L. perenne* seeds infected with *E. festucae* var. *lolii* from New Zealand, which are known to produce vertebrate toxic lolitrem B and ergovaline, as well as insect deterring peramine. We showed that alkaloid concentrations increased in summer and decreased in winter (iii) independent of dry or fresh plant weight (iv). However, alkaloid concentrations analyzed with fresh weight were lower compared to dry weight, which was previously shown for paxilline and ergot alkaloids (Bauer et al., 2018). In the study of Bauer et al. (2018) the authors showed that alkaloid concentrations for paxilline and ergot alkaloids were approximately ten times higher when analyzed with dry weight instead of fresh weight. The dried plant weight was also around 10 % of the fresh plant weight (Bauer et al., 2018). In our study the loss on drying also corresponded to the difference in alkaloid concentrations based on fresh or dry weight. That shows that the difference in alkaloid concentrations can be explained by the loss of water during drying. The study of Bauer et al. (2018) used commercially available grass seeds from German horticulture shops, hence *Epichloë* infection status of the seeds were unknown before (Bauer et al., 2018). These results show that using fresh

weight concentrations lead to an underestimation of toxic alkaloid concentrations. Most studies use dry weight to measure alkaloid concentrations, with some exceptions due to specific constraints, e.g. because of limited sample amount (König et al., 2018) or parallel measurements, which require fresh plant material (Fuchs et al., 2017a). Nevertheless, alkaloid concentrations in dried plant material are more accurate, because of possible varying water contents in the plants.

Alkaloid concentrations in dried plant material decreased after transfer of the plants from the green house to the common garden before alkaloid concentrations increased again, which may be explained by an adaptation period to common garden conditions. Another possible explanation might be that fungal growth responds delayed to a fast plant growth which is reflected in a temporary decrease in alkaloid concentrations (Fuchs et al., 2017c; Hewitt et al., 2020). Peak alkaloid concentrations in dried and fresh plant material found during late summer (September) correlate with peak abundances of main grass herbivores, like species from the insect order Orthoptera (Köhler et al., 1999), which could explain the peak in insect deterring peramine concentration in late summer (Fuchs et al., 2017b). Ryegrass stagger outbreaks occurred in summer and autumn in Australia and New Zealand, which could be explained by peaking lolitrem B concentrations (Hume et al., 2016). In the study of (Fuchs et al., 2017a) ergovaline and lolitrem B concentrations based on fresh plant weight exceeded toxicity thresholds only in the third year. Here we showed that ergovaline and lolitrem B concentrations in dried plant material exceed toxicity thresholds already in the first year. In another study it was shown that variation in lolitrem B concentrations was low over three years, whereas ergovaline concentrations varied and might be influenced by abiotic factors (Repussard et al., 2014b). Our study allowed a rough estimate to compare fresh and dry plant weight for alkaloid concentrations in *L. perenne* with a correction multiplication of 3.12 for Peramine concentrations, 3.55 for lolitrem B and 3.65 for ergovaline (fresh weight to dry weight). However, care must be taken e.g. due to climate change-mediated increase in extreme events, such as heavy rains and persistent droughts with tremendous fluctuations in plant water content (Machado and Paulsen, 2001).

III.4.4. Comparison of two alkaloid detection methods

There are different methods to detect and quantify alkaloids produced by *Epichloë* species in cool season grass species (Craig et al., 2014; Finch et al., 2013; Fuchs et al., 2013; Repussard et al., 2014c; Rudolph et al., 2018). Here, we compared two UPLC methods differing in the length of their used column and the additional detection of paxilline in method 1. Alkaloid concentrations detected with method 2 were higher for peramine and lolitrem B, whereas alkaloid concentrations detected with method 1 were higher for ergovaline (v). The limit of detection is comparable between the methods and cannot explain the differences. Both methods used the same solvents and column materials, hence the efficiency of ionization should be comparable, although the longer column in method 1 and the differing gradient changes the solvent composition slightly, which might have led to the differences in alkaloid detection. Homoperamine was used as reference to quantify lolitrem B in both methods (Fuchs et al., 2013; Krauss et al., 2020), although it is not structurally related to lolitrem B. Lolitrem B was only available in small amounts for method development, but not for a validation of the method. The substance ergovaline used for validation was only available for method 1. Hence, we assume that detection of ergovaline is more reliable with this method. Otherwise, method 2 detected higher lolitrem B and peramine concentrations. We developed method 1 originally, because we wanted to detect lolines, lolitrem B, ergovaline, peramine and paxilline within one method. Lolines belong to the 1-aminopyrrolizidines, another group of alkaloids, which can be produced by some *Epichloë* endophytes like *E. uncinata* in *Festuca pratensis* and which have insect toxic properties (Schardl et al., 2012). We used a previously published method (Rudolph et al., 2018) to add the detection of polar lolines and lipophilic paxilline, which extends the run time of the method. We had to adapt the method from Rudolph et al. (2018) due to a low solubility of lolitrem B and paxilline in aqueous solutions and a better ionization of alkaloids with formic acid instead of ammonium formate. The detection of molecules with differing molecule size and polarity can lead to methodological differences like solubility or ionization problems, which could result in differing alkaloid concentrations. Differences in chromatographic conditions (different solvent gradient and column dimensions) can lead to different

ionization efficiencies and other matrix effects, resulting in different alkaloid concentrations. In order to determine absolute alkaloid concentrations, it would be necessary to use isotope labelled reference substances, which are not available for the tested alkaloids. Due to that, toxicity thresholds from literature are also not determined with isotope labelled reference substances (if mass spectrometry is used as detection method), which means that only a rough comparability of detected alkaloid concentrations with toxicity thresholds is possible. In a previous study we compared alkaloid concentrations in seed mixtures with two different methods (Krauss et al., 2020). Alkaloid concentrations were detected with method 1 from this study and another UPLC method of the Endophyte Service of the Oregon State University. Absolute numbers of alkaloid concentrations differed in this study too, but detection of alkaloids (presence/absence) was consistent for all samples (Krauss et al., 2020). It can be assumed that other methods of alkaloid detection differ in their absolute amounts of alkaloid concentrations and can only be compared relatively. Bauer et al. (2018) for example showed that paxilline concentrations detected with HPLC-MS/MS were less than 3 % of the paxilline level detected with enzyme immunoassay (Bauer et al., 2017), but this might be explained due to the often unselective detection of immunoassays (Jensen et al., 2011). Hence, only validated methods should be used for the detection of alkaloids to compare alkaloid concentrations with other studies and toxicity thresholds from literature. This can be even more important with increasing temperatures due to climate change which can increase alkaloid production by *Epichloë* sp. (Bourguignon et al., 2015; Hennessy et al., 2016; McCulley et al., 2014).

III.5. Conclusions

We showed that (i) land-use intensity had no effect on alkaloid dry weight concentrations. We also showed that (ii) the German grasslands contained grass individuals with concentrations above the toxicity level, but not on a population level of all *L. perenne* plants per study sites. Both results confirmed findings of a previous study (König et al., 2018). Previous findings on seasonal trends in alkaloid concentration using fresh plant weight (Fuchs et al., 2017a) could be (iii) confirmed with dry plant weight experiments. We showed that (iv) alkaloid dry weight concentrations in *L. perenne* plants are approximately 3-4 times higher compared to fresh weight concentrations. Finally, we showed that (v) detection methods differed in alkaloid concentrations, due to the use of different chromatographic conditions. We assume, that the influence of the alkaloid detection method is low for general trends in a functional study, whereas to estimate intoxication risk for livestock, a validated method is important.

III.6. Supplemental material

SIII1: Study sites with alkaloid concentrations above toxicity thresholds in 2015: Number of samples with concentrations above toxicity thresholds in 2015 for peramine, lolitrem B and ergovaline and number of infected samples at the study site. Sampled in summer (July/August) 2015.

Study site	No. of samples with Peramine concentrations >2 µg/g	No. of samples with Lolitrem B concentrations >1.8 µg/g	No of samples with Ergovaline concentrations >0.3 µg/g	No of infected samples/total sample size
SEG40	2	0	1	2/20
SEG43	1	5	0	5/20
SEG44	2	3	0	3/20
SEG46	6	9	0	13/20
SEG47	2	4	0	8/20

SIII2: Differences between UPLC-methods used in dataset (3)

Name of the method	Method 1	Method 2
Publications	(Krauss et al., 2020) They used dry plant weight	(Fuchs et al., 2013; König et al., 2018) They used fresh plant weight
Detectable alkaloids	Lolitrem B, ergovaline, peramine, paxilline	Lolitrem B, ergovaline, peramine
Standards	Homoperamine, ergotamine, LSD-d3	Homoperamine, ergotamine
Substances for validation	Peramine, lolitrem B, ergovaline, paxilline	Peramine, lolitrem B
extraction	One part of the sample was reconstituted in water/acetonitrile/formic acid 50:50:0.1 (v/v/v) for the analysis of lolitrem B and paxilline the other in water/acetonitrile/formic acid 80:20:0.1 (v/v/v) for the analysis of peramine and ergovaline	Samples were reconstituted in methanol (80 %)
Analytical method	UPLC-MS/MS	UPLC-MS/MS
Column	Aquitiy UPLC column (100x2.1 mm; 1.7 µm; Waters GmbH, Eschborn, Germany), Reverse Phase	Acquitiy UPLC BEH column (50x2.1 mm; 1.7 µm; Waters GmbH, Eschborn, Germany), Reverse Phase

solvents	0.1 % formic acid dissolved in water (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B)	0.1 % formic acid dissolved in water (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B)
Flow rate	0.4 ml/min	0.3 ml/min
gradient	0–1.0 min 98% solvent A, 1.0–3.0 min to 90% solvent A, 3.0–5.0 min to 85% solvent A, 5.0–7.5 min to 80% solvent A, 7.5–10.0 min to 75% solvent A, 10.0–11.5 min to 70% solvent A, 11.5–13.0 min to 65% solvent A, 13.0–14.5 min to 50% solvent A, 14.5–16.0 min to solvent 40% A, 16.0–19.0 min to 0% solvent A, 19.0–22.0 hold 0% solvent A, 22.0–23.0 back to 98% solvent A and hold for another 2 min	from 5% to 25% solvent B in 5 min, followed by 25% to 75% solvent B in 0.5 min, then 75% to 100 % solvent B in 2.5 min
Total run time	25 min	10 min
Injection volume	10 µl for the analyses of peramine and ergovaline 5 µl for the analyses of lolitrem B and paxilline.	5 µl
Limit of detection	Limit of detection (LOD): Paxilline, lolitrem B: 0.05 ng on column Ergovaline, peramine: 0.01 ng on column.	LOD: comparable to method 1

SIII3: Mean alkaloid concentrations (in [µg/g] dry weight (mean ± SE)) per month on the five study sites in the field in 2018.

Month	Year	Peramine	Lolitrem B
April	2018	0.032 ± 0.018	0.000 ± 0.000
June	2018	0.773 ± 0.158	0.601 ± 0.113
July	2018	0.939 ± 0.233	0.367 ± 0.243
September	2018	3.227 ± 0.611	0.665 ± 0.164
November	2018	0.345 ± 0.110	0.010 ± 0.008
January	2019	0.242 ± 0.123	0.001 ± 0.001

SIII4: Mean alkaloid concentrations based on fresh or dry plant weight from April until September 2019 in the common garden experiment (mean ± SE, n=10 per time point). Date: dd.mm.yyyy, Week: calendar week. NA: not measured, nd: not detected

sample	Date	Week	Peramine		Ergovaline		Lolitrem B		Paxilline	
			fresh	dry	fresh	dry	fresh	dry	fresh	dry
1	09. April 2019	15	NA	26.21 ± 2.97	NA	0.40 ± 0.14	NA	0.29 ± 0.07	NA	0.10 ± 0.06
2	24. April 2019	17	NA	16.42 ± 1.71	NA	0.32 ± 0.12	NA	0.14 ± 0.07	NA	0.20 ± 0.10
3	07. May 2019	19	3.02 ± 0.29	8.44 ± 1.13	0.11 ± 0.03	0.48 ± 0.13	0.59 ± 0.12	1.46 ± 0.37	nd	nd
4	21. May 2019	21	NA	1.02 ± 0.10	NA	0.03 ± 0.01	NA	0.03 ± 0.004	NA	nd
5	04. June 2019	23	2.69 ± 0.31	9.29 ± 0.99	0.13 ± 0.05	0.49 ± 0.14	nd	0.05 ± 0.02	nd	nd
6	18. June 2019	25	4.21 ± 0.47	17.28 ± 2.33	0.26 ± 0.06	1.33 ± 0.30	0.01 ± 0.01	0.10 ± 0.04	nd	nd
7	02. July 2019	27	4.05 ± 0.60	13.24 ± 2.16	0.18 ± 0.04	0.65 ± 0.16	0.34 ± 0.04	1.28 ± 0.17	nd	nd
8	16. July 2019	29	7.57 ± 0.92	22.06 ± 2.76	0.34 ± 0.06	1.30 ± 0.26	0.70 ± 0.13	2.00 ± 0.32	nd	nd
9	30. July 2019	31	10.78 ± 0.700	31.81 ± 3.08	0.31 ± 0.04	1.03 ± 0.17	nd	0.03 ± 0.03	nd	0.05 ± 0.03
10	14. August 2019	33	13.46 ± 1.26	35.63 ± 3.10	0.43 ± 0.10	1.37 ± 0.28	2.51 ± 0.33	6.57 ± 1.09	nd	0.13 ± 0.07
11	28. August 2019	35	8.21 ± 0.98	28.49 ± 2.80	0.39 ± 0.08	1.43 ± 0.24	0.27 ± 0.07	0.71 ± 0.14	nd	0.32 ± 0.13
12	10. Septem-ber 2019	37	8.58 ± 0.64	22.76 ± 1.94	0.51 ± 0.08	1.43 ± 0.18	0.63 ± 0.07	nd	0.02 ± 0.02	0.56 ± 0.28
13	25. Septem-ber 2019	39	5.56 ± 1.17	16.32 ± 2.30	0.39 ± 0.09	0.81 ± 0.18	0.03 ± 0.03	nd	nd	0.07 ± 0.04

Chapter IV

Epichloë endophyte infection rates and alkaloid content in commercially available grass seed mixtures in Europe

Grass seed mixture. Photo: © Veronika Vikuk

"We cannot avoid the fact, that everything we are doing has an influence on the whole thing."

Albert Einstein

Chapter IV

Epichloë endophyte infection rates and alkaloid content in commercially available grass seed mixtures in Europe

Fungal endophytes of the genus *Epichloë* live symbiotically in cool season grass species and can produce alkaloids toxic to insects and vertebrates, yet reports of intoxication of grazing animals have been rare in Europe in contrast to overseas. However, due to the beneficial resistance traits observed in *Epichloë* infected grasses, the inclusion of *Epichloë* in seed mixtures might become increasingly advantageous. Despite the toxicity of fungal alkaloids, European seed mixtures are rarely tested for *Epichloë* infection and their infection status is unknown for consumers. In this study we tested 24 commercially available seed mixtures for their infection rates with *Epichloë* endophytes and measured the concentrations of the alkaloids ergovaline, lolitrem B, paxilline, and peramine. We detected *Epichloë* infections in six seed mixtures, and four contained vertebrate and insect toxic alkaloids typical for *Epichloë festucae* var. *lolii* infecting *Lolium perenne*. As *Epichloë* infected seed mixtures can harm livestock, when infected grasses become dominant in the seeded grasslands, we recommend seed producers to test and communicate *Epichloë* infection status or avoiding *Epichloë* infected seed mixtures.

IV.1. Introduction

Infection of crops with fungal endophytes can enhance resistance against environmental stressors, such as drought, herbivores and pathogens, and therefore have a high potential to increase yield (Franco et al., 2007; Vujanovic and Germida, 2017). Due to these beneficial traits, some endophytes have been used in seed breeding efforts to symbiotically modify their host plants (Gundel et al., 2013; Simpson et al., 2014). Economically important fungal endophytes commercially used in grass seed mixtures belong to the genus *Epichloë*, which are naturally distributed in cool season grass species and often produce vertebrate and insect toxic compounds (Song et al., 2020; Spangenberg et al., 2012). *Epichloë* endophytes are systemic endophytic fungi occurring exclusively in cool season grass species (Leuchtmann et al., 2014). The anamorphs of *Epichloë* endophytes, formerly known as *Neotyphodium* endophytes, are the most prominent producers of defensive metabolites, which are responsible for livestock intoxications, and of anti-herbivore compounds that are active against insects (Cheplick and Faeth, 2009; Hume et al., 2016; Müller and Krauss, 2005; Schardl et al., 2004). The main insect toxic or insect deterring compounds are 1-aminopyrrolizidines including lolines such as *N*-formylloline, and the pyrrolopyrazine, peramine, whereas the ergot alkaloid ergovaline, and the indole-diterpene lolitrem B and several of their precursors are toxic to livestock causing fescue toxicosis in cattle and ryegrass staggers in sheep (Liang et al., 2017; Müller and Krauss, 2005; Reddy et al., 2019; Schardl et al., 2004). Other ergot alkaloids in native grass species can cause e.g. the drunken horse syndrome (Liang et al., 2017).

Intoxication of livestock and severe economic losses have been frequently reported from New Zealand, Australia and the U.S.A (Hume et al., 2016; Young et al., 2013) but few cases have been reported from Europe (Dapprich et al., 1996; Hume et al., 2016; Lewis et al., 1997; Zabalgogeazcoa and Bony, 2008). Severe outbreaks of ryegrass staggers are especially known from New Zealand and Australia (Fletcher and Harvey, 1981; Reed et al., 2011), but have been also documented in Argentina (Odriozola et al., 1993), Chile (Butendieck et al., 1994) and South Africa (Hume et al., 2016). Single cases of ryegrass staggers in Europe have been recorded in Germany, France, the Netherlands and the United Kingdom

(Benkhelil et al., 2004; Dapprich et al., 1996; Hume et al., 2016; Lewis et al., 1997; Zabalgogeazcoa and Bony, 2008). Ryegrass staggers are caused by high concentrations of lolitrem B, produced by *Epichloë festucae* var. *lolii* infecting *Lolium perenne* (perennial ryegrass) (Hume et al., 2016), but lolitrem B can also be produced by the unnamed *Epichloë* sp. found in fine fescues as well as *Epichloë* sp. FaTG2 in *Festuca arundinacea* (tall fescue) (Christensen et al., 1993; Takach et al., 2012). When recognized in a timely manner, the intoxication is reversible (Finch et al., 2012) and the intoxication risk of humans due to consumption of animal products such as milk is considered to be low, because concentrations of alkaloids in the milk of intoxicated cows are below the toxicity threshold (Finch et al., 2013). *L. perenne* can also be infected with the allopolyploid *Epichloë hybrida* (LpTG-2) (Campbell et al., 2017), and the haploid LpTG-3 (such as AR37) that is able to provide resistance against porina larvae (*Wiseana cervinata*) through production of epoxy-janthithrems, which also have tremorgenic effects but not as potent as lolitrem B (Babu et al., 2018; Hennessy et al., 2016; Ludlow et al., 2019; Reddy et al., 2020). Fescue toxicosis is caused by the ergot alkaloid ergovaline, produced predominantly by *Epichloë coenophiala* infecting tall fescue, but has also been observed in some *Epichloë festucae* var. *lolii* isolates. Severe outbreaks of fescue toxicosis are known particularly from the US with severe economic losses (Aiken and Strickland, 2013; Hume et al., 2016), whereas reports of fescue toxicosis in Europe are scarce with one study in France observing ergovaline concentrations in tall fescue above the toxicity threshold (Repussard et al., 2014a).

Epichloë intoxication events are more common in New Zealand, Australia and the U.S.A compared to Europe because overseas grasslands are often dominated by a single non-native grass species with high endophyte infection rates. For example, the *Epichloë* infection rate of non-native perennial ryegrass is 70 % in New Zealand (Easton and Tapper, 2008) compared to 15 % in its native distribution range in Germany (Vikuk et al., 2019). To overcome vertebrate toxicity, *Epichloë* strains have been found and introduced into grass cultivars that are unable to produce the causative toxins (Johnson et al., 2013; Karpyn Esqueda et al., 2017). One of these is *Epichloë festucae* var. *loii* strain AR1, which still produces alkaloids deterring insects, but not the vertebrate toxic compounds lolitrem B and ergovaline (Stewart, 2006; Young et al., 2013). In fact, due to high herbivore pressure, New

Zealand seed companies breed perennial ryegrass with vertebrate safe endophytes as an integral part of their cultivars (Hume et al., 2020).

Perennial ryegrass and tall fescue are important grass species used for temperate grasslands due to their complementary traits such as the fast initial growth associated with the ryegrasses, and winter hardiness, high production after the second harvest year and persistence of the fescues (Spangenberg et al., 2012). Forage grass seed mixtures are used for developing pastures for agricultural purposes to feed animals (Bundessortenamt, 2018). However, turfgrasses are bred for traits that make them useful in lawns used under management practices of varying intensity (Bundessortenamt, 2019). Turfgrass mixtures are therefore not meant to feed livestock, but are used on sport fields, airports, parks, yards or riding arenas.

In other regions of the world the distribution and toxicity of *Epichloë* endophytes has received much attention (Hume et al., 2016), but very limited in European grasslands (Leyronas and Raynal, 2001; Saikkonen et al., 2000; Vikuk et al., 2019) and almost none in commercially available cultivars and seed mixtures on the European market (Jensen, 2005; Saikkonen et al., 2000). The aim of this study is to provide an overview on infection rates of grass seeds with *Epichloë* spp. and on the alkaloid content observed in a selection of commercially available forage grass and turfgrass seed mixtures. We want to demonstrate that sowing of endophyte-infected seed mixtures has the potential to introduce a risk for grazing animals, due to exposure to consuming toxic fungal alkaloids.

IV.2. Materials and Methods

IV.2.1. Seed sampling

All seed mixtures are commercially available and were bought from seed breeders between March and May 2019. All except one seed mixture contained perennial ryegrass and/or tall fescue. Composition with regard to varieties of perennial ryegrass and tall fescue and the presence of additional grasses is given in Table IV1. Samples of the seed mixtures were distributed in single-blinded and

randomized fashion to three laboratories, where they were independently analyzed. After completion of the analyses, the results were combined, and the composition of the seed mixtures was shared. Due to low infection frequencies, additional seeds were analyzed for endophyte infection. The manuscript was drafted by the two first authors without information on seed supplier names to reduce subjective judgements.

IV.2.2 Endophyte detection by multiplex PCR

Endophyte infection was performed at the Noble Research Institute, LLC, Ardmore, Oklahoma, USA, using a multiplex PCR with endophyte specific primers on DNA extracted from individual seeds randomly selected from each of the seed mixtures, as described previously (Vikuk et al., 2019). Each seed lot was tested twice. In both screens 22-24 seeds per mixture were tested and the first screen was single blinded. The PCR was performed using only the multiplex 1 primer set (Vikuk et al., 2019) with 6 µL instead of 3 µL of DNA, and the number of PCR cycles was increased to 33 cycles as the DNA template concentration was low.

Table IV1: Composition of grassland seed mixtures, bold indicate seed mixtures with infections of *Epichloë* spp. and detected vertebrate toxic alkaloids. Letters in brackets after the product name indicate if seed mixtures are mainly used as forage grass (F) or turf grass (T) according to supplier information. Supplier names are in italic.

ID	Perennial ryegrass varieties	Tall fescue varieties	Other grasses	Product
S_10*	**30 % BARCLAY II 15 % BAREURO 20% BARLICUM 20% BARLIBRO 15 % BAROMARIO**	**-**	**-**	**Regenerations-Mischung RPR, *Eurogreen, Barenbrug* (T)**
S_11	Unknown variety in unknown percentage	-	*Poa pratensis, Festuca pratensis, Dactylis glomerata, Phleum pratense, Festuca rubra, Agrostis*	Gräsermischung Weidesaat, *Kräuterwiese* (F)
S_12	25% lawn type, unknown variety 25% pasture type, unknown variety	-	20 % *Poa pratensis*, 20 % *Phleum pratense*, 10 % *Festuca rubra*	Country Horse 2117, *DSV* (F)
S_13*	14 % CARNAC 21 % EURODIAMOND 7 % DOUBLE 4n 8 % FABIAN 4n 8 % CSI CORSICA 17 % ZÜRICH	-	25 % *Poa pratensis*	Regeneration Highspeed, *UFA* (T)
S_14	-	100 % LIPALMA	-	*Camena Samen* (F)
S_15	33 % MATHILDE 34 % WADI 33 % BELIDA	-	-	Elite Gvo (ELITE Grünland Nr. 5), *Rudloff* (F)
S_16#	20 % EURODIAMOND, 15 % SIRTAKY	15 % BARCESAR, 35 % MEANDRE	15 % *Poa pratensis*	Reitbahn, *UFA* (T)
S_17*	25 % ASTONHOCKEY	20 % HYKOR	25 % *Festuca pratensis*, 20 % *Phleum pratense*, 10 % *Poa pratensis*	Country Öko 2217, *DSV* (F)
S_18	15 % BOYNE 20 % TODDINGTON 20 % INDICUS 1 15 % POLIM 15 % ARUSI 15 % GARBOR	-	-	Profi Nachsaat Gvo, *Tystofte Fonden* (F)
S_19	100 % KARATOS	-	-	*Camena Samen* (F)
S_20	-	-	7 % *Agrostis capillaris*, 3 % *Alopecurus pratensis*, 12 % *Arrhenatherum elatius*, 10 % *Cynosurus cristatus*, 10 % *Dactylis glomerata*, 15 % *Festuca rubra*, 1 % *Holcus lanatus*, 1 3 % *Phleum pratense*, 18 % *Poa pratensis*, 1 % *Trisetum flavescens*	Heuwiese für Pferde, *Appels Wilde Samen* (F)
S_21	10 % KARATOS 20 % KUBUS, 15 % TWYMAX	-	25 % *Phleum pratense* 12 % *Poa pratensis* 15 % *Festuca rubra*	Pferdeweide 1, *Camena Samen* (F)

S_22	8% PREMIUM	-	18% *Festulolium*, 18% *Phleum pratense*, 15% *Festuca pratensis*	Rotkleegras 91, *Camena Samen* (F)
S_23	100 % POLIM	-	-	*Camena Samen* (F)
S_24	**12 % BELLEVUE 20 % BOYNE 40 % STEFANI**	**-**	**18 % *Phleum pratense* 10 % *Poa pratensis***	**Pferdeweide Nachsaat, *Raiffeisen* (F)**
S_25	20 % IVANA 10 % TIVOLI 20 % SW BIRGER	20 % SWAJ	10 % *Poa pratensis* 20 % *Phleum pratense*	Pferdegreen Öko PR940, *BSV Saaten* (F)
S_26	100 % TWYMAX	-	-	*Camena Samen* (F)
S_27	28 % MATHILDE 23 % ALFAN 13 % BELIDA	-	10 % *Festuca pratensis*, 5 % *Poa pratensis*, 21 % *Phleum pratense*	Elite 20, *Rudloff* (F)
S_28	25 % MARAVA 30 % BOKSER 30 % WADI	-	15 % *Phleum pratense*	Equitana Nachsaat Gvo, *Rudloff* (F)
S_29*	10 % TURFGOLD	45 % BARCESAR, 25 % DEBUSSY 1	20 % *Poa pratensis*	Monaco-Mischung RSM, *Eurogreen* (T)
S_30	11 % TREND 5 % KARATOS 10 % TWYMAX	-	10 % *Festuca pratensis*, 11 % *Festulolium fedoro*, 7 % *Dactylis glomerata*, 5 % *Poa pratensis*, 5 % *Festuca rubra*, 14 % *Phleum pratense*	Kräuterweide, *Camena Samen* (F)
S_31	Unknown variety in unknown percentage	-	*Festuca pratensis, Poa pratensis, Poa trivialis, Festuca rubra, Phleum pratense, Alopecurus pratensis, Cynosurus cristatus, Elymus repens*	Pferdeweide-Reparatursaat, *Kräuterwiese* (F)
S_32	**15 % MARAVA 15 % BOKSER 15 % WADI**	**-**	**25 % *Phleum pratense* 20 % *Poa pratensis* 10 % *Festuca rubra***	**Equitana Universal, *Rudloff* (F)**
S_33*	**9 % COLUMBINE 7 % DOUBLE/FABIAN 12% ZURICH 5 % CSI CORSICA 12 % SIRTAKY**	**-**	**40 % *Poa pratensis*, 15 % *Festuca rubra***	**Primera Highspeed, *UFA* (T)**

IV.2.3 Alkaloid analyses

Quantitation of the alkaloids ergovaline, peramine, lolitrem B and paxilline, was performed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) using a Waters Acquity UPLC combined with a Quattro Premier triple quadrupole mass spectrometer at the University of Würzburg (Department of Pharmaceutical Biology). Seed samples were ground and prepared as described previously (Fuchs et al., 2013; König et al., 2018). For extraction about 9-10 g (25 ml in a Falcon tube) seeds per seed mixture (corresponding to approx. 2000 - 3000 *L. perenne* seeds) were ground and ground seeds (about 50 mg) were mixed with 250 µl dichloromethane/methanol 1:1 (v/v) containing the internal standards lysergic acid diethylamide-D3 (LSD-D3) (125 ng), ergotamine (500 ng) and homoperamine (500 ng). We tested one replicate per seed mixture. After centrifugation, the supernatant was recovered, and the seed pellet was reextracted with 250 µl of dichlormethane/methanol 1:1 (v/v). Two aliquots of the supernatant were evaporated under reduced pressure. One sample was reconstituted in water/acetonitrile/formic acid 50:50:0.1 (v/v/v) for the analysis of lolitrem B and paxilline, and the other in water/acetonitrile/formic acid 80:20:0.1 (v/v/v) for the analysis of peramine and ergovaline.

Chromatographic separation was performed as described previously (Rudolph et al., 2018) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. Injection volumes were 10 µl for the analysis of peramine and ergovaline and 5 µl for the analysis of lolitrem B and paxilline. Lolitrem B, ergovaline and peramine were detected according to Fuchs et al. (2013) using multiple reaction monitoring (MRM). In addition, paxilline was detected using the following specific transitions: m/z 436.3 → 130.1 and 436.3→182.1 and LSD-D3 was used as internal standard with the following specific transitions: m/z 327.1→208.1 and 327.1→226.1. Limit of quantitation (LOQ) for peramine and ergovaline was 0.01 ng on column (0.025 µg/g; 25 ppb), whereas for lolitrem B and paxilline it was 0.05 ng on column (0.125 µg/g; 125 ppb).

In parallel, the levels of lolitrem B and ergovaline were analyzed by the Endophyte Service Laboratory in Corvallis, Oregon, USA at Oregon State University as described previously (Craig et al., 2014). For ergovaline analysis,

ground seeds were extracted with chloroform, 0.001 M NaOH and ergotamine as the internal standard. After centrifugation, the organic supernatant was applied to Ergosil solid phase extraction (SPE) columns (United Technologies Corporations (UCT), Hartford, Connecticut, USA). Columns were washed with a 4:1 acetone chloroform solution, then ergovaline was eluted with methanol. Samples were dried under nitrogen then reconstituted in methanol before analysis via HPLC. For analysis of lolitrem B, ground seeds were extracted with a chloroform/methanol 2:1 (v/v) mixture. After centrifugation, the supernatant was recovered and evaporated. The samples were reconstituted in dichloromethane and purified using SPE columns (CUSIL, United Technologies Corporations (UCT), Hartford, Connecticut, USA). Columns were washed with 2.0 mL dichlormethane (DCM), followed by 0.5 mL 4:1 dichlormethane:acetonitrile (DCM:ACN) mixture. Samples were eluted with 3.0 mL of a 4:1 DCM:ACN solution then captured in an amber vial for HPLC analysis. HPLC analysis of ergovaline involved reversed-phase chromatography (Column: Perkin Elmer Brownlee, SPP C18 4.6 X 100 mm, 2.7 μ HPLC column (N9308416) or Phenomenex Kinetex C18 HPLC column; 100 Å, 4.6 X 100 mm, 2.6 μ HPLC column (00D-4426-E0)) at a flow rate of 1.8 ml/min with fluorescence detection at excitation and emission wavelengths of 250 and 420 nm, respectively. The gradient program used 35% acetonitrile and 2 mM ammonium carbonate in water as mobile phase A and acetonitrile as mobile phase B, and a linear gradient running from 99% A to 35% A from 1.0 to 1.6 min. For lolitrem B analysis, the HPLC protocol used normal phase separation (Agilent Zorbax RX-SIL column 4.6 X 250 mm, 5 μ (880975-901); guard column Security Guard column, Silica packing, Upchurch (C-135B)) using dichlormethane/acetonitrile/water (4:1:0.02, (v/v/v) as mobile phase and run at 0.5 ml/min for 15 min with fluorescence detection using excitation and emission wavelengths of 268 and 440 nm, respectively. To generate reference material for use in the calibration curve, ground seed was mixed in large batches at four target concentrations, which was validated using 98% pure ergovaline or purified lolitrem B, respectively. For both ergovaline and lolitrem B the limit of quantitation (LOQ) in plant samples was 100 ppb (0.1 μg/g).

IV.3. Results

In the 24 commercially available seed mixtures included in this study, the vertebrate toxic endophyte alkaloids ergovaline and lolitrem B were quantified by two different laboratories. In addition, paxilline, a vertebrate toxic precursor of lolitrem B, and the insect toxic alkaloid peramine were quantified by one laboratory (Table IV2). Four of the seed mixtures (S_10, S_24, S_32, S_33) were found to contain ergovaline and lolitrem B and therefore must have contained *Epichloë* infected seeds. The highest levels of lolitrem B were detected by both laboratories in S_10, a turfgrass seed mixture consisting of three different varieties of perennial ryegrass (Table IV1). Paxilline, a precursor of lolitrem B, was also observed in this seed mixture (Table IV2). The seed mixtures S_24, S_32 and S_33 all contained different varieties of perennial ryegrass and some additional cool season grasses, but no tall fescue varieties (Table IV1). Seed mixture S_33 is also a turfgrass seed mixture, but S_24 and S_32 are forage grass seed mixtures purposed for horse pastures. The insect deterring alkaloid, peramine, was detected in levels over 1000 ppb in S_10, S_24 and S_32 and at lower levels but higher than 10 ppb in S_33, S_13 and S_16 (Table IV2).

Detection for the presence of *Epichloë* using PCR identified six seed mixtures (S_10, S_13, S_16, S_24, S_30, S_33) with at least one infected E+ seed (Table IV2). In the 18 seed mixtures with no E+ infected seeds, neither ergovaline nor lolitrem B could be detected by both laboratories with the exception of S_32, which contained ergovaline, lolitrem B and peramine. Three of the E+ seed mixtures (S_10, S_24, S_33) were those for which also high levels of ergovaline and lolitrem B could be detected. Two further seed mixtures (S_13, S_16) contained a few seeds with *Epichloë* infections but did not clearly produce vertebrate toxic compounds and only low levels of peramine were detected, whereas in seed mixture S_30 no alkaloids could be detected at all. The PCR banding pattern for the single E+ seed in S_30 however suggests that the E+ seed is likely the result of a meadow fescue (*Festuca pratensis*) sample (Figure SIV1).

Epichloë festucae var. lolii that associates with perennial ryegrass is known to produce the alkaloids peramine, lolitrem B and ergovaline, but each specific

endophyte strain, may vary with the ability to produce some, or all of these alkaloids. Interestingly, our results show no evidence for *Epichloë* infected tall fescue, as none of the seed mixtures containing tall fescue varieties (S_14, S_16, S_17, 187 S_25, S_29) were shown to contain endophyte infected seeds or to produce ergovaline, the main vertebrate toxic compound of tall fescue – *Epichloë* associations (Tables IV1, IV2).

Table IV2: Endophyte detection in the Noble Research Institute and quantification of alkaloids at the University of Würzburg and the Oregon State University. Bold numbers/letters indicate that sample was infected/alkaloids were detected. University of Würzburg: one replicate (around 50 mg) of each ground seed mixture was analyzed. Oregon State University: two replicates were analyzed with the exception of samples indicated with # with only one replicate. nd = not detected; - not analyzed

	Endophyte detection Noble Research Institute					**Alkaloid detection [ppb]**					
						University of Würzburg				**Oregon State University**	
	First screen		**Second screen**		**Total [%]**	**Ergovaline**	**Lolitrem B**	**Paxilline**	**Peramine**	**Ergovaline**	**Lolitrem B**
ID	**#Seeds**	**E+**	**#Seeds**	**E+**							
S_10	**24**	**3**	24	0	**6.3**	**435**	**851**	**1540**	**1899**	**844**	**1688**
S_11	24	0	24	0		nd	nd	nd	nd	<100	<100#
S_12	24	0	24	0		nd	nd	nd	nd	<100	-
S_13	**22**	**1**	24	0	**2.2**	nd	nd	nd	**10**	<100	<100
S_14	24	0	24	0		nd	nd	nd	**<10**	<100	<100
S_15	24	0	24	0		nd	nd	nd	nd	<100	<100
S_16	**24**	**2**	**24**	**1**	**6.3**	nd	nd	nd	**65**	<100	**104**
S_17	22	0	22	0		nd	nd	nd	**<10**	<100	<100
S_18	24	0	24	0		nd	nd	nd	nd	<100	<100
S_19	24	0	24	0		nd	nd	nd	nd	<100	<100
S_20	24	0	24	0		nd	nd	nd	nd	<100	<100
S_21	22	0	22	0		nd	nd	nd	nd	<100	<100
S_22	24	0	24	0		nd	nd	nd	nd	<100	<100
S_23	24	0	24	0		nd	nd	nd	nd	<100	-
S_24	**24**	**3**	24	0	**6.3**	**709**	**240**	nd	**2286**	**425**	**991**
S_25	22	0	22	0		nd	nd	nd	nd	<100	<100#
S_26	24	0	24	0		nd	nd	nd	nd	<100	<100
S_27	24	0	24	0		nd	nd	nd	nd	<100	<100
S_28	24	0	24	0		nd	nd	nd	nd	<100	-
S_29	22	0	22	0		nd	nd	nd	nd	<100	<100
S_30	24	0	**24**	**1**	**2.1**	nd	nd	nd	nd	<100	<100#
S_31	24	0	24	0		nd	nd	nd	nd	<100	<100
S_32	24	0	24	0		**335**	**494**	nd	**1507**	**287**	**725**
S_33	**22**	**2**	**22**	**2**	**8.7**	**7**	nd	nd	**40**	**166**	**372**

IV.4. Discussion

We found with analyses of three independent laboratories that four out of 24 commercially available forage grass or turfgrass seed mixtures contained *Epichloë* endophytes, and could produce the vertebrate toxic alkaloids, ergovaline and lolitrem B. This result is especially surprising for the forage grass seed mixtures (S_24, S_32), as these are used to establish pastures that feed livestock, including sensitive animals such as horses, juveniles or diseased grazers that are more sensitive to some alkaloids (Craig et al., 2014). However, as our study only evaluated the endophyte infection and alkaloid content in the seed mixtures, we cannot conclude the potential alkaloid concentrations and possible toxicity of the pasture after seeding the infected mixtures. Assuming the endophytes are viable at sowing, endophyte-infected plants would be introduced into the environment. *Epichloë* infected agricultural grass species have a selective advantage in hot and dry environmental conditions (Bourguignon et al., 2015; Ju et al., 2006; Rudgers and Swafford, 2009) and under stressed conditions such as herbivory (Bastias et al., 2017; Dupont et al., 2015; Schardl, 1996). Climate change could result in environmental conditions that are more conducive to survival of infected grasses, which may increase their distribution to a level that endophyte-infected plants could dominate grasslands. Previous studies on grasslands in Germany and Spain showed that single individuals of cool season grass species infected with *Epichloë* can contain alkaloid concentrations above the toxicity thresholds for livestock (König et al., 2018; Soto-Barajas et al., 2019; Vikuk et al., 2019), which for ergovaline are 300-500 ppb for cattle and horses, and 500-800 ppb for sheep, with lolitrem B concentrations toxic at 1800-2000 ppb (Craig et al., 2014). In typical meadows, the infected toxic grasses are diluted by a high diversity of other species and low infection frequencies, so have been of little concern for causing toxicity. But caution should prevail as our results suggest that the distribution of *Epichloë* infected grasses within Europe could be altered due to planting seed mixtures, and that may inadvertently increase cases of animal toxicity.

The evaluation of the seed mixtures by PCR indicated that the *Epichloë* infected seed mixtures identified in this study did not contain tall fescue, as the PCR markers indicated that *Epichloë* species present was most likely in the perennial

ryegrass *L. perenne* or possibly in one case the *Festuca rubra* samples (S_32). Interestingly, the alkaloid biosynthesis marker profile of the seed mixtures analyzed here differed from that of endophyte-infected perennial ryegrass previously sampled on grasslands throughout Germany. The German perennial ryegrass samples typically lacked the marker for the *dmaW* gene and was not able to produce ergovaline (Vikuk et al., 2019), whereas seeds in the grass mixtures S_10, S_24, S_32 and S_33 contained this alkaloid. From this we conclude that the *Epichloë* infected perennial ryegrass seeds in the seed mixtures are likely not from grass native in Germany, but from grass varieties infected with other *Epichloë* isolates that are not safe for vertebrates. The same supplier that produces S_33 also produces the seed mixtures S_13 and S_16 for which 2.2% and 6.3% E+ seeds were detected, respectively, and very low levels of alkaloids. These results could either indicate low infections with vertebrate friendly *Epichloë* or some seed contamination in the production process, which was not frequent enough to clearly enhance alkaloid concentrations. As these are also turfgrass seed mixtures it seems implausible that they would lead to intoxication of livestock. However, S_16 is intended for seeding riding arenas and might therefore be grazed by horses, and the *Epichloë* infected grass might spread to horse pastures. We would therefore consider the endophyte presence in this seed mix could be a weight of concern for horse keepers. For the horse pasture seed mixture S_32 no E+ seeds could be identified, but ergovaline and lolitrem B were detected by both laboratories, as well as high levels of peramine. Interestingly, seed mixture S_28 from the same supplier also intended for horse pastures contains the same varieties of perennial ryegrass, but neither E+ seeds nor alkaloids were detected here (Tables IV1, IV2). Therefore, the potential source of seeds infected with *Epichloë* in the S_32 mixture could also be *Festuca rubra*. Infection rates show high variablity (44-92%) in *Festuca rubra* from Spain (Zabalgogeazcoa et al., 1999). But we have no information on the infection variability that might be seen in different *Festuca rubra* lines used in commercial seed lines. Ergot alkaloids and paxilline have been detected in *Festuca rubra* seeds, but at much lower concentrations than seen in *L. perenne* samples (Bauer et al., 2018).

The data on alkaloid concentrations provided here were determined analyzing the seed mixtures. The actual content of fungal alkaloids in infected *Epichloë* grasses is known to depend on environmental conditions and to be

different in the different plant parts (König et al., 2018). In addition, alkaloids were detectable in lower concentrations in the grasses grown from infected seeds than in the seeds themselves (Bauer et al., 2018). As *Epichloë* endophyte hyphae accumulate in the developing seeds once the grass enters its reproductive phase, the alkaloid content in seeds in September/October is especially relevant when grass seed straw or hay is used for feeding animals (Pirelli et al., 2016). So while the actual alkaloid concentrations in grasses developing from *Epichloë* infected seed mixtures depend on different factors and may be lower than in the seeds, these grasses contain asexually transmitted *Epichloë sp.* that have the potential to produce considerable levels of vertebrate toxic alkaloids.

In conclusion, our results demonstrate that commercially available turfgrass and forage grass seed mixtures can contain *Epichloë* infected perennial ryegrass varieties producing vertebrate and insect toxic alkaloids. We showed that endophyte infection of commercially available seed mixtures in Europe could have the potential to cause livestock toxicity, especially if left unmonitored. We therefore suggest a number of improvement measures to reduce risks of intoxication of livestock on European pastures due to *Epichloë* infected seed material:

(1) When establishing pastures for grazing animals we suggest avoiding *Epichloë* infected seed mixtures, especially with regard to seed mixtures containing perennial ryegrass varieties.

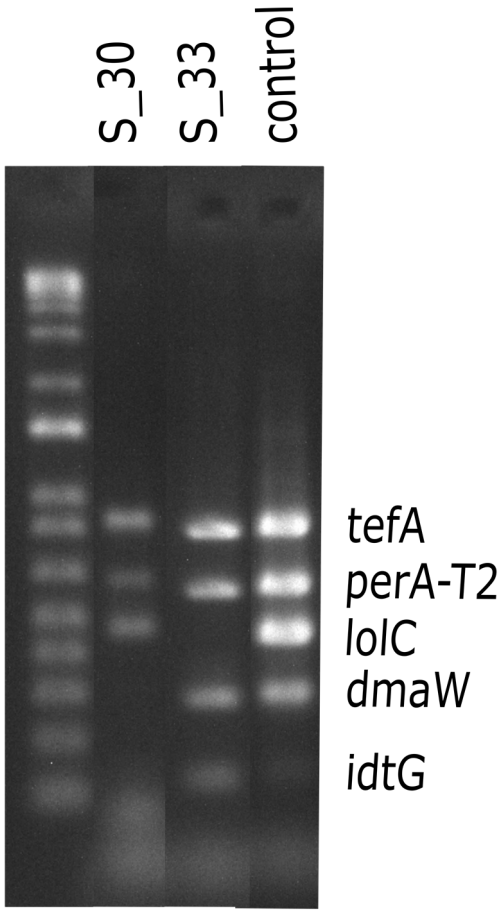
(2) Seed companies could conduct regular tests on *Epichloë* infections of the breeding and seed material and provide detailed information on the exact composition and *Epichloë* infection of the seed mixtures to consumers.

(3) The use of *Epichloë* strains incapable of producing the vertebrate toxic compounds lolitrem B and ergovaline could be utilized in Eureopean perennial ryegrass breeding, with simultaneous testing the risk of toxicosis.

(4) Finally, we like to call attention in the EU and other European states to promote research on the neglected risks of intoxications by *Epichloë* infected host grasses on European grasslands.

IV.5. Supplemental Material

Figure SIV1: A composite image representing examples of endophyte infected samples, S_30 and S_33, as tested by a multiplex PCR reaction. S_30: banding pattern characteristic for *Epichloë uncinata* in *Festuca pratensis*, S_33: banding pattern characteristic for *Epichloë festucae* var. *lolii* in *Lolium perenne*. PCR marker on the right is the 1 kb+ ladder (Thermo Fisher Scientific). On the left the PCR marker fragments for each gene are indicated, tefA = a conserved gene encoding the translation elongation factor 1-alpha; perA-T2 = a marker to the perA gene encoding peramine synthetase second thiolation domain, lolC = a loline gene marker, dmaW = an ergot alkaloid gene marker, idtG = indole-diterpene gene marker.



IV.6. Erratum Krauss, J. et al. *Epichloë* Endophyte Infection rates and Alkaloid Content in Commercially Available Grass Seed Mixtures in Europe

After the publication of the manuscript, we recognized that there is a discrepancy in Table V1 in five seed mixture compositions (S_10, S_13, S_17, S_29 and S_33), due to differences between the information in the catalog and the actual product labels. For this doctoral thesis I provide a corrected version of the manuscript. I changed the seed compositions and two suppliers in Table V1 in this doctoral thesis and deleted two sentences from the discussion, which were related to these changes. In the following I provide the text of the Erratum, which describes the changes in more detail:

The authors wish to make the following corrections to their paper (Krauss et al., 2020): The authors point out that seed mixture S_32 contained no perennial ryegrass variety NEW ORLEANS and this variety was not tested.

In the above-mentioned paper, page 4, Table 1 grass seed mixture S_33 the varieties NEW ORLEANS and MERCITWO should be replaced by ZURICH and CSI CORSICA. Additionally, the sentence in the discussion on page 8 should be deleted "The turfgrass seed mixture S_33 contains 5% of the perennial ryegrass variety NEW ORLEANS, which is listed as a top American breeding variety and could be the source of *Epichloë* infected seeds. The low percentage of this variety in the seed mixture could result in sampling variation, which may explain why 8.7% of E+ seeds were detected, and why the alkaloid quantitation results differed between the two laboratories." Furthermore, in S_10 BARMINTON should be replaced by BARLIBRO, BAROMARIO and BARLICUM. In S_13 CLEOPATRA, DICHENS and SIRTAKY/SHORTY should be deleted. For S_17 DISCUS should be replaced by ASTONHOCKEY and for S_29 DOUBLE should be replaced by TURFGOLD. For S_10 we added Barenbrug as supplier and for S_18 Tystofte-Fonden. The authors would like to apologize for any inconvenience caused to the readers by these changes.

The seed samples were taken from commercially available seed mixtures and the information in the catalog differed from the package labels. Furthermore,

the selling company and the producer of the seed mixtures were often not clearly labelled. Contaminations with *Epichloë* infected seeds can occur on several levels (breeders, producer of seed cultivars, producer of the seed mixture, trader, shop/market). Hence, it is not possible to determine where the contamination occurred, and no one can be blamed subsequently for contaminations.

Chapter V

General Discussion

Grassland in Schorfheide-Chorin, Germany, Photo: © Veronika Vikuk

Chapter V

General Discussion

Epichloë endophytes in cool season grass species can cause intoxication events in grazing animals due to their production of toxic alkaloids. Accordingly, agriculture is influenced by *Epichloë* endophytes, causing financial losses due to intoxication events. Mass intoxication events are not reported in Europe so far, but this might change in the future due to increasing temperatures or loss of biodiversity in grasslands resulting in species poor grasslands. Additionally, seeding of contaminated grass seed mixtures could contribute to the spreading of *Epichloë* endophytes. In my doctoral thesis, I firstly investigated *Epichloë* diversity in German grasslands to show which grass species are infected and to analyze if symbiotic *Epichloë* species on these grasslands are able to produce toxic alkaloids (Chapter II). Then, I focused on intoxication risks for grazing livestock in German grasslands with the example of *L. perenne* infected with *E. festucae* var. *lolii* and its dependency on the analytical detection method (Chapter III). Finally, I investigated grass seed mixtures for infections with *Epichloë* endophytes and alkaloids, because they might be a source of infections in managed grasslands (Chapter IV).

V.I. *Epichloë* endophytes in German grasslands

In my doctoral thesis, I gave an overview on infection rates with *Epichloë* endophytes in different grass species in Germany (Chapter II) and identified the corresponding symbiotic *Epichloë* species. I investigated which alkaloid pathway genes were present (genotype) and which alkaloids they produced (chemotype). Five out of 13 grass species showed infections with *Epichloë* endophytes (*Lolium perenne*, *Festuca pratensis*, *Festuca ovina*, *Dactylis glomerata* and *Festuca rubra*). I investigated *Epichloë* species in agriculturally used grass species like *L. perenne* and *F. pratensis*, which produced different vertebrate or insect toxic alkaloids. Additionally, I investigated native grass species, like *Holcus lanatus* or *Agrostis stolonifera*, which were not infected. *Festuca ovina*, another native grass, showed infections and the symbiotic *Epichloë* endophyte showed varying genotypes and alkaloids. Infection rates and alkaloid concentrations vary in native grasses and alkaloid concentrations tend to be lower than in agronomic grasses or are even not produced. (Faeth and Fagan, 2002; Leuchtmann et al., 2000; Saikkonen et al., 1998). Furthermore, stroma forming *Epichloë* endophytes, like *E. typhina* in *D. glomerata*, tend to be alkaloid free, whereas seed transmitted *Epichloë* endophytes, like *E. uncinata* in *F. pratensis*, tend to contain high concentrations of lolines (Leuchtmann et al., 2000). This can result in a higher herbivore resistance, thus alkaloid producing individuals might be favoured in seed transmitted *Epichloë* endophytes (Leuchtmann et al., 2000). Faeth and Fagan (2002) claimed that variation in alkaloid types and concentrations in native grasses might be linked to more variable environments, increased genetic heterogeneity of endophytes and grasses and the interaction between environment and variable genotypes. A varying environment is characteristic in real-world grassland systems like the Biodiversity Exploratories, where I conducted my study. Hence, genotypic variability, as I showed in *E. festucae* in *F. ovina* and genetic differences, such as the loss of ergotalkaloid pathway starting gene *dmaW* in *E. festucae* var. *lolii* in *L. perenne*, or a missing peramine reductase domain in *E. typhina* in *D. glomerata*, might be explained by varying environmental or geographic conditions. Alkaloid profiles in *Epichloë festucae* colonizing *Festuca rubra* from wild populations differed among geographic origins in a previously published study (Vázquez de Aldana et al., 2020). The authors collected *Festuca rubra* plants from wild populations in North and South

Finnland, Spain and the Faroer islands and transplanted them to each of the study regions (Vázquez de Aldana et al., 2020). Peramine content in the plants did not vary, whereas ergovaline content varied between the transplanting sites, which the authors explained with phenotypic plasticity (Vázquez de Aldana et al., 2020).

V.II. Influence of different alkaloid detection methods on alkaloid concentrations

Agricultural research often focuses on the benefits of the *Epichloë* grass symbioses, like drought resistance, insect protection and increased biomass (Hume et al., 2016; Johnson et al., 2013). Therefore, vertebrate safe *Epichloë* endophytes are developed in order to use the benefits of the fungi for agriculture (Johnson et al., 2013). However, public interest in *Epichloë* endophytes often focuses on intoxication risks of grazing animals due to alkaloids produced by *Epichloë* endophytes infecting cool season grass species. Famers are particularly worried about economic losses as a result of intoxication events (Hume et al., 2016). It is therefore necessary to detect infection rates but also alkaloid concentrations to evaluate this risk. Alkaloid concentrations are compared with toxicity thresholds from literature to estimate if concentrations are toxic for vertebrates or invertebrates. However, analytical methods to detect and quantify alkaloid concentrations differ between studies. Thus, it is not clear, if detected concentrations are always comparable with each other or with toxicity thresholds from literature. I showed that alkaloid concentrations differed, depending on the usage of fresh or dried plant weight, but also depending on different UPLC detection methods (Chapter III). I repeated a previous study (König et al., 2018) to compare the influence of land-use intensity or season on the alkaloid concentration detected with fresh or dry plant weight. I showed that land-use intensity did not influence *Epichloë* infections or alkaloid concentrations, regardless of whether fresh or dry plant weight was used. Furthermore, also the general trend of increased alkaloid concentrations in summer was constant regardless of whether fresh or dry plant weight was used. However, I showed in a common garden experiment that in direct comparison alkaloid concentrations measured with dry plant weight are

approx. three times higher than with fresh plant weight due to the missing water content in the plant. Nevertheless, in the field on real-world grasslands alkaloid concentrations on population level did not exceed toxicity thresholds, regardless of whether it was measured with fresh or dry plant weight. This shows that more controlled experiments, like potted plants in a common garden, are helpful to understand single traits, but under real-world conditions in the field these differences might decrease, because other factors, like increasing temperature or drought might be more important (Bourguignon et al., 2015; Hennessy et al., 2016; McCulley et al., 2014). An advantage of the common garden experiment compared to the field experiments in the Biodiversity Exploratories, is the better controllability in the common garden. Plants can be kept in their pots, separated from surrounding soil and from other individuals. The soil in the pots and the plant cultivar are similar, which increases the comparability. Another benefit is the availability of plant material, which can be used for experiments. In field studies plant material can be limited due to climatic factors like drought, but also due to mowing or grazing events. However, a common garden experiment can not consider all factors occuring in field conditions, which might be relevant for farmers or animals. Mace et al. (2014) showed that ergot alkaloid expression in *L. perenne* infected with *E. festucae* var. *lolii* differed significantly between tillers of a single plant, comparable to the level or even exceeding the level of variation between individual plants in a population (Mace et al., 2014). Hence, sampling individual tillers, instead of the whole plant, as I did in my field studies, might lead to a differing ergovaline concentration due to varying gene expression. Additionally, ergovaline concentrations vary during the year in *L. perenne* (Repussard et al., 2014b), but also in *F. arundinacea* plants infected with *Epichloë* spp. (Dillard et al., 2019), which I also showed in my common garden experiment. Hence, this should be considered for measurements of ergovaline concentrations in the field, if only one point in time is considered.

I used ergotamine as internal standard for the quantitation of ergovaline due to its structural similarity and its availability. Ergotamine is another ergotalkaloid which is, besides ergocristine and ergosine, typically produced by *Claviceps purpurea* (Aboling et al., 2016; Porter et al., 1987), whereas the production of ergovaline is characteristic for *Epichloë* (Shelby et al., 1997). Although a recent publication states

that ergotamine might be produced as well by *E. coenopialum* in *F. arundinacea* and *E. festucae* var. *lolii* in *L. perenne* (Song et al., 2020), the cited literature in this review (Song et al., 2020) does not support this statement (Shelby and Flieger, 1997; Shelby et al., 1997; Spiering et al., 2002). Shelby et al. (1997) showed that ergotamine was not produced by *E. coenophialum* in *F. arundinacea*, therefore it is often used as internal standard to quantify ergovaline (Fuchs et al., 2013; König et al., 2018; Rottinghaus et al., 1991; Shelby and Flieger, 1997; Spiering et al., 2002). *Claviceps purpurea* can also infect *F. arundinacea* (Porter et al., 1987), hence, detections of ergotamine might be caused by *Claviceps* contamination (Aboling et al., 2016; Shelby et al., 1997).

Additionally, I showed that different analytical detection methods can lead to different total amounts of alkaloid concentrations (Chapter III). The alkaloid concentrations detected with method 1 and method 2 were contradictory. Concentrations detected with method 2 were higher for peramine and lolitrem B, but lower for ergovaline compared to method 1. The limit of detection cannot explain the differences, because it is similar for both methods. Both methods used the same column materials and solutions, hence the efficiency of ionization should be comparable. Nevertheless, the longer column in method 1 and the differing gradient changed the solvent composition slightly, which can lead to different ionization efficiencies and other matrix effects, resulting in different alkaloid concentrations. Ergovaline used as reference substance was only available for method 1 and not for method 2. Hence, we assume that validation of ergovaline measurements might be more reliable in method 1. The availability of lolitrem B is very restricted, because it is not commercially available and only a few laboratories produce the substance and provide it in small amounts to other laboratories. Due to the limited amount of lolitrem B the validation of both methods, 1 and 2, was limited and I had to use a structurally different substance as internal standard (homoperamine) to quantify lolitrem B concentrations. Hence, differences in quantitation due to reference to a structurally different internal standard are possible. Thus, the partially restricted availability of reference substances complicates the quantitation of *Epichloë* alkaloids and leads to methodological differences in alkaloid concentrations. Nevertheless, it should be seeked for a consistent method for alkaloid quantitation in all laboratories, so alkaloid

concentrations between research groups can be compared more easily. By then, alkaloid concentrations should be stated as relative numbers, not absolute numbers.

Finally, the usage of dried plant weight should be favoured against fresh plant weight, because measurements are not diluted by the varying amount of plant water. König et al. (2018) and Fuchs et al. (2017) used fresh plant weight instead of dry plant weight. I showed that the trend of their results stayed the same, regardless of whether fresh or dry plant weight was used, but total amount of alkaloid concentrations were around three times higher if dry plant weight was used. Hence, the trends they showed were correct, but the comparison with toxicity thresholds not. But in order to estimate toxicity thresholds, it is necessary to classify, whether concentrations are already toxic for animals or not.

V.III. The role of grass seed mixtures

Grass seed mixtures can be a source of *Epichloë* infections in managed grasslands, when they are contaminated with *Epichloë* species, which produce vertebrate toxic alkaloids. Asexual *Epichloë* species are dispersed via grass seeds and it is known, that they tend to produce toxic alkaloids due to their host dependence, whereas sexual forms are not dependend on the host in their dispersal and tend to produce less toxic alkaloids (Leuchtmann et al., 2000). In six out of 24 commercially available seed mixtures, we detected *Epichloë* infections. In four seed mixtures, we detected alkaloids from *Epichloë* endophytes and three of these four were positive in the PCR detection. Two of these seed mixtures are sold as forage grass mixtures and two as turf grass mixtures (Chapter IV). A distinction is made between forage grasses, which are meant for feeding animals (Bundessortenamt, 2018) and turf grasses, which are bred for traits useful in lawns under varying management intensities like airports, parks or sport fields (Bundessortenamt, 2019). The two infected forage grass mixtures contained three different *L. perenne* varieties (S_24: Bellevue, Boyne, Stefani; S_32: Marava, Bokser, Wadi). In each of the seed mixtures one of the *L. perenne* varieties was bred as turf grass (S_24: Bellevue; S_32: Bokser), whereas the other two were bred as forage grass (S_24: Boyne, Stefani; S_32: Marava, Wadi) (Bundessortenamt, 2018, 2019). Turf grass varieties are not produced to feed

animals, but might be used due to the positive traits like drought resistance or insect herbivore resistance (Johnson et al., 2013). I have no indication, if any of the seed cultivars or contaminations at any of the producing steps of seed mixtures could be responsible for the *Epichloë* detection in our study. *Epichloë* infections in grass breeding programs are mostly ignored in Europe, although it has been detected in European cultivars (Saari et al., 2009; Saikkonen, 2000) and *Epichloë* endophytes have their origin in Europe (Kauppinen et al., 2016). It is possible that seed mixtures from unknown origin might be infected with vertebrate toxic *Epichloë* endophytes and seeding these seeds could contribute to the distribution of *Epichloë* infections in German/European grasslands and thus also to the increase of intoxication risk for grazing livestock. Hence, a routine testing for *Epichloë* infections in seeds should be mandatory for all seed breeders, to minimize the distribution of *Epichloë* infections with commercial seed mixtures. *Epichloë* infections occur naturally in European grasslands and do not pose a danger of intoxication itself. But an introduction with artificially *Epichloë* infected grass seeds can disturb the ecosystem balance and may lead to intoxication problems due to a selective advantage for grasses with *Epichloë* endophytes (Hume et al., 2020).

Alkaloid concentrations in seeds can be higher than alkaloid concentrations in the grown plant (Bauer et al., 2018), but might be a hint for infections with alkaloid producing *Epichloë* endophytes. Additionally, asexual, seed transmitted *Epichloë* infected grass seeds contain a high amount of *Epichloë* endophyte hyphae, which accumulates in the seeds once the grass enters its reproductive phase (Pirelli et al., 2016). Hence, not only seeds, but also seed containing straw or hay should be controlled for alkaloid concentrations. There is also evidence, that *Epichloë festucae* var. *lolii* survives in *Lolium perenne* seeds for 17 years, if the seeds are stored in the right conditions (<8% humidity, -5°C) (Thünen et al., 2018). Therefore, viable *Epichloë* infections are also possible in older seeds and infection rates need to be tested.

V.IV. Intoxication risks in Germany

It is necessary to detect *Epichloë* infection rates, but also alkaloid concentrations in order to evaluate intoxication risks for grazing animals with alkaloids from *Epichloë* infected grasses in grasslands. I showed that the two grass species, whose infections with *Epichloë* are responsible for mass intoxication events in New Zealand, Australia (*L. perenne*) and the USA (*F. arundinacea*) showed relatively low (*L. perenne*, 15 %) or no infections (*F. arundinacea*) in my study in Germany (Chapter II). Additionally, I showed that *E. festucae* var. *lolii* in *L. perenne* in Germany is lacking the starting gene for ergotalkaloid pathway *dmaW* (Chapter II). Hence, no vertebrate toxic ergovaline was detected. But *E. festucae* var. *lolii* was also able to produce the vertebrate toxic lolitrem B, which is responsible for ryegrass staggers in New Zealand or Australia. However, I showed, that lolitrem B concentrations on population level in all three regions in Germany did not exceed toxicity threshold, although single individuals showed lolitrem B concentrations above toxicity threshold (Chapter III). Alkaloid concentrations on five selected study sites increased in summer but did also not exceed toxicity threshold for lolitrem B on population level. However, in a monoculture-like environment with only *Epichloë* infected *L. perenne* plants, lolitrem B concentrations could exceed the toxicity threshold in September. Hence, monocultures should be avoided to minimize intoxication risks in German grasslands.

I showed that alkaloid concentrations, representing intoxication risk, were higher in late summer compared to spring, also shown in other studies before (Fuchs et al., 2017a; Jensen, 2005; König et al., 2018; Repussard et al., 2014b). In addition to maintaining diverse grasslands, monitoring for *Epichloë* infections and alkaloids in late summer might be useful. Furthermore, seeding of grass seed mixtures might be a potential source of *Epichloë* infected grasses and should therefore be monitored for infections as well. Similarly, the seeding of turf grass should be avoided on pastures (Chapter IV).

Intoxication risks for grazing cattle or horses is of financial interest for farmers, thus it reaches the most public interest. However, *Epichloë* spp. can also produce insect deterring (peramine), or insect toxic alkaloids (lolines) (Schardl et

al., 2013a). I showed that peramine concentrations exceeded toxicity threshold in my field study from July until November in a monoculture like environment with only *Epichloë* infected *L. perenne* plants and in September also on population level. In my common garden experiment peramine concentrations exceeded toxicitity threshold almost the whole year, regardless of whether fresh or dry plant weight was used. Insect-deterring or insect-toxic properties are seen as an advantage in agriculture (Johnson et al., 2013). Insect-deterring properties of peramine produced by *E. festucae* var. *lolii* in *L. perenne* for example are used in New Zealand to protect *L. perenne* plants from the Argentine stem weevil (Johnson et al., 2013). Hence, these properties might also be useful on German grasslands. However, German grasslands are more divers, hence, spreading of insect pests might be reduced, comparing it with the monoculture grasslands in New Zealand. The main insect grass pests in Germany are insects from the order Orthoptera, which have their peak of abundance in late summer (Köhler et al., 1999), which is synchronized with the peak of peramine concentration in my field and common garden study. It was shown that herbivores induce the production of herbivore specific alkaloids depending on their way of eating (Fuchs et al., 2017b). Hence, *L. perenne* infected with *E. festucae* var. *lolii* seems to be well adapted to the occurrence of insect herbivores, which shows the co-evolution of host and endophyte (Hume et al., 2020; Schardl et al., 2008).

Apart from the intoxication risks, there are opinions stating that the potential of *Epichloë* endophytes should be used for a sustainable agriculture in Europe (Kauppinen et al., 2016). Europe is a biodiversity hotspot for grasses and *Epichloë* endophytes, which could be used for breeding new forage grasses worldwide (Kauppinen et al., 2016). The idea makes sense, since grasslands represent 70 % of the worlds´ agricultural area, which are endangered by climate change and synthetic pesticides (Kauppinen et al., 2016). Microbial symbionts which improve plant resistance and plant fitness might be a more sustainable agriculture solution (Kauppinen et al., 2016). The usage of divers, species rich pastures in Europe, can be helpful to avoid intoxication risks due to *Epichloë* infections and simultaneously the benefits of *Epichloë* endophytes can be used. Additionally, it might be a benefit that *Epichloë* -grass associations are native in Europe and the disturbance of the native ecosystems might be lower than in foreign

ecosystems. Hence, *Epichloë* endophytes might be also beneficial in European grasslands. Nevertheless, monitoring of infection rates and alkaloid concentrations should be conducted, to estimate possible intoxication risks correctly.

V.V. Conclusion

I showed, which *Epichloë*-grass symbioses are present on German grasslands and how they are characterized (genotypically and chemotypically) (Research question 1, Chapter II). In Chapter II, I also showed, that dry plant weight should be used to detect alkaloid concentrations correctly and a consistent method between laboratories should be favoured, so total amounts of alkaloid concentrations can be compared. However, the trends of seasonal changes and the influence of land-use intensity stayed the same, regardless of whether fresh or dry plant weight was used (Research question 2, Chapter III). Hence, intoxication risk is generally low in Germany. Nevertheless, it is important to draw attention to the topic of *Epichloë* endophytes in cool season grass species and to not underestimate their potential threats. Especially in the context of climate change it is possible that *Epichloë* infections spread in German grasses and intoxication risks might increase in the future. I also showed that two forage grass and two turf grass seed mixtures were infected with *Epichloë* endopyhtes and detected alkaloids. This could contribute to the spreading of *Epichloë* endopyhtes in Germany. Hence, seed mixtures should be tested for *Epichloë* infections (Research question 3, Chapter IV).

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*Correspondence author (contributed *c.* 70 - 80 % to this study).

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Study design and methodology	JK	CAY/VV	MK	
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Veronika Vikuk Prof. Carolyn Young, Ph.D. Stephen T. Lee, Ph.D.

Padmaja Nagabhyru, Ph.D. Dr. Markus Krischke Prof. Dr. Martin J. Mueller

Prof. Dr. Jochen Krauss

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* Correspondence author (contributed *c.* 70 - 80 % to this study).

Author Contributions:

Conceptualization, Jochen Krauss; Data curation, Veronika Vikuk and Selina Rueb; Formal analysis, Veronika Vikuk; Funding acquisition, Jochen Krauss; Investigation, Veronika Vikuk and Selina Rueb; Methodology, Veronika Vikuk, Benjamin Fuchs, Markus Krischke, Martin J. Mueller and Jochen Krauss; Project administration, Jochen Krauss; Resources, Martin J. Mueller and Jochen Krauss; Supervision, Jochen Krauss; Validation, Veronika Vikuk and Markus Krischke; Visualization, Veronika Vikuk; Writing – original draft, Veronika Vikuk; Writing – review & editing, Veronika Vikuk, Benjamin Fuchs, Markus Krischke, Martin J. Mueller, Selina Rueb and Jochen Krauss. All authors have read and agreed to the published version of the manuscript.

Participated in	Author Initials (Responsibility decreasing from left to right)			
Study design and methodology	JK	VV	BF/MK	
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*Correspondence author, # joint first authorship (contributed *c.* 40 – 50 % to this study).

Author Contributions:

Conceptualization, KB; data curation, JK, VV, CAY and KB; funding acquisition, KB, JK; investigation, VV, CAY, MK and MJM; project administration, KB; resources, JK, CAY, MJM, and KB; visualization, KB and VV; writing—original draft preparation, JK and VV; writing—review and editing, all authors.; All authors have read and agreed to the published version of the manuscript.

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Conceptualization	KB	JK	
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12/2017	**Vikuk, V.**, König, J., Krauss, J.: Infection frequencies and alkaloid content of systemic fungal grass endophytes in *L. perenne* along a land-use intensity gradient. Poster and one-minute-lightening talk in the agriculture session. *Ecology Across Borders conference* in Ghent, Belgium. prize for best one-minute-lightening talk.

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