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# Anti-herbivory defences delivered by *Epichloë* fungal endophytes: a quantitative review of alkaloid concentration variation among hosts and plant parts

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- **Background and Aims** In the subfamily Poöideae (Poaceae), certain grass species possess anti-herbivore alkaloids synthesized by fungal endophytes that belong to the genus *Epichloë* (Clavicipitaceae). The protective role of these symbiotic endophytes can vary, depending on alkaloid concentrations within specific plant–endophyte associations and plant parts.
- **Methods** We conducted a literature review to identify articles containing alkaloid concentration data for various plant parts in six important pasture species, *Lolium arundinaceum*, *Lolium perenne*, *Lolium pratense*, *Lolium multiflorum*|*Lolium rigidum* and *Festuca rubra*, associated with their common endophytes. We considered the alkaloids lolines (1-aminopyrrolizidines), peramine (pyrrolopyrazines), ergovaline (ergot alkaloids) and lolitrem B (indole-diterpenes). While all these alkaloids have shown bioactivity against insect herbivores, ergovaline and lolitrem B are harmful for mammals.
- **Key Results** Loline alkaloid levels were higher in the perennial grasses *L. pratense* and *L. arundinaceum* compared to the annual species *L. multiflorum* and *L. rigidum*, and higher in reproductive tissues than in vegetative structures. This is probably due to the greater biomass accumulation in perennial species that can result in higher endophyte mycelial biomass. Peramine concentrations were higher in *L. perenne* than in *L. arundinaceum* and not affected by plant part. This can be attributed to the high within-plant mobility of peramine. Ergovaline and lolitrem B, both hydrophobic compounds, were associated with plant parts where fungal mycelium is usually present, and their concentrations were higher in plant reproductive tissues. Only loline alkaloid data were sufficient for below-ground tissue analyses and concentrations were lower than in above-ground parts.
- **Conclusions** Our study provides a comprehensive synthesis of fungal alkaloid variation across host grasses and plant parts, essential for understanding the endophyte-conferred defence extent. The patterns can be understood by considering endophyte growth within the plant and alkaloid mobility. Our study identifies research gaps, including the limited documentation of alkaloid presence in roots and the need to investigate the influence of different environmental conditions.

**Key words:** Symbiosis, defensive mutualism, herbivory resistance, grass, secondary metabolites, plant–endophyte interaction, plant–herbivore interaction.

## INTRODUCTION

Plant phenotypes are shaped through evolution in response to selection pressures imposed by biotic and abiotic factors, and herbivory is one of the most relevant forces (Agrawal *et al.*, 2012). The selected defensive traits in plant phenotypes under herbivory may not necessarily be encoded in plant genomes but in their symbiotic microorganisms (Ellers *et al.*, 2012; Panaccione *et al.*, 2014). Certain grass species within the subfamily Poöideae (family Poaceae) are endowed with anti-herbivore alkaloids that are synthesized by fungal endophytes of the genus *Epichloë* (family Clavicipitaceae) (Schardl *et al.*, 2004; Leuchtmann *et al.*, 2014). Within the plant–*Epichloë* symbiosis, some are considered defensive mutualisms (Clay, 1988; Wilkinson *et al.*, 2000; Schardl *et al.*, 2012, 2013a; Panaccione *et al.*, 2014; Bastias *et al.*, 2017a). Still, the effectiveness of the endophyte-mediated defensive mechanism varies across plant–endophyte associations and shows a significant context-dependency (Saikkonen *et al.*, 2010; Ueno *et al.*, 2016; Bastias *et al.*, 2017b; Bastías and Gundel, 2023). This variation may be attributed, in part, to differences in alkaloid contents among plant species, and among plant organs or parts.

*Epichloë* species differ in ploidy levels, reproductive systems, transmission modes and symptoms on plants. These endophytes grow hyphae in the intercellular spaces of aerial plant parts closely associated with meristematic buds (Christensen *et al.*, 2008; Liu *et al.*, 2017). Most – but not all (e.g. *Epichloë festucae* var. *lolii* in *Lolium perenne*) – haploid species can reproduce sexually by developing fruiting bodies (stromata) that release meiotic spores as a mode of horizontal transmission, and cause abortion of host reproductive structures (choke disease) (e.g. *Epichloë typhina* in *L. perenne*) (Schardl *et al.*, 2013*a*, 2023; Tadych *et al.*, 2014). Alternatively, *Epichloë* species are interspecific hybrids of two or three haploid species which are, in consequence, diploid and triploid, respectively. These hybrid endophytes reproduce asexually by growing hyphae in developing seeds (vertical transmission), and cause no symptoms on plant hosts (e.g. *Epichloë coenophiala* in *Lolium arundinaceum*) (Gundel *et al.*, 2011; Liu *et al.*, 2017; Zhang *et al.*, 2017). Some haploid species of *Epichloë* combine both sexual and asexual reproduction systems, and vertical and horizontal transmission modes (e.g. *Epichloë festucae* in *Festuca rubra*) (Schardl, 2010). While there have been some exceptions reported (e.g. Mc Cargo *et al.*, 2014; Soto-Barajas *et al.*, 2019), these symbiotic associations are generally specific, with plant species commonly hosting one or a few fungal species (Schardl *et al.*, 2008; Leuchtmann *et al.*, 2014). The *Epichloë*-based plant protection against herbivores is largely attributable to alkaloids (Wilkinson *et al.*, 2000; Potter *et al.*, 2008), but it may also involve other endophyte factors, as well as the endophyte-mediated induction of the plant's immune defence system (Ambrose *et al.*, 2014; Bastias *et al.*, 2017a; Fuchs *et al.*, 2017a; Cibils-Stewart *et al.*, 2022). Though not as well elucidated at the mechanistic level, resistance to pathogens and tolerance to abiotic stress factors (e.g. drought, salinity, ozone) have also been ascribed to the symbiosis with *Epichloë* species (Nagabhyru *et al.*, 2013; Buckley *et al.*, 2019; Card *et al.*, 2021; Decunta *et al.*, 2021; Ueno *et al.*, 2021; Chen *et al.*, 2022; Bastías *et al.*, 2023).

Four major chemical classes of *Epichloë*-derived alkaloids have been described: (1) 1-aminopyrrolizidines include lolines that, depending on substituents at the C-1 amine, yield different loline alkaloids; (2) pyrrolopyrazines which include the insect feeding deterrent peramine; (3) ergot alkaloids which include ergovaline, a potent toxin in mammalian systems; and (4) indole-diterpenes which include the potent tremorgen lolitrem B (Berry *et al.*, 2019; Schardl *et al.*, 2023). Although other alkaloids have been identified within these major classes (Moore *et al.*, 2015; Finch *et al.*, 2020), we here focus on these four main alkaloids because they are well characterized genetically and functionally (Schardl *et al.*, 2012, 2013*b*, 2023). The loline alkaloids [*N*-formylloline (NFL), *N*-acetylloline (NAL), *N*-acetylnorloline (NANL) and *N*-methylloline (NML)] are well known to act as plant chemical defences against herbivorous insects and nematodes through both deterrent and insecticidal effects (Wilkinson *et al.*, 2000; Schardl *et al.*, 2007; Bacetty *et al.*, 2009). Peramine is also known for its antifeedant effects on insects (Rowan, 1993; Schardl *et al.*, 2012). Ergovaline and lolitrem B are mostly known for their toxic effects on mammals (Gallagher *et al.*, 1981; Young *et al.*, 2005; Guerre, 2015; Caradus *et al.*, 2022). The variation in alkaloid profiles among fungal strains has led to the development of a forage breeding strategy aimed at improving cultivars with endophytes that produce alkaloids with insecticidal effects but not those that are harmful to mammals (Bouton *et al.*, 2002; Gundel *et al.*, 2013). This practice has been mostly performed on the economically important grass species *L. arundinaceum* (syn. *Festuca arundinacea* = *Schedonorus arundinaceus*; common name: tall fescue) and *L. perenne* (perennial ryegrass) since the common endophytes in earlier cultivars produce ergovaline and/or lolitrem B (Johnson *et al.*, 2013). This is not necessary for other commercially used species such as *Lolium pratense* (syn. *Festuca pratensis*; meadow fescue) and the annual ryegrasses *Lolium multiflorum* and *Lolium rigidum* (hereafter *L. multiflorum*|*L. rigidum*), since their endophytes do not produce alkaloids toxic for mammals or, if they do, as in *F. rubra*, grasses are mainly used as turf (Pennell *et al.*, 2010; Bylin *et al.*, 2014; Moore *et al.*, 2015).

To gain a comprehensive understanding of the variation in the endophyte-mediated defence in host plants, we reviewed the literature looking for articles that have reported data on alkaloid concentration in six of the most significant temperate pasture and forage grass species: *L. arundinaceum*, *L. perenne*, *L. pratense*, *L. multiflorum*|*L. rigidum* and *F. rubra*, in association with their common endophytes only. The annual ryegrasses *L. multiflorum* and *L. rigidum* (*L. multiflorum*|*L. rigidum*) are considered together as they both host the same endophyte species (*Epichloë occultans*) (Moon *et al.*, 2000). Given that the effectiveness of alkaloids as defences against herbivores depends on their levels in the host plant organ when attacked, we were particularly interested in evaluating their variation among plant parts. Since alkaloid concentrations can be altered by either biotic or abiotic environmental conditions (e.g. herbivory, drought) (Bultman *et al.*, 2004; Nagabhyru *et al.*, 2013; Fuchs *et al.*, 2017*c*; Bubica Bustos *et al.*, 2022), we only included data from plants that grew in the absence of treatments (control condition). We analysed data of the most studied alkaloids (lolines, peramine, ergovaline, and lolitrem B) to address the following questions: (1) How does the concentration of the different alkaloids vary among plant species? (2) How do alkaloid concentrations vary among plant parts? Improving our understanding of the distribution and abundance of *Epichloë*-derived alkaloids within and among host plants contributes to predicting how endophytes mediate plant interactions with herbivores.

## MATERIALS AND METHODS

To survey the literature on the main fungal alkaloids produced by *Epichloë* species (fungal endophytes) in grasses, we conducted a search in the Scopus$^{TM}$ database (www.scopus.com) on 1 December, 2023. The search criterion was organized by combining the following key words and conditionals: *epichloë*, OR *acremonium*, OR *neotyphodium*, AND acetamidopyrrolizidine, OR chanoclavine, OR ergot, OR ergonovine, OR ergovaline, OR lolitrem, OR n-acetylnorloline, OR n-acetylloline, OR n-formylloline, OR paxilline, OR peramine, OR terpendoles, OR terpenes, OR lolines, OR indole-diterpenes. *Acremonium* was included because, in early works, it was thought to be the genus of the fungal endophytes that were later classified as *Epichloë*/*Neotyphodium*. *Neotyphodium* was included because it was until recently used to classify the anamorphs of *Epichloë* species, but today all members of this clade are within the genus *Epichloë* (Leuchtmann *et al.*, 2014). We also screened the reference list of the selected papers to identify other relevant publications that were not detected in the first search. A total of 588 articles were obtained and reviewed to assess their suitability. Another five studies were incorporated due to knowledge of the literature in the line of this work.

To be included in the analysis, studies had to inform measurements of alkaloid concentration in plant tissues of *L. arundinaceum*, *L. pratense*, *L. perenne*, *L. multiflorum*/*L. rigidum* or *F. rubra*. We limited our work to those plant species because they are among the most studied worldwide in relation to *Epichloë* endophytes (Semmartin *et al.*, 2015). The scientific names of the grasses used in the present study were determined by consulting the Plants of the World Online database (POWO, 2023). Because the fungal endophyte species in the studies were often unspecified, we regarded the plant species as being associated with those endophyte species and strains most commonly identified in the respective host species. In the literature these are denoted as 'common', 'wild-type' or 'standard' strains of *E. coenophiala* in *L. arundinaceum*, *E. uncinata* in *L. pratense*, *E. festucae* in *F. rubra*, *E. festucae* var. *lolii* in *L. perenne* and *E. occultans* in *L. multiflorum*/*L. rigidum* (Leuchtmann *et al.*, 2014). We focused solely on symbiotic associations at the species level, excluding studies that utilized artificial inoculation to establish non-natural symbioses for scientific purposes. Studies presenting values in units that could not be converted to μg g plant $DW^{-1}$ were discarded. When alkaloid data were presented in figures, they were extracted using the software Graph Grabber (Quintessa Ltd, 2022). Differences in alkaloid levels due to varied analytical methods contribute to the between-study variability. The final database used in the analysis included 623 studies from 104 publications (Table 1). We utilized data from experiments conducted either in pots or in the field. In the latter case, the extant plants could have been either seeded or natural. However, we ensured that the sampled tissue had been pure from the specified plant species and its common endophyte, and had not come from mixtures with different species or endophyte-free plants. In cases of manipulative experiments, we utilized data from control treatments or from endophyte-symbiotic plants that remained untreated.

We considered the alkaloids that are linked to the four main chemical classes: (1) lolines (1-aminopyrrolizidines), (2) peramine (pyrrolopyrazines), (3) ergovaline (ergot alkaloids) and (4) lolitrem B (indole-diterpenes) (Schardl *et al.*, 2023). The biosynthesis of these alkaloids is well characterized at the gene level, and it is likely that intermediate molecules in the pathways to the end products also exhibit bioactive effects on herbivores (Vikuk *et al.*, 2019). In light of this observation, our study focuses on the four thoroughly characterized alkaloids. For the analysis of lolines, we used the sum of the different derivatives (NANL, NFL, NAL) since, in most cases, they were evaluated and/or reported together, and all are responsible for the anti-herbivory effects (Riedell *et al.*, 1991; Jensen *et al.*, 2009). For the analysis of the ergot alkaloids, we found two alternatives, to analyse total ergot alkaloids and/or to analyse the alkaloid ergovaline. Although a substantial number of papers report total ergot alkaloids with no clarification on the specific alkaloids included, we preferred to work with ergovaline to gain precision and because it is well known to have effects on vertebrate and invertebrate herbivores (Potter *et al.*, 2008; Schardl *et al.*, 2023). Therefore, we report the results for ergovaline here. In the case of indole-diterpenes, we considered lolitrem B because it is associated with mammalian toxicosis from *Epichloë*-infected grasses and is the most commonly measured indole-diterpene in the grasses (Young *et al.*, 2009).

The data were separated by alkaloid class and alkaloid compound and organized into four categories: work code, host species, endophyte species and plant part (Supplementary Dataset ). The host species column includes the most common scientific names for each plant species based on POWO (2023). The fungal species column includes the scientific names of the endophyte species. Based on specialized literature (e.g. Leuchtmann *et al.*, 2014), we used the scientific name of the common endophyte associated with each plant species. The combination of plant and endophyte species determined the grass–*Epichloë* symbiosis factor. The plant parts in which alkaloids were measured were classified into four distinct categories. The first two categories were below-ground biomass (roots) and above-ground biomass. When possible this category was further divided into vegetative or reproductive. In cases where there was no indication of whether the structures were vegetative or reproductive, we categorized it simply as above-ground biomass. As vegetative structures, we considered tillers, pseudostems and leaves (sheath and blade), while in reproductive structures, we included panicles (or spikes) or seeds. If explicitly indicated, leaves could have been associated with reproductive structures if they were part of a reproductive tiller (as in Ball *et al.*, 1997*a*). Therefore, our classification does not necessarily mean planta phenological stage. Alkaloid data reported for different species, populations or ecotypes from various locations within the same publication were treated as distinct case studies (*sensu* Koricheva *et al.*, 2013).

To assess the differences across grass–*Epichloë* symbioses and plant parts for each alkaloid type, we performed independent linear mixed models (LMMs) with symbiosis and plant part as crossed fixed factors and study case as a random factor by using the *lmer* function from the *lmerTest* package (Kuznetsova *et al.*, 2017) in R v.4.0.2 (R Development Core Team, 2022). Relevant model assumptions were analysed for each model using the *check_models* function from the *performance* R package (Lüdecke *et al.*, 2021); subsequently, we evaluated significant effects through Wald chi-squared tests. Furthermore, we carried out post-hoc analyses based on multiple pairwise comparisons of least-squares means using the *lsmeans* function from the *emmeans* package and *cld* function from the *multcomp* package. Data on the alkaloids ergovaline, lolitrem B and peramine were transformed using the function $\log_{10}(1 + x)$ to meet model assumptions. This transformation emphasizes variations in smaller values while mitigating variations in larger values and ensures that the result is always greater than or equal to zero. All results are presented with untransformed data.

Table 1. *List of articles that provided data for analysis of variation patterns in the concentrations of the four main fungal alkaloids lolines, peramine, ergovaline and lolitrem B across different plant–*Epichloë *symbioses. The entire list of references is provided in the* Supporting Data *Table S1*.

| Plant species | Endophyte species | Alkaloid | References |
|---|---|---|---|
| *Festuca pratensis* | *Epichloë uncinata* | Lolines | Adhikari *et al.*, 2016; Bryant *et al.*, 2010; Bylin *et al.*, 2014; Cagnano *et al.*, 2019; Justus *et al.*, 1997; Popay *et al.*, 2020; Vikuk *et al.*, 2019. |
| *Festuca rubra* | *Epichloë festucae* | Ergovaline | Gundel *et al.*, 2018; Jensen *et al.*, 2007; Leuchtmann *et al.*, 2000; Pereira *et al.*, 2021; Tanentzap *et al.*, 2014; Vázquez de Aldana *et al.*, 2004, 2007, 2010, 2020; Yue *et al.*, 1997. |
| | | Peramine | Leuchtmann *et al.*, 2000; Vázquez de Aldana *et al.*, 2004, 2010, 2020; Yue *et al.*, 1997. |
| *Lolium multiflorum*\|*Lolium rigidum* | *Epichloë occultans* | Lolines | Bastías *et al.*, 2018, 2019; Gundel *et al.*, 2018; Moore *et al.*, 2015; TePaske *et al.*, 1993; Ueno *et al.*, 2016, 2020. |
| *Lolium perenne* | *Epichloë festucae* var. *lolii* | Lolitrem B | Ball *et al.*, 1997*b*; Berny *et al.*, 1997; Bluett *et al.*, 1999; Combs *et al.*, 2014; Do Valle Ribeiro *et al.*, 1996; Eerens *et al.*, 1998; Finch *et al.*, 2018; Hahn *et al.*, 2008; Hesse *et al.*, 1999; Hewitt *et al.*, 2020; Hovermale *et al.*, 2001; Keogh *et al.*, 1996; König *et al.*, 2018; Krauss *et al.*, 2020; Lewis *et al.*, 1986; Lowe *et al.*, 2008; Oldenburg *et al.*, 1997; Reddy *et al.*, 2019; Reed *et al.*, 2000; Reed *et al.*, 2011*a*, *b*; Repussard *et al.*, 2014*b*; Soto-Barajas *et al.*, 2017, 2019; Tian *et al.*, 2013; Vassiliadis *et al.*, 2023; van Zijll de Jong *et al.*, 2008. |
| | | Ergovaline | Bluett *et al.*, 1999; Bultman *et al.*, 2004; Combs *et al.*, 2014; Easton *et al.*, 2002; Eerens *et al.*, 1998; Finch *et al.*, 2018; Hanh *et al.*, 2008; Hesse *et al.*, 1999; Hewitt *et al.*, 2020; Hovermale *et al.*, 2001; Hudson *et al.*, 2021; Krauss *et al.*, 2020; Lane *et al.*, 1997; Leuchtmann *et al.*, 2000; Lowe *et al.*, 2008; Mace *et al.*, 2014; Reed *et al.*, 2000, 2011, 2016*a*, *b*; Repussard *et al.*, 2014*a*; Soto-Barajas *et al.*, 2017, 2019; Spiering *et al.*, 2002; Sutherland *et al.*, 1999; TePaske *et al.*, 1993; Tian *et al.*, 2013; Vassiliadis *et al.*, 2023; van Zijll de Jong *et al.*, 2008. |
| | | Peramine | Ball *et al.*, 1997*a*; Bluett *et al.*, 1999; Breen *et al.*, 1992; Easton *et al.*, 2002; Eerens *et al.*, 1998; Fuchs *et al.*, 2013; Hesse *et al.*, 1999; Hewitt *et al.*, 2020; Hudson *et al.*, 2021; Keogh *et al.*, 1996; Krauss *et al.*, 2007, 2020; König *et al.*, 2018; Leuchtmann *et al.*, 2000; Lowe *et al.*, 2008; Moore *et al.*, 2015; Reed *et al.*, 2000, 2016; Soto-Barajas *et al.*, 2017, 2019; Spiering *et al.*, 2002; Sutherland *et al.*, 1999; Tian *et al.*, 2013, 2019; Vassiliadis *et al.*, 2023; van Zijll de Jong *et al.*, 2008. |
| *Lolium arundinaceum* | *Epichloë coenophiala* | Lolines | Baldauf *et al.*, 2011; Belesky *et al.*, 1989, 2009; Brosi *et al.*, 2011; Cibils-Stewart *et al.*, 2023; Dinkins *et al.*, 2023; Helander *et al.*, 2016; Jokela *et al.*, 2016; Leuchtmann *et al.*, 2000; Malinowski *et al.*, 1999; McCulley *et al.*, 2014; Pennell *et al.*, 2010; Petroski *et al.*, 1989; Piano *et al.*, 2005; Popay *et al.*, 2020; Siegrist *et al.*, 2010; Simeone *et al.*, 1998; Simons *et al.*, 2008, TePaske *et al.*, 1993. |
| | | Ergovaline | Agee *et al.*, 1994; Arechavaleta *et al.*, 1992; Baldauf *et al.*, 2011; Belesky *et al.*, 1989, 2009; Brown *et al.*, 2009; Christensen *et al.*, 1998; Dillard *et al.*, 2019; Garner *et al.*, 1993; Goff *et al.*, 2012; Grote *et al.*, 2023; Jackson *et al.*, 2015; Jensen *et al.*, 2007; Ji *et al.*, 2014; Jokela *et al.*, 2016; Kenyon *et al.*, 2018; Lane *et al.*, 1999; Lea *et al.*, 2014; Leuchtmann *et al.*, 2000; Lyons *et al.*, 1986; McCulley *et al.*, 2014; Najafabadi *et al.*, 2010; Pennell *et al.*, 2010; Petigrosso *et al.*, 2020; Piano *et al.*, 2005; Repussard *et al.*, 2014; Roylance *et al.*, 1994; Salvat *et al.*, 2001; Shelby *et al.*, 1997; Siegrist *et al.*, 2010; Simeone *et al.*, 1998; TePaske *et al.*, 1993; Vazquez de Aldana *et al.*, 2001; Walker *et al.*, 2015; White *et al.*, 2001; Yates *et al.*, 1988; Yue *et al.*, 1997; Zbib *et al.*, 2014. |
| | | Peramine | Baldauf *et al.*, 2011; Cibils-Stewart *et al.*, 2023; Krauss *et al.*, 2020; Leuchtmann *et al.*, 2000; Moore *et al.*, 2015; Roylance *et al.*, 1994; White *et al.*, 2001. |

## RESULTS

Overall, concentrations of *Epichloë*-derived alkaloids differed among plant–endophyte symbioses and/or plant parts (Table 2). Concentrations of lolines varied across symbioses depending on the plant part (Fig. 1), whereas that of peramine varied among symbioses regardless of the plant part (Fig. 2; Table 2). Concentrations of ergovaline varied among symbioses and depended on the plant part (Fig. 3). The concentration of lolitrem B, which was associated only with *L. perenne*, differed between plant parts (Fig. 4; Table 2).

Data on loline alkaloid concentrations in above-ground vegetative and reproductive tissues were retrieved for *L. arundinaceum*, *L. pratense* and *L. multiflorum*|*L. rigidum* symbioses, whereas in above-ground unclassified tissues were only available for *L. arundinaceum*. Loline alkaloid concentration data in below-ground tissues were retrieved for *L. arundinaceum* and *L. pratense* but not for *L. multiflorum*|*L. rigidum* (Fig. 1). In reproductive tissues, loline alkaloid concentrations were 13.4- and 7.5-fold higher in *L. pratense* and *L. arundinaceum* than in *L. multiflorum*|*L. rigidum* (Fig. 1) ($t_{(37.3)} = 4.470$, $P < 0.001$ and $t_{(9.28)} = 3.819$, $P = 0.025$, respectively). Conversely, the concentration of lolines in vegetative tissues did not differ among these symbioses. (Fig. 1). In below-ground tissues, loline alkaloid concentrations showed no significant differences between

Table 2. *Results of linear mixed models evaluating the effects of plant–*Epichloë *symbiosis, plant part and their interaction on the concentrations of the alkaloids lolines, peramine, ergovaline, and lolitrem B. Chi-square statistic values ($\chi^2$), degree of freedom (DF) and* P-*values are shown. Significant* P-*values are in bold.*

| Source | ***Epichloë*-derived alkaloid** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lolines | | | Peramine | | | Ergovaline | | | Lolitrem B | | |
| | $\chi^2$ | DF | ***P*** | $\chi^2$ | DF | ***P*** | $\chi^2$ | DF | ***P*** | $\chi^2$ | DF | ***P*** |
| Symbiosis (S) | 7.257 | 2 | **0.027** | 8.379 | 2 | **0.015** | 16.835 | 2 | **<0.001** | – | – | – |
| Plant part (P) | 19.621 | 3 | **<0.001** | 3.141 | 2 | 0.208 | 30.245 | 2 | **<0.001** | 15.794 | 2 | **<0.001** |
| S × P | 10.071 | 3 | **0.018** | 1.529 | 2 | 0.466 | 22.668 | 3 | **<0.001** | – | – | – |

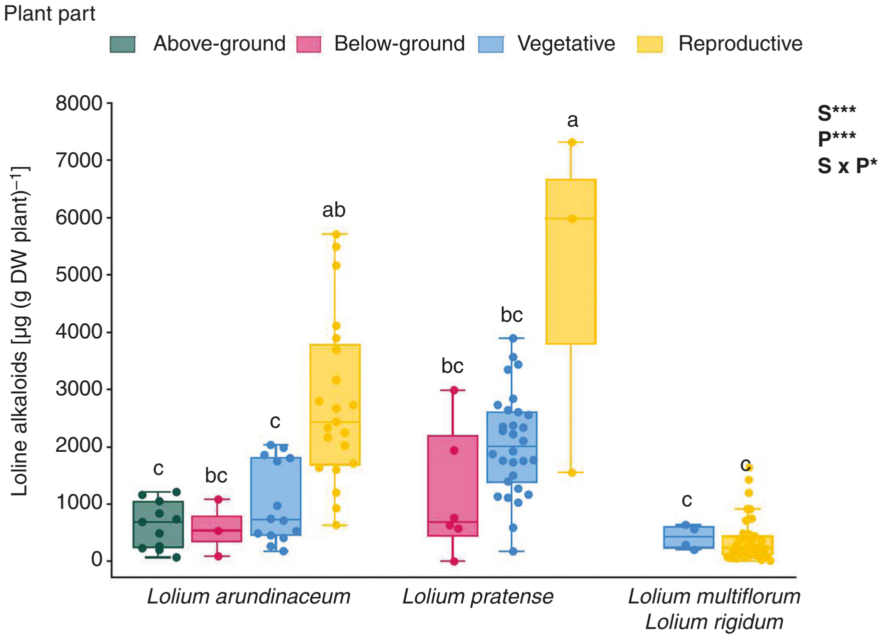

Fig. 1. Concentration of *Epichloë*-derived loline alkaloids in different plant–endophyte symbioses (indicated through host species name) and plant parts. The category ‘above-ground’ is used to aggregate all data from studies that did not define the tissue type, either vegetative or reproductive. Points around the boxplots are independent studies ($N = 132$). Loline alkaloids correspond to the sum of *N*-acetylnorloline (NANL), *N*-formylloline (NFL) and *N*-acetylloline (NAL). Asterisks denote significant effects of symbioses (S), plant part (P) or their interaction (S × P) as shown in Table 2. For vegetative and reproductive plant parts, distinct letters on boxplots indicate significant differences ($P < 0.05$) based on post-hoc multiple comparisons of means. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. DW, dry weight.

symbioses (Fig. 1). Additionally, loline alkaloid concentrations in above-ground tissues were similar to those in other plant parts for *L. arundinaceum*, but were 4.4-fold lower than in reproductive tissue ($t_{(26.0)} = -3.853$, $P = 0.026$).

Data on peramine concentration in above-ground vegetative, reproductive and unclassified tissues were retrieved for *L. arundinaceum* and *L. perenne*, whereas in above-ground vegetative tissues, data were also available for *F. rubra* (Fig. 2). Retrieved data on peramine concentration in below-ground tissues were insufficient for quantitative analysis. As the concentration of peramine varies among symbioses, rather than among plant parts or their interaction (see above), the post-hoc contrasts were explicitly directed at differences among symbioses. The concentration of peramine averaged 3.5-fold higher in *L. perenne* than in *L. arundinaceum* ($t_{(85.8)} = 3.47$, $P < 0.005$) and peramine concentration in *F. rubra* showed intermediate levels (Fig. 2).

Data on ergovaline concentration in above-ground vegetative and reproductive tissues were retrieved for *L. arundinaceum*, *F. rubra* and *L. perenne* whereas data on unclassified above-ground tissues were only available for *L. arundinaceum* and *L. perenne* (Fig. 3). Retrieved data on ergovaline concentration in below-ground tissues were insufficient for quantitative comparisons. In reproductive tissues, the concentration of ergovaline was about 3.8- and 13.5-fold higher in *L. perenne* than in *L. arundinaceum* ($t_{(229.6)} = 3.539$, $P = 0.014$) and *F. rubra* plants ($t_{(217)} = 4.524$, $P < 0.001$) respectively (Fig. 3). Conversely, the ergovaline concentration in unclassified above-ground tissues was not different among *L. arundinaceum* and *L. perenne* (Fig. 3). In vegetative tissue, the concentration of ergovaline was about 5.7-fold higher in *L. arundinaceum* than in *F. rubra* plants ($t_{(211.8)} = 3.901$, $P = 0.004$), whereas it showed an intermediate level in *L. perenne* (Fig. 3).

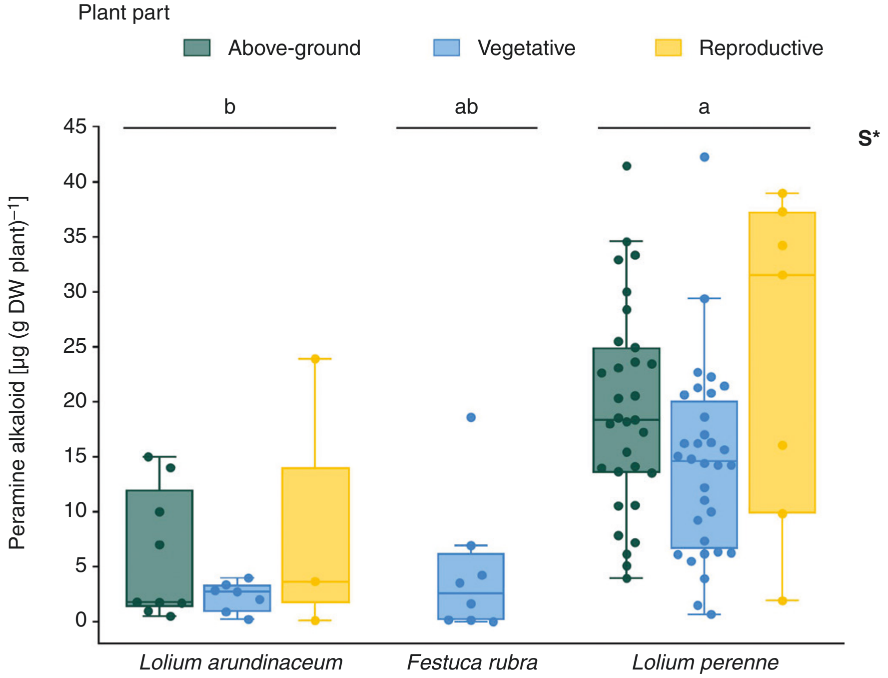

Fig. 2. Concentration of *Epichloë*-derived peramine alkaloid in different plant–endophyte symbioses (indicated through host species name) and plant parts. The category ‘above-ground’ is used to aggregate all data from studies that did not define the tissue type, either vegetative or reproductive. Points around the boxplots are independent studies ($N = 97$). Asterisks denote significant effects of symbiosis (S) only, as shown in Table 2. Distinct letters on boxplots indicate significant differences ($P < 0.05$) among symbioses based on post-hoc multiple comparisons of means. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. DW, dry weight.

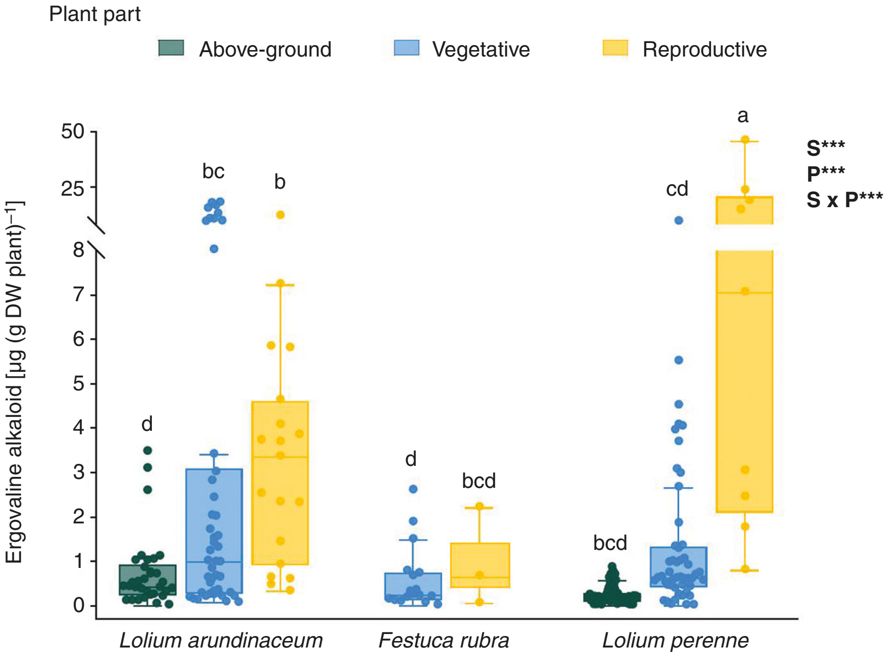

Fig. 3. Concentration of *Epichloë*-derived ergovaline alkaloid in different plant–endophyte symbioses (indicated through host species name) and plant parts. The category ‘above-ground’ is used to aggregate all data from studies that did not define the tissue type, either vegetative or reproductive. Points around the boxplots are independent studies ($N = 246$). Asterisks denote significant effects of symbioses (S), plant part (P) or their interaction (S × P) as shown in Table 2. For vegetative and reproductive plant parts, distinct letters on boxplots indicate significant differences ($P < 0.05$) based on post-hoc multiple comparisons of means. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. DW, dry weight.

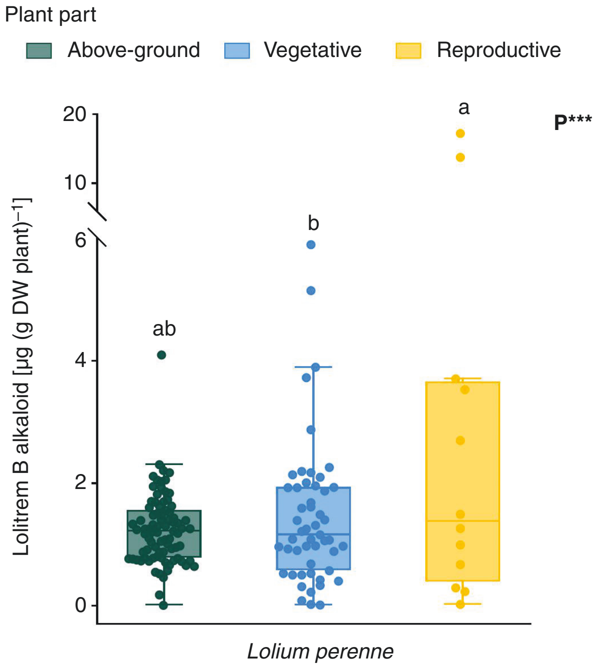

Fig. 4. Concentration of *Epichloë*-derived lolitrem B alkaloid in the symbiosis between *Lolium perenne* and the common strain of the endophyte fungus *Epichloë festucae* var. *lolii*. The category 'above-ground' is used to aggregate all data from studies that did not define the tissue type, either vegetative or reproductive. Points around the boxplots are independent studies ($N = 146$). Asterisks denote significant effects of plant part (P) only, as shown in Table 2. Distinct letters on boxplots indicate significant differences ($P < 0.05$) among plant parts based on post-hoc multiple comparisons of means. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. DW, dry weight.

Data on lolitrem B concentration were retrieved only for the *L. perenne* – *E. festucae* symbiosis occurring in above-ground vegetative, reproductive and unclassified above-ground tissues (Fig. 4). Data on lolitrem B concentration in below-ground tissues were insufficient for quantitative analysis. The concentration of lolitrem B was 1.6-fold higher in reproductive than in vegetative tissues ($t_{(142.6)} = 3.918$, $P < 0.001$). The concentration of this alkaloid in unclassified above-ground tissues was not different than in reproductive and vegetative tissues (Fig. 4).

## DISCUSSION

Through the compilation and analysis of published data, we present a synthesis of the variation in the four major alkaloids derived from *Epichloë* fungal endophytes across different hosts and plant parts. The concentrations of most alkaloids varied among plant species and between plant parts. These variations may stem from differences in fungal species-specific growth and/or alkaloid biosynthesis rates within plants, the regulation of alkaloid production by the plant, and the ability of alkaloids to mobilize between plant tissues.

The concentrations of loline alkaloids in reproductive and vegetive tissues were higher in both *L. pratense* and *L. arundinaceum* than in *L. multiflorum*/*L. rigidum*. The difference in alkaloid concentrations may result from the variations in size between these plant species. Whereas *L. pratense* and *L. arundinaceum* are perennial plants, *L. multiflorum* and *L. rigidum* are annuals. Although initially the sizes can be comparable, perennial grasses tend to accumulate more biomass than annual grasses. As the plant size increases at the crown level (i.e. the plant base that connects roots and shoots, containing apical and axillary meristematic buds that will give rise to tillers and inflorescences, respectively), it is possible that the mycelium of the endophytic fungus also increases. All this together may explain the observed positive association between the concentration of *Epichloë*-derived alkaloids and plant size (Ball *et al.*, 1997*a*). In the leaves, endophyte hyphae are generally more abundant in sheaths than in blades but in *L. multiflorum*/*L. rigidum*, hyphae are mostly located at the base of leaf sheaths (Moon *et al.*, 2000; Christensen *et al.*, 2002). Considering that most of the vegetative tiller (rolled leaf sheaths and their corresponding blades) is free of endophytic mycelia in *L. multiflorum*/*L. rigidum*, it is reasonable to expect lower alkaloid contents per unit of plant biomass in this plant species compared to *L. pratense* and certain *L. arundinaceum*, in which *Epichloë* hyphae are usually distributed throughout the whole leaf (Justus *et al.*, 1997; Christensen *et al.*, 2002). Although the localized distribution of the endophyte mycelia in annual hosts could eventually compromise the resistance to herbivores, there is evidence demonstrating a superior resistance in plants with endophytic symbiosis in comparison to their non-symbiotic counterparts (e.g. Ueno *et al.*, 2016; Bastias *et al.*, 2017b). Dissimilarities in the alkaloid synthesis rate between *Epichloë* species might have also contributed to the variations in loline alkaloid concentration among plant species. While no studies have compared loline alkaloid biosynthesis rates between the fungal species associated with *L. pratense*, *L. arundinaceum* or *L. multiflorum*/*L. rigidum*, it has been documented that differences exist in the production of lolines among plant–endophyte associations (Freitas *et al.*, 2020). Further studies are needed to investigate whether there are differences in the rates of loline alkaloid biosynthesis associated with distinct host plant species.

Peramine concentrations were higher in *L. perenne* than in *L. arundinaceum* independently of the plant part. According to this, the relative contribution of peramine to the level of plant resistance to insects might be higher in *L. perenne* than in *L. arundinaceum*. Peramine stands as the only characterized alkaloid that exclusively provides protection against insects within the *L. perenne*–common endophyte associations. However, in the context of the *L. arundinaceum*–wild type endophyte association, this protective activity can be supplemented by loline alkaloids, which also exhibit exclusive bioactivity against insects (Wilkinson *et al.*, 2000; Schardl *et al.*, 2007). Due to the relevance of peramine for herbivorous insect resistance in *L. perenne*, it is likely that insect herbivory has selected associations within this plant species with traits that result in elevated alkaloid concentrations. In agreement with this, it was shown that the same *Epichloë* strain produced higher levels of peramine per unit of plant biomass in *L. perenne* than in *L. arundinaceum* (Freitas *et al.*, 2020). As in the case of lolines, the amount of *Epichloë* mycelial biomass could also have contributed to the plant species differences in peramine concentrations, since certain plant genotypes of *L. arundinaceum* have been described with absence of *Epichloë* hyphae within leaf blades (the *in planta* correlation between peramine concentration and *Epichloë* mycelial biomass is generally positive)

(Hinton and Bacon, 1985; Christensen *et al.*, 1998; Popay *et al.*, 2003*a*; Takach *et al.*, 2012). Peramine was the only alkaloid whose concentration did not vary between plant parts. This finding agrees with previous evidence showing the systemic distribution of peramine in plants (Schardl *et al.*, 2004). The explanation has been associated with the hydrophilic nature of peramine, which allows the alkaloid to be transported via leaf fluids to plant sections that are not colonized by fungal hyphae (Keogh *et al.*, 1996; Ball *et al.*, 1997*a*; Koulman *et al.*, 2007; Hewitt *et al.*, 2020). As with loline alkaloids, the widespread presence of peramine in different plant parts probably explains the effectiveness of this alkaloid in controlling different insect herbivores (Bastias *et al.*, 2017a).

Ergovaline and lolitrem B are known to be responsible for causing severe disorders in mammals (Bouton *et al.*, 2002; Gundel *et al.*, 2013; Johnson *et al.*, 2013). There is also evidence for these two alkaloids to be involved in plant resistance to herbivorous insects (Rowan, 1993; Ball *et al.*, 1997*b*; Potter *et al.*, 2008; Graff *et al.*, 2020). Our results showed that both alkaloids were more concentrated in reproductive than vegetative parts in some plant hosts. As they both are hydrophobic compounds, they are primarily associated with plant parts where the fungal mycelium is present (Ball *et al.*, 1997*a*; Spiering *et al.*, 2005). In accordance with previous studies (Ball *et al.*, 1995; Keogh *et al.*, 1996; Repussard *et al.*, 2014), their concentrations increase with the plant passage from vegetative to reproductive stages. Given their relatively low mobility, the accumulation of these alkaloids may result from both a high mycelial biomass and/or a high rate of alkaloid synthesis within reproductive tissues. In alignment with the biomass-associated prediction, there exists a general trend of elevated endophyte mycelial biomass as plants transition into the reproductive ontogenetic phase (e.g. di Menna and Waller, 1986; Puentes *et al.*, 2007; Rogers *et al.*, 2011; Fuchs *et al.*, 2017*c*). Regarding the biosynthesis-associated prediction, the high lolitrem B concentration in reproductive tissues is supported from genetic results showing that the endophyte gene *ltmM* encoding an enzyme in the alkaloid synthesis pathway was highly expressed in floral organs of *L. perenne* (May *et al.*, 2008). An approach that holds potential for elucidating the underlying mechanisms behind the observed elevated alkaloid concentrations in reproductive plant parts would be comparing the relationship between alkaloid concentration and endophyte mycelium in seeds for both relatively immobile alkaloids (e.g. ergovaline) and relatively mobile alkaloids (e.g. peramine). If alkaloid mobility plays a significant role in plant part accumulation, it follows that the alkaloid-to-mycelium ratio would be greater for more mobile alkaloids compared to less mobile ones – at least in those plant parts where endophyte hyphae are virtually absent (e.g. in roots). This is because the former compounds are not solely generated *in situ* (within the seed) but are also transported from the plant's green tissues, as discussed by Ueno *et al.* (2020).

The occurrence of alkaloids in below-ground tissues has not been as well documented as in above-ground tissues. Only data on loline alkaloid concentration in below-ground tissues were sufficient to perform comparative and quantitative analyses. However, it is worth mentioning that individual studies have reported the occurrence of ergovaline, lolitrem B and peramine in roots (Azevedo and Welty, 1995; Ball *et al.*, 1997*a*; Justus *et al.*, 1997; Vassiliadis *et al.*, 2023). In agreement with our results, alkaloids in below-ground tissues normally reach lower concentrations than in above-ground tissues, but the magnitude of reduction depends on the alkaloid type and association (Vassiliadis *et al.*, 2023). Since endophyte mycelia are practically absent in roots (Hinton and Bacon, 1985; Azevedo and Welty, 1995), the alkaloid presence in these tissues relies mainly on the translocation from above-ground tissues and not on *in situ* production. Despite the reduced concentrations of *Epichloë*-derived alkaloids in below-ground tissues, the levels may be high enough to be effective in controlling root insect herbivores (Caradus and Johnson, 2020; but see Bastías *et al.*, 2021). For instance, the presence of common endophytes in *L. perenne* and *F. pratensis* plants reduced the population sizes of the root aphid *Aploneura lentisci* and the larval growth of the root-feeding scarab *Costelytra giveni* (Popay *et al.*, 2003*b*; Popay and Cox, 2016). It would be interesting to investigate how herbivory, whether exerted on roots or on leaves, can alter the translocation patterns of alkaloids between below-ground and above-ground structures. Subsequently, such alterations may influence the levels of endophyte-conferred resistance in host plants.

## CONCLUSIONS AND PERSPECTIVES

Our work provides a general picture of variation patterns in the concentration of the main fungal alkaloids among different hosts and plant parts. These patterns can be understood by taking into account factors such as the growth dynamics of endophytic fungi within the host plant, the particular mobility characteristics of the alkaloid compounds and the regulatory mechanisms employed by the plants in relation to endophytes. However, there are several open questions to be addressed.

First, our study focused on plant parts that are not necessarily associated with specific plant phenological stages. Recent transcriptomic analyses have revealed variations in the plant's regulation of endophyte growth and functions based on gene expression profiles across different plant organs and stages (Dinkins *et al.*, 2017; Schmid *et al.*, 2017; Nagabhyru *et al.*, 2019). Therefore, as the regulation of hyphae proliferation and endophyte metabolic activity can vary between vegetative tillers depending on the plant stage (e.g. if the plant is at tillering or at anthesis), a similar variation can be expected in alkaloid concentration (e.g. Ball *et al.*, 1997*a*). Moreover, these variations are likely to be associated with the environmental conditions that plants experience during winter and spring–summer seasons, strongly determining the described seasonal changes in endophyte mycelia contraction and alkaloid production (di Menna & Waller, 1986; Puentes *et al.*, 2007; Fuchs *et al.*, 2017*c*).

Second, accumulating evidence shows that alkaloid concentration can be influenced by environmental conditions of plant growth such as level of resources, soil water availability, salinity, temperature and herbivory (Bultman *et al.*, 2004; Rasmussen *et al.*, 2007; Repussard *et al.*, 2014; Hennessy *et al.*, 2016; Graff *et al.*, 2020; Chen *et al.*, 2022). Besides examining how general and consistent are those effects, it is essential to investigate the underlying mechanisms of the induction of alkaloids by the action of herbivores that differ in feeding habits (e.g. defoliation by mammals, chewing insects and sap-sucking insects) (Bultman *et al.*, 2004; Sullivan *et al.*, 2007; Fuchs *et al.*, 2017*b*; Bubica Bustos *et al.*, 2022).

Finally, the degree of protection against herbivores conferred by *Epichloë* may not only vary based on the growth patterns of the endophyte that influence alkaloid distributions within the plant but also due to the specific array of alkaloids present in the plant. Endophytes producing more than one alkaloid are expected to broaden the spectrum of herbivores under control conditions compared to those producing a single alkaloid (Bastias *et al.*, 2017a). Under this premise, endophytes producing more than one alkaloid should be more prevalent in natural populations, provided their presence does not entail greater production costs for the host plants (Semmartin *et al.*, 2015). However, these individual-based characteristics become not only more complex but also more eco-evolutionarily relevant when scaled up to include diversity at the population level (Vikuk *et al.*, 2019). To enhance our understanding of the role of *Epichloë*-derived alkaloids in plant resistance to herbivores, it is crucial to further explore the diversity of endophytes associated with each plant species and the chemical diversity of these fungal endophytes, and to extend this type of study to other plant species.

## SUPPLEMENTARY DATA

Supplementary data are available at *Annals of Botany* online and consist of the following.

Table S1: Reference list from articles reported in Table 1.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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