


# A fungal endophyte of a palatable grass affects preference of large herbivores

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**Abstract** Temperate grasses frequently acquire resistance to herbivores through a symbiosis with epichloid fungi that produces alkaloids of variable deterrent effects. However, in those cases without apparent endophyte negative effects on domestic herbivores, it is not clear whether plant consumption or preference is affected or not. We performed three experiments with 1-year-old steers (*Bos taurus*, Aberdeen Angus) and the annual grass *Lolium multiflorum*, infected or not by *Epichloë occultans* to evaluate preference and to identify the underlying tolerance mechanisms. The first experiment evaluated steer preference for *L. multiflorum* cultivated in plots with three endophyte infection frequencies (low, medium and high), and investigated the role of canopy structure and plant nutritional traits on preference. The second experiment evaluated preference for chopped grass, offered in individual trays with contrasting infection frequencies (low and high), to discard possible effects associated with canopy structure and to focus on nutritional traits. The third experiment was performed with a tray + basket design that separated visual and olfactory stimuli from nutritional traits. High endophyte infection frequencies reduced consistently animal preference, even after short (~10 min) feeding events. However, we did not find significant evidence of plant structural, nutritional, visual or olfactory traits. Our results discarded several potential mechanisms; therefore, the dissuasive effect of fungal endophytes on animal consumption might be related to other mechanisms, including, likely, alkaloids and changes on grass metabolome.

**Key words:** *Epichloë*, fungal endophytes, herbivore preference, *Lolium multiflorum*, *Neotyphodium*, ryegrass.

## INTRODUCTION

Large herbivores usually display forage selectivity revealing complex decision-making mechanisms that maximize intake efficiency (Bailey *et al.* 1996). For many herbivores, intake decisions are influenced by plant abundance and structural and nutritional traits (i.e. spines, thorns, prostrate habit, secondary compounds) (Senft *et al.* 1987; Bailey *et al.* 1996). Most grasses lack strong morphological and antinutritional traits, but they can affect herbivores' preference through variation of other less apparent traits. Composition of structural carbohydrates, lignin, dry matter concentration, nitrogen and soluble compounds is an important driver of voluntary intake and preference in domestic herbivores (Smit *et al.* 2006; O'Donovan & Delaby 2008; Curran *et al.* 2010). Likewise, structural factors such as canopy height, tiller density and standing biomass also affect animal preference (Cid & Brizuela 1994; Bailey *et al.* 1996).

Approximately 20–30% of C3 pooid grasses harbour asymptomatic and vertically transmitted fungal

endophytes which may confer defences against herbivores by the synthesis of harmful alkaloids, concentrating the alkaloids in sheaths, developing inflorescences, and mature seeds (Leuchtman 1992; Clay 1996; Schardl *et al.* 2012; Saikkonen *et al.* 2013; Young *et al.* 2013). These endophytes are responsible for producing four alkaloid types – ergots, lolitrem, lolines and peramines – and some of them have severe effects on animal performance, grazing behaviour or voluntary intake (Howard *et al.* 1992; Porter & Thompson 1992; Jones *et al.* 2000; Leuchtman *et al.* 2000; Schardl *et al.* 2013).

The association between *Lolium multiflorum* Lam. (Italian ryegrass) and *Epichloë occultans* does not produce harmful alkaloids to domestic herbivores (TePaske *et al.* 1993). Annual *Lolium* sp. populations infected with *E. occultans* exhibit peramines (Sugawara *et al.* 2006), N-acetylornolines and N-formylornolines (Moore *et al.* 2015) in variable concentrations according to plant organs, phenological stage and geographical origin. These types of alkaloids have not been described as toxic for cattle, but they have been described as modifiers of the preference of rodents and arthropods for infected seeds and plants (Omacini *et al.* 2001; Uchitel *et al.* 2011; Ueno *et al.*

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2016). Nevertheless, in addition to fungal alkaloids, several studies suggest that epichloid endophytes may exert further ecophysiological and metabolomic effects (e.g. different silica absorption, flavonoid and volatile compound synthesis, lignin concentration) with potential impacts on herbivores' preference (Rasmussen *et al.* 2008, 2009; Huitu *et al.* 2014; Li *et al.* 2014; Dupont *et al.* 2015). As documented for domestic herbivores consuming other grass species (Oliveira *et al.* 2004; Zabalgoeazcoa *et al.* 2006; Soto-Barajas *et al.* 2016), patterns of plant nutritional values and digestibility associated with endophyte presence in *L. multiflorum* might influence animal preference.

*Lolium multiflorum* has increased its distribution range since the 17th century and became naturalized in many temperate ecosystems worldwide. In South American grasslands, *L. multiflorum* populations bear high endophyte incidence (up to a 100% of individuals infected in managed systems, Gundel *et al.* 2009). Continuous grazing management during the last century reduced native cool-season grasses in these grasslands. This reduction caused forage scarcity during winter and favoured the invasion and further naturalization of *L. multiflorum*, which became essential for livestock production (Jacobo *et al.* 2000). Hence, elucidating the impacts of the potential resistance provided by *E. occultans* on domestic herbivores could contribute to improve these livestock production systems.

We conducted a set of complementary experiments to assess the possible influence of the association between *L. multiflorum* and *E. occultans* on preferential consumption by steers. We hypothesized that the presence of endophyte deters herbivores through changes to plant structural, nutritional and/or olfactory traits. In the first experiment, we tested preference by offering to steers cultivated *L. multiflorum* plots with different endophyte frequency. In this experiment, we also investigated canopy structure and nutritional traits as potential sources affecting preference. The second and third experiments consisted on feeding experiments in trays with chopped grass with different infection frequencies intended to assess plant nutritional and olfactory traits without canopy structure influence.

## MATERIALS AND METHODS

### Experimental site, grass populations and steers

We conducted three experiments to assess the of preference of *L. multiflorum* by steers (Aberdeen Angus) in Concepción del Uruguay experimental station of the National Institute for Agricultural Technology (INTA), from March 2013 to October 2014. This area belongs to the Río de la Plata grasslands (Soriano 1991). Climate is humid, with a

mean annual rainfall of 1200 mm uniformly distributed throughout the year, and mean temperatures of 18°C.

We grew forage with high and low endophyte infection frequency (calculated as the proportion of infected seeds in a sample) from two wild populations of *L. multiflorum* (Lucero and Feliciano). The infection frequency was determined by sampling 50 seeds of each population and endophyte status (high and low infection). Seeds were treated with NaOH for 8 h, stained with Rose Bengal and examined under microscope. Positive samples showed the presence of non-branching intercellular hyphae (Bacon & White 1994). Both populations belong to different areas of the Río de la Plata grasslands and they are naturally infected with *E. occultans*. Lucero belongs to the Inland Pampa (Carlos Casares, Buenos Aires Province, 35°55'S, 61°15'W) whereas Feliciano belongs to the Mesopotamic Pampa (Soriano 1991) (Feliciano, Entre Ríos Province, 30°26'S, 58°23'W). Both populations were reproduced and preserved by INTA. Lucero endophyte-infected seeds were obtained from the endophytic individuals of an original population with ≈53% infection, and the endophyte-free seeds, from the non-infected plants of the same original population. Even though in this study we did not quantify alkaloid plant concentration, Ueno *et al.* (2016) recently reported that plant concentration of loline derivatives (N-acetylornoline, N-formylloline and N-acetylloline) in infected plants ranged between 200 and 300 µg g<sup>-1</sup> of plant in this population. Feliciano endophyte-infected seeds were obtained from an original population with 100% infection, and the endophyte-free seeds, from the same original population from which the endophyte was removed by an ageing treatment. Briefly, the ageing treatment consisted of storing seeds in paper bags at room temperature for 2 years until the endophyte viability was eliminated (Beltramino *et al.* 2005). Grass seed stock was maintained by annual cultivation in the INTA experimental station. Infection frequency of seeds was determined in the laboratory by checking 30 seeds by the stained method described above (Bacon & White 1994). Lucero-infected and non-infected lots had 95% and 0% infection, respectively, whereas Feliciano had 92% and 0%.

All the experiments were performed with 1-year-old steers (Aberdeen Angus) of ≈220 (±13) kg. Animals were selected to be similar in weight, size and behaviour to reduce interference among them due to dominance effects. Two or three days before the experiment, the steers were fed on a corn-based diet on trays, and the day before the experiments, feeding was restricted.

### Plant and animal measurements

Plants offered in the experiments were nutritionally and structurally characterized. We analysed those traits usually affecting preference by domestic herbivores (Bailey *et al.* 1996; Jung *et al.* 1997) that, in turn, can be also modified by the endophyte (Miranda *et al.* 2011; Soto-Barajas *et al.* 2016). Nutritional characterization included non-structural (cell solubles) and structural carbohydrates (neutral and acid detergent fibres – NDF and ADF – and lignin) (Van Soest *et al.* 1991) and nitrogen content (Kjeldahl digestion). High concentration of ADF, NDF and lignin denotes

lower nutritional values, whereas high solubles and nitrogen concentration denote high nutritional values. In Experiment 1 (see below), we characterized, previously to the offer, above-ground biomass, tiller density and canopy height. Biomass and tiller density were estimated using two 30 × 30 cm frames per plot. Canopy height was estimated by placing a plastic disc (~25 cm of diameter) at random positions (four per plot) and recording disc distance to the soil surface. Biomass samples were weighed after harvest (fresh weight) and after oven-drying at 60°C for 48 h to determine water content and dry weight. All canopy measurements were taken at least 20 cm inside the patch edges to avoid border effect. In Experiments 2 and 3 (see below), plant structure at reproductive stage was evaluated by quantifying the dry weight of culms, blades and sheaths, and inflorescences (five individuals per population and infection status).

Preference was quantified through voluntary intake as the difference between offered and remaining biomass after consumption events (Macon *et al.* 2003; Smit *et al.* 2006). In the experiment in plots, we also recorded the frequency of visits to individual plots of *L. multiflorum* with different endophyte infection level (see below, Experiment 1).

### Experiment 1

To evaluate the preference of *L. multiflorum* plots with different infection frequency (low, medium and high), we delimited four blocks within an area of 500 m<sup>2</sup>. In each block, the original vegetation was removed, three plots (4 m<sup>2</sup>) were delimited and each one randomly received one of the three different treatments (Appendix S1). A month before sowing (March 2013), the area was treated with glyphosate (8 L ha<sup>-1</sup>) to remove the original vegetation. Plots were 3 m away from the fences dividing blocks and from neighbouring plots. They were fertilized with 150 kg ha<sup>-1</sup> of commercial di-ammonium phosphate with potassium (40N, 30P, 6K) and 100 kg ha<sup>-1</sup> of urea. Two months after sowing, we applied a selective herbicide for broadleaf species (Tordon D30, 4 L ha<sup>-1</sup>) to promote the growth of *L. multiflorum*. Seeds belonged to Lucero population with the corresponding endophyte infection frequency (high: ~95.3% and low: ~0%). The intermediate infection frequency was obtained by mixing seeds of high and low infection frequencies in equal proportions (medium: ~41.5%). When the experiment finished, new seeds were collected and endophyte infection was rechecked by sampling 30 seeds from each plot. The infection was 65.5% for the high frequency, 36% for the medium and 0% for the low ( $P < 0.05$ ).

In October 2013, forage was offered in one feeding trial to three steers to evaluate whether the endophyte affected preference. The frequency of visits was measured by recording the position of each of the three animals within the block every 2 min and for a total period of 1 h (Laca 1998; Solomon *et al.* 2014). Therefore, the frequency of visits of a given treatment was the proportion of the records on that treatment over the total number of records. Because animals needed a period to adapt to the plot set up, records began after ≈15 min since the animals entered into the plots.

### Experiment 2

We performed a 2 × 2 factorial experiment of preference consisting of offering two *L. multiflorum* populations (Lucero and Feliciano) each with two endophyte infection frequencies (high and low). Biomass used was cultivated in a neighbouring paddock, in 2 × 5 m plots, with 20 kg seed per ha fertilized with 80 kg di-ammonium phosphate with potassium (40N, 30P, 6K) after sowing and 100 kg urea per ha when tillering. As we performed the same experiment at two phenological stages (reproductive and vegetative), we repeated this procedure in May 2013 and 2014.

Forage was offered to the steers in November 2013 (reproductive stage) and in October 2014 (vegetative stage). We harvested and chopped grass biomass (pieces ~10 cm long) and placed 1 kg of fresh weight in individual plastic trays (45 × 45 cm, 25 cm height). The experiment was performed in two adjacent barnyards where trays with each *Lolium* type were located on the bare soil, and consisted of eight consecutive feeding rounds of 15 min each (time enough for the steers to choose between treatments but not to empty the trays). After 15 min, trays were removed and the remaining forage was weighed and saved for dry weight determination.

### Experiment 3

This experiment was performed in October 2014 and the offered forage was prepared as in Experiment 2. We only used the Lucero population with both contrasting endophyte infection frequencies (low and high). Because our objective was to investigate an indirect background effect mediated by olfactory or visual influence, we added inside the trays a nylon mesh basket (30 × 30 × 20 cm) (see picture in Appendix S2). Therefore, we offered forage in a mesh basket surrounded by a background forage which could be smelt and seen but not eaten. The mesh basket contained 500 g of fresh weight while the background in the plastic tray contained 1 kg of *Lolium* biomass. We performed a 2 × 2 factorial experiment with offer and background as factors in all possible combinations. In each round, steers could eat from four different baskets, randomly located in a barnyard as in Experiment 2, and the remaining forage was also treated similarly as in Experiment 2 to evaluate preference.

### Data analysis

In Experiment 1 (plots), preference was analysed by a linear mixed model where consumption and frequency of visits were used as dependent variables, the endophyte infection status (low, medium and high) was used as fixed variable, and plots were used as random variables. Characterization of canopy structure (biomass, tiller density, canopy height) and biomass chemical composition (cell solubles, neutral and acid detergent fibres, lignin and N) were analysed by analyses of variance, where endophyte infection was used as source of variation.

In Experiment 2 (trays, simple offer), preference was analysed by a linear mixed model where consumption was the dependent variable; population, infection frequency and the interaction between them were fixed variables; and phenological stage was a random variable (because each phenological stage experiment was carried out in different years). Plant structure (weight of culms, blades and sheaths, and inflorescences) and nutritional content (cell solubles, neutral and acid detergent fibres, lignin and N) at reproductive stage were analysed by analyses of variance where endophyte infection, population and their interaction were used as sources of variation.

In Experiment 3 (trays, offer + background), preference was analysed by a linear mixed-model analysis where consumption was the dependent variable; background, offer and their interaction were fixed variables; and barnyards were the random variable. Temporal autocorrelation was evaluated in the three experiments to assess the possible time and learning effects in the animal response. Analyses were performed with R (R Core Team 2013).

## RESULTS

Canopy and plant structure and nutritional composition of *L. multiflorum* did not vary with endophyte infection frequency ( $P > 0.05$ , Tables 1–3). We did not detect differences in plant morphology and

**Table 1.** Structural characterization of plots cultivated with *Lolium multiflorum* bearing different endophyte infection frequency (high, medium and low)

Treatment	Above-ground biomass (g m <sup>-2</sup> )	Tiller density (tiller m <sup>-2</sup> )	Height (cm)
High	1997.1 ± 179.4	185.6 ± 11.7	51.7 ± 3.1
Medium	2103.7 ± 143.6	180.8 ± 10.1	51.0 ± 3.1
Low	2384.3 ± 310.8	198.1 ± 6.6	49.1 ± 2.9

There were not significant differences between treatments ( $n = 4$ , mean ± SE).

**Table 2.** Structural characterization of *Lolium multiflorum* plants from two populations (Lucero and Feliciano) with low (~0%) and high (~80%) endophyte infection frequencies at two phenological stages

	Lucero		Feliciano	
	Low	High	Low	High
Vegetative stage				
Inflorescences	0.15 ± 0.01	0.13 ± 0.03	0.18 ± 0.03	0.14 ± 0.02
Culms	0.37 ± 0.02	0.35 ± 0.01	0.36 ± 0.03	0.33 ± 0.02
Blades + sheath	0.48 ± 0.03	0.52 ± 0.02	0.46 ± 0.05	0.54 ± 0.04
Reproductive stage				
Inflorescences	0.40 ± 0.01	0.38 ± 0.04	0.38 ± 0.02	0.41 ± 0.03
Culms	0.20 ± 0.02	0.23 ± 0.03	0.24 ± 0.01	0.22 ± 0.01
Blades + sheath	0.39 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.37 ± 0.02

Values are expressed as the proportion of above-ground biomass, and there were no significant differences between populations and infection frequency (mean ± SE;  $n = 3$ ).

nutritional composition between Lucero and Feliciano populations ( $P > 0.05$ , Tables 2,3) either.

Steer preference in Experiment 1 showed greater frequency of visits in low-infected plots (40% of total visits) than in high-infected ones (25% of total visits) ( $P = 0.003$ , Fig. 1a). These differences, however, were not detected in the consumed biomass ( $P = 0.77$ , Fig. 1b).

Steer preference in Experiment 2 (trays) revealed greater consumption for *L. multiflorum* with low endophyte infection than with high infection at both stages, vegetative and reproductive ( $P = 0.03$ , Fig. 2). Only consumption of Feliciano did not vary significantly between infection frequencies at vegetative stage. In turn, steers preferred Lucero over Feliciano ( $P = 0.001$ ) irrespective of the endophyte frequency (population × endophyte interaction  $P = 0.6$ , Fig. 2).

Finally, Experiment 3 also revealed steer preference for low endophyte-infected grasses ( $P = 0.03$ ), and it was not affected by the endophyte infection frequency of the background ( $P = 0.23$ , Fig. 3).

## DISCUSSION

Our results showed that the symbiosis between *L. multiflorum* and *E. occultans* reduces the preference of the steers. Three different experimental approaches revealed, consistently, a greater preference for *L. multiflorum* plants with low endophyte infection frequency. However, changes in preference were not mediated by changes in plant biomass, structure, protein and fibre content, or volatile compounds.

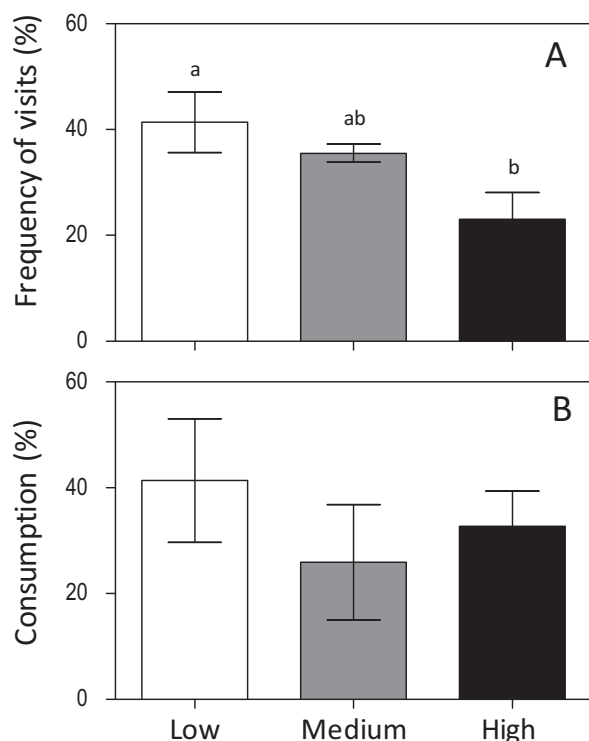
In coincidence with previous evidence, the greater preference for endophyte-free plants was not related to differences in plant structural or nutritional traits, usually influencing cattle selectivity (Oliveira *et al.*



**Table 3.** Nutritional characterization of *Lolium multiflorum* biomass with different endophyte infection frequency (high and low)

	Cell solubles	ADF (%)	NDF (%)	Lignin (%)	N (%)
Experiment 1 (plot)					
Lucero low	23.8 ± 1.7	37.3 ± 1.4	65.2 ± 2.4	4.15 ± 0.2	1.07 ± 0.05
Lucero high	20.4 ± 1.9	38.1 ± 1.8	68.3 ± 2.5	4.19 ± 0.2	1.29 ± 0.08
Experiment 2 (tray)					
Lucero low	17.8 ± 0.8	42.4 ± 1.0	71.5 ± 0.6	5.76 ± 0.4	1.53 ± 0.04
Lucero high	19.5 ± 1.0	41.8 ± 0.9	71.0 ± 1.3	5.25 ± 0.2	1.54 ± 0.07
Feliciano low	18.1 ± 1.0	42.6 ± 0.5	72.2 ± 1.1	5.50 ± 0.1	1.58 ± 0.05
Feliciano high	17.5 ± 0.9	41.7 ± 0.3	72.3 ± 0.9	6.50 ± 0.8	1.45 ± 0.19

Values indicate cell solubles, ADF, acid detergent fibres; NDF, neutral detergent fibres; N, total nitrogen. Experiment 1 was performed with Lucero population (mean ± SE;  $n = 3$ ), and Experiment 2 was performed with Lucero and Feliciano populations (mean ± SE;  $n = 4$ , data corresponding to reproductive stage). No significant differences were found between populations or infection frequency.



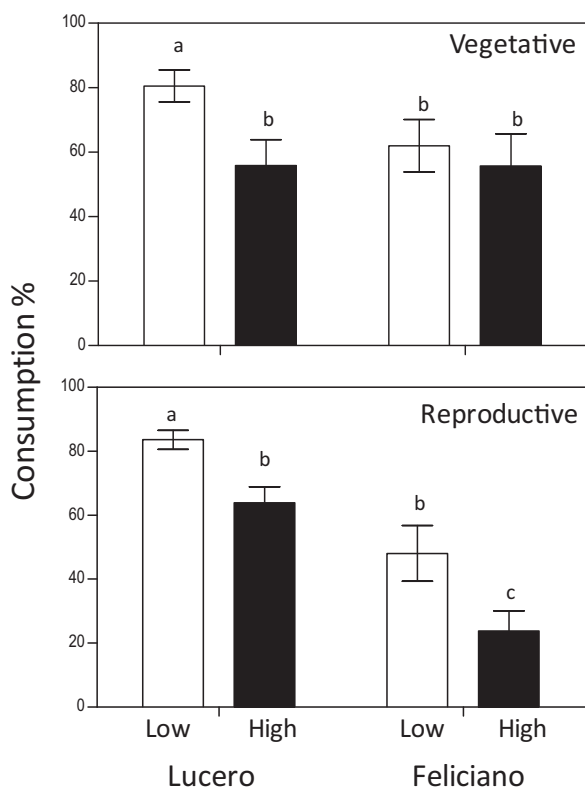
**Fig. 1.** Steer preference for *Lolium multiflorum* plots with different endophyte infection frequencies (low, medium and high). (a) Frequency of visits to each plot expressed as the proportion of total visits. (b) Consumption estimated as the fraction of biomass removed from each plot. Different letters indicate significant differences ( $P < 0.05$ ).

2004; Soto-Barajas *et al.* 2016). Endophyte effects on nutritional traits have been scarcely studied and results are variable for different species; nevertheless, while *Festuca rubra*- and *Lolium perenne*-infected plants have lower contents of neutral detergent fibres (NDF) and lignin (Zabalgoeazcoa *et al.* 2006; Rasmussen *et al.* 2008; Soto-Barajas *et al.* 2016),

*Schedonorus arundinaceus* plants do not exhibit nutritional differences accounted for the endophyte (Johnson *et al.* 2012).

The preference documented, for the first time in domestic herbivores, in this study suggests that the endophyte may deter herbivores even when alkaloids do not cause an apparent toxic effect like those reported for similar symbiosis (e.g. *S. arundinaceus*–*Epichloë coenophialum*, Schardl *et al.* 2013). This reduced preference of domestic herbivores for infected plants could play an important role in the maintenance of the endophyte (Hernández Agramonte & Semmartin 2016) as domestic grazing represents a major pressure in temperate systems (Oesterheld *et al.* 1992). Mechanisms behind this effect are still vague and might be related to both alkaloid-related and alkaloid-independent mechanisms like differences in the odour print (volatiles) (García Parisi *et al.* 2014), to an increase in silica content (Huitu *et al.* 2014) or to deep metabolic changes (Rasmussen *et al.* 2009). Furthermore, as the endophyte promotes the secondary metabolism of the plant (e.g. synthesis of lignin, flavonoids and anthocyanins) (Rasmussen *et al.* 2009; Dupont *et al.* 2015), these mechanisms might also be responsible for these plants' reduced preference as they can reduce nutritional quality or alter tissue coloration.

The promotion of volatile compound emissions by epichloid endophytes has been proposed to protect plants from herbivores (García Parisi *et al.* 2014; Li *et al.* 2014). However, the empirical evidence is limited to properly evaluate protective volatiles' extent and ubiquity. The composition and intensity of volatiles vary between plants with contrasting endophyte levels, but their magnitude and direction depend on plant species (García Parisi *et al.* 2014; Li *et al.* 2014). In our study, we did not find evidence of dissuasive effects of volatiles produced by the symbiosis on domestic herbivores, which suggests the absence

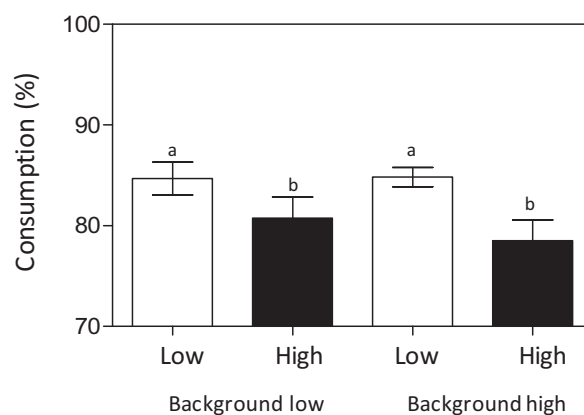


**Fig. 2.** Steer consumption of *Lolium multiflorum* biomass from two populations (Lucero and Feliciano) with low (~0%) and high (~80%) endophyte infection frequencies, at vegetative and reproductive stages. Consumption was estimated as the fraction of biomass removed from the total biomass offered in trays. Different letters indicate significant differences ( $P < 0.005$ ).

of a different volatile imprint or, at least, the absence of a signal, strong enough in the background forage to counteract volatiles emitted by the offered forage.

Our results also revealed a plant intraspecific effect on preference that was independent of the endophyte status and phenological stage. At both stages (vegetative and reproductive) of Experiment 2, steers consistently consumed more Lucero population than Feliciano. Even though intraspecific variation of forage nutritional traits (e.g. fibre fractions) is documented to affect animal preference or voluntary intake (Shewmaker *et al.* 1997; Burns *et al.* 2001), our results suggest that variations other than nutritional traits affect preference. Furthermore, the lack of a significant reduction of preference in Feliciano endophytic plants at the vegetative stage supports the existence of complex interactions between the host and the endophyte (Rasmussen *et al.* 2009; Johnson *et al.* 2012; Helander *et al.* 2016).

In conclusion, our results add new evidence about the importance of this fungal endophyte–grass association in domestic herbivores. Specifically, we found



**Fig. 3.** Animal consumption of *Lolium multiflorum* biomass with low and high endophyte infection offered in mesh baskets which were immersed in bigger trays containing a background of the same grass with low or high endophyte infection levels. Background biomass was inaccessible for the steers, although it could be smelt. Consumption was estimated as the fraction of biomass removed from the basket. Different letters indicate significant differences ( $P < 0.05$ ).

that: (i) the endophyte reduced the preference of Aberdeen Angus steers, (ii) this effect was consistent in two *L. multiflorum* populations with different intrinsic preference, (iii) the preference of the steers was not related to morphological, nutritional or olfactory traits typically influencing palatability. Further research to assess the potential effect of the endophyte on voluntary intake is necessary to evaluate whether the lower preference of endophytic plants would reduce animal production and to disentangle the mechanisms underlying the differences in animal preference.

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## REFERENCES

- Bacon C. W. & White J. F. J. (1994) *Biotechnology of Endophytic Fungi of Grasses*. CRC Press, Boca Raton, FL.  
 Bailey D. W., Gross J. E., Laca E. A. *et al.* (1996) Mechanisms that result in large herbivore grazing distribution patterns. *J. Range Manage.* **49**, 386–400.  
 Beltramino H. J., Medvescigh J. C., De Battista J. & Costa M. (2005) Efecto del hongo endófito *Neotyphodium occultans*

- en la producción de semillas de raigrás anual. *Rev. Cient. Agropecu.* **9**, 25–31.
- Burns J. C., Fisher D. S. & Mayland H. F. (2001) Preference by sheep and goats among hay of eight tall fescue cultivars. *J. Anim. Sci.* **79**, 213–24.
- Cid M. S. & Brizuela M. A. (1994) Respuesta de bovinos y ovinos en pastoreo mixto en relación a la estructura de la pastura y la selectividad animal. *Rev. Arg. Prod. Anim.* **14**, 161–73.
- Clay K. (1996) Interactions among fungal endophytes, grasses and herbivores. *Res. Popul. Ecol.* **38**, 191–201.
- Curran J., Delaby L., Kennedy E., Murphy J. P., Boland T. M. & O'Donovan M. (2010) Sward characteristics, grass dry matter intake and milk production performance are affected by pre-grazing herbage mass and pasture allowance. *Livest. Sci.* **127**, 144–54.
- Dupont P. Y., Eaton C. J., Wargent J. J. *et al.* (2015) Fungal endophyte infection of ryegrass reprograms host metabolism and alters development. *New Phytol.* **208**, 1227–40.
- García Parisi P. A., Grimoldi A. A. & Omacini M. (2014) Endophytic fungi of grasses protect other plants from aphid herbivory. *Fungal Ecol.* **9**, 61–4.
- Gundel P. E., Garibaldi L. A., Tognetti P. M., Aragón R., Ghersa C. M. & Omacini M. (2009) Imperfect vertical transmission of the endophyte *Neotyphodium* in exotic grasses in grasslands of the Flooding Pampa. *Microb. Ecol.* **57**, 740–8.
- Helander M., Phillips T., Faeth S. H. *et al.* (2016) Alkaloid quantities in endophyte-infected tall fescue are affected by the plant-fungus combination and environment. *J. Chem. Ecol.* **42**, 1–9.
- Hernández Agramonte I. M. & Semmartin M. (2016) The role of grazing intensity and preference on grass-fungal endophyte symbiosis in a Patagonian steppe. *J. Arid Environ.* **134**, 122–4.
- Howard M. D., Muntifering R. B., Bradley N. W., Mitchell G. E. & Lowry S. R. (1992) Voluntary intake and ingestive behavior of steers grazing Johnstone or endophyte-infected Kentucky-31 tall fescue. *J. Anim. Sci.* **70**, 1227–37.
- Huitu O., Forbes K. M., Helander M. *et al.* (2014) Silicon, endophytes and secondary metabolites as grass defenses against mammalian herbivores. *Front. Plant Sci.* **5**, 478.
- Jacobo E. J., Rodríguez A. M., Rossi J. L., Salgado L. P. & Deregibus V. A. (2000) Rotational stocking and production of Italian ryegrass on Argentinean rangelands. *J. Range Manage.* **53**, 483–8.
- Johnson J. M., Aiken G. E., Phillips T. D., Barrett M., Klotz J. L. & Schrick F. N. (2012) Steer and pasture responses for novel endophyte tall fescue developed for the upper transition zone. *J. Anim. Sci.* **90**, 2402–9.
- Jones T. A., Ralphs M. H., Gardner D. R. & Chatterton N. J. (2000) Cattle prefer endophyte-free robust needlegrass. *J. Range Manage.* **53**, 427–31.
- Jung H. G., Mertens D. R. & Payne A. J. (1997) Correlation of acid detergent lignin and Klason lignin with digestibility of forage dry matter and neutral detergent fiber. *J. Dairy Sci.* **80**, 1622–8.
- Laca E. A. (1998) Spatial memory and food searching mechanisms of cattle. *J. Range Manage.* **5**, 370–8.
- Leuchtman A. (1992) Systematics, distribution, and host specificity of grass endophytes. *Nat. Toxins* **1**, 150–62.
- Leuchtman A., Schmidt D. & Bush L. P. (2000) Different levels of protective alkaloids in grasses with stroma-forming and seed-transmitted *Epichloë/Neotyphodium* endophytes. *J. Chem. Ecol.* **26**, 1025–136.
- Li T., Blande J. D., Gundel P. E., Helander M. & Saikkonen K. (2014) *Epichloë* endophytes alter inducible indirect defenses in host grasses. *PLoS One* **9**, e101331.
- Macono B., Sollenberger L. E., Moore J. E., Staples C. R., Fike J. H. & Portier K. M. (2003) Comparison of three techniques for estimating the forage intake of lactating dairy cows on pasture. *J. Anim. Sci.* **81**, 2357–66.
- Miranda M. I., Omacini M. & Chaneton E. J. (2011) Environmental context of endophyte symbioses: interacting effects of water stress and insect herbivory. *Int. J. Plant Sci.* **172**, 499–508.
- Moore J. R., Pratley J. E., Mace W. J. & Weston L. A. (2015) Variation in alkaloid production from genetically diverse *Lolium* accessions infected with *Epichloë* species. *J. Agric. Food Chem.* **63**, 10355–65.
- O'Donovan M. & Delaby L. (2008) Sward characteristics, grass dry matter intake and milk production performance as affected by timing of spring grazing and subsequent stocking rate. *Livest. Sci.* **115**, 158–68.
- Oesterheld M., Sala O. E. & McNaughton S. J. (1992) Effect of animal husbandry on herbivore-carrying capacity at a regional scale. *Nature* **356**, 234.
- Oliveira J. A., González E., Alonso P. C. & Costal L. (2004) Effects of endophyte infection on dry matter yield, persistence and nutritive value of perennial ryegrass in Galicia (north-west Spain). *Span. J. Agric. Res.* **4**, 558–63.
- Omacini M., Chaneton E. J., Ghersa C. M. & Müller C. B. (2001) Symbiotic fungal endophytes impact on insect host-parasite interaction webs. *Nature* **409**, 78–81.
- Porter J. K. & Thompson F. N. (1992) Effects of fescue toxicosis on reproduction in livestock. *J. Anim. Sci.* **70**, 1594–603.
- R Core Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.
- Rasmussen S., Parsons A. J., Fraser K., Xue H. & Newman J. A. (2008) Metabolic profiles of *Lolium perenne* are differentially affected by nitrogen supply, carbohydrate content, and fungal endophyte infection. *Plant Physiol.* **146**, 1440–53.
- Rasmussen S., Parsons A. J. & Newman J. A. (2009) Metabolomics analysis of the *Lolium perenne-Neotyphodium lolii* symbiosis: more than just alkaloids? *Phytochem. Rev.* **8**, 535–50.
- Saikkonen K., Gundel P. E. & Helander M. (2013) Chemical ecology mediated by fungal endophytes in grasses. *J. Chem. Ecol.* **39**, 962–8.
- Schardl C. L., Young C. A., Faulkner J. R., Florea S. & Pan J. (2012) Chemotypic diversity of *Epichloë*, fungal symbionts of grasses. *Fungal Ecol.* **5**, 331–44.
- Schardl C. L., Young C. A., Pan J. *et al.* (2013) Currencies of mutualisms: sources of alkaloid genes in vertically transmitted epichloae. *Toxins* **5**, 1064–88.
- Senft R. L., Coughenour M. B., Bailey D. W., Rittenhouse L. R., Sala O. E. & Swift D. M. (1987) Large herbivore foraging and ecological hierarchies. *Bioscience* **37**, 789–99.
- Shewmaker G. E., Mayland H. F. & Hansen S. B. (1997) Cattle grazing preference among eight endophyte-free tall fescue cultivars. *Agron. J.* **89**, 695–701.
- Smit H. J., Tamminga S. & Elgersma A. (2006) Dairy cattle grazing preference among six cultivars of perennial ryegrass. *Agron. J.* **98**, 1213–20.

- Solomon J. K. Q., Macoon B. L., David J. V., Rhonda C. & Ward S. (2014) Cattle grazing preference among tetraploid and diploid annual ryegrass cultivars. *Crop Sci.* **54**, 430–8.
- Soriano A. (1991) Río de la Plata grasslands. In: *Ecosystems of the World. Natural Grasslands. Introduction and Western Hemisphere* (ed. R. T. Coupland) pp. 367–407. Elsevier, Amsterdam.
- Soto-Barajas M. C., Zabalgoceazcoa I., Gómez-Fuertes J., Gónzales-Blanco V. & Vázquez de Aldana B. R. (2016) *Epichloë* endophytes affect the nutrient and fiber content of *Lolium perenne* regardless of plant genotype. *Plant Soil* **405**, 265–77.
- Sugawara K., Inoue T., Yamashita M. & Ohkubo H. (2006) Distribution of the endophytic fungus, *Neotyphodium occultans* in naturalized Italian ryegrass in western Japan and its production of bioactive alkaloids known to repel insect pests. *Grassland Sci.* **52**, 147–54.
- TePaske M. R., Powell R. G. & Clement S. L. (1993) Analyses of selected endophyte-infected grasses for the presence of loline-type and ergot-type alkaloids. *J. Agric. Food Chem.* **4**, 2299–303.
- Uchitel A., Omacini M. & Chaneton E. J. (2011) Inherited fungal symbionts enhance establishment of an invasive annual grass across successional habitats. *Oecologia* **165**, 465–75.
- Ueno A., Gundel P. E., Omacini M., Ghersa C. M., Bush L. P. & Martínez-Ghersa M. A. (2016) Mutualism effectiveness of a fungal endophyte in an annual grass is impaired by ozone. *Funct. Ecol.* **30**, 226–34.
- Van Soest P. J., Robertson J. B. & Lewis B. A. (1991) Carbohydrate methodology, metabolism, and nutritional implications in dairy cattle. *J. Dairy Sci.* **74**, 3583–97.
- Young C., Hume D. E. & McCulley R. L. (2013) Fungal endophytes of tall fescue and perennial ryegrass: pasture friend or foe? *J. Anim. Sci.* **91**, 2379–94.
- Zabalgoceazcoa I., García Ciudad A., Vázquez de Aldana B. R. & García Criado B. (2006) Effects of the infection by the fungal endophyte *Epichloë festucae* in the growth and nutrient content of *Festuca rubra*. *Eur. J. Agron.* **24**, 374–84.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

- Appendix S1.** Picture and diagram of Experiment 1.  
**Appendix S2.** Tray and perforated basket used in Experiment 3, offer + background.