Fungal endophytes modify plant–herbivore interactions by producing toxic alkaloids that deter herbivory. However, studies have neglected the direct effects herbivores may have on endophytes. Antifungal properties and signaling effectors in herbivore saliva suggest that evolutionary pressures may select for animals that mitigate the effects of endophyte-produced alkaloids. Here, we tested whether saliva of moose (*Alces alces*) and European reindeer (*Rangifer tarandus*) reduced hyphal elongation and production of ergot alkaloids by the foliar endophyte *Epichloë festucae* associated with the globally distributed red fescue *Festuca rubra*. Both moose and reindeer saliva reduced the growth of isolated endophyte hyphae when compared with a treatment of distilled water. Induction of the highly toxic alkaloid ergovaline was also inhibited in plants from the core of *F. rubra*'s distribution when treated with moose saliva following simulated grazing. In genotypes from the southern limit of the species’ distribution, ergovaline was constitutively expressed, as predicted where growth is environmentally limited. Our results now present the first evidence, to our knowledge, that ungulate saliva can combat plant defences produced by a grass–endophyte mutualism.

1. Introduction

Chemical defences that limit the damage plants sustain from herbivores should be under strong positive selection. A common way that plants obtain these compounds is through symbioses with endophytic fungi, many of which produce herbivore-deterring alkaloid compounds [1]. Some of these endophytes are systemic, including *epichloae* found in many Pooidae [2]. Typically growing intercellularly throughout above-ground plant tissue, fungal endophytes of grasses establish symbiotic relationships with their hosts ranging from parasitic and antagonistic to mutualistic [1]. In the mutualistic relationship, the host provides habitat and nutrients for the endophyte [1], while the fungus synthesizes anti-herbivore toxic alkaloids [3,4] and confers tolerance to stresses such as drought [5]. While most studies have focused on grass–endophyte and grass–herbivore interactions, direct effects of herbivores on endophytes have been ignored. Large herbivores feed by biting down on grasses to their basal meristems, exposing the region where endophytes reside. This raises the possibility that herbivores can combat defensive mutualisms between plants and endophytes.

Evolutionary pressures should favour herbivores that mitigate the effects of secondary metabolites produced by foliar endophytes. Limited evidence of antifungal activity in herbivore saliva, primarily through lactoferrin peptides and lysozymes [6,7], suggests that this may arise if compounds reduce fungal growth and subsequently lower alkaloid production. Defences may also be mitigated if salivary enzymes directly inhibit signals that activate induced wound responses [8–10]. Although fungal inhibitors and disruptors of defence signalling may have also arisen independently of endophytes [9,11], this does not detract from their potential impact on plant–herbivore interactions.
2. Material and methods

(a) Saliva collection

Moose (Alces alces) and European reindeer (Rangifer tarandus tarandus) saliva were collected from mouths of anaesthetized adult males during medical procedures at the Toronto Zoo and Zoo Sauvage de St-Félicien (Canada) and immediately frozen (−20°C). Prior to application of saliva onto endophyte colonies, samples were centrifuged to remove debris and the supernatant was filtered through 0.20 µm. Distilled water was similarly treated. No filtration was performed when saliva and water were directly applied to plants.

(b) Endophyte growth

We tested whether application of reindeer and moose saliva reduced in vitro growth of Epichloë festucae colonies relative to a control treatment of distilled water. We isolated E. festucae from F. rubra, grown from natural populations in northern Sweden [14], by cutting the base of the youngest fully extended leaf in each plant. Sections were surface sterilized and embedded in potato dextrose medium with 60 mg L⁻¹ of penicillin G and streptomycin. Plates were stored in the dark at 24°C for two weeks. We then assigned 22–25 colonies per treatment, with five to nine replicates of three plant genotypes, and recorded the initial radius of each plate. Saliva was applied and we measured colony radius relative to the baseline every 12 h over 2 days, repeating this experiment with 8 h measurements.

(c) Alkaloid production

We simulated herbivory and saliva application on 32 F. rubra plants infected with E. festucae to test whether application of saliva bears ecological significance for the plant–endophyte symbiosis. We used two simulated herbivory treatments consisting of fortnightly and monthly clipping (n = 12 for each) and a control of no herbivory (n = 8). Clipped plants received either distilled water or moose saliva. The 32 plants represented 22 isolates (putatively different genets), originating from either the core of F. rubra’s distribution in Europe, mainly Sweden [14], or its natural southern limit in southern Ontario, Canada. Summer water deficit along the drier and warmer edge of F. rubra’s distribution probably limits this species’ growth (electronic supplementary material). Plants were harvested after growing in a greenhouse for eight weeks and measured for ergovaline concentrations, the primary ergot alkaloid synthesized by E. festucae, using high-performance liquid chromatography at the Endophyte Service Laboratory, Oregon State University.

(d) Analyses

We fitted linear models within a Bayesian framework to test whether saliva application reduced: (i) fungal growth in situ, and (ii) alkaloid production in planta. Saliva and cutting treatment (for (ii)) were model predictors, and we allowed the effect of saliva to vary among plant genotypes/isolates (for (i) and (ii)), cutting treatment (for (ii)) and plant origin (for (ii)). For growth (i.e. (i)), we assumed that change in colony radius \( \frac{dR}{dt} \) was a sum of these effects and modelled the integrated form of this function, also allowing treatment effects to vary among time periods, such as if saliva only reduced growth immediately after application. All parameters were estimated using Markov chain Monte Carlo sampling, and we inferred support for hypotheses if 95% credible intervals (CIs) for effects of saliva and alkaloid production were negative and excluded zero (see the electronic supplementary material).

3. Results

Both moose and reindeer saliva reduced hyphal growth (95% CIs for all: \(-0.022 \text{ to } -0.001\), electronic supplementary material, table S1). In our first experiment, we found moose saliva reduced in situ growth between 12 and 36 h after application, while colonies treated with reindeer saliva grew more slowly 12–24 h after application (figure 1). In the second trial...
with higher temporal resolution, colonies in the moose and reindeer treatments showed similar reductions in growth rate 8–32 and 16–24 h after saliva application, respectively (see CIs in the electronic supplementary material, table S1).

Compared with distilled water, moose saliva applied after experimental clipping reduced ergovaline concentrations within European plants by 41–70% (95% CI: −1.22 to −0.52). The magnitude of the reduction was similar in the low and high herbivory treatments (figure 2a; electronic supplementary material, table S2). In the absence of saliva, defoliation actually induced a defensive response within European plants, with ergovaline concentrations roughly doubling relative to uncut controls (figure 2a). This clearly demonstrates that saliva uniquely alters the plant metabolite profile.

Ergovaline was consistently expressed when we considered North American plants from the margins of *F. rubra*’s native distribution. Concentrations were higher than moose-treated European plants by at least 39% and were not reduced by saliva or elevated by cutting-only relative to uncut controls (figure 2b). Despite the consistency, however, concentrations in water-treated plants were always lower than those of the same cutting frequency from the more favourable European populations (95% CIs for difference between treatments always less than 0).

4. Discussion

We found, to our knowledge, the first evidence that herbivores can reduce the effectiveness of grass–endophyte mutualisms in combating herbivory. Herbivore saliva reduced fungal growth, but in mature plants with established hyphal networks, toxicity was only reduced where defence responses were induced. This suggests that herbivore saliva primarily interferes with induced defences, though slowing early hyphal elongation in infected plants may also be functionally important. We are unaware of previous studies demonstrating that saliva reduces the growth of foliar endophytes, despite considerable evidence for its antifungal properties [6,7,11].

Our study suggests that herbivores may have evolved strategies to reduce the high fitness cost imposed by alkaloid production. The endophyte we study probably originated with Pooidae 30–40 Myr ago [2] and would have accompanied the transition of grasses to a dominant vegetation type ca 10 Myr thereafter [15]. Within another few million years, large animals began diversifying upon these new grasslands habitats [15] and would have placed selective pressures upon endophytes to maximize persistence of their host, such as by deterring herbivores with toxic alkaloids. As other feeding niches, such as forest browsers, would have been occupied, grassland herbivores would have required a way to either detoxify their forage [16], and/or directly interfere with alkaloid production. This may be especially relevant for animals that repeatedly visit the same plants. Data from mammals that have avoided exposure to endophytes would further strengthen tests of an evolved response and help reject the hypothesis that salivary compounds only impact plant defences by coincidence.

Defensive strategies, and their subsequent response to saliva, differed according to the environment from which plants were sourced. Ergovaline was always elevated in plants from southern Ontario, regardless of prior cutting or saliva application, but was inducible in European plants in the absence of saliva. According to widely supported plant defence theories (i.e. resource availability hypothesis and protein competition model), the discrepancy between populations may arise because constitutive defences are evolutionarily favoured in marginal environments [17,18]. Plants near *F. rubra*’s southern limit may have therefore evolved permanent upregulation as an adaptation for water-stressed conditions. By contrast, populations adapted to more favourable sites invest more in growth, diverting resources towards defence production only when induced by damage [19]. Although the same biochemical pathway underlies alkaloid synthesis in both populations [12], the inducible response in European plants would require a signal to upregulate alkaloid production and this additional step may be altered by salivary components [8–10].

Differences in defensive strategies associated with our two study populations probably persisted when exposed to common conditions in the glasshouse because they have evolved under strong genetic control. Fine et al. [20] found that investment in plant defences differed between trees growing on white-stand and clay forests in Amazonia and could not be altered with reciprocal transplants, suggesting that the observed differences were genetic adaptations to habitat. Genetic adaptation to local conditions has also been demonstrated for fungal symbionts of plants [21]. More work is now needed to determine how fungal genotype, plant genotype and their interaction drive defensive strategies of host and endophyte populations.

In conclusion, our study shows that chemical inhibitors of inducible defences exist in the saliva of large mammalian

**Figure 2.** Mean concentrations of ergovaline (ppm) ± 95% CIs in (a) European (mainly Sweden) and (b) southern Ontario-derived populations of *F. rubra* infected with *E. festucae* and exposed to simulated herbivory and saliva. Lines between points plotted only to highlight interactions.
herbivores. This overturns the perception that large mammalian herbivores simply modify their foraging patterns or develop coping mechanisms for poor-quality forage [16], rather than actively ‘combating’ plant defences. Although the exact mechanism of ergovaline suppression is unknown in our study, we suggest that salivary secretions are more important in large mammals than previously appreciated.

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