Epichloë endophytes of *Poa alsodes* employ alternative mechanisms for host defense: insecticidal versus deterrence

By: Tatsiana Shymanovich, Ashleigh M. Musso, Nadja B. Cech, and Stanley H. Faeth

Shymanovich, T., Musso, A.M., Cech, N.B. & Faeth, S.G. *Epichloë* endophytes of *Poa alsodes* employ alternative mechanisms for host defense: insecticidal versus deterrence. *Arthropod-Plant Interactions* (2019) 13(1): 79-90. https://doi.org/10.1007/s11829-018-9635-8

Abstract:

Some cool-season pooid grasses partner with symbiotic fungal endophytes in the *Epichloë* genus for defense against insect herbivores via fungal alkaloids. *Poa alsodes*, North American woodland grass, independently hosts two species of *Epichloë* that vary by produced alkaloids. *E. alsodes* produces insecticidal *N*-acetylnorloline. *E. schardlii* var. *pennsylvanica* (*E. schardlii* hereafter) has the gene for peramine, an insect-deterring alkaloid, production, but peramine was not detected. We tested the effects of the two endophytes on survival, feeding preference, and plant damage by the generalist herbivore, *Spodoptera frugiperda*. No larvae survived when feeding on plants harboring *E. alsodes*. In contrast, survival was only slightly reduced by plants harboring *E. schardlii*. However, larvae that fed on *E. schardlii* infected plants experienced delayed development and reduced pupal mass. Uninfected plants and plants infected with *E. schardlii* were damaged severely when single larvae fed upon them, whereas larvae fed negligibly on plants infected with *E. alsodes*. Preference did not match performance. Larvae strongly avoided feeding on *E. schardlii* but not *E. alsodes*-infected leaves where survival was zero. When *E. schardlii* was experimentally removed, larval leaf choices suggested that this endophyte is responsible for deterrence. High levels of *N*-acetylnorloline were detected from *E. alsodes* infected plants. Peramine was not detected in the experimental plants harboring *E. schardlii*, so it remains unclear what mechanisms caused avoidance and developmental delays. The two endophytes may protect their common host in different ways: *E. alsodes* by larval mortality and *E. schardlii* by deterring feeding and negative effects on development.

Keywords: Endophytic alkaloids | Larval mortality | Larval performance | Feeding preference | *N*-acetylnorloline

Article:

Introduction

Co-evolution between plants and their insect herbivores has resulted in multiple plant defenses against attack and, in turn, counter defenses by herbivores (Agrawal 2011; Mello and Silva-Filho 2002; Schardl and Chen 2001). Most plants have evolved a wide spectrum of allelochemicals that act defensively against insect herbivores by either (1) deterring herbivores from feeding or (2) reducing growth and survival if feeding occurs (e.g., Berenbaum 1995; Bowers 1990). In turn, some insect herbivores may evolve to use plant allelochemicals as attractants or evolve mechanisms to avoid or de-toxify harmful allelochemicals, or even sequester plant toxins for defense against their own natural enemies (e.g., Faeth and Saari 2012). Some plants, however, instead of making their own allelochemicals rely on those produced by symbiotic microbial partners for chemical defense against insect and vertebrate herbivores. Notably, some cool-season grasses in the subfamily Pooideae harbor *Epichloë* species of endophytic fungi that produce secondary metabolites, alkaloids, which provide anti-herbivory protection for their hosts (Clay and Schardl 2002; Panaccione et al. 2014; Schardl 2010). Alkaloid compounds affect neuroreceptors, causing various neurotoxic effects on animals and may also deter herbivores from feeding (Schardl and Chen 2001). In turn, insect herbivores may evolve avoidance, resistance, or even the ability to sequester fungal alkaloids as their own defense against their predators and parasites (Cheplick and Faeth 2009; Faeth and Saari 2012).

Epichloë species may produce one or more alkaloids from within four major classes: ergot alkaloids, pyrrolizidines (lolines), indole-diterpenes (lolitrems), and pyrrolopyrazine (with a single compound, peramine). Individual alkaloids within these classes often have specific toxic effects depending on the type of herbivore (i.e., vertebrate vs. invertebrate). Loline alkaloids and peramine are well-known for their insecticidal or insect deterring effects, while lolitrems and ergot alkaloids often have potent toxic effects on vertebrates (Siegel et al. 1990; Wilkinson et al. 2000). However, some ergot alkaloids, such as ergopeptine, ergovaline, and ergonovine may also have insecticidal effects (Panaccione et al. 2014; Potter et al. 2008; Schardl et al. 2012; Shymanovich et al. 2015). Recent molecular genetics and chemoprofiling studies show that each endophyte species may produce a unique cocktail of different alkaloids, often from more than one group, which may act simultaneously and even synergistically with each other (Schardl et al. 2013a, b). Thus, individual plants within a given grass species that host different endophyte species may have dissimilar alkaloid compounds that confer diverse levels and types of protection against herbivores (Charlton et al. 2014; Leuchtmann et al. 2014; Oberhofer and Leuchtmann 2012; Sullivan and Faeth 2008). Recent studies show that many native grass species harbor more than one *Epichloë* species, some even within the same population, but normally have only one infection per plant (e.g., Iannone et al. 2012, Oberhofer and Leuchtmann 2014; Saari and Faeth 2012).

Poa alsodes A. Gray (grove bluegrass) is a woodland grass species native to northeastern North America that harbors two distinct *Epichloë* species with different alkaloid profiles (Shymanovich.et al. 2017). *Epichloë alsodes*, a newly described species, is widely distributed among *P. alsodes* populations from North Carolina to the Canadian border and has genes for the loline, ergot alkaloid, and peramine biosynthetic pathways. However, only the loline pathway is functional and produces the loline alkaloid *N*-acetylnorloline. The ergot alkaloid pathway is blocked at the first step, so chanoclavine I, the first intermediate product in the pathway, is not produced. The peramine gene is present in *E. alsodes* but is non-functional due to mutations. The

second endophyte species, *E. schardlii*, has a much more limited distribution. *E. schardlii* is found only in a few *P. alsodes* populations in Pennsylvania, and it was described as a subspecies, *E. schardlii* var. *pennsylvanica*, to distinguish it from another grass host, *Cinna arundinacea*, isolate (Ghimire et al. 2011; Shymanovich et al. 2017). *E. schardlii* has only the peramine gene and no other alkaloid genes. Despite the presence of an apparently functional gene, and gene mutations have not yet been found, but peramine has not been detected in *P. alsodes* samples infected by *E. schardlii* using LC-MS (Shymanovich et al. 2017).

The goal of this study was to test if the two endophyte species infecting *P. alsodes* provide protection against a generalist insect herbivore. We used the generalist pest, *S. frugiperda* (fall armyworm) in preference and performance assays with grasses infected with one of the two endophyte species or plants that were endophyte free. The fall armyworm has been used extensively as a bioassay herbivore in experiments to test for the protective effects of endophytes (e.g., Ball et al. 2006; Clay and Cheplick 1989; Crawford et al. 2010; Hardy et al. 1985). Based on our previous alkaloid analyses, we predicted that *E. alsodes* endophyte would provide insecticidal protection through the production of *N*-acetylnorloline, but that the *E. schardlii* endophyte would have no effects on larval survival and development because it does not appear to produce alkaloids.

Methods

Host plants and endophytes

To minimize the effects of variation in plant genotype, *P. alsodes* seeds used for the experiments were collected from five natural populations in Pennsylvania, USA, in 2012–2013 (Shymanovich et al. 2017). Maternal plants were previously tested by PCR genotyping to detect *Epichloë* infection (Shymanovich et al. 2017). Eleven naturally uninfected (hereafter E-) maternal plants from four populations, 11 maternal plants from three populations infected with *E. alsodes*, and 13 maternal plants from three populations infected with *E. schardlii* were used as a seed source for each infection group, respectively. Plants were grown from seeds in 3 dL pots with Metro mix-360 (Sun Gro Horticulture Canada Ltd) in the greenhouse with natural light at 25 °C/20 °C day/night temperatures and were watered/fertilized [20: 20: 20 (N: P: K), with micronutrients] (Southern Agricultural Insecticides, Inc.) twice a week. When plants were 3 months old, they were tested with a Phytoscreen Immunobloting Kit (Agrinostics, GA) to confirm infection status and then were transferred to a growth-chamber.

Insect herbivore

Spodoptera frugiperda (Lepidoptera: Noctuidae) is a generalist herbivore pest species that feeds on many different host plants but prefers grasses (Sparks 1979) and has been observed feeding on *P. alsodes* in the field (Shymanovich, personal observation). We purchased eggs (lot #I_111714Sf) from Bio-Serv Company (Flemington, NJ, transported under USDA permit #P526P-14-03123). This source population of armyworms was originally collected from the continental US and maintained in the lab for 16 years by Bio-Serv. Egg clutches were placed into a tray with standard lepidopteran diet (Bio-Serv Company) in a 25 °C chamber to hatch.

Larval performance experiment

To test the effects of infection by *E. alsodes* and *E. schardlii*, we performed a laboratory feeding experiment. First instar, neonatal larvae were individually enclosed in plastic containers (Plant Con, MP Biomedicals, LLC, Solon, OH) with wet paper towels and were fed one of three diets of leaf clippings from (1) naturally uninfected plants (36 total), (2) plants infected with *E. alsodes* (37 total), and (3) plants infected with *E. schardlii* (51 total). Twenty larvae were randomly assigned to each diet and received *ad libitum* leaf clippings mixed from multiple plants within each plant type to randomize effects of plant genotype. Containers were placed in a growth-chamber with no light and 25 °C (similar to López-Edwards et al. 1999). Larval survival, larval mass, and plant biomass consumed were recorded every 3 days. To estimate dry plant biomass consumed, at each feeding, wet leaf material provided was recorded, and after each feeding, the remaining leaf biomass air-dried and then weighed. A portion of wet material was weighed, air-dried and re-weighed to find the wet/dry biomass coefficient. During late larval stages, we monitored larvae daily for pupation and days to adult emergence and weighed pupae. For each larva that survived to pupal stage, sex was determined with a microscope using the following traits: males have two protuberances in a middle of the fifth segment; females have a small line close to the suture between the fourth and fifth segments. Leaf clipping samples from each feeding (*E. alsodes*-four samples, *E. schardlii* and E− groups-six samples each) were freeze dried, extracted, and then analyzed for *N*-acetylnorloline, chanoclavine I, and peramine alkaloids with LC-MS as described in Shymanovich et al. (2015, 2017).

Individual plant damage experiment

To test if infection by the two *Epichloë* species protects their host grasses from herbivory, we conducted a laboratory experiment where the amount of plant biomass consumed by armyworms was compared among grasses infected with the two endophytes and uninfected plants. Before the experiment, each individual plant dry leaf biomass was estimated. To estimate biomass, the total leaf length was measured for each plant. The mean g/cm coefficient was estimated from nine plants from the three plant groups by measuring total leaf length, cutting, and then drying and weighing them. Single 1-day old, first instar larvae were placed on individual live plants and enclosed with clear plastic cups that had the ends removed and covered with fine mesh cloth for air exchange. There were 31 replicates for each of the three plant groups. Enclosures were placed into a growth-chamber (Adaptis A1000, Controlled Environments Limited, Manitoba, Canada) with 15/9 h day/night period at 26 °C. Plants were watered as needed from the top of enclosures so as to not disturb the larvae. When pupation started, enclosures were checked daily. If a pupa was formed, then the date of pupation, pupal mass, and sex (determined as described above) were recorded, and remaining plant leaf biomass was cut and freeze dried for measurements. Because plants continued to grow during the experiment, we estimated additional biomass due to growth and added this biomass to the initial biomass estimated before the experiment. To estimate additional growth, a linear regression model was used for 40 undamaged plants from all three infection groups (adjusted R-squared = 0.6334, F-statistic = 68.39 on 1 and 38 DF, P-value < 0.001). The coefficients obtained from this model were used for the linear formula: *Final potential biomass* = − 0.1657 + 1.777* *Initial biomass*. Consumed dry biomass was calculated as a difference between final potential dry biomass and dry biomass remaining at pupation. Additionally, visual estimates of individual plants' damage were recorded. To validate the

biomass estimates, visually estimated damage to the plants and mathematically estimated values were used for the linear regression model and showed highly significant correlations (adjusted R-squared = 0.7044, F-statistic = 220.3 on 1 and 91 DF, P-value < 0.001). Leaf samples from 10 random individual plants from each of the infection group were collected from three leaves per plant, clipped, mixed, and freeze-dried. Later samples were extracted and analyzed by LC-MS as described in Shymanovich et al. (2015, 2017). *N*-acetylnorloline levels were measured via the published methods, and samples were also checked for the presence of peramine and chanoclavine I using positive control plant samples as described in Shymanovich et al. (2017).

Larval feeding preference on leaves with natural infections

To test whether armyworm larvae prefer or avoid plants based upon endophyte infection generally and *Epichloë* species specifically, we conducted two laboratory choice experiments using different aged larvae. In each experiment, 30 single larvae were enclosed in containers with wet paper towels with leaf pieces from the three infection groups (*E. alsodes, E. schardlii*, and E− plants) similarly as described in Shymanovich and Faeth (2018). In each trial, two 5 cm long pieces of leaves from different plants from each of three infection groups were used (six pieces total). The pair of 5 cm pieces from each infection group were placed in random order equidistant from each other and equidistant from the center of the container. Containers were placed into the growth-chamber at 26 °C and 15/9 h day/night light regiments. In the first experiment, individual 2-day-old larvae were placed in the center of the container and allowed to feed for 48 h. We then estimated how much biomass from each infection group was consumed. Because two 5 cm pieces (or 10 cm total) of each infection type were presented to larvae, we determined the percent consumed by dividing the total length eaten in each infection group by 10 and multiplying by 100. In the second similar experiment, we used older, 5-day-old larvae. These larvae were allowed to feed for 24 h in the same experimental setup. We then calculated the percent leaves consumed for each infection group (as above). For statistical analysis, only data from those larvae that survived to the end of the treatment were used. This resulted in a total of 29 replicates for the 2-day-old larvae experiment and 12 replicates for 5-day-old larvae experiment.

Larval feeding preference on infected and naturally and experimentally uninfected leaves (manipulated infections)

To separate effects of the endophyte infection and plant genotype on larval leaf type preferences, we heat treated (4 h soaking in 1.5 ml tubes, 12 min in water bath at 55 °C) half of the seeds infected with *E. scharldlii* to experimentally remove the endophyte. These seeds as well as naturally uninfected (E−) and untreated seeds with *E. schardlii* infections were germinated. Four-month-old plants were tested for infection status with a Phytoscreen Immunobloting Kit (Agrinostics, GA). Infection status for all E− and *E. schardlii* infected plants was confirmed. Based on imminoblot results, heat treated plants were separated into two groups—those that were heat treated but remained infected (HeatSch+) and those that were heat treated with the endophyte removed (HeatSch-). To confirm that the endophyte was indeed removed from HeatSch-plants, we extracted DNA with a ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research) and diluted it to 5 ng/g. We prepared reaction mixes with Power SYBR Green PCR Master Mix according to manufacturer instructions with tubulin *B* primers IS-NS-5′ and TUB-

2W-3′ and tested them on Step One Plus real-time PCR machine (Applied Biosystems) as described in Jia et al. (2015).

After both infection status tests, we determined that there were nine naturally E− plants originating from four mother plants from four populations in Pennsylvania, 12 untreated *E. schardlii* infected plants originated from five mother plants from five populations, 12 HeatSch + plants originating from two mother plants from two populations, and eight HeatSch- from four mother plants from four populations. For this experiment, an additional batch of fall armyworm eggs was received from Frontiers Scientific Services (acquired from Bio-Serv Company) lot#I_030316Sf. Eggs were hatched and larvae were fed on oat (*Avena sativa*) leaves. We used a similar experimental design as in the previous choice experiments just with four diets: E−, HeatSch−, HeatSch+, *E. schardlii*. The first experiment (30 replicates) used 2 -old larvae and continued for 48 h. The second experiment (30 replicates) used four-day-old larvae and continued for 24 h. At the end of each experiment, percent leaf consumed and larval survival were recorded. For statistical analyses, we used data from only larvae that were alive at the end of the experiment ($n = 28$ for each experiment). Leaf samples from each individual plant were analyzed for peramine, *N*-acetylnorloline, and chanoclavine I as described in Shymanovich et al. (2017).

Statistical analyses

All analyses were performed with R i386 3.3.2 software with R Commander and Dunn Test packages (R development core team, 2008). In the larval performance and plant damage experiments, to test differences in survival for larvae on three diets, we used Kruskal–Wallis rank sum tests with Bonferroni pairwise comparisons. Survival by sex for larvae on uninfected and *E. schardlii* diets was tested with Kruskal–Wallis rank sum tests. For larval mass comparisons in the larval performance experiment, ANOVA II tests with diet and sex as factors were used for each measurement period. Assumptions of normality and homogeneity of variance were checked by the Shapiro–Wilk tests and Levene's tests, respectively, and both assumptions were met. To make repeated measurements adjustments for larval mass differences from day 7–15, Hotelling's *T2* tests (E− vs. *E. schardlii* groups) were performed for each sex separately. Only larvae that survived to the pupal stage and checked for sex were used for Hotelling's *T2* tests and for ANOVA II. Also for 7-day-old larvae mass comparisons, ANOVA I was used with diet as a factor because sex did not have significant effect at this stage ($P = 0.23$). To compare pupal mass by treatment in the larval performance and plant damage experiments, ANOVA II were used with diet and sex as factors (all assumptions met). To compare days to pupation and days to adult emergence in the both experiments (assumptions of normality not met) non-parametric Kruskal–Wallis rank sum tests were separately used for diet and sex as factors. If sex had significant effect, then female and male larvae were analyzed separately. To compare dry leaf biomass consumed by a single larvae in the larval performance experiment, we used Kruskal–Wallis rank sum test with Bonferroni pairwise comparisons (data not-normal). For pupated larvae only in larval performance experiment, to compare dry leaf biomass consumed we also used ANOVA II with diet and sex as factors (all assumptions met). In the plant damage experiment, sex was not determined for any larvae feeding on *E. alsodes* plants, and biomass consumed was not directly measured but estimated. Therefore we used ANOVA I with multiple comparisons by Tukey contrasts with infection type as a factor (all assumptions met), to test for

differences for estimated biomass consumed by a single larvae when feeding on plants with different infections. To compare percent consumed of an individual plant biomass in the plant damage experiment, we used Kruskal–Wallis rank sum test with Bonferroni pairwise comparisons (data not-normal). In the larval preference experiments, percent of leaf area consumed was tested with Kruskal–Wallis rank sum tests with Bonferroni pairwise comparisons as data were not normal and contained many zeros.

Results

Larval survival

Plants harboring the two *Epichloë* infections had very different effects on larval survival. Within 10 days, all larvae that were fed *E. alsodes* infected plant material died in the larval performance experiment (Fig. 1a). Likewise, no larvae survived on the *E. alsodes*-infected plants in the plant damage experiment by the time that larvae in other treatments were beginning to pupate (Fig. 1b). In contrast, the effects of *E. schardlii* infection on larval survival in both experiments were weaker than on larvae fed *E. alsodes*-infected material (Fig. 1a, b). In the larval performance experiment, larvae survival on the *E. schardlii* diet was less than for larvae fed with uninfected (E−) leaf clippings ($P = 0.039$) (Fig. 1a). Larval mortality when fed with *E. schardlii*-infected material was observed mainly on the early to middle larval stages, whereas mortality on E− diet was observed in the pupal stages. In the plant damage experiment, larvae on *E. schardlii* plants had similar survival as those on uninfected plants ($P = 0.082$) (Fig. 1b). Survival of larvae when feeding on E−or *E. schardlii* leaf material did not differ by sex in the both experiments ($P = 0.094$ and $P = 0.19$).

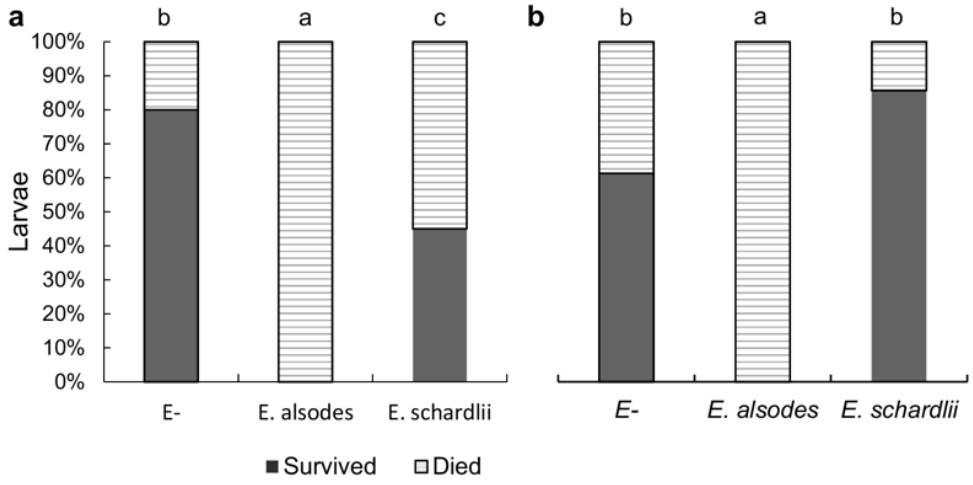

Figure 1. Percent of larvae survived and died from larval performance (**a**) (n = 20 for each group) and individual plant damage experiments (**b**) (n = 31 for each group) fed on naturally uninfected (E−) *Poa alsodes*, infected with *Epichloë alsodes* or with *E. schardlii* leaf clippings (**a**) or plants (**b**). Different letters indicate significant differences, $P < 0.05$

Larval and pupal mass

Plant material infected by the two *Epichloë* species had different effects on larval and pupal mass in the larval performance experiment (Fig. 2).The mean mass of larvae fed with *E. alsodes* plant material on the seventh day was greatly reduced compared to the other groups (Fig. 2a, b), ($P < 0.001$). By the tenth day, these larvae had stopped feeding, and their sex was not determined because they did not survive to pupation. At each measurement, mean mass of larvae fed uninfected (E−) leaf clippings was greater than those larvae fed infected *E. schardlii* clippings for both sexes (Fig. 2a, b). For these groups, sexes did not differ in mass until the tenth day of larval development ($P < 0.05$) and throughout the pupal stage ($P \leq 0.05$). Males had greater mean mass than females as expected from other studies (Vélez et al. 2014). Overall comparisons of larval mass across days 7–15 separately for females and males ($P = 0.027$ and $P = 0.012$, respectively) also showed differences for the E− verus *E. schardlii* diets. As expected larvae fed with *E. schardlii* infected leaves had reduced pupal mass compared to those fed E− leaves ($P = 0.011$), and for females this difference was greater (Fig. 2a). However, in the plant damage experiment, mean pupal mass in E− and *E. schardlii* groups was not affected by plant infection status or by sex ($P = 0.22$ and $P = 0.44$, respectively) (data not shown).

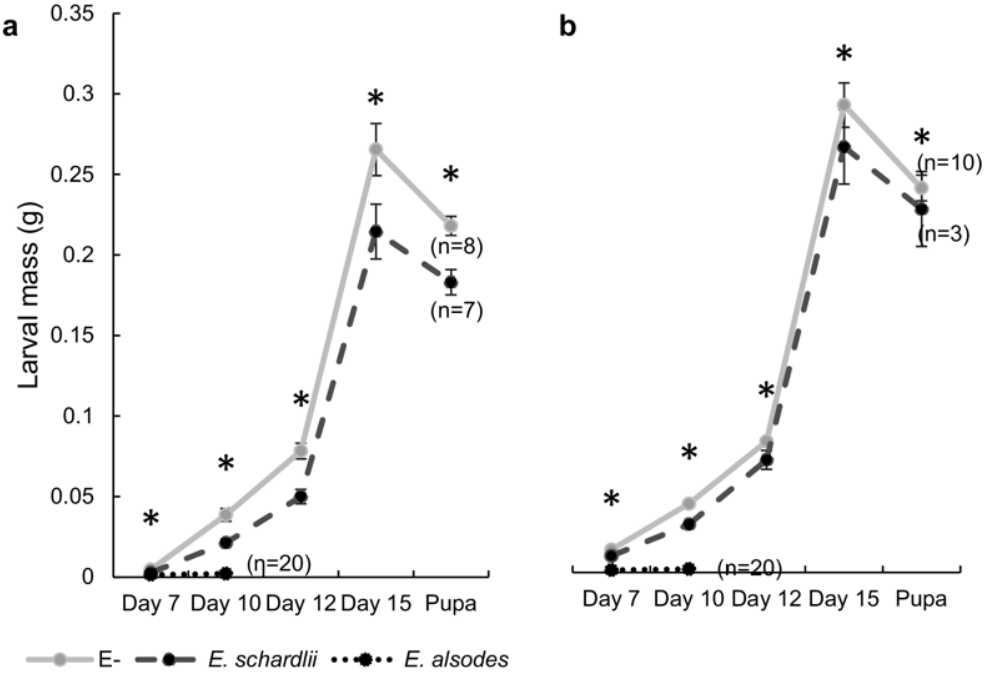

Figure 2. Mean (± SE) mass for female (**a**) and male larvae (**b**) from days seven to fifteen and mean (± SE) pupal mass when feeding on leaf clippings from uninfected plants (E−), plants with *Epichloë schardlii*, and plants with *E. alsodes*. For larvae feeding on *E. alsodes* infected leaves, sex was not determined because none survived to the pupal stage when determination was possible. Asterisks indicate significant effect of diet, $P < 0.05$

Developmental time

In the larval performance experiment, larval development time to pupation was longer for larvae feeding on *E. schardlii* infected leaves than on E− leaves ($P = 0.019$) (Fig. 3a). Sex had no effect on time to pupation ($P = 0.72$). Similar results were found in the plant damage experiment: larvae

on the *E. schardlii* diet had longer times to pupation ($P < 0.001$) (Fig. 3b), and sex did not have a significant effect ($P = 0.42$).

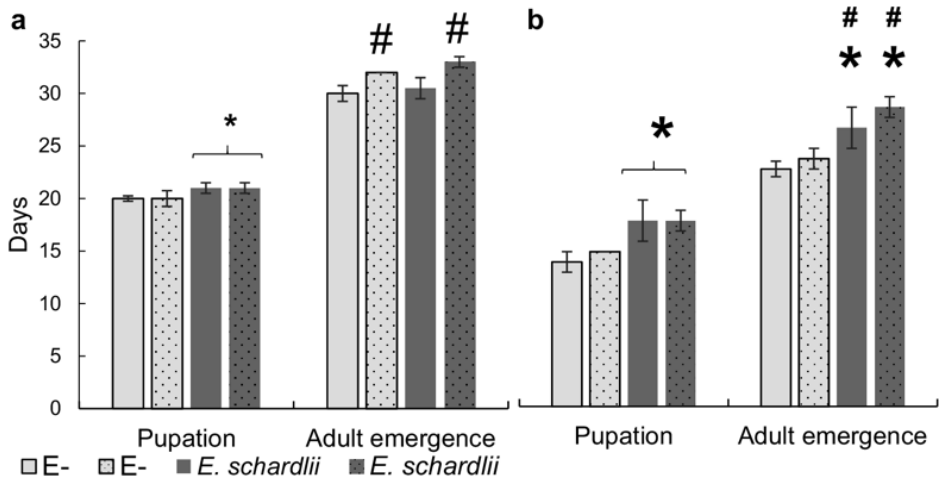

Figure 3. Median (± IQR) days to pupation and to adult emergence for female and male larvae from larval performance experiment (**a**) and individual plant damage experiment (**b**) when feeding on *Poa alsodes* leaf clippings (**a**) or individual plants (**b**) naturally uninfected (E−) or with *Epichloë schardlii* endophyte. ♀ =Tfemale larvae, ♂ = male larvae. Asterisk sizes indicate significant differences between feeding groups ($P < 0.05$, < 0.001, respectively). # sizes indicate significant differences between sexes ($P < 0.05$, < 0.001, respectively)

In the larval performance experiment, diet did not affect the total development time from larva to adult emergence (Fig. 3a) ($P = 0.95$), but total development time did vary by sex ($P = 0.001$), with longer total development times for males. In contrast, in the plant damage experiment, larvae feeding on *E. schardlii* infected plants had longer total development times (Fig. 3b) than larvae feeding on E− plants ($P < 0.001$). Similar to the larval performance experiment, males had longer development times ($P = 0.049$). In this experiment, mean time to adult emergence for females feeding on E− plants was 22.8 ± 0.6 (SD) days, while mean time for female emergence when feeding on *E. schardlii* infected plants was 27.4 ± 1.7 (SD) day. Males showed a similar delay in emergence when feeding on *E. schardlii* infected plants (Fig. 3b).

Leaf biomass consumed and individual plant damage

In the larval performance experiment, median dry leaf biomass consumed by a single larva depended on whether plant material was infected or not, and if infected, by the endophyte species (Fig. 4a). Overall, larvae consumed less biomass if plant material was infected by either endophyte species compared to endophyte-free plant material. Furthermore, larvae consumed less plant material infected by *E. alsodes* than infected by *E. schardlii*. Due to early mortality, larvae on *E. alsodes* diet consumed very small amounts of leaves compared to *E. schardlii* ($P = 0.003$) and especially E− diets (more than two orders of magnitude less) ($P < 0.001$). Larvae feeding on *E. schardlii* infected leaves also consumed less than those feeding on E− leaves ($P < 0.001$) (Fig. 4a). This reduction in amount consumed was also partially due to higher mortality.

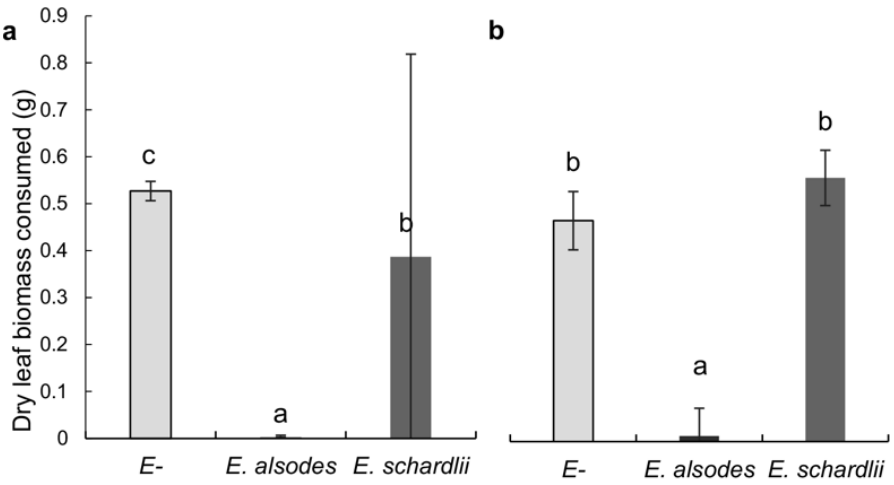

Figure 4. Median (± IQR) dry plant biomass consumed in larval performance (**a**) ($n = 20$ for each group) and estimated mean (± SE) dry leaf biomass consumed in plant damage experiment (**b**) ($n = 31$ for each group) by a single larvae fed with *Poa alsodes* uninfected (E−), infected with *E. alsodes* or with *E. schardlii* diets. Different letters indicate significant differences, $P < 0.01$

Moreover, for larvae feeding on E− and *E. schardlii* diets that survived to pupation, the amount of leaf biomass consumed differed depending on diet ($P < 0.001$) and larval sex ($P = 0.05$). On average, surviving larvae feeding on E− leaves consumed more than those feeding on *E. schardlii* infected leaves: females 0.53 ± 0.01 g (mean ± SD) and 0.43 ± 0.04 g respectively; males 0.55 ± 0.04 g and 0.47 ± 0.05 g, respectively. These consumption results correspond with the observed pupal mass differences for armyworms feeding on E− and *E. schardlii* infected leaves.

In the individual plant damage experiment, the estimated dry leaf biomass consumed was similar for larvae feeding on E− plants and on plants with *E. schardlii* infection ($P = 0.55$) (Fig. 4b). These results also correspond with observation of no difference in pupal mass results when feeding on these two groups of plants. Exploring individual plant damage from a single larvae, we observed only a few small holes in the leaves of plants with *E. alsodes* infection at the completion of the experiment, and estimated median damage was negligible, 4%. In contrast, median percent of leaf damage was high for E− plants and plants infected with *E. schardlii*, 43% and 50%, respectively (Fig. 5).

Larval feeding preference

When given the choice among leaves with either one of *Epichloë* infections or uninfected leaves, naïve 2-day and 5-day-old larvae were equally likely to choose and consume *E. alsodes* infected as E− leaves, even though larvae did not survive on the former in our larval performance and plant damage experiments (Fig. 6a, b). However, fall armyworm larvae avoided consuming leaves with *E. schardlii* infection more so than E− and *E. alsodes* infected leaves. Consumption of these leaves was lower for both naïve 2-day and 5-day-old larvae than the E− and *E. alsodes* infected leaves (Fig. 6a, b).

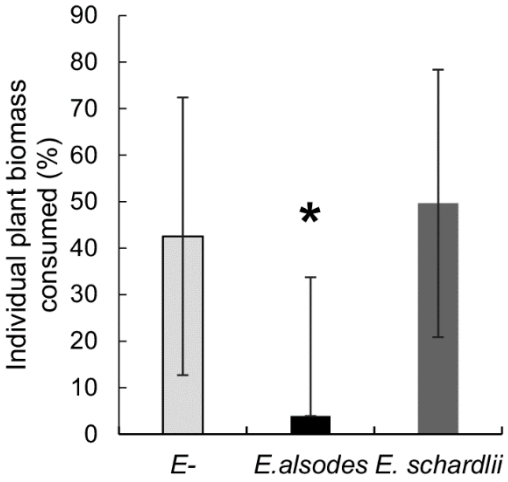

Figure 5. Median (± IQR) percent of an individual *Poa alsodes* plant biomass consumed by a single larvae depending on *Epichloë* spp. infection: E− uninfected plants, plants with *E. alsodes*, plants with *E. schardlii* infection ($n = 31$ for each group). Asterisk indicate significant difference, $P < 0.001$

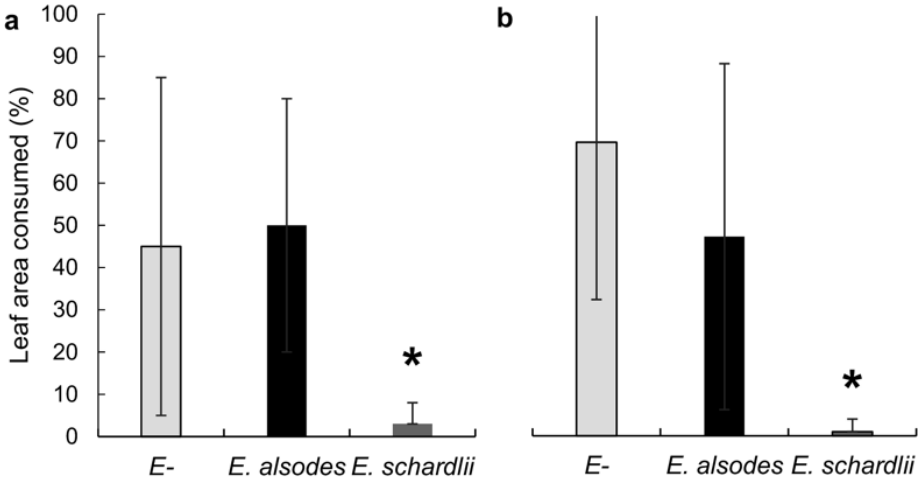

Figure 6. Larval feeding preference measured as median (± IQR) percent of leaf area consumed for uninfected (E−) *Poa alsodes* leaves, leaves with *Epichloë alsodes* infection, leaves with *E. schardlii* infection in two experiments with 2-day-old (**a**) and 5-day-old larvae (**b**). Asterisk indicates significance of differences, $P < 0.01$ ($n = 29$ for 2-day-old larvae, and $n = 12$ for 5 day old larvae experiments). For 5-day-old larvae, difference between E− and *E. alsodes* groups was not significant, $P = 0.25$

When given a choice among leaves that were naturally uninfected (E−), with *E. schardlii* experimentally removed (HeatSch-), heat treated but without removing *E. schardlii* (HeatSch+), and naturally infected *E. schardlii*,2 day-old larvae preferred to feed on leaves from the HeatSch− group, and E− leaves were their next choice ($P = 0.023$) (Fig. 7a). For 4 day-old larvae, difference in consumption of E− and HeatSch- was not significant ($P = 0.24$) (Fig. 7b). In both experiments, larvae avoided feeding on leaves with *E. schardlii* or HeatSch + relative to other leaf groups. Also there was no difference in preference of fall armyworm larvae between

HeatSch + and naturally *E. schardlii* infected leaves in either experiment ($P = 1.00$), thus indicating no extraneous experimental effects of heat treating seeds on larval preference.

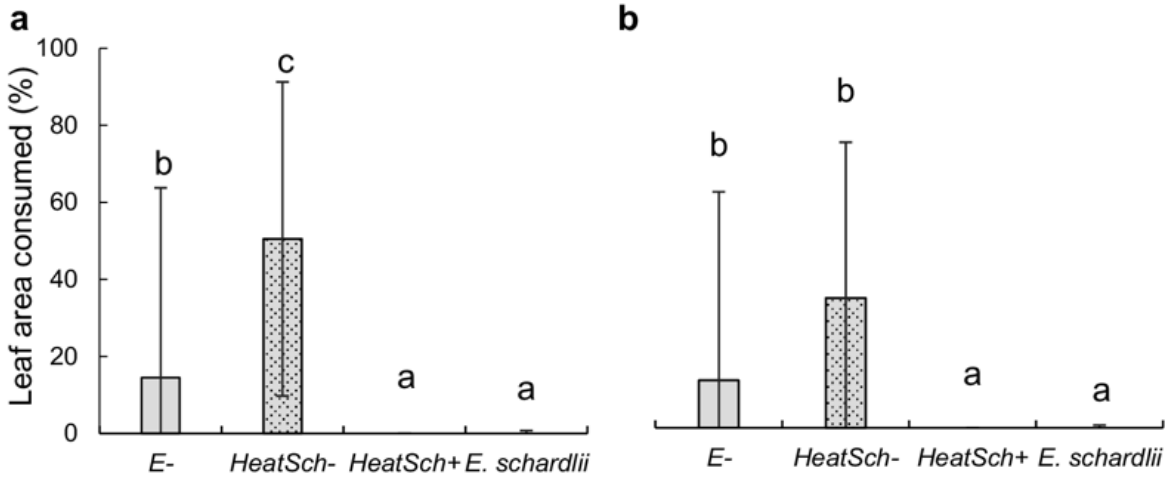

Figure 7. Larval feeding preference measured as median (± IQR) percent of leaves consumed for naturally uninfected (E−) *Poa alsodes* leaves, leaves from plants were *E. schardlii* infection was removed via seed heat treatment HeatSch−, leaves from plants were *E. schardlii* infection was not removed after seed heat treatment HeatSch+, leaves with natural *E. schardlii* infection in two experiments with 2-day-old (**a**) and 4-day-old larvae (**b**) ($n = 28$ for each experiment). Different letters indicate significant differences, $P < 0.05$

Endophyte alkaloids in leaf tissues

N-acetylnorloline was detected only from *E. alsodes* infected leaf material fed to larvae in the larval performance experiment. From four consecutive feeding samples, detected *N*-acetylnorloline concentrations were 2800, 2300, 2300, and 3350 µg/g. Chanoclavine I and peramine were not detected from *E. alsodes, E. schardlii*, or E− leaf samples. In the plant damage experiment, similar results were obtained. In leaf tissues from E− plants and plants with *E. schardlii*, no fungal alkaloids, such as *N*-acetylnorloline, peramine, and chanoclavine I, were detected. *N*-acetylnorloline was detected from all ten individual plant samples with *E. alsodes* endophyte. *N*-acetylnorloline concentrations ranged from 980 to 3400 µg/g of dry material with mean (± SD) 2578 ± 739 µg/g. Peramine and chanoclavine I were not detected from *E. alsodes* infected plant tissues. Analyses of all plants used for the feeding preference experiments with manipulated infections did not detect peramine, *N*-acetylnorloline, and chanoclavine I in either group.

Discussion

Our experiments demonstrate that both *Epichloë* endophytes in *P. alsodes* have negative effects on fall army worm survival and development, and may act to protect the plant from generalist herbivores. However, the two endophytes appear to have different modes of action in their anti-herbivore effects: insecticidal versus deterrence. The *E. alsodes* endophyte is highly toxic to fall army worm larvae. No larvae survived in the larval performance experiment beyond 10 days of feeding. Likewise, in the plant damage experiment, larvae consumed small amounts of leaves

and died soon thereafter. Clearly, grove bluegrass infected with *E. alsodes* harbors at least one powerful insecticidal compound associated with the *E. alsodes* endophyte. The likely candidate for the insecticidal properties of plants infected with *E. alsodes* is the loline alkaloid, *N*-acetylnorloline. *N*-acetylnorloline is the only fungal alkaloid detected from plant tissues of *E. alsodes* infected plants. Like other loline alkaloids, *N*-acetylnorloline is known for its insecticidal effects (Popay et al. 2009). Popay et al. (2009) showed that the concentrations of 400–1600 µg/g were effective against argentine stem weevil larvae feeding on meadow fescue. In our study, *N*-acetylnorloline concentrations ranged from 980 to 3400 µg/g, which should be highly toxic to herbivore larvae.

In contrast, plants infected with the *E. schardlii* endophyte did not have consistent negative effects on fall armyworm survival. Larvae feeding on *E. schardlii* plant material showed decreased survival in the larval performance experiment compared to larvae reared on E− plant material, but not nearly to the extent of plants infected with *E. alsodes*, where survival was nil. In the plant damage experiment, larval survival on *E. schardlii* infected plants was similar to larvae on uninfected plants. Thus, effect of *E. schardlii* diet on fall armyworm survival may depend on the environmental factors, as treatment conditions (temperature, light, clipped vs. fresh plant material) differed between the two experiments. In addition to effects on larval survival, infection by the *E. schardlii* endophyte was associated with reduced biomass, increased time to pupation and delayed adult emergence of the fall armyworm. Our larval development experiments on *P. alsodes* plants infected with *E. schardlii* corroborate those of Crawford et al. (2010) with *Cinna arundinacea* plants. Presumably their *C. arundinacea* infected plants hosted a very similar isolate of *E. schardlii* (Ghimire et al. 2011; Shymanovich et al. 2017). They found fall armyworm survival did not differ when feeding on infected versus uninfected *C. arundinacea* plants. Also similar to our study, Crawford et al. (2010), found that larvae feeding on infected plants showed reduced larval and pupal mass and delayed development compared to those feeding on uninfected plants. Reduced pupal biomass and delayed development time results in reduced fitness for fall army worm as well as for other insect species and may result in reduced population densities (Dmitriew and Rowe 2011; Vélez et al. 2014) that may protect perennial grove bluegrass plants in the next growing season.

It is unclear what alkaloids or other alleochemical compounds or traits (e.g., nutritional or morphological) of plants infected with the *E. schardlii* endophyte are responsible for larval survival effects, reduced pupal biomass, delayed development, and feeding deterrence. *Epichloë schardlii* does not have genes for loline, ergot or indole-diterpenes alkaloids, so that the presence of insecticidal alkaloids such as *N*-acetylnorloline or ergovaline are not possible, but we still chemically re-checked this. Peramine, an alkaloid commonly found in *Epichloë* infected grasses (e.g., Berry et al2015; Cheplick and Faeth 2009) and known to have insect deterring properties (e.g., Panaccione et al. 2014; Schardl et al. 2013a) would seem the likely candidate. Molecular genetic studies show the presence of three major domains of the peramine gene in *E. schardlii*, and no mutations were detected by sequencing, so that the peramine gene should be functional (Shymanovich et al. 2017). However, previous peramine chemical analyses of plant material infected with the *E. schardlii* were performed independently by two different laboratories using LC-MS, and neither detected peramine. It is unlikely that peramine concentrations in plant tissues were below the LC-MS detection limit, given that peramine was detected in control samples of *Elymus canadensis* and *Festuca arizonica* with *Epichloë* endophytes (Shymanovich

et al. 2017). Peramine levels of about 300 ppm (300 µg/g) or higher are necessary to negatively affect insects (Siegel et al. 1990), and it is unlikely that LC-MS, an analytical technique highly sensitive to alkaloids even at the 1 ppb level, would have been unable to detect peramine at these concentrations (Jarmusch et al. 2015). Indeed, due to the absence of peramine in plants infected with the *E. schardlii* endophyte, we predicted that fall armyworm larvae would perform as well on these plants as on E− plants. Peramine absence was confirmed for plants used in this study. Thus, it is unknown what chemical compounds or other properties of host plants associated with the *E. schardlii* endophyte are responsible for the negative effects on larvae and pupae. It is possible that there is some other alternative alkaloid product in the peramine biosynthetic pathway that we did not assess.

It is also possible that other allelochemical, physical or nutritional properties of the *P. alsodes* host plant genotypes that are associated with *E. schardlii* have negative effects on generalist herbivores, rather than effects mediated by the endophyte. Specific host plant genotypes are known to be associated with certain endophytre strains or species (e.g., Saikkonen et al. 2004, 2010), and the properties of the host plants themselves may confer resistance to insect herbivores (Shymanovich and Faeth 2018). However, endophyte removal demonstrated that larval leaf avoidance matches with *E. schardlii* infection and does not depend on plant genotypes. Larvae avoided feeding on both *E. schardlii* and HeatSch + leaves but did not avoid feeding on HeatSch− leaves that originated from the same mother plants. However, we observed some evidence that genotypes of E− plants and plants infected with *E. schardlii* may vary from each other in terms of preference by armyworms. 2 day-old larvae differentially preferred leaves from endophyte removed plants when compared with naturally uninfected plants. This suggests that naturally uninfected plants have other traits that make them less preferred by 2 day-old larvae compared to plant genotypes that had their endophytes experimentally removed.

In our choice tests on *P. alsodes* plants with natural infections, fall armyworm feeding preferences did not match their performances. Apparently, larvae have not adapted to avoid the highly toxic *E. alsodes*, and surprisingly avoided plants infected with *E. schardlii*, which is far less toxic. This preference–performance mismatch may be related to their broad diet across many plant species, which inhibits strong preferences for choosing or feeding upon host plants with specific chemical defenses that either stem from the host itself or its endophytic symbionts. Similar results were described for *Melanoplus bivittatus* (two-striped grasshopper) that did not discriminate diets with lethally toxic solanine and tomatine alkaloids (Harley and Thorsteinson 1967). Similarly, some other insect species do not discriminate highly toxic bait when a non-toxic alternative is available (Michaud 2003).

Our insect preference results are not congruous with those by Crawford et al. (2010) involving *P. alsodes* plants purportedly infected with *E. alsodes*. This is likely so because their endophyte infected plants were expected to contain loline alkaloids (*E. schardlii* does not produce lolines). In their experiments, all insects, fourth instar *S. frugiperda* (fall armyworm), *Schistocerca americana* (American grasshopper) larvae, and final-instar/adult *Rhopalosiphum padi* (bird cherry oat aphids), preferred feeding on endophyte free *P. alsodes* plants compared to endophyte-infected plants. In our study, we did not find strong larval preference for E− plants (Fig. 6 a, b), although 5-day-old larvae had not significantly higher preference for E− plant material compared to plant material infected with *E. alsodes* (Fig. 6b). These differences might

be explained by larval age. We used two (first instar) and 5-day-old (second instar) larvae whereas Crawford et al. (2010) used fourth instar larvae, which are usually 8–10 days-old (http://entnemdept.ufl.edu/creatures/field/fall_armyworm.htm). As larvae age, they may become more discriminating in diet preference. It is also possible that these differences in results may be due to a variation in *S. frugiperda* strains that differ in their ecological and behavioral characters (Pashley 1988). Variation in the response of fall armyworm to endophyte infected plants has been found in other studies. Bultman et al. (2009) found that *S. frugiperda* larvae avoided tall fescue plants with the *E. coenophialum* isolate AR542 that produces *N*-acetylnorloline. However, Ball et al. (2006) showed that fall army worm larvae did not avoid tall fescue plants infected with the same isolate. Alternatively, the study by Crawford et al. (2010) may have involved a different strain of endophyte infecting their plants (initially collected in the Indiana, USA). Nonetheless, it appears that preference of the fall armyworm, a generalist herbivore, does not match well with its performance on plants infected with *E. schardlii*or*E. alsodes*.

Our study showed that endophytes in *P. alsodes* may provide defenses against generalist insect herbivores. The two *Epichloë* species hosted by *P. alsodes* vary in their alkaloid profiles, and thus may have different modes of action against generalist herbivores. In the case of *E. alsodes* infecting *P. alsodes*, this mode appears to be via strong toxicity to fall army worm larvae, whereas for *E. schardlii* infecting *P. alsodes* the mechanism appears to be deterring larvae from feeding. In natural populations, these differences in endophyte species and strains within a common host grass can cascade upward to affect population dynamics of the host, host plants interaction with other species, the effectiveness of natural enemies of plant herbivores (Saari and Faeth 2012), and community diversity (e.g., Cheplick and Faeth 2009; Faeth and Saari 2012).

Acknowledgements

We thank the University of North Carolina at Greensboro Biology Department; Dr. Stanley Faeth National Science Foundation grant# DEB 0917741 for funding, Dr. Carolyn Young and Dr. Nikki Charlton from the Noble research Institute for identifying infections and alkaloid genes profiling of mother plants, Dr. Johnathan Scheerer from College of William & Mary, Williamsburg, VA for *N*-acetylnorloline standard synthesis, Anna and Anastasia Shymanovich for help with growing plants and growth estimates, Angie Larsen for help with statistics, and Daniel Foil and Dr. Daniel Todd from the Triad Mass Spectrometry Facility at the University of North Carolina at Greensboro for assistance with mass spectrometry analysis.

Author contributions

TS and SHF conceived and designed the experiments, TS performed the experiments and analyzed the data, NBC and AMM performed chemical analyses, TS and SHF wrote the manuscript; other authors provided editorial advice.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Agrawal AA (2011) Current trends in the evolutionary ecology of plant defence. Funct Ecol 25:420–432

Ball OJ, Coudron TA, Taper BA, Davies E, Trently D et al (2006) Importance of host plant species, *Neotyphodium* endophyte isolate, and alkaloids on feeding by *Spopoptera frigiperda* (Lepidoptera: Noctuidae) larvae. J Econ Entomol 99:1462–1473

Berenbaum MR (1995) The chemistry of defense: theory and practice. Proc Natl Acad Sci 92:2–8

Berry D, Takach JE, Schardl CL, Charlton ND, Scott B, Young CA (2015) Disparate independent genetic events disrupt the secondary metabolism gene *perA* in certain symbiotic *Epichloë* species. Appl Environ Microbiol 81:2797–2807

Bowers MD (1990) Recycling plant natural products for insect defense. In: Evans DL, Schmidt JO (eds) Insect defenses: adaptive mechanisms and strategies of prey and predators. State University of New Your Press, Albany, pp 353–383

Bultman TL et al (2009) Influence of genetic variation in the fungal endophyte of a grass on an herbivore and its parasitoid. Entomol Exp Appl 130:173–180

Charlton ND, Craven KD, AfkhamiM E, Hall BA, Ghimire SR, Young CA (2014) Interspecific hybridization and bioactive alkaloid variation increases diversity in endophytic *Epichloë* species of *Bromus laevipes*. FEMS Microbiol Ecol 90:276–289

Cheplick GP, Faeth SH (2009) Ecology and evolution of the grass-endophyte symbiosis. Oxford University Press, New York

Clay K, Cheplick GP (1989) Effect of ergot alkaloids from fungal endophyte-infected grasses on fall armyworm (*Spodoptera frugiperda*). J Chem Ecol 15:169–182. https://doi.org/10.1007/Bf02027781

Clay K, Schardl C (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am Nat 160:S99–S127. https://doi.org/10.1086/342161

Crawford KM, Land JM, Rudgers JA (2010) Fungal endophytes of native grasses decrease insect herbivore preference and performance. Oecologia 164:431–444. https://doi.org/10.1007/s00442-010-1685-2

Dmitriew C, Rowe L (2011) The effects of larval nutrition on reproductive performance in a food-limited adult environment. PLoS ONE 6:e17399

Faeth SH, Saari S (2012) Fungal grass endophytes and arthropod communities: lessons from plant defence theory and multitrophic interactions. Fungal Ecol 5:364–371. https://doi.org/10.1016/j.funeco.2011.09.003

Ghimire SR, Rudgers JA, Charlton ND, Young C, Craven KD (2011) Prevalence of an intraspecific neotyphodium hybrid in natural populations of stout wood reed (*Cinna arundinacea*) from eastern North America. Mycologia 103:75–84

Hardy TN, Clay K, Hammond AM (1985) Fall armyworm (Lepidoptera: Noctuidae): a laboratory bioassay and larval preference study for the fungal endophyte of perennial ryegrass. J Econ Entomol 78:571–575

Harley KLS, Thorsteinson AJ (1967) The influence of plant chemicals on the feeding behavior, development, and survival of the two-striped grasshopper, *Melanopus bivittatus*(Say), Acrididae: Orthoptera. Can J Zool 45:305–319. https://doi.org/10.1139/z67-043

Iannone LJ, Novas MV, Young CA, DeBattista JP, Schard lCL (2012) Endophytes of native grasses from South America: biodiversity and ecology. Fungal Ecol 5:357–363. https://doi.org/10.1016/j.funeco.2011.05.007

Jarmusch AK et al (2015) Comparison of electrospray ionization and atmospheric pressure photoionization liquid chromatography mass spectrometry methods for analysis of ergot alkaloids from endophyte-infected sleepygrass (*Achnatherum robustum*). J Pharm Biomed Anal 117:11–17

Jia T, Shymanovich T, Gao Y-B, Faeth SH (2015) Plant population and genotype effects override the effects of *Epichloë* endophyte species on growth and drought stress response of *Achnatherum robustum* plants in two natural grass populations. J Plant Ecol 8:633–641

Leuchtmann A, Bacon CW, Schardl CL, White JF, Tadych M (2014) Nomenclatural realignment of *Neotyphodium* species with genus *Epichloe*. Mycologia 106:202–215. https://doi.org/10.3852/106.2.202

López-Edwards M, Hernández-Mendoza JL, Pescador-Rubio A, Molina-Ochoa J, Lezama-Gutiérrez R, Hamm J, Wiseman B (1999) Biological differences between five populations of fall armyworm (Lepidoptera: Noctuidae) collected from corn in Mexico. Florida Entomol 58:254–262

Mello MO, Silva-Filho MC (2002) Plant-insect interactions: an evolutionary arms race between two distinct defense mechanisms. Braz J Plant Physiol 14:71–81

Michaud J (2003) Toxicity of fruit fly baits to beneficial insects in citrus. J Insect Sci 3:8

Oberhofer M, Leuchtmann A (2012) Genetic diversity in epichloid endophytes of *Hordelymus europaeus* suggests repeated host jumps and interspecific hybridizations. Mol Ecol 21:2713–2726. https://doi.org/10.1111/j.1365-294X.2012.05459.x

Panaccione DG, Beaulieu WT, Cook D (2014) Bioactive alkaloids in vertically transmitted fungal endophytes. Funct Ecol 28:299–314

Pashley DP (1988) The current status of fall armyworm host strain. Florida Entomol 71:227–234

Popay A, Tapper B, Podmore C (2009) Endophyte-infected meadow fescue and loline alkaloids affect Argentine stem weevil larvae. NZ Plant Prot 62:19–27

Potter DA, Stokes JT, Redmond CT, Schardl CL, Panaccione DG (2008) Contribution of ergot alkaloids to suppression of a grass-feeding caterpillar assessed with gene knockout endophytes in perennial ryegrass. Entomol Exp Appl 126:138–147. https://doi.org/10.1111/j.1570-7458.2007.00650.x

R Development Core Team (2008) R: a language and environment for statistical computing. R Foundation for Statistical Computing,Vienna. ISBN 3-900051-07-0, URLhttp://www.R-project.org

Saari S, Faeth SH (2012) Hybridization of *Neotyphodium* endophytes enhances competitive ability of the host grass. New Phytol 195:231–236. https://doi.org/10.1111/j.1469-8137.2012.04140.x

Saikkonen K, Wali P, Helander M, Faeth SH (2004) Evolution of endophyte-plant symbioses. Trends Plant Sci 9:275–280. https://doi.org/10.1016/j.tplants.2004.04.005

Saikkonen K, Wäli PR, Helander M (2010) Genetic compatibility determines endophyte-grass combinations. PLoS ONE 5(6):e11395. https://doi.org/10.1371/journal.pone0011395

Schardl CL (2010) The epichloae, symbionts of the grass subfamily pooideae. Ann Mo Bot Gard 97:646–665. https://doi.org/10.3417/2009144

Schardl CL, Chen F (2001) Plant defences against herbivore attack. Wiley, Chichester. https://doi.org/10.1002/9780470015902.a0001324.pub2

Schardl CL, Young CA, Faulkner JR, Florea S, Pan J (2012) Chemotypic diversity of epichloae, fungal symbionts of grasses. Fungal Ecol 5:331–344. https://doi.org/10.1016/j.funeco.2011.04.005

Schardl CL, Florea S, Pan J, Nagabhyru P, Bec S, Calie PJ (2013a) The epichloae: alkaloid diversity and roles in symbiosis with grasses. Curr Opin Plant Biol 16:480–488. https://doi.org/10.1016/j.pbi.2013.06.012

Schardl CL et al (2013b) Currencies of mutualisms: sources of alkaloid genes in vertically transmitted epichloae. Toxins 5:1064–1088. https://doi.org/10.3390/toxins5061064

Shymanovich T, Faeth SH (2018) Anti-insect defenses of *Achnatherum robustum*(sleepygrass) provided by two *Epichloë* endophyte species. Entomol Exp Appl 166(6):474–482. https://doi.org/10.1111/eea.12692

Shymanovich T et al (2015) Alkaloid variation among epichloid endophytes of sleepygrass (*Achnatherum robustum*) and consequences for resistance to insect herbivores. J Chem Ecol 41:93–104

Shymanovich T, Charlton ND, Musso AM, Scheerer J, Cech NB, Faeth SH, Young CA (2017) Interspecific and intraspecific hybrid *Epichloë* species symbiotic with the North American native grass *Poa alsodes*. Mycologia 109(3):459–474. https://doi.org/10.1080/00275514.2017.1340779

Siegel MR et al (1990) Fungal endophyte-infected grasses—alkaloid accumulation and aphid response. J Chem Ecol 16:3301–3315. https://doi.org/10.1007/Bf00982100

Sparks AN (1979) A review of the biology of the fall armyworm. Florida Entomol 62:82–87

Sullivan TJ, Faeth SH (2008) Local adaptation in *Festuca arizonica* infected by hybrid and nonhybrid *Neotyphodium* endophytes. Microb Ecol 55:697–704. https://doi.org/10.1007/s00248-007-9312-4

Vélez A, Spence rT, Alves A, Crespo A, Siegfried B (2014) Fitness costs of Cry1F resistance in fall armyworm, *Spodoptera frugiperda*. J Appl Entomol 138:315–325

Wilkinson HH, Siegel MR, Blankenship JD, Mallory AC, Bush LP, Schardl CL (2000) Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. Mol Plant Microbe In 13:1027–1033