

## **Bottom-up regulates top-down: the effects of hybridization of grass endophytes on an aphid herbivore and its generalist predator**

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### **Abstract:**

The ecological consequences of hybridization of microbial symbionts are largely unknown. We tested the hypothesis that hybridization of microbial symbionts of plants can negatively affect performance of herbivores and their natural enemies. In addition, we studied the effects of hybridization of these symbionts on feeding preference of herbivores and their natural enemies. We used Arizona fescue as the host-plant, *Neotyphodium* endophytes as symbionts, the bird cherry-oat aphid as the herbivore and the pink spotted ladybird beetle as the predator in controlled experiments. Neither endophyte infection (infected or not infected) nor hybrid status (hybrid and non-hybrid infection) affected aphid reproduction, proportion of winged forms in the aphid populations, aphid host-plant preference or body mass of the ladybirds. However, development of ladybird larvae was delayed when fed with aphids grown on hybrid (H+) endophyte infected grasses compared to larvae fed with aphids from non-hybrid (NH+) infected grasses, non-hybrid, endophyte-removed grasses (NH-) and hybrid, endophyte-removed (H-) grasses. Furthermore, adult beetles were more likely to choose all other types of grasses harboring aphids rather than H+ infected grasses. In addition, development of ladybirds was delayed when fed with aphids from naturally uninfected (E-) grasses compared to ladybirds that were fed with aphids from NH+ and NH- grasses. Our results suggest that hybridization of microbial symbionts may negatively affect generalist predators such as the pink spotted ladybird and protect herbivores like the bird cherry-oat aphids from predation even though the direct effects on herbivores are not evident.

**Keywords:** hybridization | symbionts | endophytes | herbivores | predators

**Article:**

Ecosystems are regulated by a range of biotic and abiotic factors that can be classified as either top–down or bottom–up processes (Roughgarden et al. 1994). A major focus of ecology is to disentangle the relative importance of these top–down and bottom–up species interactions in structuring communities (Hunter and Price 1992). The outcome of interspecific interactions often depends upon genetic factors of the interacting species (Thompson 1999, 2005, Gomulkiewicz et al. 2003). Genetic variation can arise from multiple sources. Generally, research on interspecific interactions focuses on variation originating by mutation and subsequently shaped by natural selection, migration and drift (Thompson 1999, 2005). Although less acknowledged and researched, genetic variation can also be created rapidly by interspecific hybridization.

In plants, hybridization may have widespread ecological consequences at the individual and population level, including changes in plant defensive chemistry, altered resistance to parasites and disease and the ability to persist in marginal environments (Moullia 1999, Pilson 1999, Rieseberg et al. 2003). Hybridization in plants can also lead to community and ecosystem level effects, including changes in biodiversity, food web structure and ecosystem processes (Hochwender and Fritz 2004).

Unlike hybridization in free-living organisms, the ecological consequences of hybridization in symbionts, particularly microbial partners that are purported to interact mutualistically with their host-plants, are largely unknown. Yet, hybridization among microbes may have widespread ecological consequences. For example, hybrid fungal pathogens may exhibit increased host range and virulence. Brasier et al. (1999) found that a persistent and damaging *Phytophthora* fungal pathogen of alder is a hybrid. Furthermore, aggressive genotypes of Dutch elm disease, a devastating pathogen of elm trees in North America that permanently altered plant community structure, may have resulted from hybridization of two different strains (Brasier et al. 1998). Schardl and Craven (2003) reviewed other cases of hybrid fungal pathogens and argue that hybridization can result in ‘superpathogens’, with high degrees of virulence and broad host ranges. Alternatively, hybridization in microbes may lead to more specialized symbionts with reduced virulence and to more mutualistic interactions with their hosts (Schardl and Craven 2003, Selosse and Schardl 2007).

The best known examples of hybridization in microbial symbionts are the systemic, asexual, endophytic *Neotyphodium* fungi in cool-season grasses (Craven et al. 2001a, b, Schardl and Craven 2003, Moon et al. 2004, Selosse and Schardl 2007). Hybridization occurs when the sexual form (or teleomorph) *Epichloë* co-occurs with plants already infected with *Neotyphodium* or another *Epichloë* strain and hyphae fuse in a parasexual process to form dikaryon cells (Schardl and Craven 2003). Hybridization is thought to provide genetic variation that may 1) be adaptive in changing and stressful environments (Schardl and Craven 2003, Selosse and Schardl 2007) and 2) increase the type and quantity of alkaloids that increase host-plant resistance to herbivores (Clay and Schardl 2002, Schardl and Craven 2003, Moon et al. 2004). In general, increased resistance to herbivores is viewed as the major mechanism by which systemic endophytes such as *Neotyphodium* and *Epichloë* provide benefits to their host

grasses (Cheplick and Faeth 2009). That more than two thirds of *Neotyphodium* endophytes within and among grass species are hybrids has been used as de facto evidence that hybrid *Neotyphodium* are better adapted than non-hybrid species and strains (Schardl and Craven 2003, Oberhofer and Leuchtman 2012).

However, in addition to affecting herbivores, alkaloids produced by *Neotyphodium* endophytes may also have negative bottom–up effects on the natural enemies (predators and parasites) of herbivores (Saikkonen et al. 2010, Faeth and Saari 2012). Indeed, just like plant-produced alkaloids, fungal alkaloids may have stronger negative effects on generalist predators than herbivores themselves because 1) many herbivore species evolve to tolerate or even require plant alkaloids for development and 2) some herbivore species may sequester plant allelochemicals for their own defense (Pasteels 2007, Faeth and Saari 2012). Thus, supposedly “acquired defenses” (sensu Cheplick and Clay 1988) of grasses via endophytic alkaloids could instead act to decrease resistance if natural enemies of herbivores are less effective in controlling herbivore populations on infected plants. If so, then endophyte infection has a positive and indirect top–down effect on herbivores through reduced predation. A few studies have shown that endophyte infection and presumably their alkaloids can negatively affect predators (de Sassi et al. 2006) and parasites (Omacini et al. 2001, Bultman et al. 1997) and may result in increased herbivore diversity and abundances (Jani et al. 2010). Recent studies have also shown that hybrid endophytes often harbor more genes for alkaloid synthesis (Schardl et al. 2012). However, no studies to our knowledge have tested the hypothesis that genetic variation of plant symbionts due to hybridization 1) increases resistance to herbivores or, 2) affects preference and performance of natural enemies of the herbivores.

We studied the effects of hybridization of microbial symbionts on bottom–up and top–down processes by testing feeding preference and performance of herbivores and their natural enemies. Furthermore, we examined whether hybridization increases alkaloid levels. In our experiments, we used *Festuca arizonica* (Arizona fescue) as the host-plant, *Neotyphodium* endophyte as the symbiont and the aphid *Rhopalosiphum padi* as the herbivore and the ladybird beetle, *Coleomegilla maculata* as the predator. Previous studies have suggested that endophyte–plant genotype combinations are stable and unique because of endophyte haplotype–plant genotype compatibility (Lehtonen et al. 2005, Sullivan and Faeth 2008, Cheplick and Faeth 2009). Thus, hybrid and non-hybrid endophytes are likely associated with specific plant genotypes. Therefore, we experimentally removed the endophyte from one half of the plants naturally infected with hybrid and non-hybrid endophytes to test the effects of infection and plant genetic background on aphids and ladybird beetles.

Accumulating studies of *Neotyphodium*-infected native grasses indicate that infection can deter and reduce performance of invertebrate herbivores but the effects are highly variable depending on invertebrate and host grass species, plant and endophyte genotype, and environmental factors (Faeth 2002, Tintjer and Rudgers 2006). For Arizona fescue, infection generally does not increase, and sometimes decreases resistance to herbivores (Saikkonen et al. 1999, Faeth 2009)

but these studies have not considered the effects of hybridization of endophytes on herbivores. Based upon these previous findings, we did not expect infection alone to have strong negative effects on aphids. However, based upon Schardl's and Craven's (2003) hypothesis, we predicted that aphids would prefer and perform better on plants infected with non-hybrid endophytes than hybrid endophytes and that plants infected with hybrid endophytes would have higher levels of alkaloids. Because *Neotyphodium* endophytes have been demonstrated to have negative effects on predators, presumably via alkaloids sequestered in prey (Saikkonen et al. 2010, Faeth and Saari 2012), we predicted the negative effects of endophytes would be transmitted to the third trophic level. Thus, we expected that ladybird beetles would be negatively affected by infection and associated alkaloids. We also predicted that the loss of infection and endophyte infection related plant genotypic effects in the experimentally-removed, endophyte-free plants may have effects on herbivores that may also cascade to their predators.

## **Material and methods**

### **Arizona fescue and *Neotyphodium***

Arizona fescue *Festuca arizonica* is a dominant, perennial native grass in the southwestern USA and is frequently infected by hybrid (H+) and non-hybrid (NH+) *Neotyphodium* endophytes, *N. tembladerae* and *N. huerfanum*, respectively. The hybrid form of the endophyte in Arizona fescue has resulted from hybridization between co-occurring *Epichloë typhina* and *E. festucae* (Iannone et al. 2012). Because of the close relationships and shared biological properties, *Neotyphodium* genus name will soon disappear and the group will be absorbed in the genus *Epichloë* (Miller et al. 2011). In DNA sequencing, multiple gene copies indicate *Neotyphodium* hybridization (H+) while single gene copies indicate a nonhybrid (NH+) *Neotyphodium* species (Schardl and Craven 2003, Sullivan and Faeth 2004). Five different genotypes of H+ endophyte and three different genotypes of NH+ endophyte have been found in natural Arizona fescue populations (Sullivan and Faeth 2004).

### **Plant and endophyte material**

Seeds from plants with known endophyte infection categories (H+, NH+) and naturally endophyte free (E-) plants were collected from natural populations in Arizona. Endophytes were removed via heat treatment (Siegel et al. 1984) from half of the NH+ and H+ seeds. Then E-, NH+, H+ seeds and seeds with their endophyte infections experimentally-removed (H- and NH-) were planted using a completely randomized design in the field in Flagstaff, Arizona USA in 2001. Seeds produced by these plants were collected in autumn 2010 and stored in -23°C for six months before the experiment began in February 2011. Seeds were sown in 1-dl pots in regular potting soil and grown in a growth chamber with a photoperiod of 16 h light: 8 h dark at 28:23°C (D:N). Within each infection category, we used seed mixtures from at least five maternal plants to randomize the variation in maternal genotypes within each infection category. In these mixtures, the seeds originating from infected maternal plants (H+ and NH+) contained a

random set of endophytic haplotypes (five different haplotypes of H<sup>+</sup> endophytes and three different haplotypes of NH<sup>+</sup> endophyte). Instead of randomizing the variation in maternal genotypes, it would have been better to know exactly which endophyte genotypes were within each infection category. However, this was not possible at the time of the experiment. By randomizing the plant genotypes within endophyte infection categories, we ensured that the results of the endophyte infection related plant genotypes can be generalized. Each endophyte-treatment combination was replicated 20 times for a total of 100 plants. Plants were watered when needed and fertilized once a week with a liquid fertilizer (16:9:22 – N:P:K, with micro nutrients). Infection status of the plants was verified using an immunoblot assay to detect monoclonal antibodies specific to *Neotyphodium*. The NH<sup>+</sup> and H<sup>+</sup> infection status of the plants was verified from a random sample of 10 NH<sup>+</sup> and 10 NH<sup>-</sup> plants using the method described in Sullivan and Faeth (2004) and from a random sample of five NH<sup>+</sup> and five H<sup>+</sup> plants by sequencing the PCR products. All tested plants were confirmed as the correct infection (infected or not) and hybrid status (H<sup>+</sup> or NH<sup>+</sup>).

### **Effects on aphid populations**

To test the effects of endophyte infection and hybrid status of the endophyte on herbivore population size, we reared the bird cherry–oat aphids *Rhopalosiphum padi* on 20 plants each of the five plant categories (E<sup>-</sup>, H<sup>+</sup>, NH<sup>+</sup>, H<sup>-</sup> and NH<sup>-</sup>). Aphids were collected from natural grass populations in Greensboro, NC, USA. The bird cherry–oat aphid is a generalist aphid that feeds on grasses and cereal crops and is often used in bioassays for detecting the effects of endophytes and their alkaloids on herbivores (Cheplick and Faeth 2009). Because the bird cherry–oat aphid survives and reproduces on endophyte infected Arizona fescues (Saari unpubl.), it was chosen as a model herbivore in this study that includes a higher trophic level – the ladybird predators. However, the previous study (Saari unpubl.) did not consider the effects of hybridization of endophytes on the bird cherry–oat aphid resistance of the host. The aphids were maintained on seedlings of wheat for several generations in natural light and at room temperature. When the 100 experimental plants were about one month old, they were placed in enclosures and five adult aphids were introduced onto the leaves of each individual plant. The plants with aphids were then placed into a randomized complete block design of 20 blocks so that one plant belonging to each endophyte infection category (E<sup>-</sup>, NH<sup>+</sup>, NH<sup>-</sup>, H<sup>+</sup> and H<sup>-</sup>) was present in each block. The number of wingless and winged aphids was determined every three days for three weeks. Winged forms were counted separately because increasing proportion of winged forms in an aphid population is often correlated with deteriorating host quality (Braendle et al. 2006). At the end of the experiment, the number of tillers and the heights of the plants were measured and used as covariates in the statistical analysis.

### **Bird cherry–oat aphid feeding preference**

To test the effects of endophyte infection, hybrid status and plant genotype within infection categories, we conducted aphid choice tests on Arizona fescue tillers. We conducted the

following eight pairwise comparisons to test: 1) the effect of infection, NH<sup>+</sup> versus NH<sup>-</sup>, H<sup>+</sup> versus H<sup>-</sup>, NH<sup>+</sup> versus E<sup>-</sup>, H<sup>+</sup> versus E<sup>-</sup>; 2) the effect of plant genotype, E<sup>-</sup> versus NH<sup>-</sup>, E<sup>-</sup> versus H<sup>-</sup>, H<sup>-</sup> versus NH<sup>-</sup>, and, 3) the effect of hybrid status, H<sup>+</sup> versus NH<sup>+</sup>. For each trial (n = 9 for each pairwise comparison), one fresh tiller from each of the pair was placed symmetrically on a petri dish at room temperature. One naïve aphid that had been reared on oats was placed centrally in each petri dish and allowed to roam freely to choose between the two tillers. The choice of each aphid was recorded at 1, 5 and 24 h. If aphids did not choose between either of the two tillers, the preference was recorded as 0.

### **Effects of infection and hybridization on ladybird development**

To test the effect of endophyte infection, hybrid status of the endophyte and plant genotype within endophyte infection categories on the third trophic level, we fed aphids that were reared on E<sup>-</sup>, H<sup>+</sup>, NH<sup>+</sup>, H<sup>-</sup> and NH<sup>-</sup> plants to ladybird beetles. Offspring of five female ladybird beetles, randomly chosen from a colony of beetles (original stocks were provided by Dr. Lundgren, USDA/ARS North Central Agricultural research laboratory, Brookings, SD, USA and Dr. Michaud, Kansas State University, Kansas, KS, USA), were included in the experiment. Five randomly selected, one-day-old ladybird larvae from each of the five females were weighed. These five ladybirds were then randomly placed on each of the five plant types (E<sup>-</sup>, NH<sup>+</sup>, NH<sup>-</sup>, H<sup>+</sup> and H<sup>-</sup>) harboring aphid populations. The plants were arranged in a randomized complete block design with five blocks so that each of the blocks contained ladybird beetle larvae originating from one adult female. In total, 25 ladybird larvae were included in the experiment. Each plant was replaced with another plant within the same infection category to ensure that aphids were available ad libitum at all times.

The larvae were weighed every three days until they formed pupae. After weighing, the ladybird larvae were placed back on the experimental plants. The hatched adults were weighed and the width of pronotum and the distance between the eyes of the ladybirds were measured to estimate size. Development time from egg to adult was recorded and the sex of each adult ladybird was determined.

### **Ladybird beetle feeding preference**

To test the effects of infection, hybrid status and plant genotype within infection categories on preference by predators, we conducted a ladybird beetle choice test on Arizona fescue plants. We conducted pairwise comparisons to test, 1) the effect of infection, NH<sup>+</sup> versus NH<sup>-</sup>, H<sup>+</sup> versus H<sup>-</sup>, NH<sup>+</sup> versus E<sup>-</sup>, H<sup>+</sup> versus E<sup>-</sup>; 2) the effect of plant genotype, E<sup>-</sup> versus NH<sup>-</sup>, E<sup>-</sup> versus H<sup>-</sup>, H<sup>-</sup> versus NH<sup>-</sup>, and, 3) the effect of hybrid status, H<sup>+</sup> versus NH<sup>+</sup>. The eight different infection category comparisons were set up within each of twenty blocks. For each infection category comparisons, two Arizona fescue plants of different infection categories with comparable height and number of tillers were used. Each plant harbored *R. padi* aphid populations.

The plant pairs were placed 4 cm apart in an enclosure to reduce any external cues that might be used by the adult ladybirds. The category/infection status of the two plants was unknown to the investigator to eliminate any bias in placing the beetle and recording preference. For each replicate (n = 20), one ladybird beetle was placed equidistant between the two plants in a small, clear container. The container was opened and the beetle was allowed to choose between the two plants. When the beetle made contact with the tillers of a particular plant, the choice and time to choose (from opening the container) was recorded. A different and naïve individual ladybird beetle was used for each replicate trial. The number of aphids on each plant, the number of tillers, and the height of each plant (size comparison) were recorded.

### **Alkaloid analyses**

To test the hypothesis that hybridization affects alkaloid production in infected grasses, peramine content of a random sample of five plants from all infection categories (n = 25) was analyzed by J. Strickland, University of Kentucky, USDA-ARS via the methods described in Faeth (2002).

### **Statistical analysis**

For the aphid population experiment, the total number of aphids and proportion of winged aphids were analyzed using a randomized block, repeated measures ANOVA, with endophyte status as the between-subject factor and time of sampling as the within-subjects factor. Interaction between time and endophyte status was also included, with plant height and number of tillers included as covariates. Since the total number of aphids was a count variable which did not follow assumptions of normality and equal variance, it was modeled as a Poisson response using a generalized linear model. However, effects tests can be interpreted in a similar manner to those of an analysis of variance. For the ladybird development experiment, ladybird weight and development time were modeled as normally distributed responses using analysis of variance, again with endophyte and hybrid status as factors in a randomized complete block design. Time of sampling was included as a repeated measure for analyzing ladybird weight, but not for development time (from egg to adult), because developmental time was measured only once. In the models for ladybird weight and development time, sex, back width and eye width were included in the model as covariates. Simultaneous confidence intervals for estimating differences between pairs of status groups were computed using Scheffe's procedure. Simultaneous confidence intervals allow 95% confidence that all intervals simultaneously contain their true mean difference.

The ladybird preference experiment employed a discrete choice conditional logit model (Train 2009) to assess preferences of choosing between two plants with different endophyte statuses. In addition to endophyte status, number of aphids, number of tillers, plant height and time were investigated for association with preference. Odds ratios were used to compare preference for different endophyte status, and p-values were computed using  $\chi^2$ -tests. Odds are a way to express probability of choosing a particular status, and odds ratios compare odds for two different status.

If the odds ratio associated with two status equals 1, then they are equally likely to be chosen, while an odds ratio significantly different from 1 suggests that one status is more likely to be chosen than another. It is important to note that the odds reported in the results represent the overall odds of choosing a particular status across all alternatives with which it was paired in the experiment, and not the specific odds of choosing a particular status within a pair.

## **Results**

### **Effects of infection status and hybridization on aphid populations**

Endophyte infection, hybrid status and plant genotype did not affect the total number of aphids ( $F_{4,69} = 1.50$ ,  $p = 0.21$ ) or the proportion of winged forms in the aphid populations ( $F_{4,49} = 1.24$ ,  $p = 0.30$ ). These factors did not vary across the three week study period (number of aphids:  $F_{20,1} = 7.78$ ,  $p = 0.28$ ; proportion of winged aphids:  $F_{20,435} = 0.82$ ,  $p = 0.69$ ). However, the total number of aphids increased during the course of the experiment ( $F_{5,1} = 302.47$ ,  $p = 0.04$ ).

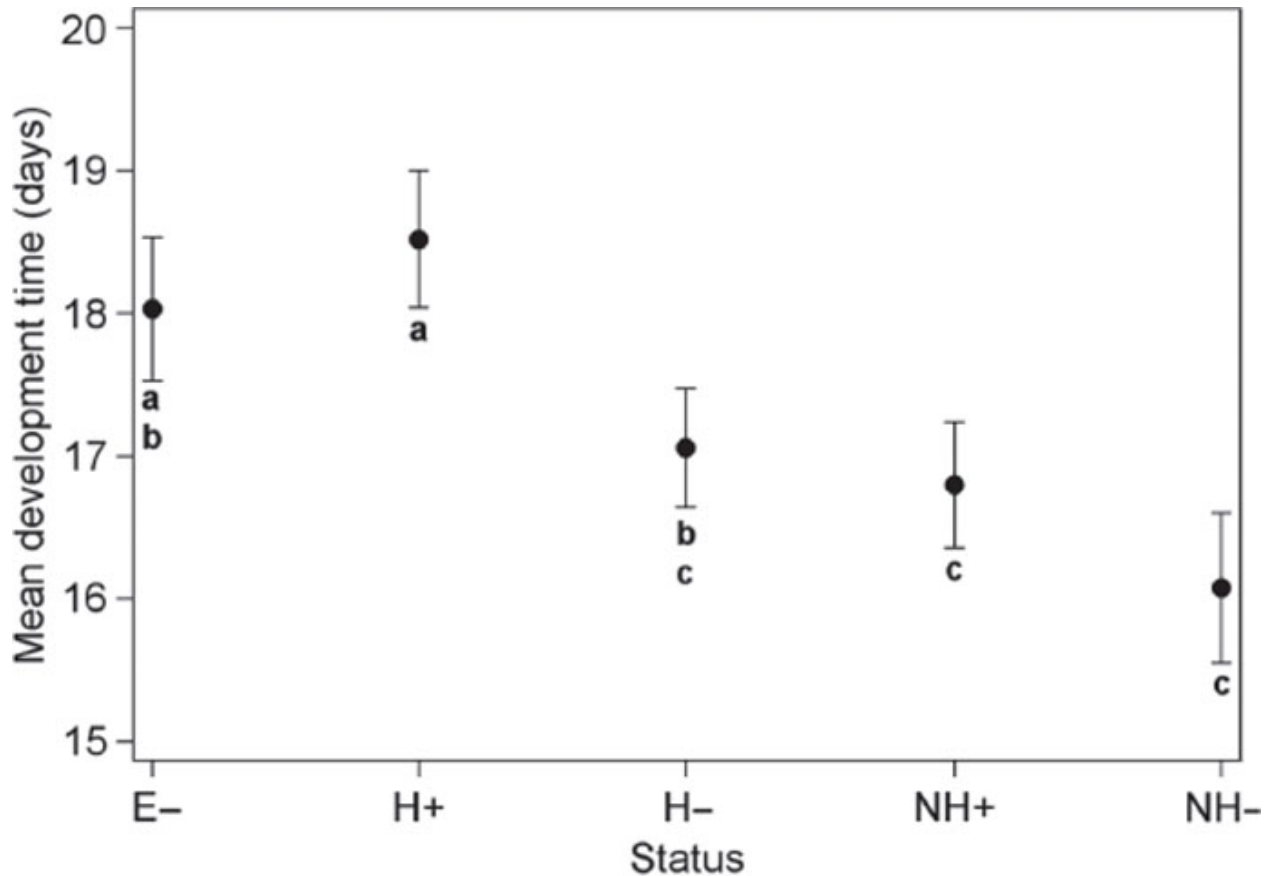
### **Bird cherry–oat aphid feeding preference**

Endophyte infection status, hybrid status and endophyte-associated plant genotypes affected host-plant preference of aphids. The odds of aphids choosing E<sup>-</sup> plants was more than three times the odds of choosing H<sup>+</sup> plants, although this effect was not statistically significant (OR = 3.03,  $p = 0.09$ ). Likewise, the odds of aphids choosing NH<sup>+</sup> plants was more than three times greater than the odds of choosing H<sup>+</sup> plants, but again this effect was not statistically significant (OR = 3.66,  $p = 0.06$ ). No other pairwise comparisons were statistically significant.

### **Effects of infection and hybridization on ladybird weight and development**

Endophyte infection (NH<sup>+</sup> versus NH<sup>-</sup>, H<sup>+</sup> versus H<sup>-</sup>, NH<sup>+</sup> versus E<sup>-</sup>, H<sup>+</sup> versus E<sup>-</sup>) and hybrid status of the endophyte (H<sup>+</sup> versus NH<sup>+</sup>) did not affect the weight of the ladybird beetles ( $F_{4,10} = 1.07$ ,  $p = 0.42$ ). However, development time of the ladybirds from egg to adult was affected by endophyte status and hybrid status ( $F_{3,51} = 189.52$ ,  $p < 0.0001$ , Fig. 1). Development of ladybirds that fed upon aphids from H<sup>+</sup> plants was delayed on average by about 1.5 days compared to ladybirds that fed upon aphids from H<sup>-</sup> plants (95% simultaneous confidence interval: 0.4 to 2.5 days), by about 1.7 days compared to ladybirds feeding on NH<sup>+</sup> plants (95% simultaneous confidence interval: 0.6 to 2.8 days) and by almost 2.5 days compared to ladybird beetles feeding on NH<sup>-</sup> plants (95% simultaneous confidence interval: 1.4 to 3.5 days). Ladybird beetles took on average about 1.2 days longer to develop when consuming aphids that had been feeding on E<sup>-</sup> plants compared to ladybirds that had been feeding on aphids from NH<sup>+</sup> plants (95% simultaneous confidence interval: 0.2 to 2.3 days) and about two days longer compared to those feeding on NH<sup>-</sup> plants (95% simultaneous confidence interval: 0.7 to 3.2 days). No other pairwise comparisons were statistically significant.

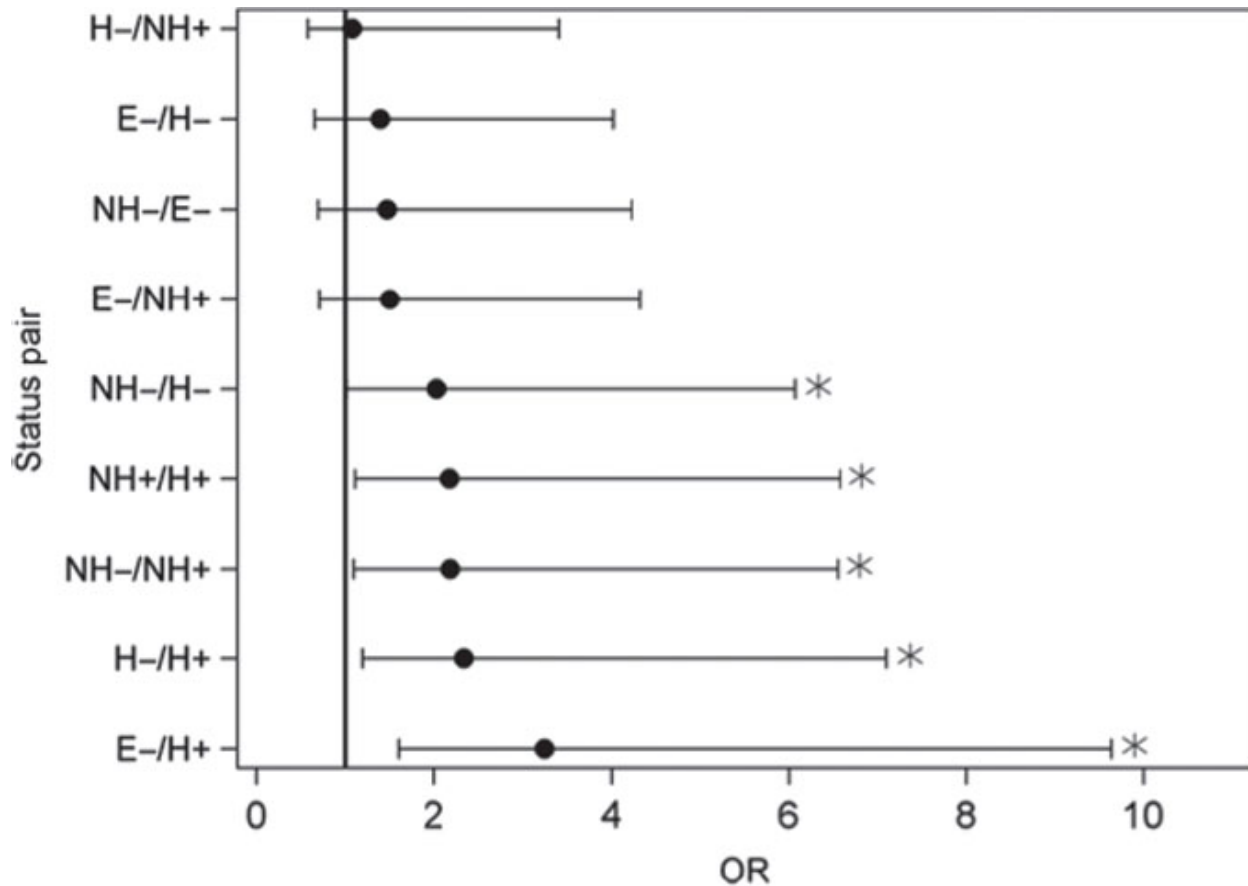




**Figure 1.** The effects of endophyte infections (E- naturally endophyte free, H+ hybrid endophyte infected, H- manipulatively hybrid endophyte free, NH+ non-hybrid endophyte infected, NH- manipulatively non-hybrid endophyte free) of Arizona fescue on the development time (from egg to adult) of the pink spotted ladybird beetles. Estimated means with 95% confidence intervals. Lower case letters indicate statistically significant differences among endophyte status.

### Ladybird beetle feeding preference

Endophyte infection category of the host plant affected the host-plant preference of the ladybird beetles ( $\chi^2$  (4 DF) = 9.61,  $p = 0.05$ ). Ladybird beetles were more likely to choose all other infection categories (E-, H-, NH+) than to choose H+ (Fig. 2). The odds of choosing E- were 3.24 times the odds of choosing H+ ( $p < 0.001$ ); the odds of choosing H- were 2.34 times the odds of choosing H+ ( $p = 0.017$ ); and the odds of choosing NH+ were 2.17 times higher than the odds of choosing H+ ( $p = 0.028$ ). Furthermore, odds of beetles choosing NH- plants were higher than the odds of choosing NH+ plants (OR = 2.18,  $p = 0.024$ ), and the odds of choosing NH- plants were higher than the odds of choosing H- plants (OR = 2.03,  $p = 0.040$ ). Neither the number of aphids on the plants, the number tillers, the height of the plant, nor the duration of a trial were statistically significant predictors of host-plant preference of the ladybirds.



**Figure 2.** Odds ratios, with 95% confidence limits, comparing pairs of endophyte infection status (E- naturally endophyte free, H+ hybrid endophyte infected, H- manipulatively hybrid endophyte free, NH+ non-hybrid endophyte infected, NH- manipulatively non-hybrid endophyte free) on the preferences of the pink spotted ladybird beetles for grasses harboring aphid populations. For each pair, the interval represents the odds of choosing the first status divided by the odds of choosing the second status. An interval that does not include 1 indicates a statistically significant ( $p < 0.05$ ) difference in preference (indicated by asterisks).

### Peramine produced by the endophytes

Both NH+ and H+ plants produced peramine. However, the level of peramine produced did not vary statistically between NH+ and H+ plants ( $t(18) = 0.55$ ,  $p = 0.59$ ). Endophyte free plants (E-, NH- and H- plants) did not produce any peramine, as expected.

### Discussion

#### Endophytes and aphids

Although endophyte infected grasses are commonly thought to be protected against herbivores via endophytic alkaloids (Clay and Schardl 2002, Faeth 2002, Saikkonen et al. 2010), our results with the bird cherry-oat aphid and Arizona fescue do not support the concept of herbivore

defense via endophyte infection (Cheplick and Clay 1988, Clay and Schardl 2002). We did not find negative effects of either NH<sup>+</sup> or H<sup>+</sup> endophytes on reproduction of aphids, proportion of winged forms in aphid populations or the host-plant preference of aphids. These results are consistent with those from earlier studies where preference and performance of other insect herbivores were not affected by the endophyte infection in Arizona fescue (Saikkonen et al. 1999, Tibbets and Faeth 1999, Faeth 2009). However, these studies did not consider the effect of hybridization of endophytes on herbivore resistance. Our results suggest that *Neotyphodium*-infected Arizona fescue, regardless of H<sup>+</sup> or NH<sup>+</sup> infection, is not particularly deterrent or toxic to bird cherry–oat aphids. Accordingly, the levels of peramine were not shown to differ between H<sup>+</sup> and NH<sup>+</sup> plants. Furthermore, plant genotypes associated with H<sup>+</sup> and NH<sup>+</sup> Arizona fescues did not affect the host-plant preference or performance of aphids in our experiments (i.e. no differences among E<sup>-</sup>, NH<sup>-</sup> and H<sup>-</sup> plants).

The bird cherry–oat aphid has often been used in bioassays to test the effects of endophytes and their alkaloidal mycotoxins on herbivore resistance because it is highly sensitive to levels of alkaloids in infected perennial ryegrass and tall fescue (Hunt and Newman 2005, Krauss et al. 2007, Meister et al. 2006). However, peramine, the only alkaloid known to be produced by *Neotyphodium* in Arizona fescue (Faeth et al. 2002) is not particularly effective against the bird cherry–oat aphid at low levels (Siegel 1990) but may be highly effective against other herbivorous insects (Cheplick and Faeth 2009). Regardless of possible undetected alkaloid or other biochemical differences between H<sup>+</sup> and NH<sup>+</sup> Arizona fescues, these do not translate into differences in herbivore resistance, at least to the bird cherry–oat aphid.

### **Endophytes and ladybird beetles**

Although we did not find evidence for effects of endophyte infections, hybridization of the endophytes or plant genotype on aphid preference and performance, our experiments are the first to demonstrate that hybridization of microbial plant symbionts affects preference and performance of the natural enemies of herbivores. The development of ladybird beetles was delayed when consuming aphids from H<sup>+</sup> plants compared to H<sup>-</sup>, NH<sup>+</sup> and NH<sup>-</sup> infected plants. Furthermore, this difference in performance is reflected in preference by ladybird beetles – generally, adult beetles avoided aphids feeding on H<sup>+</sup> plants over aphids feeding on all other plant types in our experiment.

The delayed development of ladybird beetles on H<sup>+</sup> and E<sup>-</sup> plants, compared to the development time of beetles on other plants, may translate into lower fitness of these generalist predators. Conditions experienced during early insect development have strong downstream effects on the adult phenotype, and therefore on fitness. When juvenile nutritional conditions are good, animals tend to mature at an earlier age (Day and Rowe 2002). This trait has been demonstrated to strongly correlate with fitness in a range of species, including beetles, even when delayed development, like in our experiment, is not associated with lowered adult weight (de Sassi et al. 2006, Dmitriew and Rowe 2011).

The mechanisms by which endophytes affect higher trophic levels are still poorly understood (Saikkonen et al. 2010, Faeth and Saari 2012). Similar to other plant allelochemicals (Jeffries and Lawton 1985, Pasteels 2007, Witte et al. 1990), alkaloids originating from endophyte infections may affect natural enemies of herbivores more than the herbivores themselves (Omacini et al. 2001, Faeth and Saari 2012). Many invertebrate herbivores tolerate or compensate for suboptimal nutritional diets (Simpson and Simpson 1990) and others store, sequester and use plant noxious compounds for their own defense against natural enemies (Soetens et al. 1998, Dobler 2001). Likewise, invertebrates feeding on endophyte-infected grasses have also been suggested to sequester and use endophytic alkaloids in their defense against predators and parasites (Faeth 2002, Cheplick and Faeth 2009). These population level effects may translate into consequences at the community level. For example, Faeth and Shochat (2010) and Jani et al. (2010) found that parasite and predator richness declined on infected plants of two native grasses, Arizona fescue *Festuca arizonica* and sleepygrass *Achnatherum robustum*, and herbivore abundance was greater on infected plants.

We do not know the mechanism for the negative effects of the H<sup>+</sup> endophyte on ladybird beetles, as we did not detect differences in the amount of peramine produced by H<sup>+</sup> and NH<sup>+</sup> infected plants. It is possible that H<sup>+</sup> plants are producing some alkaloids harmful to insects that went unmeasured in our study, although only peramine has been detected in infected Arizona fescue so far (Faeth et al. 2002, Schardl et al. 2012). For example, recent evidence suggests that the *idtG* gene (one gene of many in a complex biosynthetic pathway) for lolitrem B (an indole diterpene) is found in both H<sup>+</sup> and NH<sup>+</sup> Arizona fescue (Schardl et al. 2012, C. Young pers. comm.). Furthermore, endophyte infection is associated with several biochemical, physiological and morphological changes in host grasses (Rasmussen et al. 2008) that may explain the effect found in the ladybirds.

Our results may provide insight into solving a puzzle regarding the distribution of infection and hybridization in Arizona fescue. Contrary to the endophyte hybrid hypothesis (Schardl and Craven 2003) and empirical evidence that hybrids are more common across grass species and within grass populations (Oberhofer and Leuchtman 2012, Iannone et al. 2012), H<sup>+</sup> is far less common than NH<sup>+</sup> in natural Arizona fescue populations (Hamilton et al. 2009). Even though H<sup>+</sup> plants perform (Hamilton et al. 2010) and compete (Saari and Faeth 2012) better than NH<sup>+</sup> plants under stressful moisture and nutrient conditions, such as those often found in their natural habitats, they are less frequent than NH<sup>+</sup> plants. Our results provide a possible explanation for the relative rarity of H<sup>+</sup> plants: natural enemies of herbivores such as ladybirds avoid H<sup>+</sup> plants because development time and, presumably, fitness are reduced. H<sup>+</sup> plants may have higher herbivore loads, and thus reduced fitness, because predators avoid these plants, creating “enemy-reduced space” for herbivores (Jani et al. 2010). Although additional experiments under field conditions are needed, our results suggest that the wide variability in endophyte – host-plant – herbivore interactions observed in natural populations may result from unanticipated effects of endophytes on the third trophic level.

## **The effects of plant genotypes associated with endophyte infections on herbivores and predators**

We did not find direct effects of the host-plant genotypes associated with endophyte infections on herbivores. Even so, our findings suggest that the endophyte infection related plant genotypes may affect ladybirds because ladybird larvae developed more slowly when feeding on aphids that had been growing on E- compared to NH- plants. This result is in line with previous studies that suggest that certain plant genotypes are likely co-adapted with their fungal symbionts (Lehtonen et al. 2005, Sullivan and Faeth 2008, Cheplick and Faeth 2009). In general, the effects of plant genotypes on herbivore community structure are well known (Crutsinger et al. 2006) but the significance of within-species plant genotype diversity for trophic interactions has not been well studied. Johnson (2008) found that plant genotypes can have a direct impact at the third trophic level, affecting the abundance and richness of predators of aphids in a natural system. But others (Vinson 1999) suggested that within-species variation in plant genotype only has weak effects on natural enemies of herbivores.

### **Conclusions**

Our results suggest that hybridization of microbial symbionts may have a negative bottom-up effect on a predator and consequently, a positive, indirect top-down effect on an herbivore. Direct effects of hybridization of endophytes on herbivores were not evident in this experiment. Both variation in endophyte and plant genotypes can affect higher trophic levels. If predators of herbivores are viewed as allies of the host-plant by reducing herbivore loads, and the H+ endophyte is negatively affecting predators, then H+ infection may shift the interaction between the endophyte and the host towards antagonism along the mutualistic – antagonistic spectrum. The traditional view of endophytes as defensive plant mutualists via herbivore defense (Clay and Schardl 2002) may not always hold if the third trophic level is included. Our findings indicate that studying multi-trophic level consequences of genetic variation of microbial plant symbionts may be essential to understanding the ecology of plant-symbiont interactions and the consequences for structure of communities. Microbial plant symbionts should be considered as an important influence shaping the bottom-up and top-down forces of communities.

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