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HERBIVORY MEDIATES GRASS–ENDOPHYTE RELATIONSHIPS: COMMENT

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In the recent paper by Koh and Hik (2007), the pattern of *Neotyphodium* spp. endophyte infection frequency is shown to be related with the gradient of grazing pressure by two native herbivores, pikas (*Ochotona collaris*) and hoary marmots (*Marmota caligata*) in Yukon Territory (Canada). The grazing gradient is determined by the behavior of herbivores, being high "on" boulder fields and low "far" from there. The mechanism invoked to explain the pattern of infection frequency is the relative fitness of endophyte-infected and uninfected plants along this gradient. According to the defensive mutualism and optimal defense theories (Clay 1993, Zangerl and Rutledge 1996), infected hosts with alkaloid-based deterrents would be favored where grazing was consistently high, while uninfected plants or infected plants without deterrents would be favored where herbivory was low or absent. To reinforce the hypothesis, a trial was carried out to evaluate pikas selectivity for endophyte-infected and uninfected plants sampled from "on + near" and "far" from boulder fields. Herbivores were able to discriminate endophyte-infected and uninfected *Festuca altaica* plants from boulder fields, but they did not discriminate when plants came from meadows. In summary, the authors conclude that grazing pressure and herbivory selectivity is the major factor generating the pattern in endophyte infection frequency. Hence, endophyte infection is viewed as an adaptive trait in the environment with high grazing pressure.

In our opinion, Koh and Hik (2007) present little information supporting that differences in endophyte infection frequency and herbivore avoidance of infected grasses were mainly a consequence of the distinct longer term grazing history of the grasses mediated by the deterrence fungus effect. We propose that the effect of herbivores on infection frequency might as well be mediated through selection of phenotypic traits not related to antiherbivory (e.g., compensatory growth ability [Karban and Baldwin 1997, Sullivan et al. 2007]), or through an interaction with other environmental factors that can affect both partners to a different extent (Hill et al. 2005, Rasmussen et al. 2007). A usual observational approach to study broad ecological patterns is to find out simple relationships between the distribution and abundance of organisms and any environmental factor. However, this procedure may result in oversimplifications leading to wrong conclusions (see the case of shared enemies discussed by Holt and Lawton 1994). For the specific case of *Neotyphodium* endophytes, ecological mechanisms underlying patterns of endophyte infection frequency are quite complex (Faeth 2002). In fact, Koh and Hik (2007) acknowledge that "variations at the population level in the deterrence ability of infected tillers, frequency of infection, and hyphal densities are thought to be produced by multiple factors (genetic and environmental)" among which they cite "differences in grazing pressure." Different theoretical and modeling approaches have indicated that the endophyte infection frequency in local populations may result from some of the following mechanisms: (1) relative fitness of endophyte-infected and uninfected plants, (2) vertical transmission efficiency of endophyte in infected plants, and (3) migration of infected and uninfected individuals among connected local populations (Clay 1993, Ravel et al. 1997, Saikkonen et al. 2002, Gundel et al. 2008). Here, we discuss each of those mechanisms and confront them with the arguments presented by Koh and Hik (2007). Our aim is to show that relative fitness may or may not be related to herbivory deterrence and that the other two mechanisms have been almost completely neglected.

1. The relative fitness of endophyte-infected and uninfected related plants has been always used to explain natural variations in endophyte infection frequency in grass populations (Clay 1993, Clay and Schardl 2002).— By this mechanism, it is predicted that the endophyte infection frequency tends to be high if endophyte enhances the host plant fitness or, alternatively, the endophyte goes to extinction if it depresses the fitness of the plants (Clay 1993). An ecological trait can be considered adaptive if it has a direct impact on fitness in natural environments. In the case of the endophyte infection, to consider it as adaptive based solely on the observed correlation between infection frequency and grazing level may be problematic. Even when the correlation is observed, it is difficult to determine whether the ecological trait (endophyte infection) per

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se contributes directly to variation in fitness under grazing pressure, or whether this relationship reflects indirect selection via correlated traits (see Holt and Lawton 1994, Sullivan et al. 2007). It is known that endophyte modifies plant functional traits (physiology, development and morphology), which in turn may affect components of fitness, such as date of flowering, growth rate and number of buds or tillers (Malinowski and Belesky 2000, Cheplick and Cho 2003, Vila-Aiub et al. 2005). As a consequence, endophyte may enhance plant fitness under variations in water and nutrients availability or toxic elements, among others (West et al. 1993, Bonnet et al. 2000, Malinowski and Belesky 2000, Lewis 2004, Zabalgogeazcoa et al. 2006). Hence, environmental differences in patterns of selection on functional traits attributed to variations in alkaloid concentration may in fact be produced by endophyte effects on several fitness components of the host fitness (Faeth 2002).

Invoking the same mechanism (relative fitness) and with the same experimental approach used by Koh and Hik (2007), other studies have found correlations between patterns of endophyte infection frequency and different environmental factors (Novas et al. 2007, Bazely et al. 2007). These studies have in common two lines of reasoning. First, in one extreme of the correlation, the association between high endophyte infection frequency and any environmental factor indicates a higher fitness of endophyte-infected plant over uninfected ones. Thus, it is thought that endophyte infection confers fitness advantage to such environmental factors. Second, in the other extreme of the correlation, the energetic cost of maintaining an endophyte that does not confer fitness advantage to the environmental factor is used to explain the lack of infection. Koh and Hik (2007) themselves state that "since there may be metabolic costs to harbor endophytes and to produce secondary metabolites, there could be selection against a deterrent system for infected grasses where there is no herbivory." The most conspicuous issue in the original paper is the absence of a gradient of grazing pressure; therefore, the proposed relationship between both variables is not supported. Moreover, Faeth et al. (2006) found for another native species, *Achnatherum robustum*, a negative relation between infection frequency and concentration of endophyte-produced alkaloids, demonstrating that high levels of infection are not necessarily driven by this anti-herbivory trait. The intrinsically complex nature of the grass–endophyte system in natural environments is likely to result from the high genetic diversity and varying selective pressures due to the biotic and abiotic conditions continuously changing in time and space (Faeth 2002).

These examples demonstrate that, while the correlation approach provides a broad screening about the association between variables, drawing cause–effect conclusions must be cautiously treated. Therefore, to determine whether herbivory selection might be favoring endophyte infection in different environments, studies have to be conducted to examine the influence of this trait on plant fitness in environments that differ in grazing pressure but keep all other environmental variables constant. Not only Koh and Hik (2007) did not discuss or even consider this methodological drawback, but they didn't consider other biological complicating factors, such as competition with other plants, mycorrhizal associations and also herbivory by invertebrates animals likely to be driving differential fitness between infected and uninfected plants along the grazing gradient (Marks et al. 1991, Clay et al. 2005, Omacini et al. 2006). In fact, Faeth (2002) after reviewing existing literature concluded that endophyte benefits are more related to increasing competitive ability rather than anti-herbivory effect. Environment "on" boulder fields seem to be quite different from the rest of the environments, differing both in abiotic variables (physic and chemical properties) and, also, in biotic interactions (e.g., total plant cover).

2. Neotyphodium *endophytes are transmitted vertically through the host plant seeds, since there is no contagious spread and they do not travel by pollen (Clay and Schardl 2002, Faeth 2002).*—The efficiency of transmission in host plants depends on the proportion of endophyte infected individuals in relation to the total number of new individuals produced. Failure in vertical transmission is likely to occur under natural conditions. Several species of *Festuca* and *Lolium* have shown to produce variable proportions of uninfected tiller and seeds (Welty et al. 1994, Schulthess and Faeth 1998, Bazely et al. 2007), and noninfected seedlings produced by infected seeds have also been reported, as a result of endophyte death in viable seeds (Welty et al. 1987, Hill et al. 2005). The above examples indicate that endophytes and host plants do not often share requirements and that in fact, they have different sensitivities to environmental factors (Hill et al. 2005, Rasmussen et al. 2007). Therefore, endophyte infection frequencies could be driven by the imperfect vertical transmission rather than relative fitness of infected and uninfected plants (Ravel et al. 1997, Gundel et al. 2008). This possibility is not even mentioned by Koh and Hik (2007). In fact, endophyte-infected plants could be highly superior in terms of ecological fitness, but the endophyte be negatively affected. Notably, the authors show data suggesting that endophyte growth is negatively affected in *Festuca* plants "far" from boulder fields (i.e., lower hyphal density). This result is explained by the authors based on the metabolic cost to maintain a trait (i.e., the endophyte) that does not produce an evident benefit for the hosts; hence they state that the endophyte is selected against. We believe this is unfortunate. First, they do not prove the relative fitness of infected and uninfected plants in each site. Second, we think that the low hyphal density found in tussock plants sampled from "far" sites of boulder fields in fact supports the idea of different requirements for endophytes and their host plants.

3. Migratory processes have been modeled simulating the movement of endophyte-infected and/or uninfected propagules between populations and consequently affecting the endophyte persistence and infection frequency (Saikkonen et al. 2002).—Koh and Hik (2007) acknowledge the fact that "In the absence of a toxic effect, a condition thought to be important for mutualism and the persistence of *Neotyphodium* spp. in natural populations of grasses (Clay and Schardl 2002), infection in grasses could be maintained by the spatial structure of metapopulations, so that local-level selection favors infected plants relative to uninfected plants (Saikkonen et al. 1998)." However, the role of migration processes still remains unexplored. Due to the lack of a real grazing gradient, intermediate level of endophyte infection in "near" stands suggests the existence of an ecotone; thus, this level of infection could be caused by mass effects from both patches "on" and "far." In this ecotone transition area, seeds with a high proportion of infection coming from "on" to "near" would mix with seeds with a low proportion of infection migrating from "far" to "near." Presumably, in the Yukon Territory (Canada), boulder fields and meadows differ in patch size and topography (Koh and Hik 2007). This might be crucial for persistence of particular grass biotypes (infected and uninfected) with differences in reproductive and dispersal ability (Cousens and Mortimer 1995). Moreover, grazing selective pressure may impact on seed production and dispersal through tiller, flower, and seed consumption, and seed accumulation or burial in nesting areas.

The cafeteria-style feeding trial carried out by Hok and Hik (2007) was suitable to assess the preference for uninfected plants by the herbivores (pikas), but it did not provide conclusive evidence that the selective grazing was generating the observed endophyte infection frequency. The question whether the pattern of endophyte infection frequency is due to the grazing pressure and selective herbivory by pikas (selectivity viewed as a cause) or is only a consequence of the status of the endophytes in the plants from different sites (selectivity viewed as a consequence), remains unsolved. Furthermore, the use of hyphal density as indicative of the alkaloid status in plant is a simplistic approach prone to failure because the relationship between hyphal density and infection frequency with alkaloid level is still far from being consistent (Spiering et al. 2005, Rasmussen et al. 2007, also see Faeth et al. 2006). Although the deterrent effect of endophyte-infected native plants on native herbivores may constitute a novel discovery (Koh and Hik 2007), the existence of native species populations with high endophyte infection level in absence of toxicity is still intriguing (Faeth 2002, Faeth et al. 2006).

In summary, it is not our objective to underestimate the importance of selective grazing by herbivores as a driving force of this particular endophyte infection frequency along transects from boulder fields to meadows in the Yukon Territory. However, we intend to call the attention that other environmental factors could be, in addition, affecting or even interacting with grazing to produce such specific pattern of endophyte infection frequency. Along transects, other factors may be varying in relation to topography and generating environmental gradients. These variations might generate not only changes in abiotic conditions but also in species interaction. Thereafter, it does not seem appropriate to consider herbivore grazing as the environmental factor generating selection pressure over the grass population, without considering the others. Moreover, Hok and Hik (2007) avoided addressing failures in the mechanism of endophyte vertical transmission, and the effect of migration processes in the infection frequency. We believe that, although those issues have been recurrently neglected, they are critical to understand not only the persistence and abundance of endophytes in grass populations but also to comprehend the symbiotic nature of the interaction between partners (grass and endophyte) in natural systems.

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HERBIVORY MEDIATES GRASS–ENDOPHYTE RELATIONSHIPS: REPLY

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The ecology and evolution of endophytes is a relatively recent topic of inquiry and there are still only a few experimental studies under natural field conditions on which to attempt to develop more general models. Gundel et al. (2008) have commented on our report (Koh and Hik 2007) that vertebrate herbivores can mediate the relationship between systemic fungal endophytes (*Neotyphodium* spp.) and a host native grass (*Festuca altaica*). While they presented both specific and more general objections to our methodology and our interpretation of results, it is worth remembering that our original report was intended to stimulate some debate about the role of herbivory in determining patterns of endophyte infection in *Festuca*, and we have clearly been successful in this respect.

There are more points of consensus between our original report and Gundel at al. (2008) than their comment might suggest. They are in agreement with us

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that the importance of grazing by herbivores as a driving force of this particular endophyte infection frequency along transects from boulder fields to meadows in the Yukon Territory should not be underestimated. We are also in agreement with them that attention must be paid to other environmental factors that could also be affecting or even interacting with grazing to produce such specific pattern of endophyte infection frequency.

In this reply, we will clarify several statements that Gundel et al. (2008) interpreted somewhat differently than we had intended, and emphasize why we believe that we are able to separate causal from correlative interpretations of our data in a way that supports our conclusion that grazing was the primary determinant of infection frequency. We will also provide additional information about other attributes of the grass–endophyte relationship at our study site.

Gundel et al. (2008) stated that the conclusions in our original paper were that (1) "differences in endophyte infection frequency" and (2) "herbivore avoidance of infected grasses" "were mainly a consequence of the distinct longer term grazing histories of the grasses mediated by the deterrence fungus effect." However, what we argued in our original report was that differences in infection frequencies were the result of the grazing history experienced by the grasses as a result the behavioral characteristics of the herbivore that constrained their activity closer to geologically stable boulder fields. Thus grasses "far" from boulder fields were not exposed to grazing pressures regardless of any effect that anti-herbivore deterrents found in infected grasses may have had. However, grasses "on" the boulder fields were exposed to grazing, and therefore must be subjected to different selection pressures. In contrast to the Gundel et al. (2008) interpretation, we argued that behavioral constraints and not herbivore selectivity generated the differential grazing pressure (and infection frequency) between grazed and ungrazed populations of grasses in our study.

Second, we suggested that herbivores avoided infected grasses only from "on" (rather than all infected grasses, including those from "far," as suggested by Gundel et al. [2008]) where endophytes were found to have significantly greater hyphal densities relative to grasses from "far." This is a subtle but important distinction to consider since herbivores did not avoid infected grass if that grass was from "far", but did avoid infected grasses from "on" (see Fig. 3 in Koh and Hik 2007). The experimental evidence from the feeding trials and hyphal density counts strongly suggested that infected grasses in situ "on" boulder fields should be protected from grazing compared to their uninfected conspecifics. This is not speculation but an experimentally observed property of the natural system we examined (Koh and Hik 2007).

Gundel et al. (2008) stated that the "most conspicuous issue in the original paper" is the absence of a gradient of grazing pressure. The basis for the consideration of a strong grazing gradient at our site is well documented (McIntire and Hik 2002, 2005, Karels et al. 2004, Morrison et al. 2004) and similar patterns have been observed at other sites occupied by pikas (Huntly 1987, Roach et al. 2001). In our original paper we focused on patterns at both endpoints and the middle of this grazing gradient.

Gundel et al. (2008) were also concerned that we did not correctly interpret causal from correlative relationships, and suggested some options to determine if herbivory selection might favor endophyte infection in differing environments. Specifically, they recommend this could be done with environmental variables held constant while differing grazing pressure and they suggest that we did not "even consider this methodological drawback." In our circumstance, the challenge was to avoid altering the system for the convenience of measurement since this would have compromised the integrity of the relationships that were being examined. In a natural setting, it is simply impossible to impose a constant and thus artificial environment over the entire study area where the interactions between the key elements of the system are to be measured. Therefore, we randomly selected boulder fields from different parts of the valley that were occupied by the herbivores. In this context, we isolated and maintained the grazing gradient as a constant while the environment varied. Site selection to randomize abiotic variables was done deliberately. Slope, aspect, wind exposure, soil moisture, soil depth, soil temperature, and water regime varied for each of the sites (*unpublished data*). We found that infection rates were remarkably consistent for all "on" boulder field and "far" from boulder field areas over time. Since the environment varied while we held the grazing gradient constant, we argued that patterns in infection frequency at these sites should be related to long term grazing patterns and associated selection pressures.

Gundel et al. (2008) correctly inferred that "on" boulder field should be different from "far" in terms of abiotic (physical and chemical) and biotic interactions (e.g., total cover). We used Pearson's correlation coefficients and nonparametric multiplicative regression (NPMR) analyses (McCune 2006) to examine the relationship between infection frequency (response variable) and abiotic or biotic predictors that, at our site, vary over space, including slope, aspect, soil moisture, soil depth, and plant cover (*unpublished data*). NPMR uses a forward stepwise "free search" to create a series of response models from different combinations of the measured predictor variables. A cross-validated R^2 then determines the predictive quality of those models (McCune 2006). The estimated response for a given

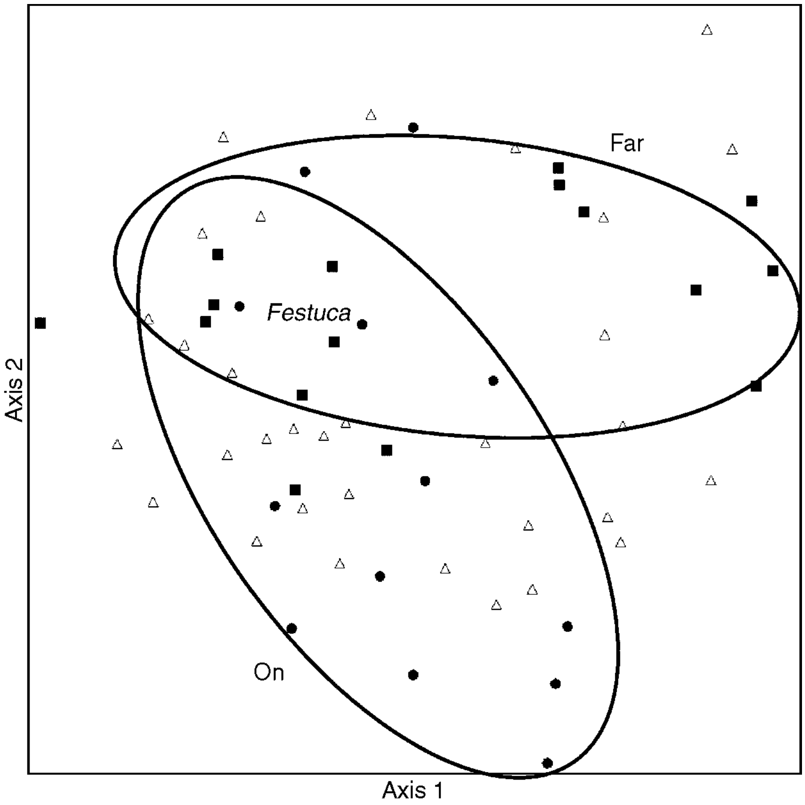

FIG. 1. Nonmetric multidimensional scaling (NMS) plot ordination of plant species composition data for 1 × 1 m quadrats ($n = 60$) located at varying distances from boulder fields: "on" boulder fields, 0–5 m (lower left in ordination, solid circles); "near" boulder fields, 5.1–15 m (scattered throughout ordination, open triangles); and "far" from boulder fields, 60–80 m (upper area in ordination, solid squares). Ordination was performed with the full species data set; however, only the position of *Festuca altaica* is shown here.

location (the target point) inside the predictor space for a given set of predictor variables used a locally linear model and was determined by a weighted least-squares regression. A Gaussian function weighed points closer to the target point more heavily than points that were more distant. The statistical significance of the best model produced was determined with a Monte Carlo randomization test (McCune 2006).

Infection frequency was very poorly related to any combination of the measured abiotic and biotic variables. Of the 353 models generated, a single variable, slope, was suggested as the best predictor for infection frequency. However this model produced a cross-validated R^2 of 0.03742 and was statistically insignificant (Monte Carlo randomization test, model $P = 0.286$).

On the basis of a couple of indices, infection frequency did not appear to be related to potential for competition at our site. We recorded vascular plant species cover and infection frequency of *Festuca altaica* ($n = 23$) in quadrats (1 × 1 m) and found that neither the total amount of non-*Festuca* cover or the ratio between *Festuca* and total cover were significantly related to infection frequencies (Pearson correlation, non–*Festuca* cover, $r = 0.06$, $P = 0.208$; Pearson correlation, *Festuca* total cover, $r = 0.06$, $P = 0.199$. An ordination of plant community data using non-metric multi-dimensional scaling (PCOrd 5.12; MJM Software Design, Gleneden Beach, Oregon, USA) showed a discernable pattern in the distribution of the plant community with distance from boulder field, but the presence of *Festuca altaica* did not appear to show an affinity for any particular distance class (Fig. 1).

Gundel et al. (2008) suggested that, without knowing the relative fitness of infected and uninfected plants at each site, we would be unable to ascertain whether or not metabolic costs vary. We have established several experiments to address questions similar to those posed by Gundel et al. (2008), including examination of the demography of infected and uninfected *Festuca altaica* along a grazing gradient and the role of competition in grass–endophyte relations. For example, we have surveyed infected and uninfected tussocks that were marked and clipped to ground level along the grazing gradient in the previous year to examine if infection status and distance from boulder field would be related to differences in tussock regrowth (measured as the

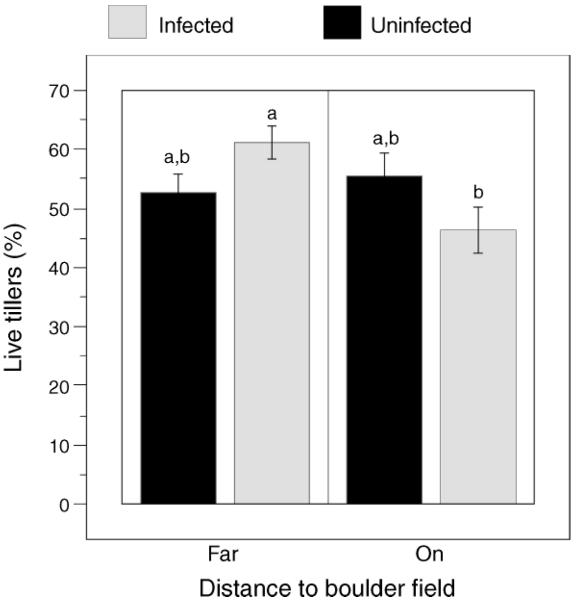

FIG. 2. Percentage (mean ± SE) of live tillers 0–5 m from boulder fields ("on") and 60–80 m from boulder fields ("far") for infected (gray bars) and uninfected (black bars) *Festuca altaica* tussocks (infected "on," $n = 31$; infected "far," $n = 35$; uninfected "on," $n = 36$; uninfected "far," $n = 51$) in July 2005 from plants clipped to ground level in July 2004. Means that share a letter were not significantly different (Tukey-Kramer comparison of means, $P > 0.05$).

percentage of tillers emerging from a previously clipped tussock). These measurements were conducted without knowledge of the infection status of the tussock. Data were analyzed using a 2 × 2 cross-factorial ANOVA with infection status and location (on or far) as fixed factors. Neither infection status (ANOVA, $F_{1,149} = 0.0147$, $P = 0.9036$) or location (ANOVA, $F_{1,149} = 2.831$, $P = 0.0946$) had an effect on tussock regrowth. However there was a significant interaction between infection status and location (ANOVA, $F_{1,149} = 6.2305$, $P = 0.0136$). Infected plants "far" from boulder fields with relatively lower hyphal densities (Koh and Hik 2007) had significantly higher levels of regrowth compared to infected plants "on" boulder fields (Fig. 2). Interestingly, uninfected plants from both locations were intermediate to the infected plants from "far" and "on" (Fig. 2). This suggests that infected plants "on" boulder fields may incur higher metabolic costs to host the higher densities of endophytes compared to infected plants "far" from boulder fields where hyphal densities were relatively lower. Infected plants "far" from boulder fields may have had better regrowth than uninfected plants because host plants, with lower hyphal densities, may have benefited from the presence of endophytes when clipped (Clay and Schardl 2002). These new results and those from our original paper are consistent with predictions based on optimal defense theory (Zangerl and Rutledge 1996) and those previously made by Faeth (2002) that the relationship between grasses and *Neotyphodium* spp. should vary with grazing if that relationship is based on mutualism.

We concur with Gundel et al. (2008) that transmission efficiency, along with many other variables, may play a role in determining infection frequency of host grasses by *Neotyphodium* endophytes at the population level. However, we think it is unlikely to be a critical factor at our alpine sites. Here, infection frequency as a result of transmission efficiency would be immediately altered by differences in germination rates and juvenile survivorship between infected and uninfected individuals. In any case, infection frequency of adult individuals would then be subjected to the selection pressures associated with the specific levels of herbivory and other environmental variables at each site or location. For instance, while Ravel et al. (1997) were able to account for changes in infection frequency as a result of imperfect transmission, variations as a result of subsequent selection pressures were not incorporated into their model.

We are still exploring the role of various biotic and abiotic factors in our alpine system, however given the consistency of our results at multiple sites over a variety of environments along similar grazing gradients we stand by our original conclusion that vertebrate herbivory was the primary determinant of infection frequency in meadows inhabited by collared pikas in the Yukon. We hope future studies will clarify how the various mechanisms operate to influencing grass–*Neotyphodium* interactions in other natural environments.

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