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FUNGAL ENDOPHYTE INFECTION IN AN ALPINE MEADOW: TESTING THE MUTUALISM THEORY

by

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ABSTRACT

Neotyphodium are fungal endosymbionts of grasses that reproduce asexually by infecting the host's seed. This relationship has traditionally been considered mutualistic, with the fungus improving host fitness by alleviating important stresses. To determine the importance of biotic and abiotic stresses in mediating the endophyte-grass interaction, I investigated the relationship between grazing pressure by collared pikas and *Neotyphodium* sp. infection frequency in the grass *Festuca altaica* in an alpine meadow. I conducted a factorial design experiment combining endophyte infection, grazing history, fungicide and fertilizer. Leaf demography and herbivory damage were monitored every two weeks. In areas with chronic grazing history, infected plants were significantly less productive than uninfected tussocks, but there was no difference at low grazing history. There was no effect of infection on the likelihood of herbivory. Contrary to predictions of the mutualism theory, the *Neotyphodium sp.* / *F. altaica* symbiotum varied from parasitic to neutral across our gradient of interest.

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CHAPTER 1

GENERAL INTRODUCTION

Endophytic fungi

The term endophyte refers broadly to any organisms living within plants (Clay and Schardl 2002), but it is more commonly applied to fungal symbionts asymptomatically established within the roots or in the above ground structures of their host. Here I will use the word symbiosis to describe an intimate association between two individuals of different species and in which one lives on or inside the other (Townsend et al. 2003), with no assumption as to whether the interaction is mutualistic, commensalistic or parasitic; the pair is referred to as a symbiotum. The sparsely branched hyphae of these fungi run along cell walls where they feed on amino acids and sugars released into the apoplast (Clay and Schardl 2002). While endophytic fungi have been found in all woody plants screened for such infection, as well as in many algal and moss species (Arnold et al. 2000, Saikkonen et al. 1998), the bulk of scientific studies has been concerned with systemic infection in grasses by members of the Clavicipitaceae family (Ascomycota). One genus in particular, *Epichloe* (tribe Balansieae*)* and their anamorphic form *Neotyphodium* have attracted much attention because of their widespread infection of cool-season grasses (Clay and Schardl 2002), which have important economic value due to their use as forage for livestock (Hoveland 1993).

Reproductive cycles

Typically, clavicipitaceous fungi are regarded as parasitic to their cool-season grass host because of their sexual lifecycle. After a period of systemic growth, the fungi, such as *Epichloe* spp., produce fruiting bodies which partly inhibit or completely abort the plant’s own reproductive effort. Conidia, and later ascospores, are then released to infect neighbouring hosts; this is horizontal transmission (Clay and Schardl 2002). In their anamorphic form, these fungi appear limited to vertical transmission; hyphae grow inside the developing seeds of the host without interfering with them and the fungus disperses along with its host, thus colonizing the next generation. The anamorph forms of *Epichloe* spp. have been grouped under the genus *Neotyphodium*. Even in these species however, horizontal transmission cannot be entirely ruled out. Because endophyte-free grass seedlings have been experimentally inoculated with *Neotyphodium* strains (Kasai et al. 2006) it has been suggested that insect feeding on infected plants (E+) might transport viable hyphae to another, endophyte-free (E-) plant (Saikkonen et al. 1998). Additionally, mycelial *Neotyphodium* spp. producing conidiophores, and therefore capable of sporulating, can be readily grown in the laboratory; studying E+ *Poa ampla*, Tadych et al. (2007) confirmed that mycelial development could also occur naturally and their results suggested that water may facilitate horizontal transmission of *Neotyphodium* spp. Contrary to primarily horizontal dispersers like *Epichloe* spp. which have been readily classified as parasitic (Leuchtmann 1997), investigation into the nature of the relationship linking *Neotyphodium* spp. to their cool-season grass hosts has yielded mixed evidence.

Mutualism theory

The fitness of vertically transmitted symbionts is intrinsically tied to that of their host. Ecological and evolutionary theory predict that these symbionts should not persist in a host population if they are detrimental to the fitness of their host (Lipsitch et al. 1995). For example, if an E+ host plant was disadvantaged by the infection, its progeny should also be affected and natural selection would work against the infected lineage, decreasing the endophyte's frequency of occurrence in the host population (Clay and Schardl 2002, Lipsitch et al. 1995). Following this logic, and because *Neotyphodium* spp. infection rate in many pooid grass populations is high, the prevailing conceptual framework has been that *Neotyphodium* spp. and their cool-season grass host form a stable obligate mutualistic interaction (Saikkonen et al. 1998, Sullivan and Faeth 2004), with the fungus providing services such as herbivory deterrence and drought resistance in exchange for shelter, photosynthates and dispersal (Faeth and Fagan 2002, Malinowski and Belesky 2000). This relationship is well documented (Clay et al. 2005, Clay and Schardl 2002, Fortier et al. 2000) but a meta-analysis of endophyte literature examining 227 peer-reviewed studies showed that 82% of publications focused on *Lolium perenne* (perennial ryegrass) and/or *Lolium arundinaceum* (tall fescue, formerly *Festuca arundinacea*), two economically important, but also artificially selected and introduced agricultural grasses (Saikkonen et al. 2006). This underscores a real bias in grass-endophyte literature and has led many research groups to look more closely at infection patterns in natural systems.

Mutualism contested

Conventional mutualism theory predicts that the effects of *Neotyphodium* spp. on plant fitness should be exclusively beneficial; thus, infection frequencies of near 100% should be observed in any infected population, and intermediate ratios should be transient states (Clay 1993). However, this pattern is rarely observed in natural systems, where apparently stable grass populations exhibit intermediate infection ratios ranging from <5% to 70-80% and correlation with environmental or biotic stress has only been observed in a few cases (Bazely et al. 2007, Lewis et al. 1997, Novas et al. 2007). While it has been suggested that loss of endophyte infection in only 10% of seeds produced by an infected plant (through harsh environmental condition or simply through failure of the fungus to colonize during seed development) would be enough to maintain incomplete infection levels in a host population (Ravel et al. 1997), recent reviews have questioned the defensive mutualism theory altogether and now consider the interaction to span the entire continuum between mutualism and parasitism (Ahlholm et al. 2002, Saikkonen et al. 1998, Schulthess and Faeth 1998). In this scenario, the exact nature of each symbiotum is determined by environmental factors, biotic stress levels and the genotype of both symbionts (Ahlholm et al. 2002, Clay and Schardl 2002, Faeth et al. 2002), while strict defensive mutualism is restricted to a few rare cases (Faeth and Fagan 2002).

Services provided by *Neotyphodium* spp.

Fungal endophytes benefit strongly from their association with their cool-season grass host. Not only does the plant provide protection from desiccation as well as habitat, it also appears to supply the nutrients and photosynthates necessary for fungal growth (Clay and Schardl 2002). Most importantly, plants assume the greater part of dispersal and propagation costs (Clay and Schardl 2002, Faeth and Fagan 2002). In return, *Neotyphodium* spp. symbionts are expected to provide services to alleviate the principal stresses affecting the plant. Many such services have been reported in the literature: herbivory deterrence against both vertebrate (Durham and Tannenbaum 1998) and invertebrate grazers (Bultman et al. 2004) as well as plant pathogens (Kuldau and Bacon 2008), improved competitive abilities (Brem and Leuchtmann 2002), drought resistance (Arechavaleta et al. 1992), and mineral stress tolerance (Malinowski and Belesky 2000).

Alkaloids

These services are generally attributed to production of several families of alkaloid compounds by the endophyte. Ergot alkaloids are potent chemicals associated with vertebrate herbivory deterrence, and have been linked with severe fescue toxicosis in grazing livestock (Porter and Thompson 1992). Lolitrems can act as tremorgens against sheep (Sampaio et al. 2008), while peramines and lolines are strongly associated with resistance to invertebrate herbivory (Kuldau and Bacon 2008). Only one or two of these are normally produced by one endophyte-grass symbiotum, and the concentrations found in plant tissue vary widely, with certain symbiotum sometimes even producing no detectable alkaloid (Faeth et al. 2002).

Mineral stress tolerance

Greenhouse studies have often noted a significant increase in alkaloid production in E+ grasses under high N conditions (Arechavaleta et al. 1992, Krauss et al. 2007), which has spurred many research groups to investigate the relationship between N economy and endophyte infection. Studies on the effect of N availability on grass/endophyte symbiota have noted a significant difference in plant response to variations in ambient N according to infection status (Lyons et al. 1990, Malinowski and Belesky 2000); this has been attributed to modified enzyme activity in E+ lineages. In a study by Lyons et al. (1990), these lineages had a greater glutamine synthetase activity then their E- counterparts; this enzyme is responsible for NH_4+ reassimilation and appears to operate independently of available N, which corroborates observations of more efficient N use in E+ plants (Arechavaleta et al. 1992, Richardson et al. 1999). Much effort has focused on the interaction between N availability and *Neotyphodium* spp. because alkaloids, which are a serious concern in agronomic systems, have a high N content, but other nutrients, such as phosphorous (P), magnesium (Mg) and copper (Cu) have also been shown to vary between E+ and E- grasses (Arechavaleta et al. 1992, Saker et al. 1998, Zabalgogeazcoa et al. 2006).

In contrast with their response to available N, which appeared positively correlated with the benefits of infection, *Neotyphodium* spp. consistently appear to confer greater benefits to their hosts in low P conditions, with E+ grasses showing significantly longer root systems and higher root exudate activity, as well as significantly higher P, Mg, potassium (K) and calcium (Ca) tissue content (Malinowski and Belesky 2000, Zabalgogeazcoa et al. 2006). The opposite was true in high P conditions, where *Neotyphodium* spp. became an apparent parasite to their host (Malinowski and Belesky 2000). Variations in Ca concentration inside reproductive plant tissue have been shown to depend on infection status, potentially because Ca is implicated in ergot alkaloid synthesis as a coenzyme for DMATase[1] along with N and P (Malinowski and Belesky 2000, Zabalgogeazcoa et al. 2006). Phenotypic response of E+ lineages to nutrient availability in greenhouse trials is often noted as highly dependent on both *Neotyphodium* spp. and host genotype (Faeth et al. 2002). In contrast, Zabalgogeazcoa et al. (2006), studying 5 different genotypes from a natural semiarid grassland in western Spain, only found a significant interaction between infection and lineage for 1 of 10

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[1] Dimethylallyltransferase

nutrient analysed (Mg). Cheplick and Feath (2009) noted that, at present, there is not enough evidence to conclude that endophytes provide their host with greater nutrient stress tolerance in natural systems without the support of further experimental evidence.

Herbivore deterrence

The herbivore deterrence properties conferred to grasses by their endophytic symbionts are probably the most well known and studied service provided by the fungi to their hosts. Toxicity of certain cool-season grasses to herbivores has been well-known to scientists for over a century. Cheplick and Faeth (2009) noted Hance's 1876 report of the severe impact of "drunken horse grass" on grazing cattle in Mongolia, however, it was not until Bacon et al. (1977) that the connection was formally made between fungal infection and fescue toxicity syndrome. Since then, a wide variety of invertebrate and vertebrate herbivores have been tested for sensitivity to endophyte infection in forage grasses (Saikkonen et al. 2006).

Invertebrates -

Resistance of E+ grasses to herbivory by invertebrate pests has been widely studied both because of their economic importance and for their ease of use in experimental studies. In a literature review of infection effect on insect herbivores, Saikkonen et al. (1998) found increased herbivore resistance in E+ forage grasses in 66% of studies concerning *L. arundinaceum* and 71% of studies on *L. perenne*. Specifically, the bird cherry-oat aphid *Rhopalosiphum padi* has been consistently shown to be severely affected by endophytic infection (Bultman and Bell 2003, Clement et al. 2005). Davidson and Potter (1995) found a negative response in *R. padi* as well, but also in the greenbug aphid *Schizaphis graminum* as well as in five species of leafhoppers and flea beetles. These decisive results in invertebrate herbivores have been widely used to confirm the defensive mutualism theory, although the concept was originally founded on evidence from vertebrate herbivores.

Vertebrates -

Collating data from the American cattle industry, Hoveland (1993) conservatively estimated economic losses due to endophyte infected forage to reach up to US$609 million per year in the United-States alone. Since then, adverse effects of E+ forage have been documented in many large non-native herbivores. Cattle grazing in heavily infected pastures showed impaired thermoregulation, copper deficiency and, in extreme cases, development of gangrene in the tail and feet (fescue foot) (Porter and Thompson 1992, Saker et al. 1998). These effects are generally believed to derive from increased vasoconstriction caused by ergot alkaloids and are considered symptoms of fescue toxicosis (Paterson et al. 1995). Horses are also susceptible to this condition, with broodmares being particularly at risk of increased gestation time, increased rate of miscarriage and agalactia (Cross et al. 1995). Other large species known to perform poorly on infected forage include sheep and alpaca, with the alkaloid lolitrem B acting as a strong tremorgen in ovids and causing symptoms colloquially known as "ryegrass staggers" (Sampaio et al. 2008). The serious consequences of E+ forage for livestock have led to the engineering of new *Neotyphodium* spp. strains which still confer greater water and mineral stress resistance to the host grass without being toxic to herbivores. Conversely, the impact of wild endophyte/grass symbiota on native herbivores is much less clear and few studies have managed to determine whether large native herbivores of the same scale as livestock (e.g. *Rangifer tarandus*) are similarly affected by endophyte infection in their diet, or if infection plays a role in diet selection (Granath et al. 2007). It is possible that the lower population densities and larger ranges of these species diminish net herbivory pressure on any single grass population, and consequently any advantages associated with defensive mutualism.

An E+ diet may have strong effects on small mammals as well. Durham and Tannenbaum (1998) demonstrated that the percentage of offspring surviving to 21 days was 5 times lower in *Microtus ochrogaster* (prairie vole) fed an E+ *L.*

arundinaceum seed diet than for E- and control diets. Furthermore, breeding pairs fed the E+ diet took 3 times as long to produce their first litter as other breeding pairs. The depressed food intake of voles fed with E+ seeds suggests that in the wild, voles may simply avoid E+ forage and favour less toxic food items. In that case, the availability of E+ forage would only affect population dynamics in times of high density when higher quality food is scarce and individuals are forced to consume more toxic forage. Outside of the laboratory, Fortier et al. (2000) found that *Microtus pennsylvanicus* (meadow vole) were equally abundant in E+ and E- *L. arundinaceum* plots, but that sex ratio varied significantly depending on infection status of *L. arundinaceum*: female voles were present in significantly lower densities in E+ plots, while males were equally present between the two. Interestingly, female occurrence in E+ plots tended to increase with population density, but these females displayed a slower rate of sexual maturation, which supports Durham and Tannenbaum's (1998) hypothesis that *Neotyphodium* spp. infection in forage may only affect the population dynamics of small herbivores when their density is high. In another field study, only *Sigmodon hispidus* (cotton rat) occurred equally between E+ and E- stands, whereas *Blarina brevicauda* (short-tailed shrews), *Reithrodontomys humulis* (eastern harvest mice) and *Microtus pinetoruns* (pine vole) were all captured significantly more often in E- stands (Coley et al. 1995). Overall, evidence suggests that fungal infection in forage grass can affect small mammalian herbivores severely, particularly in no-choice situations, which may arise in wild populations when density peaks and alternative forage is less available.

Study species

Neotyphodium sp.

Neotyphodium spp. are foliar endophytes of cool-season grasses. They belong to the Balansieae tribe of the Clavicipitaceae family (Ascomycota) and are generally regarded as anamorph forms of *Epichloe* spp. (White et al. 2000). At our study site, the fungal endosymbiont of *Festuca altaica* was identified as part of the *Neotyphodium* genus using a commercial western blot detecting monoclonal antibodies specific to that genus (Phytoscreen Test, Agrinostics, Watkinsville, GA, USA) (Koh et al. 2006, Koh and Hik 2007). For the purpose of this study we have assumed that the positive signal obtained with this test was due to a single species of *Neotyphodium*. Although identification to the species level remains to be done, the general patterns of host-specificity in these fungal species suggests that this may be a previously un-described species of *Neotyphodium*.

Festuca altaica

Festuca spp. are perennial bunchgrasses of the Poaceae family (subfamily Pooideae). They have a cosmopolitan distribution, with the majority of species living in cool-climate regions. Fescues are closely related to ryegrasses and have been principally studied for their association with fungal endophytes in *Neotyphodium* and *Epichloe*. *Festuca altaica* Trin. (northern rough fescue, Figure 1-1a) is a densely tufted bunchgrass with culms standing between 25 and 100cm high (Cody 1996). Leaf sheaths and leaf bases can persist for many years at the base of the plant, creating very large tussocks. *F. altaica* is found in open woods, alpine grasslands and tundra regions, most commonly in amphi-beringian regions (Cody 1996).

Mammalian herbivores: Ochotona collaris, Marmota caligata and microtines

The collared pika (*Ochotona collaris*, Nelson 1893, order Lagomorpha, Figure 1-1b) is a small (160g) generalist herbivore inhabiting boulder field slopes in the alpine and subalpine regions of northern British Columbia, Yukon and Alaska (Broadbooks 1965). During the long winter, collared pikas remain active under the snowpack and survive on dried vegetation accumulated in large haypiles cached within the boulder field matrix (MacDonald and Jones 1987). Around mid-July, collared pikas stop foraging strictly for immediate nutrition and begin “haying” intensively. Pikas are behaviourally confined to boulder field patches and are rarely seen >10 meters from the boulder field edge (Huntly 1987, McIntire and Hik 2002, Morrison et al. 2004). Two factors contribute to cause this behaviour: (1) predation risk by raptors and foxes increases rapidly far from the shelter of the boulder field (Huntly 1987, Morrison et al. 2004), and (2) pikas have notably low heat tolerance and rely heavily on the rock matrix to cool down between haying forays (Smith 1974). The strong gradient in grazing pressure caused by these limiting factors was documented by Koh and Hik (2007, 2008), who reported a steady decline in herbivory on *Festuca altaica* with distance from boulder field, going from 13% of tussocks showing herbivore damage at distances of 0-3m from boulder field and reaching 0% at a approximately 60m from boulder field.

Marmota caligata (hoary marmot, Figure 1-1c) is the largest North American marmot species. Their range extends from Idaho, Montana and Washington to Alaska and the Yukon Territory in Canada (Karels et al. 2004). At the northern extent of their range, hoary marmots hibernate for approximately 8 months during the winter (Patil 2010). During the growing season, hoary marmots burrow in and near boulder fields; they feed preferentially in meadows adjacent to these boulder fields (< 12m) and may occasionally utilize *F. altaica* out to 30 meters or more (Karels et al. 2004).

An initial investigation of vole species assemblages at my field site (H. Wheeler, unpublished data) suggests two species which are found over a wide range of alpine habitats: *Cleithrionomys rutilus* and *Microtus oeconomus*. This observation was consistent with previous studies which found these species in both alpine tundra and subalpine shrub, and also at lower elevations in forested communities (Boonstra and Krebs 2006). *Microtus miurus* and *M. longicaudus* were more restricted in their range, inhabiting high alpine areas (over 1800m elev.) and low shrubby alpine (below 1650m elev.) respectively. All landscapes were associated with a high level of variability both in species present and population size.

Study site

My field work was carried out in a subarctic alpine meadow (4km^2) interspersed with boulder field and located in the Ruby Range, east of Kluane Lake, YT, Canada (61°13’N, 138°16’W, elev. 1700-1900m), with slopes facing east and west (Figure 1-2). Snow cover allows for a growing season of approximately 70 days, ending with the first snowfall, usually after the third week of August (D.S. Hik, unpublished data). At my study site, McIntire and Hik (2005) documented a change in plant community between 0 and 6 m from boulder fiel. They denoted slightly lower species richness away from boulder field: graminoid species richness (SR) decreased slightly while dwarf shrub SR increased and forbs SR remained constant. They only covered a small portion (6 m) of the gradient included in my study (60m), and although Koh and Hik (2008) showed that plant community does change across the 60m, *F. altaica* density was constant at both ends of the gradient. Mitchell et al. (2009) showed that at my field site, edaphic conditions such as soil depth and nitrate availability are extremely variable over short distances; this was illustrated by Mitchell (2006) in a conceptual diagram (Figure 2-9, p.51). Pikas and hoary marmots, the most common herbivores, are mostly constrained to discrete boulder field patches and focus grazing effort on a 10-20m wide strip of vegetation along boulder field edge (Karels et al. 2004, McIntire and Hik 2002, Morrison et al. 2004). The physical structure of the boulder fields has likely been stable over the past century (Price 1991), therefore we can assume that behavioural constraints on herbivores have remained constant. Consequently selection pressures have likely remained different in magnitude on the *F. altaica* / *Neotyphodium* sp. symbiotum at each end of the gradient (Koh and Hik 2007).

Koh and Hik (2007) demonstrated the existence of an endophytic component to the grass-herbivore relationship between collared pikas and *F. altaica* at our study site. Extensive sampling of *F. altaica* tussocks across the herbivory gradient described above revealed significantly higher *Neotyphodium* sp. infection rates for *F. altaica* growing near or on the boulder field (high grazing history) compared to tussocks sampled in alpine meadows with low grazing history. Using open-style cafeteria trials, Koh and Hik (2007) showed that pikas had a significant preference for E- forage if presented with E+ and E- *F. altaica* from locations on boulder fields. In contrast, pikas did not select forage according to infection status when forage was taken in the meadow. Because nutritional value (measured as N-content) was constant across all forages, they concluded that pika preference was likely due to chemical deterrence in E+ *F. altaica* on boulder field (Koh and Hik 2007). However, the nature of this deterrent remains unclear and preliminary screening for ergot alkaloids was negative. Overall, these results thus pointed towards defensive mutualism between grass and fungus in high grazing history locations and a more neutral interaction in low grazing history areas (see also Koh and Hik 2008).

Objectives

The general aim of my thesis was to document the interaction linking the native cool-season bunchgrass *F. altaica*, its *Neotyphodium* sp. endosymbiont and the native herbivore *O. collaris*. To do this, I performed a factorial experiment combining 4 main factors and all interactions: (1) endophyte infection (E-/E+), (2) grazing history (high/low) (3) fertilization (ambient/+NPK) and (4) fungus removal (control/fungicide).

(1) My first objective was to determine whether the correlation between infection frequency and herbivory pressure was due to defensive mutualism between *Neotyphodium* sp. and *F. altaica*. Results from a previous study at our field site (Koh and Hik 2007) showed that there was a correlation between herbivore pressure and *Neotyphodium* sp. infection in *F. altaica*; this appeared to be caused by a fitness advantage to E+ lineages due to herbivory deterrence properties provided by the fungus. Their feeding trial results suggested that on boulder fields, herbivore pressure was strong enough that *Neotyphodium* sp. might have been selected for herbivore deterrence, therefore giving E+ grass lineages an advantage over E- lineages (Koh and Hik 2007). In Chapter 2, I examined the difference in natural grazing pressure on E+ and E- experimental tussocks. I monitored herbivory on tillers treated under the factorial-design experiment throughout the field season. In mid-August, I recorded relative herbivory on whole tussocks. Several greenhouse studies involving invertebrate herbivores have suggested that N availability positively affects herbivore deterrence in E+ grasses (Arechavaleta et al. 1992, Bultman et al. 2004, Krauss et al. 2007), therefore I also investigated whether herbivory on infected plants varied with fertilization treatments.

(2) Secondly, I determined if the persistence of endophyte infection in host populations located in areas of low grazing history was mediated by nutrient availability. As per the strict interpretation of mutualism theory, if vertically transmitted fungal endophytes persist over time in a host population, it can be concluded that the physiological weight they represent is equal or less than the advantage they represent for their host. The study conducted by Koh and Hik (2007) at our field site indicated persistent levels of infection in *F. altaica* tussocks located in meadows (~30%), where herbivory is known to be extremely low. Due to slow nutrient cycling, tundra environments are notably nutrient-poor environments (Hobbie 1992). I hypothesized that in areas not affected by high grazing history, the dominant stress to *F. altaica* would be nutrient availability. In Chapter 3, I investigated the response of E+ and E- plants to fertilization in areas where there appears to be no other stressor for plants, as well as in areas chronically stressed by herbivory pressure. To do this, I monitored leaf demography bi-weekly for 3 tillers per treated tussock within the factorial design experiment. Leaf senescence rate and vegetative reproduction were also recorded. Finally, I also investigated the difference in soil nutrient availability between boulder field and meadow sites by deploying Plant Root Simulator probes (Western AG Innovations, Saskatoon, Canada) at each end of the grazing gradient.

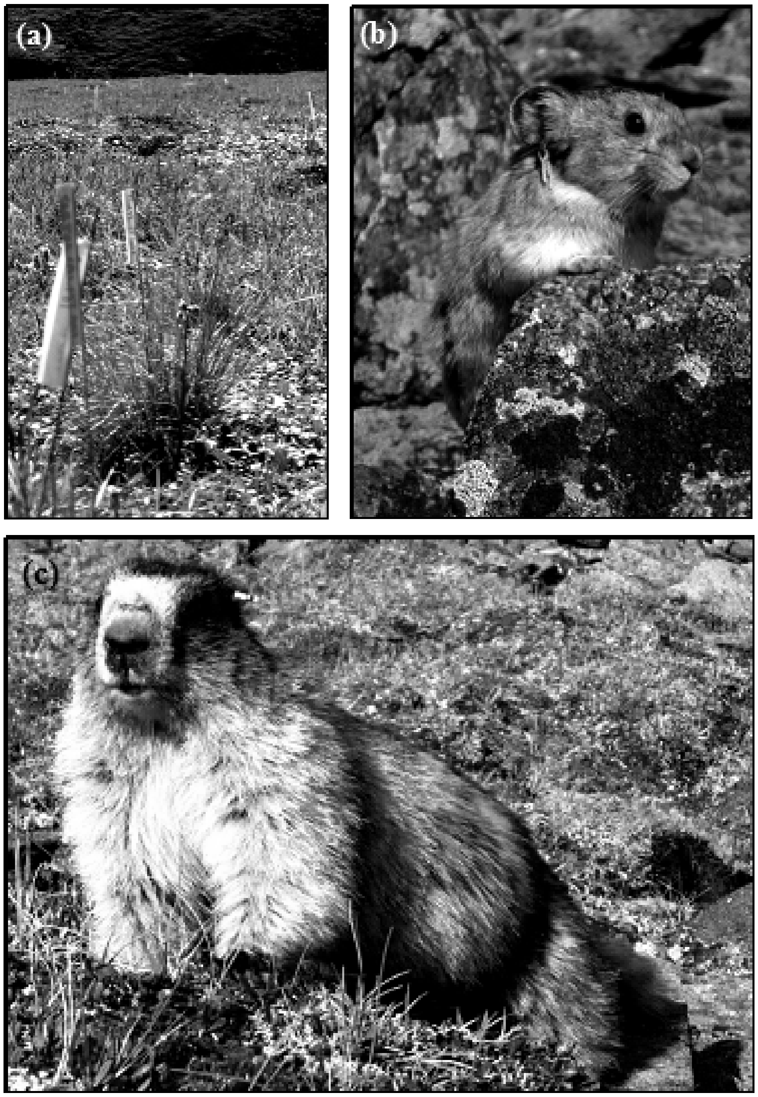

Figure 1-1 Study species: (a) *Festuca altaica* (Northern rough fescue) (b) *Ochotona collaris* (Collared pika) (c) *Marmota caligata* (Hoary marmot)

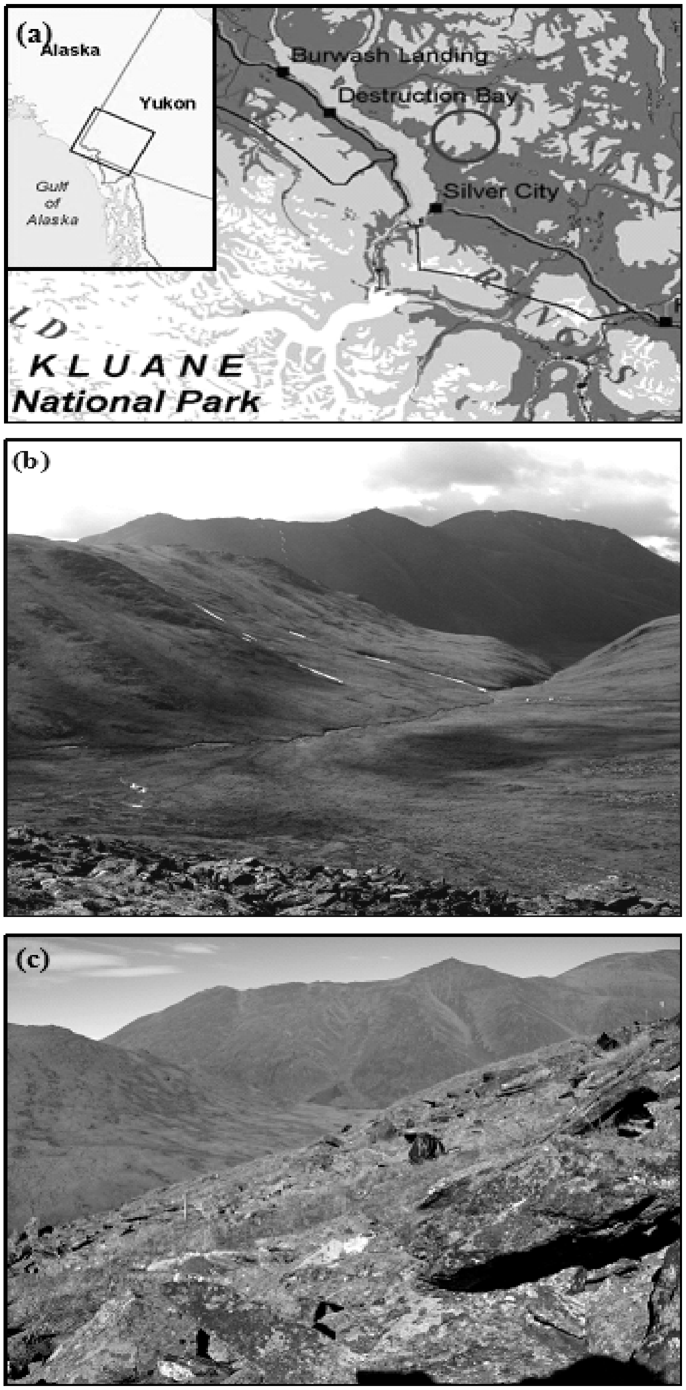

Figure 1-2 Field site: (a) Ruby Range, YT, Canada, (b) alpine meadow interspersed with boulder field and (c) boulder field.

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CHAPTER 2

SPATIAL COVARIATION BETWEEN GRAZING AND *Neotyphodium* sp. INFECTION IN AN ALPINE MEADOW: TESTING THE DEFENSIVE MUTUALISM THEORY

Introduction

The term endophyte may refer broadly to any organism living inside plants (Wilson 1995), but for fungi it most often refers to *Epichloe* and *Neotyphodium*, which colonize cool-season grasses such as fescues and ryegrasses without causing obvious symptoms or injury (Kuldau and Bacon 2008). Fungal endophytes of *Lolium arundinaceum* (tall fescue, formerly *Festuca arundinacea*) and *L. perenne* (perennial ryegrass) have been widely studied for their serious physiological effects in grazing ruminants, which can occasionally lead to substantial economical losses (Bacon et al. 1977, Hoveland 1993). Their sparsely branched hyphae develop along the cell walls of above-ground tissues, where they rely on amino acids and sugars released into the apoplast for nourishment (Clay and Schardl 2002). Although horizontal transmission is theoretically possible through mycelial growth and subsequent sporulation, or through transmission of active hyphae by insect herbivores (Kasai et al. 2006, Tadych et al. 2007), current knowledge suggests that *Neotyphodium* spp. disperse primarily vertically by extending their hyphae into the plant’s seeds, thereby infecting the next generation of hosts (Clay and Schardl 2002, Kuldau and Bacon 2008). Because reproduction and dispersal of the fungus depend on the host’s reproductive success, ecological and evolutionary theory predict that the interaction between the two should be mutualistic (Lipsitch et al. 1995). It therefore follows that if the symbiont is beneficial to its host, endophyte infection frequencies in host population must be at or near 100%, but if the net effect of *Neotyphodium* spp. infection is a decrease in host fitness, infected lineages should not persist in stable systems and infection frequency should be near 0% (Clay and Schardl 2002, Lipsitch et al. 1995). Based on extensive evidence from highly infected agronomic systems (Belesky et al. 1988, Hoveland 1993, Porter and Thompson

1992), the prevailing conceptual framework has been that in exchange for habitat and photosynthates, *Neotyphodium* spp. provide potent alkaloids which act as herbivory deterrents against vertebrate and invertebrate predators. This pattern is known as defensive mutualism.

The defensive mutualism theory is strongly supported by evidence from livestock grazing on endophyte infected (E+) *L. arundinaceum*, which includes observations of impaired thermoregulation, copper deficiency, and, in extreme cases, development of gangrene in the tail and feet of cattle (Paterson et al. 1995, Porter and Thompson 1992, Saker et al. 1998). Similar reactions have been documented for horses (Cross et al. 1995) and ovids (Sampaio et al. 2008). On the other hand, these observations may present a biased view of the situation, since a majority of studies have been concerned with one or both of the introduced and highly-selected agronomic grasses *L. arundinaceum* and *L. perenne*, and their interaction with equally introduced and selected livestock species (Saikkonen et al. 2006).

Natural systems have yielded more equivocal evidence: the predictions of the traditional mutualism theory such as high infection frequency in stable systems and correlation of infection frequency with environmental stress have been difficult to corroborate. When comparing long term exclosures with populations of *Festuca arizonica* (arizona fescue) exposed to natural levels of grazing by deer, elk and cattle, Schulthess and Faeth (1998) did not find the gradient in infection frequency that would have been expected if *Neotyphodium* spp. had been beneficial to plant lineages exposed to herbivory but neutral or detrimental to plants not exposed to this stress. On the other hand, the *Neotyphodium* sp. infection frequency of *F. rubra* (red fescue) was positively correlated with grazing pressure in the St. Kilda islands (Scotland, UK), but Bazely et al. (1997) could not confirm their results on the Hebridean islands of Benbecula and Rum. In Sweden, *F. rubra* infection frequency was again correlated with the intensity of reindeer grazing, but the co-occurring *F. ovina* (sheep’s fescue) was not (Granath et al. 2007). Saona et al. (2010) reported another caveat in the *F. rubra*/*Neotyphodium* sp. relationship: grazing history by greylag and Canada geese was significantly correlated with infection prevalence, but only at dry sites. Mesic and moist sites showed no correlation of infection frequency with grazing history.

There is also evidence from greenhouse studies which suggests that nutrient availability affects herbivore deterrence properties in infected forage grasses (Arechavaleta et al. 1992, Davidson and Potter 1995). The bioprotective alkaloids which grant endophytes their herbivore deterrence properties have been consistently detected in greater concentration in fertilized plants; this has been attributed to the high N content of these compounds (Bacon 1993, Richardson et al. 1999).

Small mammals, like other herbivores, make dietary choices based on energy, water and nutrient considerations (Durham and Tannenbaum 1998), which includes avoidance of harmful or costly compounds (Freeland and Janzen 1974). Conover (2003) found that *Microtus pennsylvanicus* (meadow voles) normally fed a diet of E- ryegrass leaves avoided E+ forage when presented with a choice. This trend was also found in managed *L. arundinaceum* fields, where Coley et al. (1995) found that only *Sigmodon hispidus* (cotton rat) occurred equally between E+ and E- stands, whereas *Blarina brevicauda* (short-tailed shrews), *Reithrodontomys humulis* (eastern harvest mice) and *Microtus pinetoruns* (pine vole) were all captured significantly more often in E- stands. On the other hand, a similar study of *L. arundinaceum* stands by Fortier et al. (2000) did not find a significant difference in the population density of *M. ochrogaster* (prairie vole) between E+ and E- stands, but their results indicated that while males did not differentiate between E+ and E- stands, females were more likely to be found in E- stands. Female voles found in E+ stands displayed a slower rate of sexual maturation, which is consistent with the toxic effects of E+ forage documented in no-choice lab experiments (Durham and Tannenbaum 1998).

Collared pikas (*Ochotona collaris*) are small alpine herbivores that, because of spatio-behavioural constraints, create herbivory gradients around their territories which can be assumed to have remained constant over centuries due to the geological properties of the site (Huntly 1987). Koh and Hik (2007) surveyed a subarctic alpine meadow and documented a *Neotyphodium* sp. infection gradient stretching from a pika-inhabited boulder field, where *Festuca altaica* Trin. (northern rough fescue) exhibited ~70% infection, into alpine meadows (approximately 60m), where infection declined to ~30%. Using biomass from plants previously screened for *Neotyphodium* sp. infection using a commercial field immunoblot assay specific to that genus (Phytoscreen Test, Agrinostics, Watkinsville, GA, USA), the authors performed open-style cafeteria feeding trials which revealed that when *F. altaica* was harvested in high grazing history areas, collared pikas consistently chose E- leaves ($p=0.0006$), but when presented with forage from the meadow, pikas did not differentiate between E+ and E- (Koh and Hik 2007). Because they also found forage quality (measured as N content) to be equal across all treatments, this pattern suggested that in areas of high grazing history, a strong defensive mutualism has developed between *Neotyphodium* sp. and *F. altaica*, but where there is less grazing, the endophyte has developed to provide different services to its host in order to persist in the host population.

Our objective was to experimentally determine which factors may be affecting *Neotyphodium* sp. infection frequency at each end of the grazing history gradient described above. More specifically, we aimed to determine the effects of endophyte infection and soil nutrient conditions on the herbivory sustained by *F. altaica* tussocks. Our hypothesis was that in areas of high grazing history, where the grass/endophyte relationship is thought to have developed into defensive mutualism, fungus removal would increase herbivory on *F. altaica*, whereas fertilization would increase *Neotyphodium* sp. deterrent properties. In areas of low grazing history, we predicted that herbivory would not vary with fungus removal, but would increase with fertilization regardless of infection because of higher forage quality.

Methods

Ochotona collaris

Ochotona collaris Nelson (Lagomorpha, collared pika) is a small (160g) larder-hoarding and generalist herbivore inhabiting boulder field slopes in the alpine and subalpine regions of northern British Columbia, Yukon and Alaska (Broadbooks 1965). Collared pikas intensively exploit vegetation in their territory throughout the growing season. Early in the summer, new shoots and flowers are consumed directly, but in mid-July they begin to harvest vast quantities of vegetation and stockpile it into food caches (haypiles). Protected by the rocky matrix of boulder fields, these haypiles sustain pikas through the following winter, during which they remain active under the snow pack. Pikas are behaviourally confined to boulder field patches and are rarely seen >10 meters from the boulder field edge (Huntly 1987, McIntire and Hik 2002, Morrison et al. 2004). Two factors contribute to cause this behaviour: (1) predation risk by raptors and foxes increases rapidly far from the shelter of the boulder field matrix (Huntly 1987, McEachern et al. 2006, Morrison et al. 2004), and (2) pikas have notably low heat tolerance and rely heavily on the rock matrix to cool down between haying forays (Smith 1974).

Study site

Our study was carried out in a subarctic alpine meadow (4km^2) interspersed with boulder field and located in the Ruby Range, east of Kluane Lake, YT, Canada (61°13’N, 138°16’W, elev. 1700-1900m), with slopes facing east and west. The growing season lasts approximately 70 days, beginning one to two weeks after snow melt, approximately mid-June, and ending with the first snowfall, usually after the third week of August (D.S. Hik, unpublished data). While the plant community changes from boulder field to meadow, the perennial bunchgrass *F. altaica* is ubiquitous across the gradient (McIntire and Hik 2005, Koh and Hik

2007, 2008). *F. altaica* is an amphi-beringian fescue species which forms densely tufted bunches with culms standing between 25 and 100cm high; it is generally found in open woods, alpine grasslands and tundra regions (Cody 1996). Mitchell et al. (2009) showed that at my field site, edaphic conditions such as soil depth and nitrate availability are extremely variable over short distances. The physical structure of the boulder fields has likely been stable over the past century (Price 1991), therefore we can assume that behavioural constraints on herbivores have remained constant. Consequently, selection pressures have likely remained different in magnitude on the *F. altaica* / *Neotyphodium* sp. symbiotum at each end of the gradient (Koh and Hik 2007). While pika populations may have experienced year-to-year fluctuations, thereby changing the immediate intensity of grazing, it is unlikely to have affected the overall herbivory pressure experienced by a tussock over the course of its lifetime (several decades).

Experimental design

In order to test our hypotheses, we performed a full factorial experiment combining 4 factors and interactions: (1) infection (E+/E-), (2) grazing history (high/low), (3) fertilization (ambient/+NPK) and (4) fungicide (control/ fungicide)[†], for a total of 16 treatments.

Using a field immunoblot assay to detect monoclonal antibodies specific to *Neotyphodium* spp. (Phytoscreen test, Agrinostics, Watkinsville, GA, USA) (Koh et al. 2006, Saari et al. 2010), we created a pool of *F. altaica* tussocks of known infection status on west and east-facing slopes, respectively, for which tiller number and tussock area were recorded (N=991).

At each site, high herbivory treatment tussocks were selected in boulder field occupied by pikas, and low herbivory treatment tussocks were in meadows, approximately 60m from the nearest boulder field; this is the distance at which

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[†] Within the scope of this experiment, the word “control” is used throughout the text only in reference to tussocks or tillers not treated with fungicide. Tussocks or tillers treated with water throughout the experiment are referred to as “untreated”.

herbivory is lowest, according to Koh and Hik's (2007) survey of *F. altaica* tussocks. Manipulative treatments were assigned to experimental tussocks randomly with restrictions to minimize any potential contamination due to slope drainage. A total of 34 tussocks were assigned to each treatment, for a total sample size of 544 tussocks. Final sample size was fixed based on the number of tussocks we could feasibly measure in under 7 days and on the expected effect size from the results of Koh and Hik's (2007) feeding trials. Further details on initial screening and treatment attribution are given in appendix A-1.

A standard water soluble fertilizer (PlantProd®, 20-20-20 NPK All-Purpose Fertilizer) was used for fertilization treatment, and applied 3 times at two week intervals (beginning mid-June) for a total of 2.054g of nitrogen per plant[‡]. We used Tilt 250E for the fungus removal treatments (Syngenta Crop Protection Inc., Calgary, AB, Canada, active ingredient: propiconazole). Propiconazole has been shown to eradicate *Neotyphodium* spp. effectively both in seeds and seedlings (Faeth and Sullivan 2003, Hill and Brown 2000). A full-dose treatment was applied mid-June (Tilt 250E concentration = 2.5mL/L), when growth had just begun, and a follow-up dose of 1.2mL/L was applied 2 weeks later. All chemical solutions were applied in 1L volume, including control water treatments and combined fertilizer and fungicide treatments.

We verified the effectiveness of the fungicide treatment one month after initial application. Stem clippings were taken from the bottom of a young leaf in E+ control and fungicide treated experimental tussocks, they were then analyzed using a field immunoblot assay. These immunoblot cards indicate endophyte presence quantitatively via the intensity of the colour signal (Koh et al. 2006), and each signal on the card was qualified as absent, weak or strong, according with colour intensity. The percentage of absent or weak signals went from 7.5% (n=40) in infected controls to 83.8% (n=80) in knock-out tussocks. We also collected samples from a subset of experimental E+ tussocks at the end of the experiment

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[‡] 2% NH_4-N and 18% Urea $(NH_2)_2$-CO

and analysed the immunoblot signal colour value quantitatively. The results of this analysis are reported in detail in Chapter 3, and the fungicide treatments were considered to be effective.

Experimental monitoring

Within each experimental tussock, 3 individual tillers were identified with wire at the base of the stem and subsequent herbivory damage to each leaf was noted, beginning three days after the first application of chemical treatments. These were monitored every 2 weeks for a total of 4 observation rounds throughout the growing season. Once a tiller was recorded as grazed, it was considered as such for the remainder of the season. Grazing by invertebrate herbivores was not taken into account but was not common in this or previous studies at this site (Hik et al. 2003, Koh and Hik 2007). At the end of the growing season, all experimental tussocks were visually evaluated for presence or absence of grazing damage at the tussock-level.

Data analyses

Herbivory recorded for our monitored tillers was expressed as a 0-3 score per tussock reflecting the likelihood (probability p) that one monitored tiller had at least one leaf showing damage by herbivory within that tussock. To facilitate further analysis, the probability p was logit transformed such that $\text{logit}(p)=\log(p/1-p)$ and was analysed using a generalized linear mixed model assuming a binomial distribution. The random structure of the model, which included site effects, was selected using Akaike's Information Criterion scores for all plausible structures. Fixed effects, including infection, grazing history, fungicide and fertilizer, as well as all possible interactions, were tested using the GLIMMIX procedure (SAS 9.2, SAS institute, Cary, NC, USA). Least square means and confidence interval estimates were computed on the logit scale and back transformed for presentation. Odds of grazing $[p/(1-p)]$ were the basis of interpretation of all treatment effects. Any two probabilities of grazing were compared through the ratio of their odds:

$$\log\left(\frac{p_1/(1-p_1)}{p_2/(1-p_2)}\right)$$

The same procedure was followed for the whole-tussock assessment of herbivory damage: here probability p was the likelihood of any tiller in the tussock showing herbivory damage.

P-values for all statistical tests performed were interpreted using $\alpha=0.05$, with no adjustment (Bonferroni or otherwise) for the number of tests performed. It has become standard in ecological studies using multiple statistical tests for a single dataset to control for Type I error by reducing the significance threshold proportionally with the number of tests to be performed (Perneger 1998). More recently, mathematical, logical and practical objections have been raised against this practice (Moran 2003). Following Moran’s (2003) recommendation, we report only exact p-values and compare them to the accepted 0.05 cut-off.

For tussocks not treated with fertilizer or fungicide, we also calculated the total proportion of all tillers having at least one leaf damaged by herbivory. This was expressed as a proportion of all monitored tillers and for each interval during the season in order to illustrate patterns in the timing of herbivory depending on grazing history and endophyte infection.

Results

Tiller-level herbivory

There was a significant three-way interaction between grazing history, infection and fertilization on the probability of herbivory on tillers ($F_{1,670}=5.32$, p=0.0213, Table 2-1) which appeared primarily driven by the difference in the grazing pressure on E+ versus E- tillers in low grazing history areas and high nutrient conditions ($t_{1,718.2}=2.13$, p=0.0333, Table 2-2). In these conditions, herbivory was found to be 1.98 [1.06, 3.71] ($\bar{x}$ [95% C.I.]) times more likely for E+ tillers than E- (Table 2-2). In low grazing history areas, the probability of herbivory on E- tillers remained constant across both fertilization treatments, but the same probability for E+ tillers increased significantly with fertilization (Figure 2-1).

As expected, grazing history alone had a significant effect on the probability of herbivory ($F_{1, 670}$=17.38, p<0.0001). Tillers from *F. altaica* plants on boulder field had a 0.25 ± 0.04 probability of experiencing herbivory, whereas the probability for tillers in the meadow was only 0.15 ± 0.03. This pattern conformed to assumptions of high herbivore pressure on the boulder field versus meadow. Fertilization was another main factor with a significant effect on probability of grazing damage ($F_{1, 670}$=10.84, p=0.001), such that tillers grown at ambient nutrient levels had a 0.16 ± 0.03 ($\bar{x}$ ± 95% C.I.) probability of experiencing grazing damage while tillers treated with an NPK solution had a 0.24 ± 0.04 probability. Contrary to our prediction, infection did not appear to affect the probability of herbivory ($F_{1, 670}$=0.00, p=0.9695).

Tussock-level herbivory

At the tussock level, no significant interactions were found between main effects but grazing history and fertilizer were each affected the likelihood of grazing independently (respectively, $F_{1, 522}$=13.97 p=0.0002, $F_{1, 522}$=34.71 p<0.0001), while infection again did not appear to influence the likelihood of herbivory ($F_{1, 522}$=1.74, p=0.1883). Fertilized tussocks were 2.94 [2.05, 4.21] times more likely to experience herbivory to at least one tiller than tussocks grown at ambient nutrient levels. Similarly, tussocks located in high grazing history areas were 1.98 [1.38, 2.83] times more likely to show grazing damage. These results are consistent with our predictions, except for the lack of effect of infection or of any interaction between infection and nutrient availability on the probability of herbivory.

Timing of herbivory

The proportion of untreated tillers with signs of herbivory demonstrated different trends depending on infection status and the grazing history of the area. Figure 2-2 illustrates how, on boulder field, the proportion of damaged tillers increases at a constant rate through the growing season. However, the proportion of damaged E- tillers appeared to increase more slowly. In low grazing history areas, E+ and E- tillers were grazed at similar rate, with a slight divergence in pattern in the first interval of the season. At that time, more E- tillers had grazing damage but E+ tillers matched this by mid-July.

Discussion

While we verified the existence of a grazing gradient in *F. altaica*, we did not find any evidence to support the pattern of defensive mutualism we had predicted. Fertilizer significantly increased likelihood of herbivory for both indices, regardless of endophyte infection and this was most likely due to the increased nutritional value of fertilized plants. Contrary to our predictions, endophyte infection appeared to affect the probability of herbivory in low grazing history areas under high fertilization treatments, but not on boulder field where we had expected to see signs of defensive mutualism. An emerging pattern in the timing of herbivory pressure suggests that pikas may respond differently to endophyte infection in forage grasses depending on whether it is destined for caching in haypiles or for immediate consumption.

Effects of grazing history and fertilizer

Our results confirmed the existence of a grazing pressure gradient stretching from pika-inhabited boulder fields into the alpine meadows. Tussocks on boulder field were 1.92 [1.41, 2.61] times more likely to have at least one tiller damaged by herbivory than tussocks from meadows (Table 2-2).

Irrespective of the presence of fungal endophytes, fertilization treatment had a significant effect on the likelihood of herbivory for both parameters investigated. Fertilized tussocks were 2.94 [2.06, 4.21] times more likely to exhibit herbivory compared to their counterpart grown under ambient nutrient levels (Table 2-4). This may be due to longer leaves when tillers were fertilized (Table A-8b). Hudson et al. (2008) showed that collared pikas chose forage selectively according to leaf size, and higher N content is also considered an indicator of forage quality (Ball et al. 2000).

Interaction between infection, fertilization and grazing history

Analysis at the tiller-level, which reflects the likelihood of at least one leaf in one tiller being grazed within a tussock, revealed a significant three-way interaction involving infection, fertilization and grazing history ($F_{1,670}$=5.32, p=0.0231).

Boulder field –

Post-hoc comparisons showed that this interaction was not due to significant differences between E+ and E- in high grazing history areas, where we had expected to see a difference. Koh and Hik (2007) found that in cafeteria-style feeding trials, pikas consistently preferred E- *F. altaica* to E+, however, we did not detect a significant difference in the likelihood of herbivory between E+ and E- *F. altaica* on boulder field. Furthermore, several greenhouse studies have demonstrated that alkaloid production in E+ grasses is positively correlated with soil nutrient availability (Cheplick et al. 1989, Davidson and Potter 1995), and we had therefore expected fertilizer to exacerbate herbivore deterrent properties; we did not find this.

It is possible that while E+ *F. altaica* have herbivore deterrent properties (Koh and Hik 2007), we were unable to detect them *in situ* because pikas avoid or make little use of *F. altaica* altogether. However, graminoids are an important source of forage for pikas and may represent 40-50% of haypile content (Dearing 1996, Hudson et al. 2008). Alternatively, it is possible that while collared pikas have a marked preference for E- forage, as detected by Koh and Hik (2007), other herbivores, such as hoary marmots, may be exploiting *F. altaica* who do not have such a preference. In that case, indiscriminate grazing would mask any pattern in herbivory damage caused by pikas, and net herbivory on *F. altaica* would not be dependent on *Neotyphodium* sp. infection. Regardless of the mechanism, however, it appears that *Neotyphodium* sp. infection has no effect on the probability of *F. altaica* being consumed, and that the relationship between *Neotyphodium* sp. and *F. altaica* on boulder field is likely not mediated by herbivory, and therefore not defensive mutualism.

Meadow –

In areas of low grazing history and under high nutrient conditions we found that E+ tussocks were 1.97 [1.07, 3.71] times more likely to show herbivory than E- tussocks. This result contradicts our prediction that there is no effect of infection on the likelihood of herbivory in low grazing history area.

This interaction could have been caused by higher productivity in E+ versus E- in meadows; while we did detect significantly higher tiller number in E+ plants than in E- tussocks, this difference in productivity was so slight (<5 tillers, Figure 3-1) that it is unlikely to have affected herbivore foraging behaviour and therefore cannot explain the significant interaction of infection x grazing history x fertilization (Table 2-3). On the other hand, this significant interaction could well have been caused by heterogeneity in the meadow structure. Indeed, alpine meadows are boulder fields which have been grown over by a thick layer of sphagnum mosses, forbs, dwarf shrubs and graminoids. Our alpine meadows appeared as mosaics of successional stages, with patches where old boulder field structure still emerged from the vegetation matrix. Because pikas rarely venture more than 10-20m out of the boulder field where they nest and into the meadow (Huntly 1987), and because these patches were 50-60m from the nearest pika-inhabited boulder field, which was the distance identified as having the least occurrence of herbivory by Koh and Hik (2007), they still met our criteria for low grazing history. These rocks, partially emerging from the soil, are not suitable habitat for pikas, but they may provide a solid structure to tunnel and nest for microtine herbivores such as voles (tundra (*Microtus oeconomus*), long-tailed (*M. longicaudus*), singing (*M. miurus*) red backed (*Myodes rutilus*)) and northern bog lemming (*Synaptomys borealis)*. After our initial screening for *Neotyphodium* sp., it appeared that many E+ tussocks in meadows were located on these rocks, such that E+ tussocks in meadows were more likely to be grazed than E- tussocks simply by virtue of growing closer to meadow structures that may allow nesting and tunnelling by these microtine species.

The lack of infection effect in ambient nutrient condition, and the increase of herbivory pressure with *Neotyphodium* sp. infection rather than the opposite indicate that the observed pattern is unlikely to be due to a direct interaction between the herbivore and *Neotyphodium* sp. On the other hand, *Neotyphodium* sp. could be providing services alleviating a significant stress to the plant, such as soil nutrient availability, which would make E+ plants more productive or give them a higher quality as forage under high nutrient condition and thereby increase the likelihood of herbivory. This is discussed further in Chapter 3.

Timing of herbivory

The cumulative number of tillers exhibiting herbivory damage on at least one leaf was compared over time to show variation in the timing of herbivory depending on infection status (Figure 2-2). In meadows, herbivory damage appeared to accumulate at a relatively constant rate with little variation between E+ and E- tussocks. Conversely, in high grazing history areas, E+ and E- appeared to diverge over the course of the growing season.

In mid-July, collared pikas change their grazing behaviour and begin to harvest large amounts of vegetation which they accumulate in food caches (haypiles) within the boulder field matrix. This larder-hoarding behaviour allows them to survive the harsh winter months, during which they remain active under the snow pack (Huntly et al. 1986). In American pikas, the selection criteria for this winter forage were different than for summer forage. While energy, nutrient and water contents were weak predictors of winter diet selection, there was strong evidence that pikas selected forage with higher content in plant secondary compounds (PSCs) (Dearing 1996). Dearing et al. (1996) concluded that American pikas may have been taking advantage of antibacterial properties of PSCs, which would slow decomposition rates and increase preservation of the haypile through the winter. In agronomic studies, E+ *L. arundinaceum* consistently shows slower decomposition rates than E- (Lemons et al. 2005, Omacini et al. 2004), and this has been widely attributed to alkaloid production by the fungus (Siegrist et al. 2010). In our system, E+ *F. altaica* plants on boulder field tested negative to the presence of ergot alkaloids (Koh and Hik 2007), and we have found no evidence of other active chemicals. In a decomposition experiment, Siegrist et al. (2010) demonstrated that while E+ *L. arundinaceum* did have consistently slower decomposition rate than E-, alkaloid content in E+ standing dead biomass all but disappeared, and experimental alkaloid addition did not change decomposition rate; they therefore concluded that other mechanisms associated with *Neotyphodium* sp. infection may be slowing down decomposition rate. It is possible that collared pikas, similarly to the pattern suggested by Dearing et al. (1996), would learn to take advantage of such properties and selectively harvest E+ forage when building their haypiles. In this case, *Neotyphodium* sp. infection in forage grasses would have a strong effect on local herbivores.

Conclusion

While collared pikas appear to differentiate between endophyte infected and endophyte free *F. altaica* plants when these are presented to them in experimental feeding trials, this did not appear to be reflected in the likelihood of an *F. altaica* plant being grazed. We conclude, therefore, that the high endophyte infection frequency observed on boulder field locations is not caused by a selective advantage to E+ lineages due to herbivory deterrence. In meadows, there appears to be a trend towards higher likelihood of damage by herbivores on E+ plants under high nutrient conditions, and this was most likely due to the heterogeneity of meadow structure in conjunction with microtine herbivores. The prevalence of E+ tussocks near old, grown-over boulder field is an indication that factors other than pika herbivory may be dictating *F. altaica*/ *Neotyphodium* sp. interactions. Consequently, the gradient of infection frequency documented by Koh and Hik (2007) will also be influenced by other factors varying along the same spatial gradient, such as water availability, soil nutrient availability or interspecific competition.

Table 2-1 Type III tests of fixed effects obtained by a non-parametric 4-way ANOVA to test the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the probability of herbivory (y/n) on tillers at any point during the growing season. Asterisks indicate significant p-values (α=0.05).

Effect	Numerator d.f.	Denominator d.f.	F-value	Pr > F	
Fertilizer (Fe)	1	670	10.84	0.0010	✳
Fungicide (Fu)	1	670	0.27	0.6026	
Fe x Fu	1	670	0.03	0.8716	
Infection (I)	1	670	0.00	0.9695	
Fe x I	1	670	0.56	0.4545	
Fu x I	1	670	0.00	0.9562	
Fe x Fu X I	1	670	2.33	0.1276	
Grazing history (G)	1	670	17.38	<.0001	✳
Fe x G	1	670	0.29	0.5916	
Fu x G	1	670	0.06	0.8130	
Fe x Fu x G	1	670	1.12	0.2907	
I x G	1	670	1.56	0.2127	
Fe x I x G	1	670	5.32	0.0213	✳
Fu x I x G	1	670	1.76	0.1847	
Fe x Fu x I x G	1	670	3.23	0.0729	

Table 2-2 *Post-hoc* t-test comparisons and difference estimates for fixed effects having tested significant in a 4-way non-parametric ANOVA testing the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the probability of herbivory (y/n) on tillers at any point during the growing season. The second element of the comparison is the -1 element. Asterisks denote significant p-values (α=0.05). (Fe) denotes fertilizer treatment and (G) is grazing history.

Effect	Denominator d.f.	t-value	Pr>|t|		Mean difference	95% CI	
						-	+
+NPK vs ambient	670	3.29	0.001	✳	1.6776	0.45	0.61
High vs. Low	670	-4.17	<.0001	✳	1.9252	0.51	0.69
E+ vs E-							
at Fe=ambient							
at G=High	589.6	0.18	0.8568		1.0565	0.48	0.86
at G=Low	980.3	-0.8	0.4267		0.7572	0.38	0.75
at Fe=+NPK							
at G=High	436	-1.56	0.119		0.6473	0.28	0.47
at G=Low	718.2	2.13	0.0333	✳	1.9782	0.91	1.73

Table 2-3 Type III tests of fixed effects obtained by a non-parametric 4-way ANOVA to test the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the probability of a tussock having tillers damaged by herbivory. Asterisks indicate significant p-values (α=0.05).

Effect	Numerator d.f.	Denominator d.f.	F-value	Pr > F	
Fertilizer (Fe)	1	522	34.71	<.0001	✳
Fungicide (Fu)	1	522	0.26	0.6091	
Fe x Fu	1	522	0.11	0.7417	
Infection (I)	1	522	1.74	0.1883	
Fe x I	1	522	0.02	0.8828	
Fu x I	1	522	0.16	0.6918	
Fe x Fu X I	1	522	0.47	0.4948	
Grazing history (G)	1	522	13.97	0.0002	✳
Fe x G	1	522	0.37	0.5444	
Fu x G	1	522	0.01	0.9051	
Fe x Fu x G	1	522	0.68	0.4117	
I x G	1	522	0.02	0.9005	
Fe x I x G	1	522	0.22	0.6401	
Fu x I x G	1	522	0.35	0.5530	
Fe x Fu x I x G	1	522	0.20	0.6558	

Table 2-4 *Post-hoc* t-test comparisons and difference estimates for fixed effects having tested significant in a 4-way non-parametric ANOVA testing the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the probability of a tussock having tillers damaged by herbivory. The second element of the comparison is the -1 element. Asterisks denote significant p-values (α=0.05).

Effect	Denominator d.f.	t-value	Pr>|t|		Mean difference	95% CI	
						-	+
+NPK vs Ambient	522	5.89	<.0001	✳	2.94	0.88	1.27
High vs Low	522	3.74	0.0002	✳	1.98	0.60	0.86

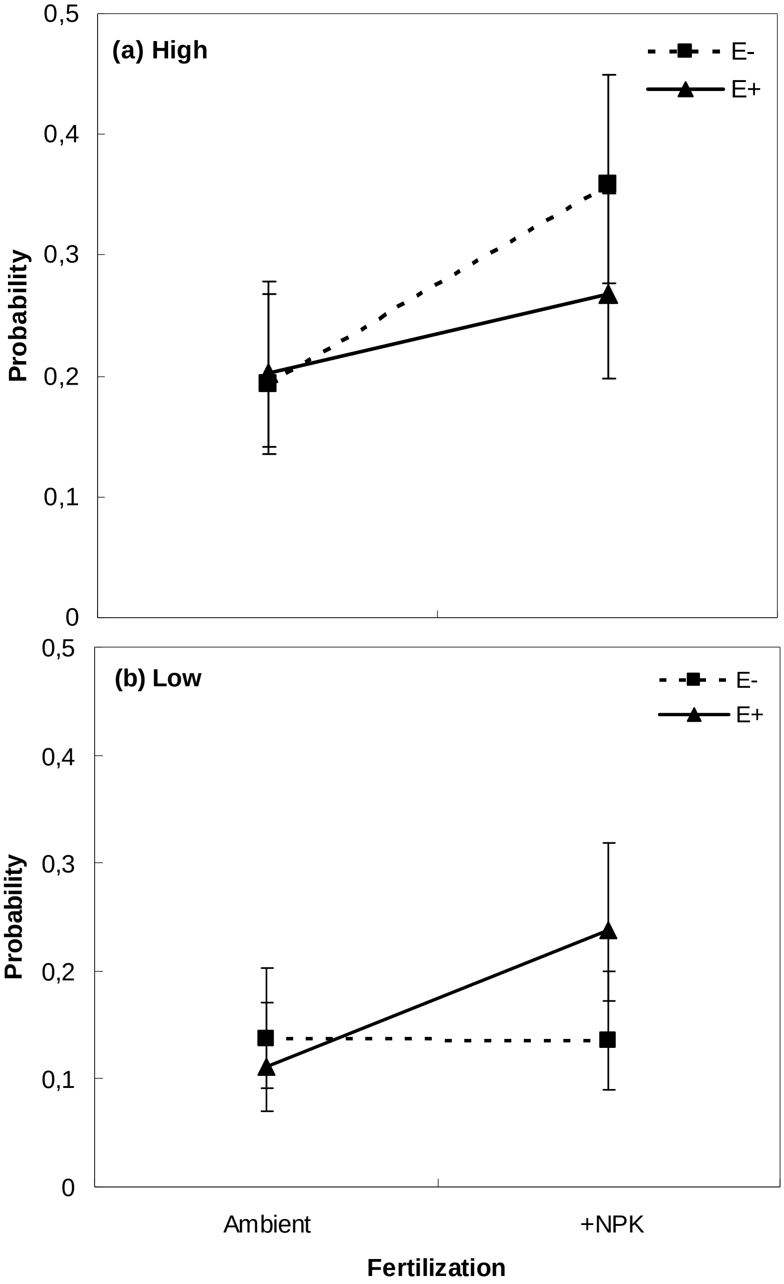

Figure 2-1 Probability of herbivory on at least one of the three monitored tillers in E- and E+ tussocks exposed to two levels of fertilization and grown in (a) high grazing history areas (E-: $N_{ambient}$=68 and N_{+NPK}=68, E+: $N_{ambient}$=67 and N_{+NPK}=68) and (b) low grazing history area (E-: $N_{ambient}$=68 and N_{+NPK}=68, E+: $N_{ambient}$=67 and N_{+NPK}=68). Error bars are 95% confidence interval.

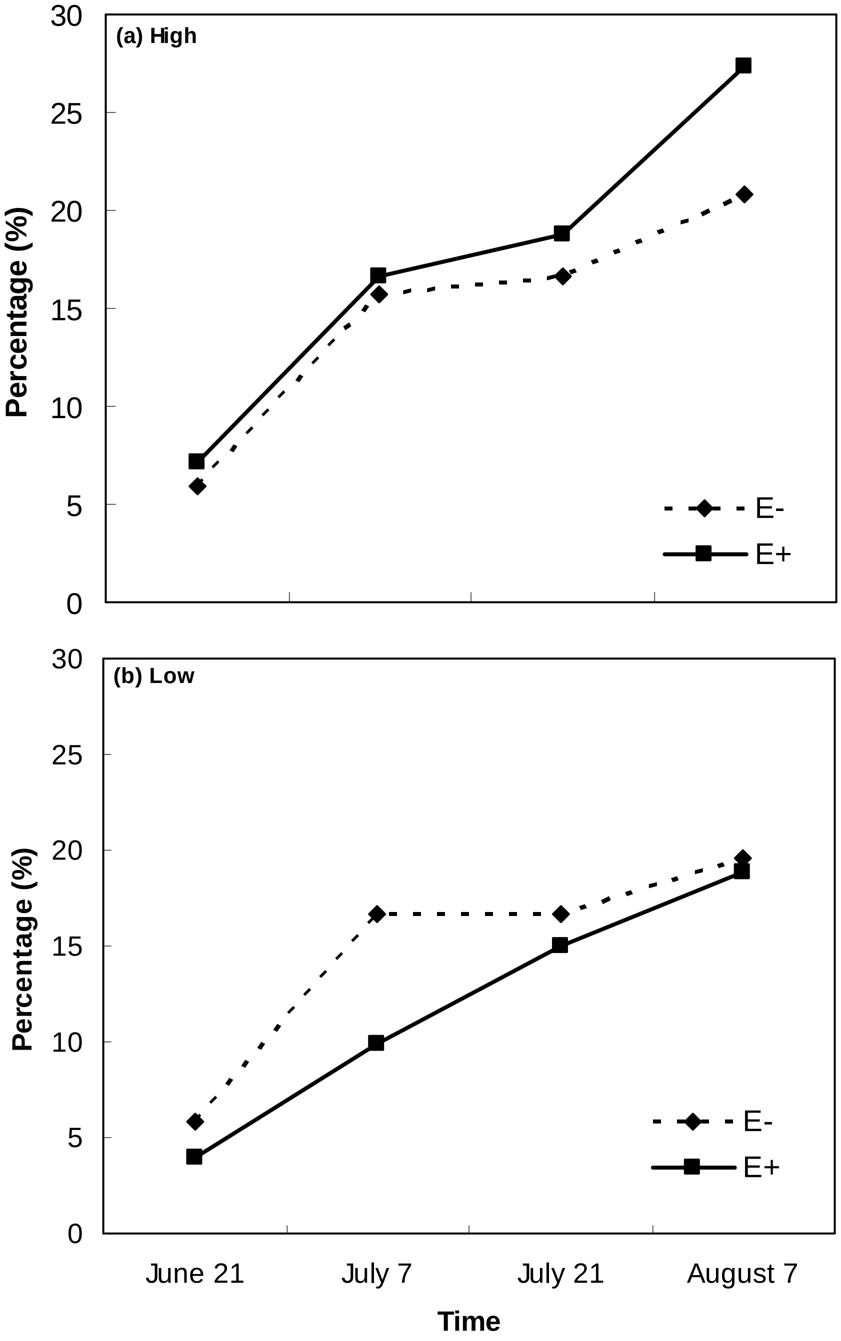

Figure 2-2 Proportion of *F. altaica* tillers from untreated plants showing herbivory damage throughout the growing season in areas of (a) high grazing history (June 21: N_{E-}=102 N_{E+}=98, July 7: N_{E-}=102 N_{E+}=96, July 21: N_{E-}=102 N_{E+}=96, August 7: N_{E-}=101 N_{E+}=95) and (b) low grazing history (June 21: N_{E-}=102 N_{E+}=102, July 7: N_{E-}=102 N_{E+}=101, July 21: N_{E-}=102 N_{E+}=100, August 7: N_{E-}=102 N_{E+}=100).

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CHAPTER 3

DEMOGRAPHY OF *Festuca altaica* INFECTED BY A FUNGAL ENDOPHYTE: EVIDENCE OF MUTUALISM?

Introduction

Fungal endophytes have been found in all plant phyla examined to date, including mosses, algae and coniferous trees (Arnold et al. 2000). In grasses, the percentage of species hosting such symbionts is estimated at 20-30% (Leuchtmann 1992). In particular, the fungal genus *Neotyphodium* (tribe Balansieae, family Clavicipitaceae, phylum Ascomycota) has attracted much attention because of its widespread infection of economically important cool-season grasses (Clay and Schardl 2002, Hoveland 1993). In these grasses, sparsely branched hyphae develop along the cell walls of above-ground tissues, where they rely on amino acids and sugars released into the apoplast for nourishment (Clay and Schardl 2002). Although horizontal transmission is theoretically possible through mycelial growth and subsequent sporulation, or through transmission of active hyphae by insect herbivores (Kasai et al. 2006, Tadych et al. 2007), current knowledge suggests that *Neotyphodium* spp. disperse primarily vertically by extending their hyphae into the plant's seeds, thereby infecting the next generation of hosts (Clay and Schardl 2002, Kuldau and Bacon 2008).

Because the fungus' dispersal depends on the reproductive success of the host, ecological and evolutionary theory predict that if the net effect of *Neotyphodium* spp. infection is a decrease in host fitness, infected lineages should not persist in stable systems and infection frequency should be near 0% (Clay and Schardl 2002). Conversely, if the symbiont provides an advantage to infected (E+) lineages, host population infection ratio should be near 100% (Lipsitch et al. 1995). Based on the confirmation of this pattern in many common grass species, such as *F. obtusa* and *Poa sylvestris* (Clay and Leuchtmann 1989), the strict mutualism theory remained the prevailing conceptual framework up to the middle of the 1990's. More recently however, a meta-analysis of endophyte literature

(N=227) found that 82% of publications reviewed focused on *Lolium perenne* (perennial ryegrass) and/or *Lolium arundinaceum* (tall fescue, formerly *Festuca arundinacea*), two economically important, but also artificially selected and introduced agricultural grasses (Saikkonen et al. 2006). In natural populations, endophyte infection patterns often appear stable at intermediate infection ratios (Bazely et al. 1997, Koh and Hik 2007, Novas et al. 2007), a state which, according to evolutionary theory, should be strictly transient. Furthermore, failure to find clear and consistent advantages to the fitness of E+ plants, and sometimes a marginally negative impact of infection in natural symbiota (Faeth and Fagan 2002, Faeth and Sullivan 2003) has led several authors to suggest that the exact position of the endophyte/host relationship along the mutualism-parasitism continuum is largely dependent on environmental factors, biotic stress levels and the genotype of both symbionts (Faeth et al. 2002, Kogel et al. 2006) while strict mutualism is restricted to a few rare cases (Faeth and Fagan 2002). Nevertheless, studies in natural systems have documented a wide variety of services apparently provided to the plant by its fungal symbiont: herbivory deterrence, drought resistance, as well as nutrient stress tolerance (Clay and Schardl 2002).

Services provided to the host by fungal endophytes are usually credited to bioprotective alkaloids and secondary compounds which are often detected in E+ plants under apparent biotic or abiotic stress (Bush et al. 1997, Saikkonen et al. 1998). These nitrogen-rich compounds are known to be strong herbivory deterrents, discouraging insects (Bultman et al. 2004), small mammals (Durham and Tannenbaum 1998) and migratory birds (Conover and Messmer 1996) from feeding on the host grass, and causing severe health problems such as fescue foot and ryegrass staggers in livestock forced to feed on it (Porter and Thompson 1992, Sampaio et al. 2008).

Defensive mutualism through herbivory deterrence was the first and is the most studied effect of *Neotyphodium* spp. on host grasses (Cheplick and Faeth 2009), however, even before *Neotyphodium* spp. had been isolated from forage grasses

(Bacon et al. 1977), nitrogen (N), phosphorous (P) and potassium (K) nutrition were known to modify the alkaloid content of tall fescue, and thus its toxic properties (Gentry et al. 1969). Because nitrogen fertilization has been shown to increase alkaloid production in E+ tall fescue (Arechavaleta et al. 1992), soil nutrient availability can strongly influence the deterrent properties of the fungus (Krauss et al. 2007). Indeed, Bultman (2004) demonstrated that while E+ and E- tall fescue responded differently to grazing history, with E+ plants showing greater resistance and E- plants showing increased susceptibility to herbivory, medium and high nutrient conditions exacerbated this pattern. On the other hand, studies in natural systems have not shown the consistent pattern linking herbivory deterrence to soil nutrient levels (Faeth et al. 2002), but nutrient availability does appear to interact with *Neotyphodium* spp. infection to affect plant growth (Malinowski and Belesky 2000).

Interestingly, fertilization experiments have consistently shown increased productivity indices in E+ grasses when compared to E- plants (Arachavaleta et al. 1989, Cheplick et al. 1989, Krauss et al. 2007). Richardson (1999) noted a 58% higher N uptake in E+ seedlings compared to E-, particularly when exposed to NH_4-N (versus NO_3-N). In contrast, the benefits of infection appear inversely related to P availability. In low P conditions, E+ plants had modified root structure and activity (Malinowski and Belesky 2000) as well as higher nutrient tissue content (Rahman and Saiga 2005, Zabalgogeazcoa et al. 2006), while the opposite was true under high P conditions. The effect of P soil content on nutrient uptake may be significant as some of these, such as calcium (Ca), are involved in ergot alkaloid synthesis (Rahman and Saiga 2005). Many other nutrients, such as zinc (Zn), copper (Cu) and manganese (Mn), have been found in different concentrations between E+ and E- plants (Monnet et al. 2001, Zabalgogeazcoa et al. 2006), although not consistently across studies.

A survey by Koh and Hik (2007) in a subarctic alpine meadow demonstrated the existence of a *Neotyphodium* sp. infection gradient stretching from boulder field, where *Festuca altaica* (northern rough fescue) exhibited ~70% infection, to alpine meadow (a gradient of approximately 60m), where infection declined to ~30%. The spatial covariation of this gradient with spatio-behavioural patterns of a common native generalist herbivore *Ochotona collaris* (collared pika) as well as the results of open-style cafeteria feeding trials where collared pikas preferentially chose E- *F. altaica* when presented with forage from the boulder field (p=0.0006) were strong indicators of defensive mutualism (Koh and Hik 2007). On the other hand, other environmental factors known to interact with fungal endophytes in cool-season grasses, such as soil nutrient availability and hydrology may realistically vary along this same gradient (Gundel et al. 2008); the former may prove particularly important as tundra systems are notably poor in available nutrients (Hobbie 1992). Koh and Hik (2007) also hypothesized that the low infection ratio observed in meadows (~30%) was due to the lower fitness of E+ plants under lower herbivory pressure; however, in the context of native grass populations, 30% infection is a relatively high infection ratio, and other studies have classified as “low infection” populations showing <5% of infection (Saona et al. 2010). The persistence of this intermediate infection ratio suggests that even when herbivory pressure is low, there may be a marginal advantage to maintaining E+ lineages.

Our objectives were to experimentally determine the factors affecting *Neotyphodium* sp. infection frequency at each end of the grazing gradient described above. First we aimed to verify the existence of the herbivory gradient. Then, more specifically, we attempted to test the effect of nutrient availability on the leaf demography of E+ and E- *F. altaica* tillers across this long-term herbivory and infection frequency gradient. We also measured soil nutrient availability across this gradient. Our hypothesis was that on boulder field endophytes are strict mutualists of *F. altaica* and provide higher plant condition and/or fitness through herbivory deterrence, while in meadows endophytes also maintain a weak mutualistic link with their host, by providing advantages to their host and mitigating the effects of soil nutrient deficiency.

Methods

Study site

Our study was carried out in a subarctic alpine meadow (4km²) interspersed with boulder field and located in the Ruby Range, east of Kluane Lake, YT, Canada (61°13'N, 138°16'W, elev. 1700-1900m), with slopes facing east and west. Growing season lasts approximately 70 days, beginning one to two weeks after snow melt, about mid-June, and ending with the first snowfall, usually after the third week of August (D.S. Hik, unpublished data). While the plant community changes between boulder field and meadow, the perennial bunchgrass *Festuca altaica* is ubiquitous across the gradient (McIntire and Hik 2005, Koh and Hik 2007). *F. altaica* is an amphi-beringian fescue species which forms densely tufted bunches with culms standing between 25 and 100cm high; it is generally found in open woods, alpine grasslands and tundra regions (Cody 1996). Mitchell et al. (2009) showed that at our field site, edaphic conditions such as soil depth and nitrate availability are extremely variable over short distances. Collared pikas, the most common herbivore, is constrained to boulder field patches by predation risk (Huntly 1987, Morrison et al. 2004) and thermoregulation (Smith 1974); they focus grazing effort on a 10-20m wide strip of vegetation along boulder field edge (McIntire and Hik 2002, Morrison et al. 2004). The physical structure of the boulder fields has likely been stable over the past century (Price 1991), therefore we can assume that behavioural constraints on herbivores have remained constant. Consequently, selection pressures have likely remained different in magnitude on the *F. altaica* / *Neotyphodium* sp. symbiotum at each end of the gradient (Koh and Hik 2007). While pika populations may have experienced year-to-year fluctuations during that time, thereby changing the immediate intensity of grazing, it is unlikely to have affected the overall herbivory pressure experienced by a tussock over the course of its lifetime (several decades).

Experimental design

In order to test our hypothesis, we performed a full factorial experiment combining 4 factors and their interactions: (1) infection (E+/E-), (2) grazing history (high/low), (3) fertilization (ambient/+NPK) and (4) fungicide (control/ fungicide)[§], for a total of 16 treatments.

Using a field immunoblot assay to detect monoclonal antibodies specific to *Neotyphodium* spp. (Agrinostics, Watkinsville, GA, USA) (Koh et al. 2006, Saari et al. 2010), we created a pool of *F. altaica* tussocks of known infection status at two experimental sites: on west and east-facing slopes, respectively, for which tiller number and tussock area were recorded (N=991).

At each site, high herbivory treatment tussocks were selected in boulder field occupied by pikas, and low herbivory treatment tussocks were in meadows, approximately 60m from the nearest boulder field; this is the distance at which herbivory was lowest in Koh and Hik's (2007) survey of *F. altaica* tussocks in 2004. Manipulative treatments were assigned to experimental tussocks randomly with restrictions to minimize any potential contamination due to slope drainage. A total of 34 tussocks were assigned to each treatment, for a total sample size of 544 tussocks. Final sample size was fixed based on the number of tussocks we could feasibly measure in under 7 days and on the expected effect size from the results of Koh and Hik's (2007) feeding trials. Further details on initial screening and treatment attribution are given in appendix A-1.

A standard water soluble fertilizer (PlantProd®, 20-20-20 NPK All-Purpose Fertilizer) was used for fertilization treatments, which was repeated 3 times at two weeks intervals (beginning mid-June) for a total of 2.054g of nitrogen per plant[**]. We used Tilt 250E for the *Neotyphodium* sp. removal treatments (Syngenta Crop

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[§] Within the scope of this experiment, the word "control" is used throughout the text only in reference to tussocks or tillers not treated with fungicide. Tussocks or tillers treated with water throughout the experiment are referred to as "untreated".

[**] 2% NH_4-N and 18% Urea $(NH_2)_2$-CO

Protection Inc., Calgary, AB, Canada, active ingredient: propiconazole). Propiconazole has been shown to eradicate *Neotyphodium* spp. effectively both in seeds and seedlings (Faeth and Sullivan 2003, Hill and Brown 2000). A full-dose treatment was applied mid-June (Tilt 250E concentration = 2.5mL/L), when growth had just begun, and a follow-up dose of 1.2mL/L was applied 2 weeks later. All chemical solutions were applied in 1L volume, including water treatments and combined fertilizer and fungicide treatments.

Hyphal density / immunoblot screening

Koh et al. (2006) found a close correlation between *Neotyphodium* spp. hyphal density and the colour intensity of the detection signal on the field immunoblot assay card, as measured by the RGB pixel value of a scan. RGB colours are expressed as red, green and blue values between 0 and 255. We collected stem clippings from a sub-sample of experimental tussocks (primarily E+ treatments) in order to assess the effectiveness of the fungicide treatment at the end of the growing season. All clippings were processed on the same card (Table A-2), which was subsequently scanned and five pixels per plant clipping were sampled (GIMP 2.6.10); green values were pooled to an average per clipping.

Experimental monitoring

Within each experimental tussock, three tillers were marked at the base with coloured wire for monitoring, beginning three days after the first application of chemical treatments. We recorded variables associated with leaf demography every two weeks beginning immediately after the first treatment application and up to mid-August, near the end of the growing season.

Soil nutrient analysis using PRS probes

Plant Root Simulator probes (Western AG Innovations, Saskatoon, Canada) are strips of anion and cation exchange resin membrane framed in plastic. They are chemically pre-treated such that their ion sorption rate closely resembles that of plant root surface. Ninety-six anion-cation probe pairs were deployed on June 29

(2008), and remained buried for 52 days. The probes were buried in sets of 2 anion-cation pairs, within 30cm of the tussock base (n=48): 12 for each herbivory/infection combination (limited to tussocks not treated with either fertilizer or fungicide). This approach allowed us to detect any difference at the large scale (boulder field versus meadow) and at the small scale (between tussock patchiness). A study by Mitchell et al. (2009) at our study site showed the patchiness of ammonium and nitrate availability over only a few meters. The probes were recovered at the end of the growing season, cleaned and sent back to Western AG Innovations for chemical analysis. The two anion-cation pairs were pooled for each tussock and the membranes were analyzed for 7 nutrients (Ca, Cu, K, Mg, Mn, P, Zn) as well as 2 forms of nitrogen (NH_4-N and NO_3-N). Chemical extraction techniques varied according to ion properties, and the values obtained were in μg/10 cm²/52 days.

Data analysis

Tiller number and tussock area were analyzed using 2-way mixed-model ANOVAs. we first selected the random effect structure using Akaike's Information Criterion scores of all plausible models using the MIXED procedure (SAS 9.2, SAS institute, Cary, NC, USA). The list of random effect included but was not restricted to site effects, as well as tussock effects in cases where the experimental unit was the tiller. Random effects with extremely small estimates were removed from the model for simplicity. If the assumptions of normality and equality of variance were not met, we then used the GLIMMIX procedure to test the chosen models for fixed effects (infection, grazing history, infection x grazing history), otherwise fixed effects were tested using the MIXED procedure.

Immunoblot signal green value, as a proxy for hyphal density, was analysed following the statistical procedure described above, with the additional fixed effect factors of fertilizer and fungicide, as well as associated interactions.

Leaf birth was considered to be the number of leaves in a given tiller at the end of the growing season minus the original number of leaves in that tiller. A leaf was considered dead when less then 1/3 of the leaf length was green. We judged that this was the point at which leaves were unlikely to contribute to any increase in tussock biomass, while being still recognizable and firmly attached to the tiller; completely dead leaves became very brittle and were easily broken away from the tiller. Statistical analysis was carried out following the same procedure as above.

Development of new leaves outside of the older leaves (and therefore out of sequence) was considered vegetative reproduction. The probability of one tiller in a tussock showing signs of vegetative reproduction was expressed as a 0-3 score per tussock reflecting the likelihood (probability *p*) that one tiller showed signs of vegetative reproduction. To facilitate further analysis, the probability *p* was logit transformed such that logit(*p*)=log(*p*/1-*p*) and was analysed using a generalized linear mixed model assuming a binomial distribution. The random structure of the model was selected using Akaike's Information Criterion scores for all plausible structures. Fixed effects were tested using the GLIMMIX procedure. Odds of vegetative reproduction [*p*/(1-*p*)] were the basis of interpretation of all treatment effects. Any two probabilities of vegetative reproduction were compared through the ratio of their odds:

$$\log\left(\frac{p_1 / (1-p_1)}{p_2 / (1-p_2)}\right)$$

Green length was averaged over all leaves within a tiller and all subsequent analyses were weighted by the number of leaves within that tiller. We concentrated our analysis on green leaf length patterns at the end of July, as this afforded the longest time possible for the plant to respond to treatments without these patterns disappearing due to leaf senescence (Figure A-7).

Senescence rate was calculated as [1-(GL4/GL3)], where GL3 and GL4 represent the average green length of a given tiller at the end of July (GL3) and mid-August (GL4). Because we were measuring leaf senescence, tillers showing net growth during this period were automatically assigned a value of zero. This ratio was weighted by the harmonic mean of the leaf number at each time step to obtain average leaf senescence rate. The same statistical procedure presented above was followed.

It was not possible for us to analyze nutrient availability using a multivariate ANOVA because of the mixed nature of our basic statistical model. Instead, we treated each nutrient as a repeated measure element at the tussock level within our statistical model, which allowed us to test our fixed model on all nutrients in a single analysis while still including important random effects such as “site”. Nutrient availability data was therefore first tested for broad, multi-nutrient patterns using proc MIXED, and single nutrients patterns were tested in *post-hoc* t-tests.

P-values for all statistical tests performed were interpreted using $\alpha=0.05$, with no adjustment (Bonferroni or otherwise) for the number of tests performed. It has become standard in ecological studies using multiple statistical tests for a single dataset to control for Type I error by reducing the significance threshold proportionally with the number of tests to be performed (Perneger 1998). More recently, mathematical, logical and practical objections have been raised against this practice (Moran 2003). Following Moran’s (2003) recommendation, we report only exact p-values and compare them to the accepted 0.05 cut-off. We considered “near significant” p-values greater than 0.05 but smaller than 0.1.

Results

Tiller number and tussock area

A non-parametric two-way ANOVA revealed a significant interaction between grazing history and infection for both tiller number ($F_{1,987}=210.03$, $P<0.0001$) and tussock area ($F_{1,987}$=986.08, P<0.0001) (Table A-3a). Figure 3-1 shows the sharp decrease of both parameters between high and low grazing locations: tiller number was 2.01 ± 0.03 ($\bar{x}$ ± 95% C.I.) times higher in boulder fields than in meadows, and tussock area was 2.04 ± 0.01 times higher (Table A-3b). However, while the morphological parameters of E- tussocks were 1.23 ± 0.02 times higher than E+ where grazing history was high, E- tussock parameters become slightly but still significantly lower than E+ in areas of low grazing history (Figure 3-1).

Hyphal density/immunoblot screening

The green value of the immunoblot signal was significantly affected by the interaction between fungicide and infection ($F_{1, 260}$=18.05, p<0.0001, Table 3-1). This relationship is shown in Figure 3-2a, where control E+ tillers have a low green value (193.4 ± 3.5) (and therefore high hyphal density) while control E- tillers have the much higher green value (232.3 [205.9, 261.9]) ($\bar{x}$ [95% C.I.]) associated with low to null hyphal density. When treated with Tilt 250E fungicide, E- tillers showed a relatively constant green value (217 [200.9, 236.1]), but E+ tillers showed a sharp increase in green value (239.6 ± 4.3). This corresponds to the expected decrease in hyphal presence following fungicide application. Fungicide alone also affected green value significantly ($F_{1, 260}$=9.82, p=0.0019, Table 3-1) and this was most likely due the unequal sample size between E+ and E-; the neutral response of the relatively few number of E- samples on our immunoblot card was not enough to outweigh the strong response of the E+ samples to fungicide in the ANOVA. We opted to sample more E+ in order to obtain better resolution in their response to fungicide. When E- were excluded, E+ tillers had a 1.24 ± 0.03 times higher green value when treated with fungicide ($t_{1, 260}$=16.47, p<0.0001, Table A-4). Similarly, infection had only a marginal effect on green value ($F_{1, 260}$=3.12, p=0.0786), but a simple *post-hoc* comparison between controls of each infection status revealed a significant difference ($t_{1, 260}$=5.46, p<0.0001), with E- samples having green values 1.3 ± 0.1 times higher than E+ tillers.

The 4-way ANOVA also showed a significant 3-way interaction between fertilizer, grazing history and infection ($F_{1,\ 260}$=4.69, p=0.0313, Table 3-1). *Post-hoc* comparisons showed that this was mainly driven by the difference between E+ tillers from high and low grazing history areas treated with fertilizer ($t_{1,\ 260}$=-3.13, p=0.0019). Figure 3-2b shows how in nutrient rich conditions, the green value of the immunoblot signal is 1.06 ± 0.03 times higher for E+ tillers grown in low grazing history areas than for their counterparts in high grazing history locations; this indicates a slightly lower hyphal density in the former.

Leaf demography

A 4-way non-parametric ANOVA of leaf birth data revealed a significant interaction between fertilizer and fungicide($F_{1,17.22}$=5.07, p=0.0376, Table 3-2), irrespective of infection status. *Post-hoc* tests showed that the significant interaction was due to a 1.44 ± 0.3 times higher leaf birth number in control tillers versus tillers treated with fungicide in high nutrient conditions (Figure 3-3). At ambient nutrient levels, there was no significant effect of fungicide on leaf birth ($t_{1,\ 23.04}$=0.35, p=0.7281, Table A-5). Each main effect (fertilizer, fungicide) also significantly affected leaf birth independently, with fertilized tillers showing 1.8 ± 0.3 times more leaf birth than tillers exposed to ambient nutrient levels ($F_{1,\ 17.42}$=66.64, p<0.0001, Table 3-2). To a lesser but significant degree, leaf birth was also 1.23 ± 0.2 times higher in control tillers compared to fungicide-treated tillers ($F_{1,\ 17.42}$=7.76, p=0.0125, Table 3-2, Table A-5).

Type III tests of fixed effects for leaf death showed that there was a significant interaction between grazing history and fertilization treatments ($F_{1,17.42}$=4.26, p=0.0392, Table 3-2). However, a closer look revealed that this was due to a change in the effect of fertilizer between the two grazing history levels: on boulder field, tillers grown at ambient nutrient level had 1.56 ± 0.2 times higher leaf mortality than their fertilized counterparts, but in meadows leaf mortality of unfertilized tiller was only 1.27 ± 0.2 times higher (Table A-5, Figure A-6). The 4-way ANOVA also found grazing history alone to have significant effect on leaf death ($F_{1,\,1580}$=4.87, p=0.0274), with tillers from low grazing history showing 1.12 ± 0.1 times more leaf mortality than those from high grazing history locations (Table 3-2). A similarly small, yet near significant effect of fungicide was detected ($F_{1,\,1580}$=2.95, p=0.0861), with control tillers having 1.09 ± 0.1 times more leaf mortality than treated tillers (Table 3-2). Overall, fertilization appeared to have the most effect on leaf mortality ($F_{1,\,1580}$=46.47, p<0.0001) such that tillers exposed to ambient nutrient levels had 1.4 ± 0.1 times more leaf mortality than tillers treated with an NPK solution (Table A-5).

Vegetative reproduction

A 4-way non-parametric ANOVA showed that the likelihood of a tiller reproducing vegetatively within a tussock was not affected by infection status ($F_{1,14.89}$=0.56, p=0.4641). The only significant effect was that of fertilizer ($F_{1,14.89}$=5.64, p=0.0314): fertilized tussock were 1.43 [1.03, 1.97] times more likely to show signs of asexual reproduction (production of new tillers) in at least one tiller.

Green length of leaves

Mean green and total length of leaves on tillers not treated with chemicals (fertilizer = ambient, fungicide = control) were plotted against time to determine which intervals would be most informative on patterns in the mean green length of tillers; we selected the 21 July measurements as the furthest from treatment application and immediately preceding onset of senescence (Figure A-7). The type III tests of fixed effects yielded by the 4-way non-parametric ANOVA revealed a significant 4-way interaction between all factors (infection x grazing history x fertilizer x fungicide) ($F_{1,\,505.7}$=5.72, p=0.0171, Table A-8a).

Post-hoc comparisons between the two levels of grazing history for each combination of fertilizer, fungicide and infection, revealed a single near-significant difference. In tillers not exposed to chemical treatments, E- tillers were near-significantly different between high and low grazing history locations ($t_{1,}$

$_{521}$=1.86, p=0.0636, Table A-8b), with average green lengths of boulder field tillers being 9 ± 9mm larger than meadow tillers (Figure 3-4); in contrast, E+ tiller green length was smaller in high grazing history areas than in low grazing history by 5 ± 9 mm ($t_{1,\ 531}$=-0.95, p=0.3428). It is interesting to note a trend towards reversal of the E+/E- relationship between the two levels of grazing history (Figure 3-4), which mirrored the clear pattern in tiller number and tussock area.

Grazing history alone also had a significant effect on average green length of tillers ($F_{1,505.7}$=8.49, p=0.0037), which was 4.5 ± 3.4 higher in boulder field tiller than in meadow tillers. Fertilizer had the greatest effect on average green length of tiller ($F_{1,506.1}$=37.42, p<0.0001). Tillers exposed to NPK fertilizer had an average green length 15.3 ± 3.4mm higher than their counterparts exposed to ambient nutrient levels.

Senescence rate

The highest order interaction revealed by the 4-way non-parametric ANOVA was fertilizer x grazing history ($F_{1,\ 538.5}$=6.07, p=0.014, Table 3-3); this was principally driven by the 1.5 ± 0.3 times higher rate of senescence in low grazing history areas than on boulder fields in high nutrient conditions ($t_{1,504}$=4.23, p<0.0001, Table 3-4).

Overall, grazing history had a significant effect on senescence rate ($F_{1,\ 538.5}$=11.75, p=0.0007), with plants located in meadows showing 1.28 ± 0.2 times more senescence than those from boulder fields. Plants not exposed to fungicide had a 1.3 ± 0.2 times higher senescence rate than their treated counterparts ($t_{1,\ 538.5}$=3.69, p=0.0003). *Post-hoc* comparisons revealed that this was due to a significant difference between control and knocked-out E+ tussocks in high grazing history areas and in high nutrient conditions: control E+ tussocks had a senescence rate 1.84 [1.24, 2.72] times higher than their knocked-out counterparts (Table 3-4). Fertilizer treatment had the most effect on senescence rate ($F_{1,\ 538.5}$=146.74,

$p<0.0001$) with plants exposed to ambient nutrient levels showing 2.4 ± 0.3 times more senescence then their fertilized counterparts.

Soil nutrient analysis using PRS-probes

A three-way ANOVA revealed significant variations in nutrient uptake by PRS-probes both at the small and larger scale. The significant three-way interaction between nutrient identity, grazing history and infection ($F_{8,\ 38}=3.02$, $p=0.01$) indicates that certain nutrients vary significantly between boulder field and meadow areas (scale: ~60m), as well as between tussocks (scale ~1-20m) (Table A-9). There was also a significant interaction of nutrient x grazing history ($F_{8,\ 38}=3.31$, $p=0.0058$), indicating that certain nutrients showed variation strictly at the larger scale.

Post-hoc comparisons listed in Table 3-5 revealed that these significant interactions were principally due to variations in two nutrients. Manganese (Mn) adsorption by Plant Root Simulator probes was significantly different between grazing history levels ($t_{1,\ 30.4}=-2.33$, $p=0.0266$), which appeared principally due to the difference in Mn adorption near E+ tussocks between boulder field and meadow areas ($t_{1,46}=-2.19$, $p=0.0334$). Probes located around E+ tussocks on boulder field had 0.25 [0.07, 0.95] times the Mn that those in meadows adsorbed (Table 3-5). Potassium (K) also varied significantly between high and low grazing history areas, but in the PRS-probes located near E- tussocks ($t_{1,\ 15}=2.4$, $p=0.0298$): E- tussocks on boulder field had 2.23 [1.08, 4.55] times more K available in the soil around them than E- tussocks located in meadows. The statistical power of our analysis was relatively low, but because this was an exploratory survey meant to point towards possible avenues of investigation, it is interesting to note the trends shown by NH_4-N and P. NH_4-N was (not significantly) 1.3 times higher in high grazing history areas around E- tussocks but showed the opposite trend around E+ tussocks; P was (not significantly) 1.83 times higher on boulder field than in meadows around E+ tussocks.

Discussion

Based on previous research at our study site (Koh and Hik 2007), we expected that in areas of higher grazing history the relationship between *F. altaica* and its endosymbiont would be strictly mutualistic, with E+ plants showing more productivity and generally better condition because of release from herbivory. In meadows, where herbivory was not expected to be a factor, we predicted a slight advantage to E+ plants with soil nutrients as the primary driver. Previous studies have shown that *Neotyphodium* spp. can provide significant advantages to their host in areas of poor soil nutrient availability (Arachavaleta et al. 1989, Cheplick et al. 1989) such as tundra (Hobbie 1992), although specific mechanisms remain highly debated (Malinowski and Belesky 2000).

An immunoblot field assay confirmed the efficacy of our fungicide treatment in E+ tussocks, although fungicide appeared unexpectedly in a significant interaction with fertilization affecting leaf birth, irrespective of endophyte infection. Overall, our results confirmed that the relationship between *Neotyphodium* sp. and *F. altaica* was different at each end of the gradient, however, the direction of this disparity was not what we had predicted. In high grazing history areas, *Neotyphodium* sp. showed signs of being a parasitic symbiont of *F. altaica*. Conditions appeared generally more difficult for *F. altaica* growing in meadows, and the interaction between the grass and *Neotyphodium* sp. appeared neutral. Contrary to our predictions, there was no evidence of an interaction between infection and nutrient conditions on plant growth patterns.

Fungicide treatment

Koh et al. (2006) observed that the green value of the immunoblot signal detected by the field immunoblot assay test is closely inversely correlated to *Neotyphodium* spp. hyphal density in stem clippings, so we were able to confirm that our fungicide treatment was effective in eliminating the fungus. As shown in Figure 3-2a, green value of knock-out E+ tussocks was clearly brought back to E- levels by the fungicide treatment.

Fungicide unexpectedly appeared as a significant effect interacting with fertilizer. Tillers treated with fungicide had a smaller increase in leaf birth in response to NPK fertilization than did their untreated counterparts, although no negative effect of fungicide treatment on leaf birth was apparent at ambient nutrient levels (Figure 3-3). This apparently adverse effect of fungicide in high nutrient conditions may be due to an unintended disruption of mutualistic interactions with other beneficial fungal symbionts such as mycorrhizal fungi. However, propiconazole, the active ingredient in Tilt 250E is only mildly detrimental to the external hyphae of arbuscular mycorrhizal fungi, and does not appear to affect their internal activity (Kjoller and Rosendahl 2000). It is worth noting that leaf birth was the only parameter suggesting a adverse effect of fungicide irrespective of infection.

Boulder field patterns

Based on our hypothesis that in high grazing history areas *Neotyphodium* sp. provides herbivore-deterrent properties to *F. altaica* (Koh and Hik 2007), and since ecological and evolutionary theory predict that a vertically transmitted endosymbiont can only profitably provide services to its host if it does so strongly enough to affect the host’s own fitness (Lipsitch et al. 1995), we had expected relief from herbivory stress to translate into improved general productivity in E+ *F. altaica* (Clay 1990). While there was a sharp difference in the morphological measurements of *F. altaica* depending on infection status, this difference was in favour of E- tussocks, with tiller number 1.23 ± 0.02 times higher in E- than in their infected counterparts (Figure 3-1). Green length of untreated tillers exhibited a similar pattern, although it was not statistically significant (Figure 3-4). Conversely, while investigating a similar correlation between infection frequency and grazing history, Bazely et al. (1997) found no variation between the growth patterns of E+ and E- *F. rubra* (red fescue); in addition, they found higher mortality rate in greenhouse-grown E- plants than in E+. In our case, the significantly higher senescence rate observed in control E+ tillers (on boulder field) when compared to their knocked-out counterparts in high nutrient conditions (Table 3-4) suggests that release from infection was beneficial to the plant.

We concluded that *Neotyphodium* sp. infection in boulder field represented a significant physiological burden for the host. While the exact mechanisms through which *Neotyphodium* spp. may represent a cost to their host have no yet been resolved, we can suppose that these might include an additional internal sink for photosynthates and amino acids, thus creating a need for higher photosynthetic activity, as well as greater internal nitrogen deficiency in cases where the fungus is exploiting the plant's resources to produce N-rich alkaloids. Had the advantage provided by the herbivory-deterrent properties of the fungus (e.g. Koh and Hik 2007) been strong enough to compensate for the physiological burden of infection in terms of fitness, the relationship would still have been mutualistic. However, another aspect of our study examining herbivore response to our experimental treatments found that herbivore damage to tussocks in high grazing history areas did not vary according to infection status (Chapter 2). This result contrasts sharply with the definite preference shown by pikas in cafeteria-style feeding trials (Koh and Hik 2007), but may be explained by *F. altaica* being a relatively less desirable food item for collared pikas compared to available forbs such as *Polygonum bistorta* and *Artemisia norvegica* (Andruchow 2000, Morrison et al. 2004). Therefore, because *Neotyphodium* sp. infection appeared to have no advantage to *F. altaica*, given the difference in plant productivity between E+ and E- tussocks, and despite the high infection frequency exhibited by high grazing history populations (~70%), we conclude that the interaction is at least slightly parasitic.

Meadow patterns

Based on the infection frequency documented by Koh and Hik (2007) (~30%), and because the physical factors at our site have likely been stable for hundreds of years (Price 1991), we expected that in meadows the *Neotyphodium* sp./ *F. altaica* relationship would be weakly mutualistic, thus allowing for the persistence of intermediate infection frequencies over long periods of time. Tundra systems have notably low soil nutrient availability (Hobbie 1992), therefore our hypothesis was that in areas where herbivory is not expected to be a factor, the main stressor to the *Neotyphodium* sp./*F. altaica* symbiosis would be nutrient availability.

Our results consistently showed that meadows were not as favourable to *F. altaica* as boulder field. *F. altaica* tussocks located on boulder field had 2.01 ± 0.03 times the number of tillers (Figure 3-1) and an average tiller green length 4.5 ± 3.4mm longer than tussocks growing in meadows. Leaf mortality and senescence rate also reflected this pattern, with tillers from meadow tussocks showing higher leaf senescence rates than their counterparts from the boulder field (Table 3-4). Therefore, we suggest that growing conditions in meadows are more stressful for *F. altaica* which is characteristically a colonizing species with relatively poor competitive abilities. However, too little is known concerning *F. altaica* ecology to predict how it will perform on boulder field or in meadows. Nonetheless, the apparent change in environment quality for *F. altaica* is interesting because mathematical models have predicted that mutualistic interactions between endosymbionts and their host are more likely to arise in marginal environments, where the plant is under more acute environmental stress (Hochberg et al. 2000).

In accordance with this theory, our data indicated a slight advantage to E+ tussocks within meadows. *F. altaica* tussocks infected with *Neotyphodium* sp. had a slightly higher number of tillers and larger tussock area than E- tussocks (Figure 3-1, Table A-3b). This disparity was further noticeable (though not statistically significant) in the average green length of experimental tussocks (Figure 3-4). This leads us to conclude that *Neotyphodium* may have a mutualistic relationship with its host in meadows, but the net advantage is so slight that only a long-term study could establish its existence with any certainty. Our single-season experiment did not detect any effect of infection on the likelihood of vegetative reproduction ($F_{1,14.89}$=0.56, p=0.4641), and therefore the difference in tiller number between E+ and E- tussocks must have arisen over a long period of time

Nutrient availability

While we found that the overall system was limited by soil nutrient availability, with average green length 15.3 ± 3.4 mm longer in fertilized plants (Table A-8b) and leaf senescence rate 2.37 ± 0.31 times higher in plants grown at ambient nutrient levels (Table 3-4), we also found that (irrespective of infection status) plants located on boulder field responded more to NPK fertilization than they did in meadows.

This pattern was principally apparent in the leaf senescence rate, which, under NPK conditions, was 1.5 ± 0.3 times greater in low than in high grazing history areas (Table 3-4). Though less strongly, leaf death also showed this pattern (Table A-5). Interestingly, while hyphal density (as estimated by the green value of immunoblot signal) appeared constant in E+ tussocks in boulder field and in meadows exposed to ambient nutrient levels, it decreased significantly in meadows under increased nutrient availability (Figure 3-2b). In previous studies where *Neotyphodium* spp. appeared to alleviate nutrient stress, fertilization usually caused an increase in endophyte expression (either hyphae or alkaloid production) (Bacon 1993, Krauss et al. 2007). This suggests that nutrient availability is not the main driver behind the interaction of *Neotyphodium* sp. and *F. altaica* in low grazing history areas.

In deploying Plant Root Simulator probes at both ends of our gradient, as well as near E+ and E- tussocks at each of these ends, we aimed to detect any larger or smaller scale variations in soil nutrient availability which might have driven the infection gradient documented by Koh and Hik (2007). However, despite the disparity in the response of plants on boulder field and in meadows to NPK fertilization, results for nutrient availability obtained from Plant Root Simulator probes revealed no clear pattern in nutrient availability across our gradient. Probes deployed on boulder field detected 75% less Manganese (Mn) near E+ tussocks than near E+ in meadows (Table 3-5). The relationship of Mn with fungal endophytes is not as well documented as that of other, more commonly studied nutrients, however Malinowski and Belesky (2000) mention that soluble Mn in the soil may be significant because of its ability to bind phosphorous (P) and thus render it unavailable. As mentioned previously, low P conditions have been associated with increased advantage to E+ plants (Malinowski and Belesky 2000, Zabalgogeazcoa et al. 2006). Potassium (K) also varied between boulder field and meadow: E- tussocks on boulder field had 2.23 [1.08, 4.55] times more K available in the soil around them than E- tussocks located in meadows (Table 3-5). K has been detected in different concentrations between E+ and E- plants depending on endophyte infection and soil P availability (Malinowski and Belesky 2000, Rahman and Saiga 2005), but there is little direct evidence of a link. These patterns are not enough to conclude that there is a meaningful difference in the nutrient availability between high and low grazing history areas. Phosphorous (P) was almost 2x more abundant near E+ in boulder fields, than in meadows ($t_{1,\ 28.8}$=1.45, p=0.1579, Table 3-5); P has been linked to many aspects of *Neotyphodium* spp. / grass interactions, such as nutrient uptake efficiency and alkaloid production (Malinowski and Belesky 2000)

Based on the similarity of the nutrient profile on boulder field and in meadows presented above, the greater response of plants located on boulder field (either E- or E+) to NPK fertilization, and the decrease in hyphal density under high NPK conditions low grazing history areas, it appears unlikely that the neutral/slightly mutualistic relationship between *Neotyphodium* and *F. altaica* noticed in meadows is driven by nutrient availability, or that it is a driving factor behind the infection ratio gradient stretching from boulder field to meadow.

Cross-gradient pattern

The prediction that vertically transmitted symbionts must attain 100% infection rate in a host population if they are beneficial to their host or disappear if they are detrimental (Lipsitch et al. 1995) has been so universally accepted that high infection frequency is now often taken as a marker for mutualism (Saona et al. 2010). However, as Cheplick and Faeth (2009) point out, this may lead researchers to mistake correlation for causation. Using a modelling approach, Saikkonen (2002) found that parasitic endophyte/grass associations could theoretically persist as part of a structured metapopulation made up of patches of different quality for the symbiotum, even in cases where the fungal endosymbiont is “trapped” by vertical transmission. Previous studies on the wild grass *Festuca arizonica* (arizona fescue) and its *Neotyphodium* spp. symbionts have demonstrated the possibility that infection in a host population can remain stable at high levels (60-100%) even when E+ plants show consistently inferior reproductive output and biomass production compared to their E- counterparts (Faeth and Sullivan 2003, Schulthess and Faeth 1998). Our results support the idea that stable parasitic *Neotyphodium* spp./grass interactions are viable in natural systems, despite the overwhelming evidence against this from agronomic systems (Clay and Schardl 2002). It is possible that in highly-managed and genetically homogeneous agronomic systems more deleterious and labile interactions, though naturally occurring, have been bred out of the most common forage grasses (Muller and Krauss 2005).

While there is agreement that the existence of geographic mosaics in the quality of environment for grass hosts and their endosymbiont is key in the explanation of infection patterns (Hochberg et al. 2000, Saikkonen et al. 2002), there is some disagreement as to whether favourable or marginal environments are more likely to foster parasitic interaction. Greenhouse experiments and mathematical models both predict that parasitism is more likely to arise under limited resource conditions, when the plant can no longer afford the physiological weight of the symbiont (Ahlholm et al. 2002, Cheplick et al. 1989, Saikkonen et al. 2002). Conversely, our results showed that, in the apparently more favourable conditions found on boulder field denoted by the greater general condition of *F. altaica* tussocks, the *Neotyphodium* sp. / *F. altaica* symbiotum showed signs of being more parasitic. In the harsher conditions of the meadow, the same relationship was neutral / slightly mutualistic. Our results agree with a model proposed by Hochberg (2000) whereby hosts in more hospitable environments are more likely to develop parasitic relationship with their endosymbionts, while in marginal and sink environments, symbiota are more likely to develop into mutualistic relationships.

Unresolved questions

The ecological gradient which we investigated here is not a single-layered ecotone restricted to a decrease in pika haying behaviour further from boulder fields (Koh and Hik 2008). Though we have restricted our study to grazing history and nutrient availability, other factors may be changing across the same gradient that could have an affect on *Neotyphodium* sp./ *F. altaica* interactions. For example, water supply deficit is a well known driver of endophyte/grass relations in agronomic species (Bacon 1993), and recent studies in natural systems have also supported this pattern (Saona et al. 2010, Wali et al. 2007). The harsh geographic relief at our study site may be creating hydrological gradients that we are unaware of, and this could in turn affect the *F. altaica* and its symbiont. Furthermore, the gradient investigated in this study is also marked by a change in plant community (McIntire and Hik 2005), and it is possible that in meadows, where highly competitive species such as *Carex spp.* are more abundant, *Neotyphodium* sp. may be providing heightened competitive abilities to its host. This is a more rarely reported benefit of *Neotyphodium* spp. infection, but studies in agronomic systems have shown that endophyte infection can significantly affect the host’s competitive abilities (Clay and Holah 1999, Rudgers et al. 2005).

Conclusion

We concluded that despite the host having a high and stable infection ratio, *Festuca altaica* / *Neotyphodium* sp. symbiota in high grazing history areas have a parasitic relationship. However, in meadows this relationship appeared to change to a slightly mutualistic relationship. We could not explain this pattern with trends in soil nutrient availability or response to NPK fertilization, and we suggest that other factors such as interspecific competition or water availability may be the main drivers behind this change in the interaction. Finally, we emphasize the fact that in the study of vertically transmitted fungal endophytes in native cool season grasses it may be misleading to use infection frequency as an indicator of the position of the interaction along the mutualist-parasite continuum.

Table 3-1 Type III tests of fixed effects obtained by a 4-way ANOVA to test the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the hyphal density of *Neotyphodium* sp. in stem clippings of *F. altaica*, as indicated by the green value of a western blot signal. Asterisks denote significant p-values (α=0.05).

Effect	Numerator d.f.	Denominator d.f.	F-value	Pr > F	
Fertilizer (Fe)	1	260	1.90	0.1693	
Fungicide (Fu)	1	260	9.82	0.0019	✳
Fe x Fu	1	260	0.03	0.8533	
Infection (I)	1	260	3.12	0.0786	
Fe x I	1	260	4.23	0.0408	✳
Fu x I	1	260	18.05	<.0001	✳
Fe x Fu x I	1	260	0.54	0.4621	
Grazing history (G)	1	260	7.26	0.0075	✳
Fe x G	1	260	1.14	0.2871	
Fu x G	1	260	1.36	0.2440	
Fe x Fu x G	1	260	1.12	0.2917	
I x G	1	260	9.67	0.0021	✳
Fe x I x G	1	260	4.69	0.0313	✳
Fu x I x G	1	260	0.39	0.5328	

Table 3-2 Type III tests of fixed effects obtained by a non-parametric 4-way ANOVA to test the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the leaf demographics of monitored *F. altaica* tillers. Asterisks indicate significant p-values (α=0.05).

	Leaf birth				Leaf death			
Effect	Denominator d.f.	F Value	Pr > F		Denominator d.f.	F Value	Pr > F	
Fertilizer (Fe)	17.42	66.64	<.0001	✳	1580	46.47	<.0001	✳
Fungicide (Fu)	17.42	7.76	0.0125	✳	1580	2.95	0.0861	
Fe x Fu	17.42	5.07	0.0376	✳	1580	0.93	0.3343	
Infection (I)	17.42	0.72	0.407		1580	0.01	0.9307	
Fe x I	17.42	0.06	0.8118		1580	0.3	0.5819	
Fu x I	17.42	0.34	0.5657		1580	0	0.9992	
Fe x Fu X I	17.42	0	0.9728		1580	0.6	0.438	
Grazing history (G)	17.42	0.81	0.3797		1580	4.87	0.0274	✳
Fe x G	17.42	0	0.9926		1580	4.26	0.0392	✳
Fu x G	17.42	0.24	0.63		1580	0.44	0.5073	
Fe x Fu x G	17.42	0.58	0.4576		1580	0.28	0.5943	
I x G	17.42	0.05	0.8269		1580	0.08	0.7831	
Fe x I x G	17.42	0.07	0.7947		1580	0.16	0.6931	
Fu x I x G	17.42	1.06	0.3177		1580	0.99	0.3196	
Fe x Fu x I x G	17.42	0.01	0.9164		1580	0.28	0.599	

Table 3-3 Type III tests of fixed effects obtained by a non-parametric 4-way ANOVA to test the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the average leaf senescence rate of monitored *F. altaica* tillers during the last two weeks of the growing season. Asterisks indicate significant p-values (α=0.05).

Effect	Numerator d.f.	Denominator d.f.	F-value	Pr > F	
Fertilizer (Fe)	1	538.5	146.74	<.0001	✳
Fungicide (Fu)	1	538.5	13.59	0.0003	✳
Fe x Fu	1	538.5	0.50	0.4796	
Infection (I)	1	538.5	0.40	0.5278	
Fe x I	1	538.5	0.42	0.5159	
Fu x I	1	538.5	0.56	0.4565	
Fe x Fu x I	1	538.5	0.18	0.6718	
Grazing history (G)	1	538.5	11.75	0.0007	✳
Fe x G	1	538.5	6.07	0.0140	✳
Fu x G	1	538.5	0.90	0.3420	
Fe x Fu x G	1	538.5	0.29	0.5878	
I x G	1	538.5	0.00	0.9493	
Fe x I x G	1	538.5	2.54	0.1118	
Fu x I x G	1	538.5	0.20	0.6521	
Fe x Fu x I x G	1	538.5	1.21	0.2717	

Table 3-4 *Post-hoc* t-test comparisons and difference estimates for fixed effects having tested significant in a 4-way non-parametric ANOVA testing the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the average leaf senescence rate of monitored *F. altaica* tillers during the last two weeks of the growing season. The second element of the comparison is the -1 element. Asterisks denote significant p-values (α=0.05). (I) denotes infection status, (G) is grazing history and (Fe) indicates fertilization treatment.

Effect	Denominator d.f.	t-value	Pr>|t|		mean difference	95% CI	
						-	+
Ambient vs +NPK	538.5	12.11	<.0001	✳	2.37	0.31	0.26
Control vs fungicide	538.5	3.69	0.0003	✳	1.30	0.17	0.20
at I=E-							
at G=High							
at Fe=Ambient	587.1	1.29	0.1979		1.29	0.43	0.65
at Fe=+NPK	528.3	1.1	0.2701		1.24	0.42	0.62
at G=Low							
at Fe=Ambient	545.8	0.57	0.5687		1.11	0.36	0.54
at Fe=+NPK	472.9	1.22	0.2248		1.25	0.40	0.58
at I=E+							
at G=High							
at Fe=Ambient	584.1	1.05	0.2929		1.23	0.42	0.63
at Fe=+NPK	511.5	3.09	0.0021	✳	1.84	0.59	0.89
at G=Low							
at Fe=Ambient	577.6	1.23	0.2184		1.28	0.43	0.64
at Fe=+NPK	501.8	0.9	0.3675		1.19	0.39	0.57
Low vs High	538.5	3.43	0.0007	✳	1.28	0.17	0.19
at Fe=ambient	573.8	0.67	0.5020		1.07	0.20	0.24
at Fe=+NPK	504	4.23	<.0001	✳	1.52	0.27	0.33

Table 3-5 *Post-hoc* t-test comparisons and difference estimates for fixed effects having tested significant in a 4-way non-parametric ANOVA testing the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on nutrient uptake in Plant Root Simulator probes (Western AG Innovations, Saskatoon, Canada). The second element of the comparison is the -1 element. Asterisks show significant p-values (α=0.05). (N) denotes nutrient and (I) denotes infection status.

Effect	Denominator d.f.	t-value	Pr>|t|		Mean difference	95% CI	
						-	+
High vs Low	4.34	-0.17	0.8716		0.97	0.34	0.52
N=Ca	11.1	-1.08	0.3027		0.78	0.31	0.52
I=E-	6.84	-0.89	0.4058		0.78	0.38	0.72
I=E+	7.02	-0.97	0.3624		0.76	0.37	0.71
N=Cu	18.2	-0.3	0.7695		0.92	0.39	0.69
I=E-	42	0.74	0.4658		0.47	1.08	3.60
I=E+	-	-	-		-	-	-
N=K	20.4	1.55	0.1356		1.53	0.67	1.18
I=E-	15	2.4	0.0298	*	2.23	1.14	2.32
I=E+	15.6	0.11	0.9154		1.04	0.53	1.10
N=Mg	9.4	0.23	0.8239		1.05	0.42	0.70
I=E-	5.41	0.32	0.762		1.08	0.51	0.98
I=E+	5.53	0.06	0.9523		1.02	0.48	0.92
N=Mn	30.4	-2.33	0.0266	*	0.48	0.23	0.43
I=E-	45.6	-0.59	0.5594		0.69	0.50	1.72
I=E+	46	-2.19	0.0334	*	0.25	0.18	0.64
N=NH_4-H	13.3	-0.19	0.8556		0.96	0.39	0.66
I=E-	10.4	0.9	0.3866		1.31	0.63	1.21
I=E+	10.8	-1.18	0.2624		0.70	0.34	0.65
N=NO_3-N	37.2	-0.72	0.4774		0.77	0.40	0.83
I=E-	47.8	-1.22	0.2277		0.38	0.30	1.49
I=E+	47.9	-0.96	0.3428		0.46	0.37	1.88
N=P	34.8	1.48	0.1471		1.60	0.76	1.46
I=E-	27.8	0.73	0.4716		1.35	0.76	1.76
I=E+	28.8	1.45	0.1579		1.83	1.04	2.43
N=Zn	10.3	0.74	0.4734		1.19	0.48	0.8
I=E-	5.59	1.57	0.1718		1.49	0.70	1.33
I=E+	5.72	-0.31	0.7677		0.92	0.44	0.82

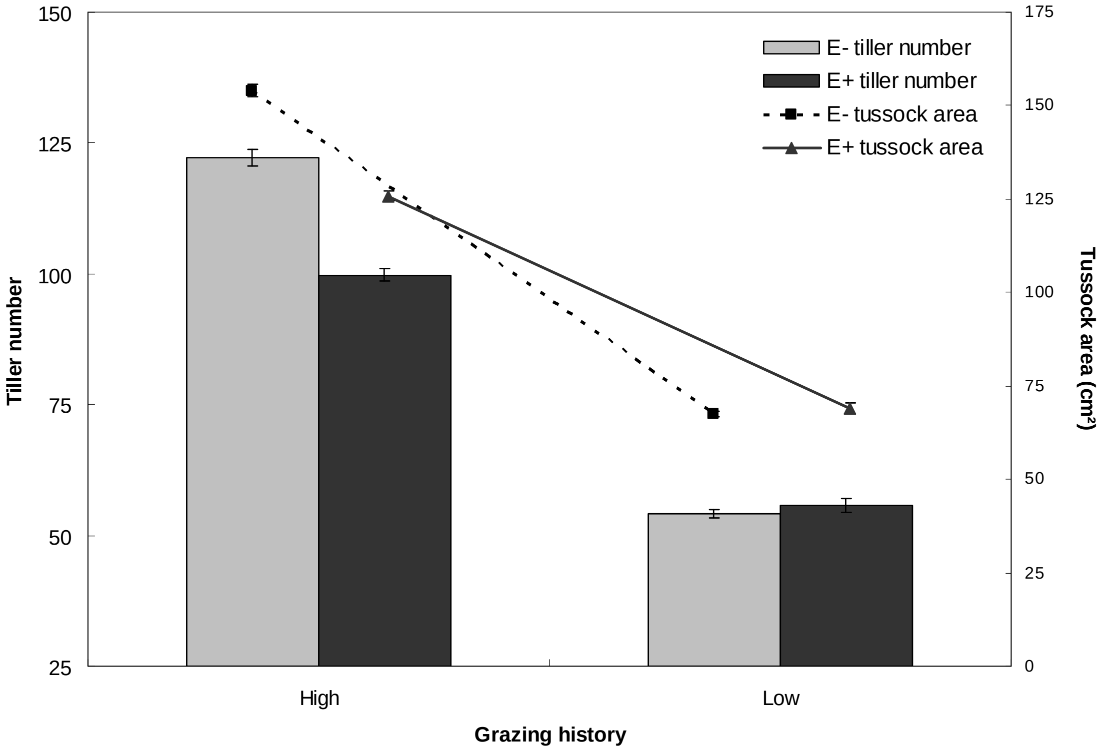

Figure 3-1 Average tiller number (bars) and average tussock area (lines) for *F. altaica* tussocks located in areas of high and low grazing history and both uninfected (E-) and infected (E+) with *Neotyphodium* sp. (High: N_{E-}=188 N_{E+}=305, Low: N_{E-}=357 N_{E+}=141). Error bars show 95% confidence intervals.

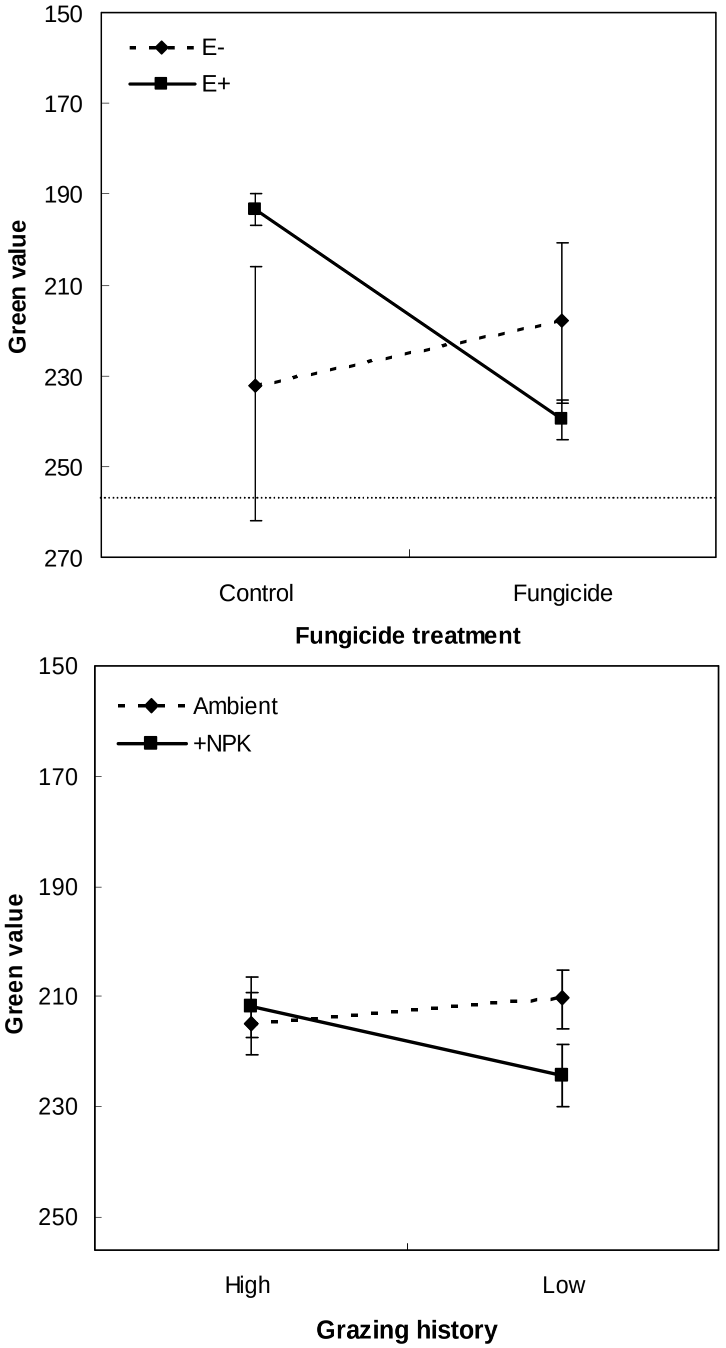

Figure 3-2 Average pixel green value of a western blot signal of *F. altaica* stem clippings used as an inversely-correlated indicator of *Neotyphodium* sp. hyphal density in the plant. Graphs show hyphal density response of (a) infected and uninfected tillers treated with Tilt 250E fungicide (Control: N_{E-}=7 N_{E+}=62 , Fungicide: N_{E-}=5 N_{E+}=64) and (b) E+ tillers grown at two levels of grazing history and exposed to different nutrient conditions (High: $N_{Ambient}$=61 N_{+NPK}=63 , Low: $N_{Ambient}$=65 N_{+NPK}=66). Dotted line represents the highest possible green value. Error bars show 95% confidence intervals.

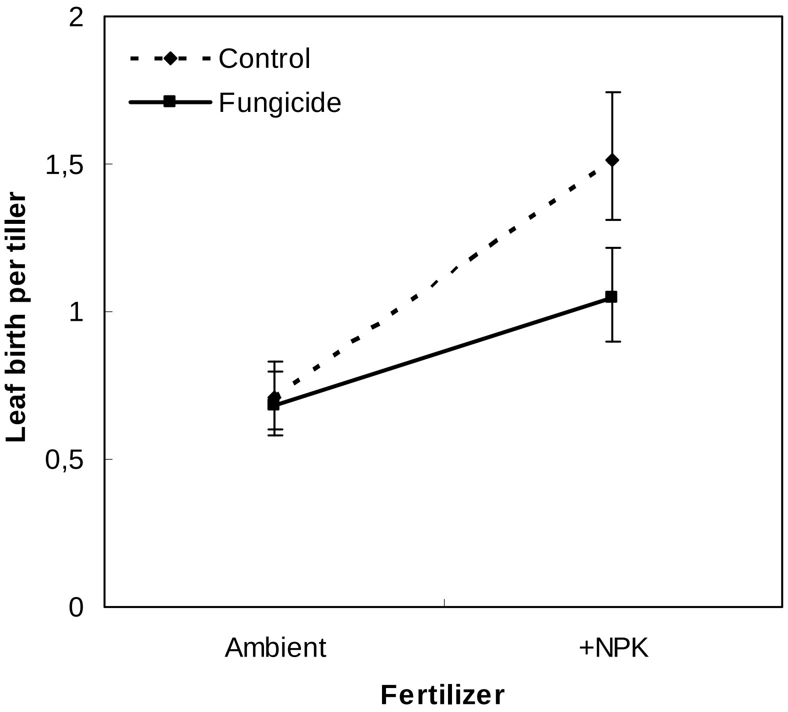

Figure 3-3 Average leaf births per tiller in *F. atlaica* tussocks treated with NPK fertilizer or grown under ambient nutrient level, and treated or not with Tilt 250E fungicide (Ambient: N_{cont}=399 N_{fung}=404, +NPK: N_{cont}=398 N_{fung}=395). Error bars are 95% confidence intervals.

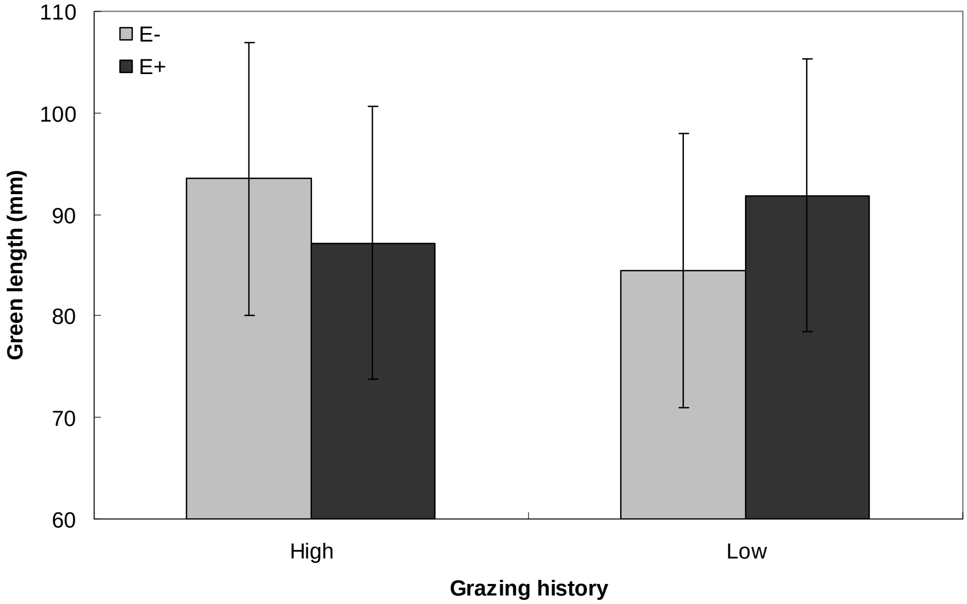

Figure 3-4 Mean green length (mm) of untreated *F. altaica* tillers infected (E+) and uninfected (E-) by *Neotyphodium* sp. in areas of high and low grazing history as measured during the last week of July (High: N_{E-}=96 N_{E+}=102, Low: N_{E-}=100 N_{E+}=102). Error bars show 95% confidence intervals.

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CHAPTER 4

CONCLUSIONS

Overview of my results

The majority of studies investigating grass/*Neotyphodium* spp. dynamics have assumed that strictly vertically transmitted endosymbionts must, in order to persist in a host population, provide their host with an advantage strong enough to affect their fitness relative to that of uninfected lineages (Lipsitch et al. 1995). Consequently, *Neotyphodium* spp. infection occurrence frequency in host populations has been widely used, both implicitly and explicitly, as indicator of the position of the grass/endophyte interaction along the parasitism-mutualism continuum (Cheplick and Faeth 2009, Saona et al. 2010, Shelby and Dalrymple 1993). This is particularly true in natural system, where evidence is scarce and the relative effects of biotic and abiotic factors on E+ and E- plants are difficult to untangle.

At my study site in an alpine meadow (Ruby Range, YT, Canada), a previous experiment demonstrated a sharp *Neotyphodium* sp. infection gradient in *Festuca altaica*, which was correlated with a gradient in grazing pressure history by collared pikas (*Ochotona collaris*) (Koh and Hik 2007). Based on these results, and the established use of infection occurrence as a measure of mutualism (Shelby and Dalrymple 1993), as well as feeding trial results, Koh and Hik (2007) concluded that the *Neotyphodium* sp. infection gradient in *F. altaica* was likely due to a higher fitness advantage to E+ plants in high grazing history areas. They considered that this was mostly likely due to the production of herbivore deterrent chemicals compared with E+ tussock grown under low grazing conditions, where it was hypothesized that the endophyte provided little to no advantage to its host.

Nevertheless, despite high *Neotyphodium* sp. occurrence in high grazing history areas, we could not confirm the defensive mutualism relationship of Koh and Hik (2007). In contrast with their feeding trial results, I found that damage by herbivores did not differ according to *Neotyphodium* sp. infection status and fertilization did not change this (Chapter 2), as had been suggested by previous studies on herbivore deterrence in *Neotyphodium* spp. strains (Krauss et al. 2007). In further contrast with my predictions, uninfected plants appeared to be in generally better condition, with larger tussock surface areas and higher tiller number (Chapter 3). Based on the apparent lack of advantage to E+ in terms of general plant condition or herbivory deterrence, I concluded that in my system, contrary to my working hypothesis, *Neotyphodium* sp. has a parasitic relationship with its host. Taking into account that this pattern was principally apparent in indices of long-term productivity, and less so in parameters indicating within-season responses, I suggest that the disadvantage to infected plants is not very strong. While this type of relationship is relatively counterintuitive in a vertically transmitted symbiont, it is not unheard of: in natural *F. arizonica* (arizona fescue)/ *Neotyphodium* spp. pairings, Faeth and Sullivan (2003) found reduced seed production and seed weight, as well as reduced general productivity in E+ individual.

In meadows, the persistence of intermediate *Neotyphodium* sp. infection frequency (~30%) (Koh and Hik 2007) appeared to indicate a marginal advantage to infected lineages. While grazing pressure is low, and therefore defensive mutualism is unlikely, I found that *Neotyphodium* sp. infection in *F. altaica* was neutral or very slightly mutualistic. Nutrient cycling is extremely slow in tundra systems (Hobbie 1992), and my hypothesis was that in areas not afflicted with chronic herbivory, the chief cause of stress for *F. altaica* would be nutrient availability; therefore, this would mediate *Neotyphodium* sp. / *F. altaica* relationships. However, while I confirmed that soil nutrient availability was significantly limiting for *F. altaica* growth both in boulder fields and meadows, I found no indication of a difference between E+ and E- plants in response to fertilization.

Having reasonably ruled out nutrient availability and grazing pressure as drivers of the *Neotyphodium* sp. / *F. altaica* symbiosis, other biotic and abiotic factors known to have an effect on *Neotyphodium* sp. endophytes must be investigated in order to fully understand the system. The infection frequency gradient in my study stretched from rocky boulder fields to mossy alpine meadow, and many parameters are likely to vary across the same spatial gradient other than grazing pressure, such as plant community structure and diversity, as well as water availability (Koh and Hik 2008).

Alternative hypotheses

Drought resistance is a widely documented benefit of many *Neotyphodium* spp. strains associated with *L. arundinaceum* and *L. perenne* (Bacon 1993, Cheplick and Faeth 2009). West et al's (1993) study on Kentucky 31 (cultivar of *L. arundinaceum*) showed a significant difference in the response of plants in E+ plots to experimental drought compared to their controls: by the end of the growing season, tiller number in E+ plots had recovered to the level of irrigated plots, whereas the same parameter in E- plots was only 62% of the tiller number in irrigated plots. While much evidence in agronomic systems supports these results (Bacon 1993), there has also been evidence of increased endophyte occurrence natural grass populations in arid climates. Considering naturally infected *Lolium* spp. populations across France, Lewis et al. (1997) found that the principal environmental predictor of infection frequency in 57 host populations was water supply deficit. In a glasshouse experiment, Saona et al. (2010) found that E- *Festuca rubra* tussocks (red fescue) outperformed E+ tussocks in all treatment but dry, nutrient-rich conditions, where E+ tussocks productivity was higher. Several mechanisms have been suggested for this effect of endophytes on plant condition: Malinowski and Belesky (2000) point towards a more developed and more efficient root system, modification of stomatal behaviour in E+ grasses which could reduce transpiration, and osmotic adjustments by way of solute accumulation to maintain turgor during water stress. Given the change in soil structure across our gradient of interest, soil hydrological properties are likely to change significantly across the same area, and it is plausible that boulder fields, where the soil layer is very thin over large boulders, represent a much dryer habitat for plants, whereas meadows, dense in sphagnum mosses which can remain waterlogged several days after a heavy rain, are significantly less stressed for water.

A less widely investigated aspect of *Neotyphodium* spp. / grass interactions is the possibility of increased competitive abilities in E+ grasses. In experimental *L. arundinaceum* plots, Clay and Holah (1999) monitored the increase in biodiversity through colonization by surrounding species: after 4 years, *L. arundinaceum* had become significantly more dominant in terms of relative biomass in E+ plots than E- tussocks in their plots; plant diversity was also significantly lower when *L. arundinaceum* was infected by *N. coenophialum*. This increase in competitive abilities appears to extend to intra-specific interaction as well: in a greenhouse experiment, 1 of 2 native woodland grass species naturally infected by *Epichloe* spp. had increased competitive abilities against E- conspecifics (Brem and Leuchtmann 2002). The evidence is, however, not unanimous; in a similar experiment to that of Clay and Holah (1999) and under artificial grazing treatment, Spyreas et al. (2001) found the opposite pattern of higher plant diversity in E+ plots. While there is no clear consensus in the literature about the effect of endophyte infection on the competitive abilities of its host, it appears that in one direction or the other *Neotyphodium* spp. do affect these abilities in some way. Composition of the plant community changes considerably across my gradient of interest: species richness tends to be lower further into the meadow, with fewer species of graminoids and increased diversity of dwarf shrubs (McIntire and Hik 2005), as well as an increase in the proportion of graminoids represented by sedges (*Carex* spp.) (Personal observations). Future research at alpine sites in the Yukon should concentrate on the potential competitive advantage conferred to E+ *F. altaica* compared to E- tussocks in areas where it is more likely to enter in interspecific competition with *Carex* spp., in meadows.

Overall, then, it appears that the gradient in *F. altaica* infection frequency is not driven by grazing history as had been previously thought. Nonetheless, it may be that while local herbivores do not mediate grass/endophyte dynamics, *Neotyphodium* spp. infection in forage does affect herbivores to a certain degree. My investigation of timing of grazing according to *Neotyphodium* sp. infection status suggested that pikas may be manipulating the preservation properties of their food cache by preferentially using E+ *F. altaica* during the haying period, while they do not appear to differentiate during the early season grazing period. This type of behaviour has been documented for *Ochotona princeps* (American pika), and confirmation of this pattern in another pika species could be an important step in understanding pika ecology. Future research on this topic should include litterbag decomposition experiments as well as chemical analysis of E+ *F. altaica* for secondary compounds. A recent study by Siegrist et al. (2010) also suggested that contrary to the long-standing assumption, increased plant matter conservation in endophyte infected grasses may not be related to alkaloid production, and that other properties conferred to the host by its symbiont may be involved, although what these might be remains to be explored.

Benefits of hindsight

The study of complex ecological interactions often requires that researchers examine higher-order interactions instead of simple one-way interactions. My experimental design, while complicated, allowed me to answer a large number of questions concerning the drivers of the *F. altaica* / *Neotyphodium* sp. symbiotum in alpine meadows. However, the drawback of testing high order interactions is that they can be very difficult to interpret and any potential insight may be wasted. When choosing the experimental design best suited for their question, researchers should carefully weight the risk of spending time and effort on a large scale experiment that could yield few decisive results versus the information that could be lost by dislocating ecological relationships which should be considered together in order to gain true insight. For this study, a full factorial experiment was the only way of testing complex hypotheses, but single or two-way experiments could have been sufficient to test my main effects. As it is often the case in ecological studies, there were more than one approach in which to ask these questions, and the final choice should reflect the researcher’s own philosophy.

Future research

My study concentrated on the effect of biotic and abiotic factors on the interaction between *Neotyphodium* sp. and its *F. altaica* adult host, but to fully understand the factors interacting to create the infection frequency gradient documented by Koh and Hik (2007) we must consider the lifecycle of the symbiotum in its entirety. Figure 4-1 shows the key steps in the *F. altaica* life cycle at which biotic and abiotic factors can intervene to promote or neutralize *Neotyphodium* sp. infection.

Seed production is a crucial step in the maintenance of endophyte / grass associations. A change in the seed production of the host is the most direct way in which *Neotyphodium* spp. can affect their own fitness, but documented effects of endophyte on host seed production have been highly variable (Cheplick and Faeth 2009). Alpine and arctic grasses such as *F. altaica* often demonstrate lagged reproduction, where bud formation occurs one or more growing seasons before the development of the culm; this is thought to be an adaptation to short and unpredictable growing seasons (Arft et al. 1999). Over the course of my project, I attempted to assess reproductive effort of experimental tussocks, but examination of sampled culms revealed an extremely low number of developed seeds. Growing seasons between 2005-2008 having been particularly cold and wet, it is very likely that seed production in *F. altaica* was delayed until a more favourable growing season, and I was therefore unable to decipher the effect of endophyte on seed production. Another important mechanism occurring at this stage of the host life cycle, and which could help explain the observed infection frequencies on boulder fields and meadows, is the efficiency of fungus transmission to the seed. According to Ravel et al. (1997), fungus failure in only 10% of seeds would be enough to maintain an intermediate infection frequency in a host population, even if the symbiotum was strictly mutualistic. In a naturally infected *L. rigidum* population, Canals et al. (2008) found that only 68% of seeds produced by an infected individual were themselves infected. In Argentina, Gundel et al (2009) found that *L. multiflorum* (Italian ryegrass) had highly variable endophyte transmission rate, particularly in humid prairies whereas *L. arundinaceum* had consistently high transmission success. Establishing the rate of endophyte transmission success to *F. altaica* seeds is a key step in untangling the factors behind the infection gradient under study.

Endophyte hyphae within seeds can also fail during seed dormancy without the seed being affected. Many endophyte infection control methods include storage in either humid, cold or dry conditions in order to kill the fungus before germination (Canals et al. 2008, Clement et al. 2005). Environmental conditions at my study site can be very severe, particularly during the short period before snow creates an insulative layer, or during snowmelt when freeze-thaw events occur daily. Extreme cold, or high humidity during snowmelt could affect *F. altaica* seeds differently depending on whether they are on well-drained, but exposed boulder fields, or sheltered and water-logged meadows. Future research at alpine sites in the Yukon should include a litterbag experiment to assess fungus overwinter survival.

While effects of endophyte infection on seed germination rate remain uncertain, with studies finding no effect (Gundel et al. 2006a), or effects dependent on host species, genotype, and environmental conditions (Gundel et al. 2006b, Wali et al. 2009), hyphal failure within the seedling remains possible. In tall fescue seedlings grown from E+ *L. arundinaceum* seeds, Hill and Brown (2000) found between 73 and 83% uninfected seedlings. Drawing from results from seven grass species, Afkhami and Rudgers (2008) showed that *Neotyphodium* spp. infection could potentially be lost at every life cycle stage; therefore, concentrating on adult plants and the factors that affect them may provide only a biased idea of the true interaction existing between *Neotyphodium* spp. and their host, and future research aiming to understand the factors driving intermediate infection frequencies in natural systems should take every lifecycle stage into consideration.

Conclusions

I found that grazing history did not appear to control *Neotyphodium* sp. infection frequency in the *F. altaica* population, despite strong spatial correlation. Furthermore, *Neotyphodium* sp. infection on boulder field appeared parasitic, a result that contradicts the long-held assumption that vertically-transmitted endosymbionts must be neutral or mutualistic to persist within a host population. In meadows, *Neotyphodium* sp. appeared as either neutral or slightly beneficial to *F. altaica*, which is consistent with my predictions, although this pattern was unrelated to nutrient availability. I suggest that other significant sources of stress may be mediating plant/fungus relations in these areas, such as water availability or competition.

Most importantly, my results showed that treating endophyte infection frequency as an indicator of mutualism may be misleading, particularly in complex natural systems where little is known about the range of possible interactions between the two symbionts. Following reviews and meta-analysis which criticized the lack of evidence from natural systems (Saikkonen et al. 2006), many researchers have now refocused their efforts on wild grass populations. However few have followed up their initial surveys with *in situ* experimental evidence, and those who have often found results putting into question the widespread assumption that strictly vertically-transmitted symbionts must be beneficial to their host to persist in a host population. In my study system, the use of infection frequency as a proxy for the fitness increase in host grass did not appear to reflect the true effect of endophyte infection on *F. altaica*, and like Gundel et al. (2008), I recommend researchers be cautious about this assumption without the support of plant performance indices such as reproductive effort or overall productivity.

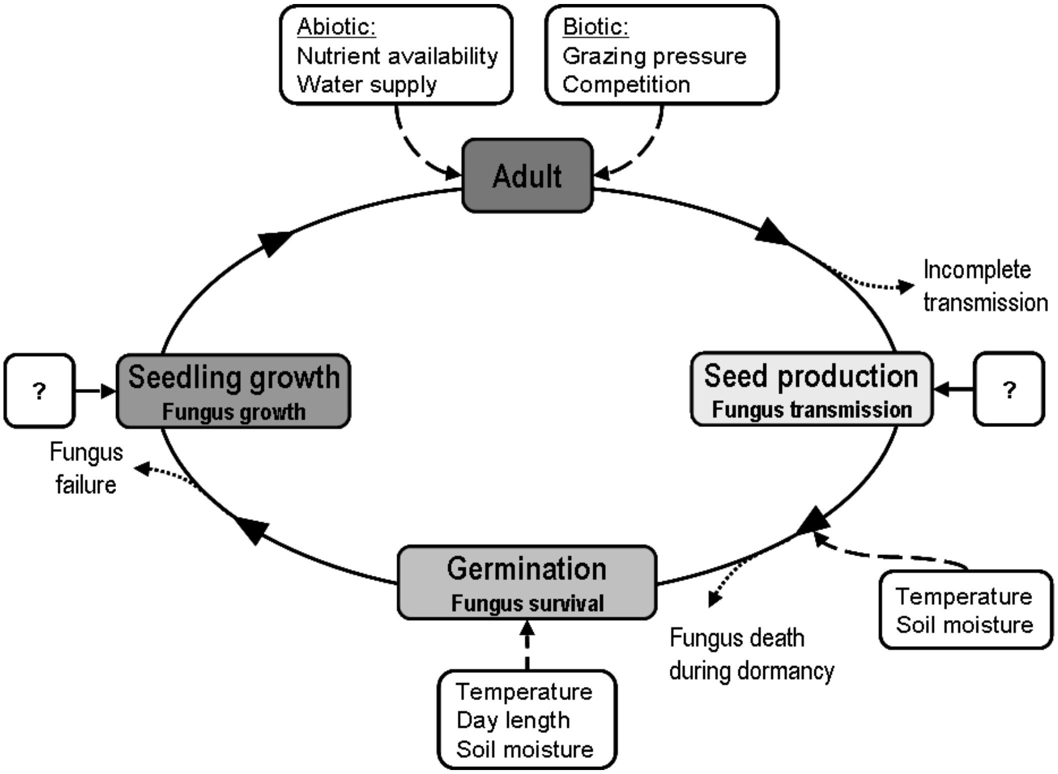

Figure 4-1 Simplified *Festuca altaica* life cycle highlighting significant stages for *Neotyphodium* sp. persistence in the host lineage and the biotic and abiotic factors which can regulate it.

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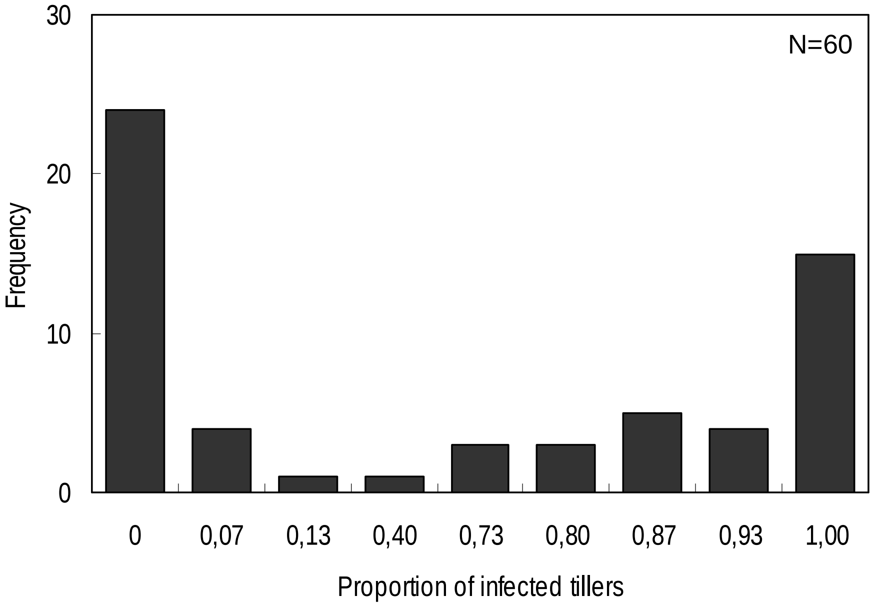
APPENDICES

A-1 Initial screening and experimental tussock selection

In the summer of 2007, clippings from the youngest leaf of one tiller were sampled for 1000 *F. altaica* tussocks. These tussocks were evenly divided between my two experimental sites, on west and east-facing slopes, respectively. At each site, the sampled tussocks were equally distributed between two levels of grazing history: boulder field and meadow, for a total of 250 sampled tussocks per site/grazing history combination.

One clipping per tussock was analysed using a Phytoscreen Test (Agrinostics, Watkinsville, GA, USA), a commercial western blot developed to detect monoclonal antibodies specific to *Neotyphodium* spp. I determined tussock infection status based on the presence or absence of the red/pink signal denoting the presence of fungal proteins. In a few cases (<5), immunoblot signal interpretation was not conclusive; these samples were excluded from the pool and therefore were not considered during treatment allocation.

In order to verify the accuracy of my sampling scheme, I also collected samples from a separate set of *F. altaica* tussocks. Stem clippings from 15 tillers were sampled from 15 tussocks at each site/grazing history combination (N=60). The frequency graph below shows the proportion of infected tillers within the 15 tiller sample; had infection been completely consistent within a single tussock, I would have expected to see tussocks with infections frequencies of either 0 or 1. The graph shows that while infection frequency was not completely consistent in all sampled tussocks, the distribution was highly skewed towards 0 and 1, therefore, I was reasonably confident that my judgement of infection status based on a single tiller was accurate in the great majority of tussocks. It is possible that partially-infected tussocks may have been assigned the wrong infection status, and this may have introduced a certain degree of noise in my data.



Chemical treatments were assigned to experimental tussocks before green-up in the summer of 2008 (10-11 June). A list of random numbers ranging from 1 to 4 and representing each combination of chemical treatments (water, fertilizer, fungicide, fertilizer and fungicide) were generated using MS EXCEL 2003 (Microsoft Corporation, Redmond, WA, USA). While one experimenter picked out a tussock of known infection status, the second followed the list and dictated treatment assignment. When a risk of contamination by slope run-off was perceived by the second experimenter (for example if the selected tussock was immediately downslope of a tussock previously assigned to fertilizer and the treatment called for was water), the first was asked to pick another tussock and the random list of treatment was followed. This protocol was followed at each site/grazing history combination until 34 tussocks had been assigned to each treatment with equal representation of E+ and E- tussocks.

Final experimental sample size (N=544) was limited by the number of E+ tussocks in meadows, as well as my ability to monitor all tussocks within a reasonably short amount of time; my target was 1 week. Tussocks selected for the experiment at each grazing history level covered a wide range of sizes (shown below).

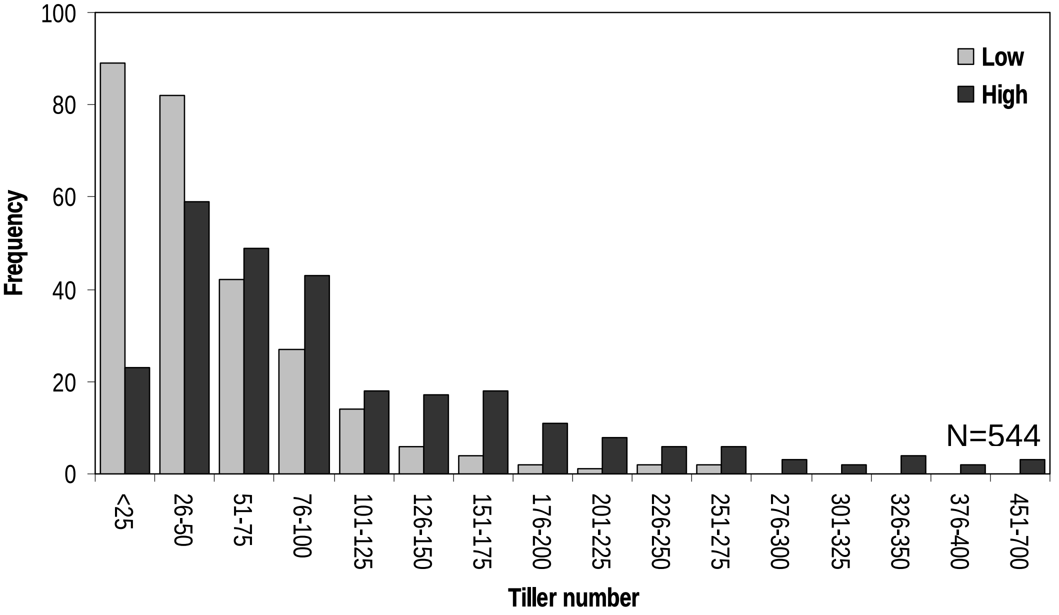

100
80
60
40
20
0
Frequency
Low
High
N=544
<25
26-50
51-75
76-100
101-125
126-150
151-175
176-200
201-225
226-250
251-275
276-300
301-325
326-350
376-400
451-700
Tiller number

Table A-2 Summary of sample sizes

Herbivory	ON								FAR							
Infection	E-				E+				E-				E+			
Fertilizer	Ambient		NPK		Ambient		NPK		Ambient		NPK		Ambient		NPK	
Fungicide	Control	Fung	Control	Fung	Control	Fung	Control	Fung	Control	Fung	Control	Fung	Control	Fung	Control	Fung
Morphological measurements																
Tiller number	188	-	-	-	305	-	-	-	357	-	-	-	141	-	-	-
Tussock area																
Leaf demography																
Birth	101	100	98	98	95	103	98	99	102	102	100	102	101	99	102	96
Death																
Green length	96	98	98	99	102	100	96	94	100	99	102	94	102	99	101	101
Senescence rate	96	93	92	90	90	96	93	94	100	99	100	100	96	97	102	87
Immunoblot signal	5	4	5	4	29	32	33	30	2	1	0	1	33	32	33	33
PRS-probes	12	-	-	-	12	-	-	-	12	-	-	-	12	-	-	-
Vegetative reproduction	101	99	98	96	95	96	96	94	102	102	100	102	100	99	102	96

Table A-3a Type III tests of fixed effects obtained by a non-parametric 2-way ANOVA to test the impact of *Neotyphodium* sp. infection, grazing history and their interaction on *F. altaica* tussock area and the number of tillers within a tussock. Asterisks indicate significant p-values (α=0.05).

Effect	Nominator d.f.	Denominator d.f.	F-value	Pr > F	
Tiller number					
Grazing history (G)	1	987	7657.75	<0.0001	✳
Infection (I)	1	987	119.8	<0.0001	✳
G x I	1	987	210.03	<0.0001	✳
Tussock area					
Grazing history (G)	1	987	9922.90	<0.0001	✳
Infection (I)	1	987	159.51	<0.0001	✳
G x I	1	987	246.52	<0.0001	✳

Table A-3b *Post-hoc* t-test comparisons and difference estimates for fixed effects having tested significant in a 2-way non-parametric ANOVA testing the impact of *Neotyphodium* sp. infection, grazing history and their interaction on *F. altaica* tussock area and tiller number within tussock. The second element of the comparison is the -1 element. Asterisks denote significant p-values (α=0.05). (G) denotes grazing history and (Fe) indicates fertilization treatment.

	Denominator d.f.	t-value	Pr>|t|		Mean difference	95% interval	
						-	+
Tiller number							
High vs Low	987	87.51	<.0001	✳	2.01	0.031	0.032
E- vs E+	987	10.95	<.0001	✳	1.09	0.017	0.017
at G=High	987	23.27	<.0001	✳	1.23	0.021	0.021
at G=Low	987	-2.12	0.0344	✳	0.97	0.025	0.026
Tussock area							
High vs Low	987	99.61	<.0001	✳	2.0401	0.028	0.028
E- vs E+	987	12.63	<.0001	✳	1.0946	0.015	0.015
at G=High	987	26.05	<.0001	✳	1.2248	0.018	0.018
at G=Low	987	-1.83	0.0676		0.9783	0.023	0.023

Table A-4 *Post-hoc* t-test comparisons and difference estimates for fixed effects having tested significant in a 4-way ANOVA testing the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the hyphal density of *Neotyphodium* sp. in stem clippings of *F. altaica*, as inversely indicated by the green value of a western immunoblot signal. The second element of the comparison is the -1 element. Asterisks denote significant p-values ($\alpha=0.05$). (Fe) indicates fertilizer treatment, (Fu), is fungicide treatment, (I), infection status and (G), grazing history.

Effect	Denominator d.f.	t-value	Pr>|t|		mean difference	95% CI	
						-	+
E- vs E+							
at Fe=ambient							
at Fu=control	260	5.46	<.0001	✳	1.28	0.11	0.12
Control vs fungicide							
at I=E+	260	16.47	<.0001	✳	1.238	0.031	0.032
Ambient vs +NPK							
at I=E+	260	-1.92	0.0555		0.975	0.025	0.025
Low vs High							
at I=E+	260	-1.37	0.1731		0.982	0.025	0.025
at Fe=ambient	260	1.17	0.2423		0.978	0.035	0.038
at Fe=+NPK	260	-3.13	0.0019	✳	1.058	0.033	0.035

Table A-5 *Post-hoc* t-test comparisons and difference estimates for fixed effects having tested significant in a 4-way ANOVA testing the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the leaf demographics of experimental *F. altaica* tillers. Asterisks denote significant p-values (α=0.05). The second element of each comparison is the -1 element. (Fe) indicates fertilization treatment and (G) is grazing history.

	Denominator d.f.	t-value	Pr>|t|		mean difference	95% CI	
						-	+
Leaf birth							
+NPK vs ambient	17.42	8.16	<.0001	✳	1.81	0.26	0.30
Control vs fungicide	17.42	2.79	0.0125	✳	1.23	0.17	0.20
at Fe=Ambient	23.04	0.35	0.7281		1.04	0.21	0.27
at Fe=+NPK	12.57	3.86	0.0021	✳	1.44	0.27	0.33
Leaf death							
Ambient vs +NPK	1580	6.82	<.0001	✳	1.40	0.13	0.14
at G=High	1580	6.06	<.0001	✳	1.57	0.21	0.24
at G=Low	1580	3.49	0.0005	✳	1.27	0.16	0.18
Control vs fungicide	1580	1.72	0.0861		1.09	0.10	0.11
Low vs High	1580	2.21	0.0274	✳	1.12	0.10	0.11

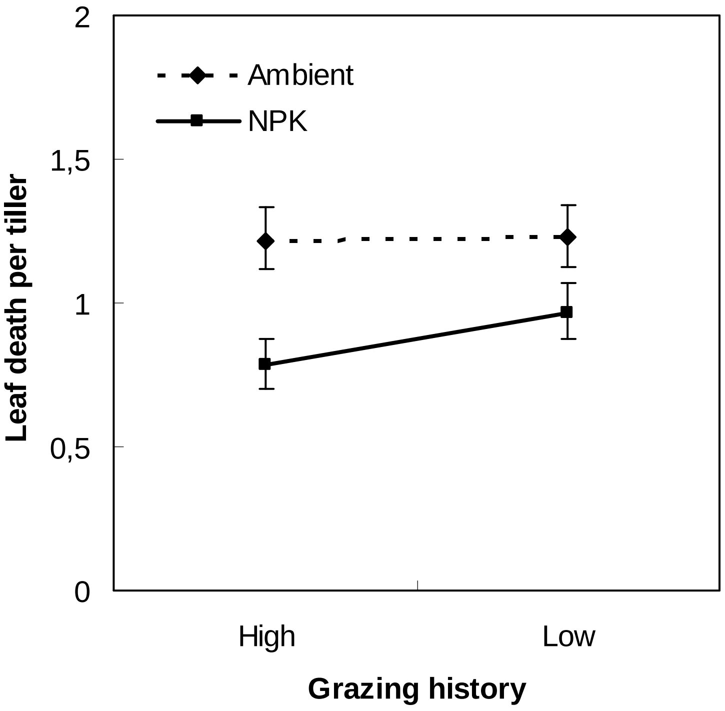


Figure A-6 Mean leaf death per tiller in *F. altaica* tillers treated with NPK fertilizer or exposed to ambient nutrient levels and located in areas subjected to high and low grazing history (High: $N_{ambient}$=399 N_{+NPK}=393, Low: $N_{ambient}$=404 N_{+NPK}=400). Error bars represent 95% confidence intervals.

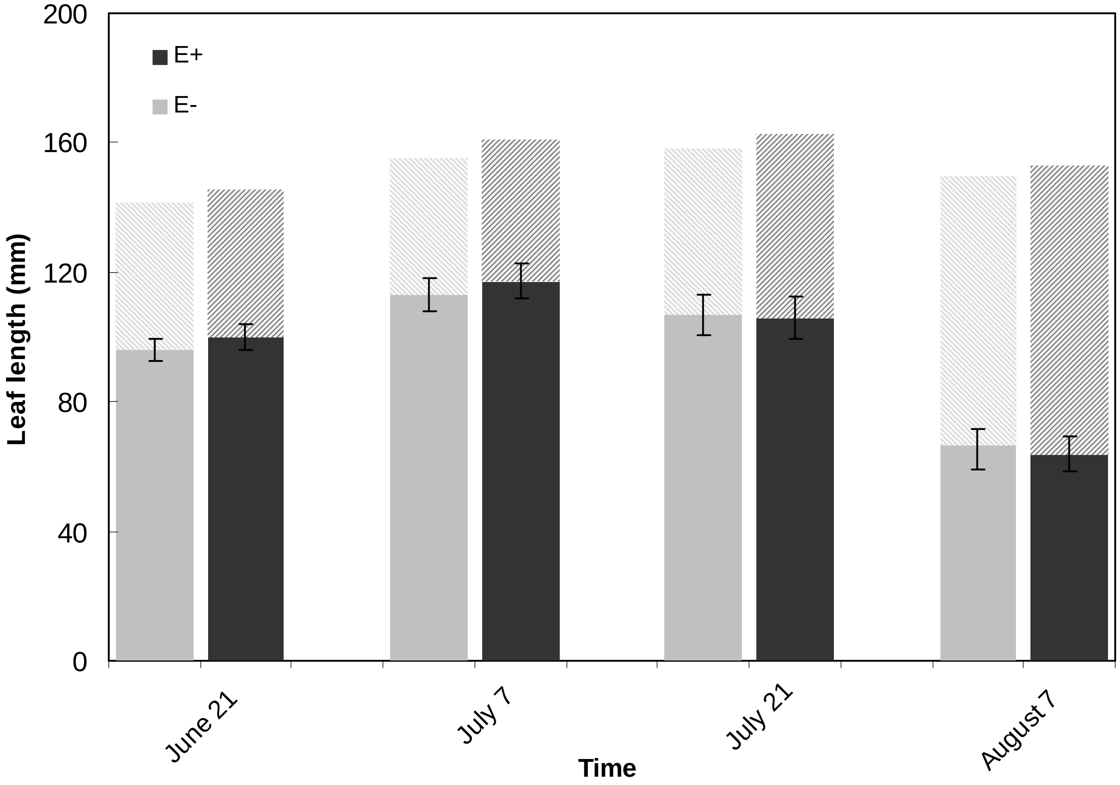


Figure A-7 Average green length (solid) and total length (stripes) of both E+ and E- untreated *F. altaica* tussocks throughout the growing season, pooled across areas of high and low grazing history (E-: N_{june7}=204 N_{july7}=203 N_{july21}=204 $N_{August7}$=202 , E+: N_{june7}= 200 N_{july7}=194 N_{july21}=196 $N_{August7}$=193). Error bars represent 95% confidence intervals of green length values.

Table A-8a Type III tests of fixed effects obtained by a non-parametric 4-way ANOVA to test the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the average green length of leaves within experimental *F. altaica* tillers. Asterisks denote significant p-values (α=0.05).

Effect	Numerator d.f.	Denominator d.f.	F-value	Pr > F	
Fertilizer (Fe)	1	506.1	37.42	<.0001	✳
Fungicide (Fu)	1	505.5	1.11	0.2932	
Fe x Fu	1	505.7	1.33	0.2502	
Infection (I)	1	505.5	0.27	0.6061	
Fe x I	1	505.5	0.18	0.6743	
Fu x I	1	505.8	0	0.9795	
Fe x Fu x I	1	505.6	0.24	0.6252	
Grazing history (G)	1	505.7	8.49	0.0037	✳
Fe x G	1	506.5	0.42	0.5193	
Fu x G	1	505.5	0.42	0.5162	
Fe x Fu x G	1	505.5	0.02	0.8836	
I x G	1	505.5	1.19	0.2752	
Fe x I x G	1	505.6	0.78	0.3764	
Fu x I x G	1	505.7	0.93	0.3352	
Fe x Fu x I x G	1	505.7	5.72	0.0171	✳

Table A-8b *Post-hoc* t-test comparisons and difference estimates for fixed effects having tested significant in a 4-way non-parametric ANOVA testing the impact of fertilization, fungicide, *Neotyphodium* sp. infection, grazing history and all possible interactions on the average green length of leaves within experimental *F. altaica* tillers. The second element of the comparison is the -1 element. Asterisks denote significant p-values (α=0.05). (Fe) indicates fertilization treatment, (Fu) is fungicide and (I) indicates infection status.

Effect	Denominator d.f.	t-value	Pr>|t|		mean difference	95% CI	
						-	+
+NPK vs ambient	521	8.88	<.0001	✳	15.33	3.39	3.39
High vs Low	521	-2.61	0.0092	✳	4.51	3.39	3.39
at Fe=ambient							
at Fu=control							
at I=E-	523	1.86	0.0636		9.06	9.57	9.57
at I=E+	531	-0.95	0.3428		-4.68	9.69	9.69
at Fu=fungicide							
at I=E-	528	0.82	0.4127		4.01	9.60	9.60
at I=E+	528	1.22	0.2233		6.00	9.67	9.67
at Fe=+NPK							
at Fu=control							
at I=E-	509	0.36	0.718		1.75	9.52	9.52
at I=E+	496	1.33	0.1851		6.38	9.45	9.45
at Fu=fungicide							
at I=E-	519	1.3	0.1932		6.34	9.57	9.57
at I=E+	534	1.47	0.142		7.21	9.64	9.64

Table A-9 Type III tests of fixed effects obtained by a non-parametric 3-way ANOVA to test the effect of location (larger scale, ~60m, by proxy: grazing history) and tussock-level variations (smaller scale, ~1-20m, by proxy: infection) and any interaction between the two on soil nutrient availability as detected by Plant Root Simulator probes (Western AG Innovations, Saskatoon, Canada). Nutrient identity was included as a repeated measure element, and was therefore included in the fixed effects. Asterisks denote significant p-values ($\alpha=0.05$).

Effect	Numerator d.f.	Denominator d.f.	F-value	Pr > F	
Grazing history (G)	1	6.85	0.47	0.5161	
Infection (I)	1	6.85	0.00	0.9554	
I x G	1	6.85	1.21	0.3077	
Nutrient (N)	8	38	2134.09	<.0001	✳
G x N	8	38	3.31	0.0058	✳
I x N	8	38	0.69	0.6944	
G x I x N	8	38	3.02	0.0100	✳