Above-ground herbivory causes rapid and sustained changes in mycorrhizal colonization of grasses

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Abstract

This study investigated the effects of grazing by rabbit and insect herbivores on root-colonization of grasses by arbuscular mycorrhizal fungi (AMF) in two lowland grasslands in southern England, UK. A temporal assessment from grazing exclosures was also made. Root samples from three grass species at each site were analyzed in terms of total mycorrhizal colonization and proportional colonization by individual mycorrhizal structures. Colonization was increased by moderate levels of rabbit grazing at both sites. The change was fast, consistent throughout the sampled field plots, and temporally sustainable. There was no significant effect of insect herbivory on total colonization but proportional colonization by different AM structures was affected on some sample dates where vertebrate herbivores had been removed, indicating a herbivore-dependent effect on the degree of benefit within the plant-fungal partnership. The results suggest that the type of herbivore and defoliation intensity to which plants are subjected are key determinants of below-ground effects upon mycorrhizal-host plant symbiosis. This information has strong implications regarding restoration and management of grassland ecosystems.

Keywords arbuscular mycorrhizal fungi, grassland, grazing, insect, rabbit.

Introduction

Grasslands (and interactions therein) are an important focus of ecological studies since they comprise a large proportion of terrestrial global ecosystems. Temperate grasslands comprise about 32% of the natural vegetation on earth and typically have a long history of co-evolution with grazing herbivores (McNaughton 1984). There is also an increasing awareness that grazing impacts directly and indirectly upon below-ground soil floral and faunal populations and food webs (e.g. Bardgett et al. 2001).

39 The European Rabbit (*Oryctolagus cuniculus* L.) has been a resident in Britain since it was
40 introduced by the Normans in the 12th Century but has experienced major population
41 fluctuations since then, with the British population numbering approximately 37.5 million in
42 1999 (Toms et al. 1999). Densities in British grasslands can vary from 0 to 100 per hectare
43 but are usually in the range 0.5 to 15 individuals per hectare (Corbet and Harris 1991). The
44 impact of rabbits is varied and seems to fall along a continuum from beneficial to detrimental,
45 dependent on habitat (grasslands versus agricultural land) and grazing intensity. They are
46 thought to have played a significant role in the shaping of the British countryside over the
47 centuries since their introduction and are considered a keystone species in the maintenance of
48 grasslands in southern England (Crawley 2004). Despite this, there has been little
49 investigation of their impacts upon below-ground parameters, contrasting to the number of
50 studies involving larger agricultural grazers such as sheep and cattle (e.g. Bardgett et al.
51 2001). Above-ground phytophagous insects represent a lower total herbivore biomass than
52 vertebrates and generally remove smaller quantities of vegetation. However, as a group they
53 are much more diverse in their modes of feeding and degree of specialism. Invertebrates can
54 influence a wide range of aspects of ecosystem functioning; for example affecting succession
55 (Brown and Gange 1999), and changing root exudation, phytomass, and activity of
56 decomposer organisms (Wardle and Bardgett 2004).
57 Rabbits and insects can have important and often contrasting impacts on plant community
58 structure and succession (e.g. Edwards et al. 2000) so it might be expected that their effects
59 on AM fungi may also differ. Insect exclusion effects take longer to become apparent (Brown
60 and Gange 1999), thus effects on AMF fungi might also take longer. However, to date,
61 experiments have only involved either invertebrate or vertebrate herbivore impacts on
62 mycorrhizas. In most grasslands, herbivory of both types is common and thus in order to

63 understand herbivore effects on multi-level interactions and ecosystem processes we must
64 consider both types of herbivory simultaneously.

65 Arbuscular mycorrhizal fungi (AMF) form a dominant component of rhizosphere
66 microfloras and can constitute up to 25% of the total microbial biomass and they are present
67 in almost all natural and semi-natural grasslands (Read et al. 1976), with significant
68 ecological importance at the level of the individual, community and ecosystem. AM fungi
69 confer a wide range of benefits upon plant hosts, including increasing the nutrient absorptive
70 capacity of plant root systems (Marschner and Dell 1994), acquisition of otherwise
71 unavailable nutrient forms (e.g. Jolicoer et al. 2002), drought resistance (e.g. Gemma et al.
72 1997), enhancements of plant growth and vigour (e.g. Koide and Lu 1992) and suppression of
73 pathogens (Newsham et al. 1995). There are also a variety of differing effects upon
74 invertebrate phytophages (Gange 2006). Mycorrhizas have also been found to be responsible
75 for determining plant biodiversity and community structure (van der Heijden et al. 1998).

76 It has often been reported in the literature that defoliation reduces root colonization by
77 mycorrhizal fungi (Gehring and Whitham 1994; Gange et al. 2002). The mechanism is
78 thought to be one of carbon limitation in the host plant (Gehring and Whitham 2002).
79 However, there is some evidence that this may not always be the case (e.g. Eom et al. 2001;
80 Kula et al. 2005; Mikola et al. 2005). In some circumstances defoliation may have no effect,
81 or even increase the degree of colonization by these fungi. Published results to date are
82 therefore, extremely varied. If carbon limitation below-ground (largely through root
83 exudation) is caused by reduced photosynthetic biomass aboveground under intense
84 herbivory, it is possible that where herbivory is at low to moderate levels it could be
85 beneficial to plant-microbial interactions through enhanced root exudation. For example,
86 Bardgett et al. (2001) found that soil microbial biomass peaked under light grazing and was
87 lower in soil from which the sward had been ungrazed or intensely grazed by sheep. It is

possible that a curvilinear response to grazing intensity exists with plant-mycorrhizal interactions such as that proposed for primary productivity (Dyer et al. 1982).

This study was carried out to ascertain whether mycorrhizal colonization within roots of grasses in two typical lowland UK grasslands was reduced, unaffected, or increased by above-ground herbivory. Furthermore, we quantified effects over time to see whether responses were transient and recorded mycorrhizal presence in terms of the types of structures encountered in order to assess whether herbivory caused a change from mutualism to parasitism along the mycorrhizal benefit continuum (Gange and Ayres 1999). We report herbivore effects on mycorrhizas using two exclosure experiments. One excluded each herbivore in a factorial design, while the second used a long-term rabbit grazing removal field site where consistency of trends could be investigated further. If removal or manipulation of levels of grazers enhances plant-AM association as has been found in some other ecosystems (Gehring and Whitham 2002) then this may provide a beneficial management strategy for increasing the ability of grasslands to tolerate drought stress with climate change, and other myco-induced alleviation such as enhanced pathogen resistance, that AM fungi can confer (REF). This work has important implications for management of natural and semi-natural grasslands since most current management strategies focus solely upon above-ground controls and response parameters without considering below-ground microbial associations. Maintenance of a positive AM-plant association in field situations is key to plant community functioning (Jeffries et al. 2003). Thus, elucidating impacts upon this below-ground interaction is essential to ecosystem conservation and restoration.

Materials and Methods

Multi-herbivore exclusion experiment

113 The field site used for the combined rabbit and insect manipulation experiment was located at
114 Royal Holloway, University of London (RHUL) in Egham, Surrey, UK. The site experienced
115 an annual rainfall of approximately 635-653mm and was a typical temperate climate old field
116 lowland grassland on a nutrient poor acidic sandy loam soil, with a soil organic matter content
117 of approximately 6-7% and average bicarbonate extractable phosphate content of 20 mg P kg^{-1}
118 and nitrogen content of 4 mg NO_3^- kg^{-1} (Wearn 2006). At RHUL there were approximately
119 5-7 rabbits ha^{-1} with approximately 20-35 herbivorous insects (of all taxa) m^{-2} recorded from
120 vortis suction sampling. Herbivorous insects were dominated by hemiptera, sminthurid
121 collembola and orthoptera (during summer), with much lower representation by lepidopteran
122 larvae and molluscs.
123 The field had been managed every year prior to the commencement of the study by
124 mowing to an even sward height of 30-40mm during spring and summer (in addition to the
125 constant rabbit presence). However, once the study had begun the experimental area
126 experienced no further anthropogenic management. The plant community before treatments
127 were started was a relatively homogeneous and fairly species-poor grassland. The sward was
128 dominated by two perennial grass species, *Anthoxanthum odoratum* (L.) and *Holcus lanatus*
129 (L.) with smaller patches of *Agrostis tenuis* (Sibth.) [= *A. capillaris* (L.)]. Forbs were at much
130 lower densities and as such were not included in this study since they were not present in
131 sufficient numbers of subplots to obtain analyzable replicate data.
132 Field manipulations of both rabbits and insects were established in April 2003. The
133 experiment consisted of four different treatments; exclusion of insects and/or rabbits, plus a
134 control with all herbivores present. A randomized block design was set up, containing six
135 replicates of each treatment. Each treatment plot measured 2 m x 2 m. Rabbits were
136 excluded with 2.5 cm wire mesh fences, extending 0.3 m below ground and 1m above.
137 Insects were excluded by applications of imidacloprid (marketed as Provado Ultimate Bug

138 Killer (Pbi, Cambridge) every four weeks. This was used at the label rate of 15 ml
139 concentrate in 1 l water. Imidacloprid has been proven as an effective systemic control of a
140 range of invertebrate phytophages (Mullins 1993) and its toxicity to vertebrates is low (Anon.
141 2006) so non-target effects on the rabbit population also grazing in the same sward were
142 negligible. The insecticide was translocated acropetally within the plant so light spray
143 application was used to avoid insecticide contact with the soil (which would be caused by
144 drenching) and ensured the chemical remained in the above-ground plant tissues. Non-target
145 fertilization affects of the insecticide upon mycorrhizal colonization were assessed in a
146 controlled trial and no significant impacts were found, neither were any effects on plant
147 community observed in the field. Efficacy of the insecticide was assessed throughout the
148 experiment by suction sampling of aboveground invertebrates in all treatments.
149 Sampling was carried out every 6-8 weeks from April 2003 to January 2005. Cores were
150 removed from the ground with a 4.5 cm diameter corer to a depth of 10 cm. Core removal
151 was randomized within each sub-plot, although mole hills, where present, were avoided.
152 Holes left by core removal were filled with sand. This was to prevent the soil from drying
153 out, and washed sand was used instead of soil from nearby, to minimize the extent to which
154 non-native organisms were introduced by back-filling. A pre-treatment sample was taken in
155 April 2003, just prior to the first insecticide application and fence erection, which provided
156 baseline data for the study. Above-ground plant biomass estimates were made by drying live
157 material taken from each core by heating to 70^{o}C for 48 hours. Root biomass was assessed by
158 extraction of clean roots from soil particles (Kelly 1975), dried as before and weighed.
159 An equal subsample of each root system was used for mycorrhizal quantification. Roots
160 from each grass species were carefully removed with forceps, rinsed with water and placed
161 into 5 ml wells in repli-dishes. Roots were only taken from the bases of identified plants
162 rather than trying to identify unattached roots by morphology. Only the younger roots were

selected since mycorrhizas could only be observed in the actively growing roots due to short lifespan of some structures, especially arbuscules.

The Quink™ root-staining method was used, based on that devised by Vierheilig et al. (1998) with modifications. Roots were cleared in 5% (w/v) KOH at room temperature and left overnight. They were then washed with tap water and transferred to 1% HCl for 15 minutes to ensure they were adequately acidified for staining. Acidified roots were transferred to staining solution (a mixture of water:HCl:Quink™ in the ratio 95:5:1) and left for an hour. Quink™ permanent blue was used as it gave the clearest results. After an hour all roots were transferred to destaining solution (glycerol:water:1%HCl in the ratio 70:23:1). Again they were left overnight, to allow excess stain to leach out of the roots.

Quantification of mycorrhizas was undertaken using the magnified intersection method of McGonigle et al. (1990). The slide was scanned methodically and counts of percentage root length colonized (%RLC) were carried out. Presence or absence of mycorrhiza was scored each time a root was crossed by a cross hair axis. In addition to a total percentage figure for mycorrhizal colonization, the types of structure encountered (vesicle/internal spore (V&S), arbuscule (A), or intraradical hypha (H)) were recorded as these can yield vital information about the state of the symbiosis occurring (Klironomos et al. 2004). Values for these structures were converted to a proportion of the total mycorrhizal presence within the root system for ease of comparison of the symbiosis across data sets. At least 100 intersections per slide were recorded to ensure accuracy of the data yielded.

Long-term grazing exclusion experiment

To examine temporal effects of rabbit exclusion, long term plots were sampled at Silwood Park near Ascot, Berkshire, in southern England. Exclosures had been set up at Silwood over a longer period of time than at RHUL, ranging from 1 year to 19 years of age. This allowed

188 both long- and short-term effects of grazing exclusion to be assessed by sampling plots and
189 adjacent grazed areas over a temporal gradient. In addition, the youngest exclosures could be
190 directly compared with data from RHUL. At Silwood, rabbit populations were greater than
191 10 individuals ha^{-1}. The sampled Silwood field plots possessed similar swards to those at
192 RHUL, except *Holcus mollis* (L.) occurred instead of *H. lanatus*. Soil characteristics were
193 also equivalent (Edwards et al. 1999). Thus the two sites were ecologically similar, so that
194 comparisons could be made between them.

195 Exclosures at Silwood Park had been erected in a similar way to those at RHUL, excluding
196 rabbits but not smaller vertebrates or invertebrate phytophages (Edwards et al. 2000). Plots
197 sampled were 1, 13, 15, 16, and 19 years post exclusion. There were no suitable plots of
198 intermediate age where no fertilization or pesticide application had taken place. The newest
199 plots could be directly compared with the field site at RHUL, whilst the remainder gave an
200 indication of how the effects may change over time as succession took place in exclosures
201 relative to the rabbit-grazed grassland. Two samples were taken at Silwood, one in early July
202 2004 and the other in early December 2004. To aid quick identification of grass species for
203 the subsequent December sample when no easily recognizable inflorescences would be in
204 view, 1 m bamboo canes were pushed into the field soil adjacent to known species at the end
205 of the July sampling. Samples of the three grass species described above were removed from
206 exclosures and adjacent grazed grassland (randomly selected where abundant, semi-randomly
207 selected where only a few individuals were present) with a 4.5 cm x 10 cm corer. Cores were
208 also taken from four intensely grazed areas (with a sward height of 10-16 mm compared with
209 50-90 mm from moderately grazed grassland), adjacent to four of the sampled plots.
210 Mycorrhizal quantification followed the same method as stated for the RHUL root samples.
211
212 Data Analysis

All mycorrhizal data were transformed prior to statistical analysis using an arc sine transformation (Zar 1999). Repeated measures analysis of variance (ANOVA) was performed on biomass and colonization data from RHUL using the UNISTAT® statistical package. Data for each grass species were analyzed according to dates with rabbit and insect exclusion as main effects. Set-point dates were also individually analyzed (for proportional colonization) with separate ANOVAs to further elucidate insect effects. Because pairs of plots in each age group were sampled at Silwood, but plots of the same age were spatially separated (thus local environmental factors could have influenced data), a mixed within-subjects factorial design analysis of variance model (Keppel et al. 1980) was performed to elucidate the effects of grazing and plot age, and to identify if any interaction terms existed between the two.

Results

Efficacy of the insecticide was found to be high. Invertebrate phytophage densities in insecticide treatments were reduced by 94.75% (± 5.25%) from approximately 25 m^{-2} to almost zero. Thus, insect exclusion treatments were successful (Wearn 2006).

At RHUL, the initial sward was mown to approximately 30 mm prior to the study so foliar biomass was low (Fig. 1). Sward heights and biomass subsequently increased and varied seasonally but not between insect exclusions. Mean heights were 400-600 mm inside exclosures by July 2004 (some inflorescences of *H. lanatus* up to 900 mm) and 80-110 mm outside exclosure fences. The impact of rabbit herbivory by this time was dramatic ($p < 0.001$), causing reductions in foliar biomass compared with exclosures (Fig. 1). Root biomass was not found to be significantly reduced by grazing treatments relative to ungrazed controls ($p > 0.1$, data not displayed).

237 In all three grass species, total mycorrhizal colonization levels (Fig. 2a, b, c) were
238 consistently higher where the above-ground vegetation was grazed by rabbits, irrespective of
239 the presence or absence of insects (all $p < 0.001$). There were no significant effects of insect
240 exclusion on total colonization levels over the duration of the study (all $p > 0.3$). Fig. 2 shows
241 that the response of the AM fungal community was rapid (within eight weeks) following
242 removal of mowing and erection of exclosures. There was also a strong seasonal effect ($p <$
243 0.001) on total colonization patterns. Peaks (although not in the same month each year) were
244 in summer or early autumn, with winter and spring minima.
245 When proportions of AM structures within roots were considered (Fig. 3, 4, 5), for the
246 experimental duration as a whole there were no significant treatment effects ($p > 0.1$).
247 However, when set-point dates were individually analyzed, significant differences between
248 proportional representation by arbuscules (Fig. 3a, b, c) were found in September 2003,
249 January 2004, and April 2004 for *A. odoratum* and *A. tenuis* (all $p < 0.01$). These differences
250 occurred where insects alone were present. Proportional colonization of *H. lanatus* showed
251 significant differences only in the last two of these dates (both $p < 0.01$) with significantly
252 lower arbuscular proportions where only insect herbivores were present above-ground.
253 Proportions of vesicles and spores (Fig. 4a, b, c) were correspondingly greater in '- rabbits +
254 insects' treatment plots in January 2004 for all three grasses (*A. odoratum* and *H. lanatus* $p <$
255 0.01, *A. tenuis* $p < 0.001$) and in April 2004 for *A. tenuis* ($p < 0.01$). Proportions of hyphae
256 (Fig. 5a, b, c) showed much more similar levels within roots between treatments. Overall at
257 RHUL, *H. lanatus* appeared to demonstrate the least response to above-ground insect attack,
258 whilst *A. tenuis* showed the most.
259 At Silwood, moderately grazed sward heights averaged between 50-90 mm (mean 73 mm).
260 Unfortunately *A. odoratum* was absent from the 1 year exclosures. There was no significant
261 effect of exclosure age on colonization of *A. odoratum*, *A. tenuis*, or *H. mollis* in either sample

(Table 1). There was however, a highly significant positive effect of grazing on mycorrhizal colonization of two out of three grass species at each sampling time (Table 1). A greater colonization of grazed samples relative to adjacent ungrazed exclosures existed in the majority of soil samples. Although shown as non-significant in Table 1a, the July data for *H. mollis* did show a statistically weak positive effect of grazing ($p = 0.1$). Overall, mycorrhizal colonization of *A. odoratum* showed the most consistent positive effect of rabbit grazing. There were no significant interaction terms between grazing and plot age.

Samples taken from intensely grazed areas at Silwood had a sward height of between 10 mm and 16 mm whilst ungrazed swards were approximately 450-500 mm. Mean colonization levels for the intensely grazed sward were used for analysis, as all species in the intensely grazed sward areas showed very similar levels of colonization. Plots of differing ages could be directly compared to adjacent grazed areas since it had been established that there was no significant effect of exclosure age on colonization patterns. Two distinct response types could be identified in mycorrhizal samples from high grazing intensities. In the first response type there was a lower total AM colonization under intense grazing than where moderate grazing occurred ($p < 0.001$), with no significant change in proportional representation of internal mycorrhizal structures in the roots at any grazing intensity ($p > 0.8$). Thus, in this scenario total but not proportional colonization appeared to be grazing intensity-dependent. In the second response type no significant difference in colonization between highly and moderately grazed swards occurred ($p = 0.9$), but there was a higher proportional representation by hyphae ($p < 0.05$) and lower representation by arbuscules ($p < 0.01$) within roots at the high grazing intensity.

Discussion

286 It is clear that rabbits have dramatic effects on AM colonization in the field while
287 invertebrates have considerably less effect. Moderate grazing by rabbits had a rapid and
288 persistent positive effect on mycorrhizal colonization of roots of the three grasses studied.
289 Furthermore, proportional colonization by different mycorrhizal structures was altered by
290 invertebrate grazing where vertebrate herbivores had been removed, indicating effects upon
291 the form of the symbiosis that were dependent on the type of herbivore.

292 In the RHUL field site at the onset of the experiment, Holland and Detling's (1990) theory
293 of carbon limitation seemed to hold true, which was that removal of nearly 90% of the above-
294 ground foliage from the sward by a combination of both grazing and mowing resulted in so
295 much loss of biomass that carbon-limitation below-ground came into play. At such high
296 intensity defoliation the majority of carbon resources were likely to have been allocated to the
297 shoots for regrowth. Subsequently, AM colonization was low because of the low availability
298 of photosynthate below-ground to stimulate and maintain the symbiosis with the host plants.
299 After the release from mowing, the degree of mycorrhizal colonization of both the grazed and
300 ungrazed plots showed a significant increase, though the increase was always greater in
301 grazed plots. By the eighth week of the RHUL field experiment, colonization levels in rabbit
302 grazed plots were already approximately 30% greater than in excluded plots, indicating that
303 the AM response was rapid. After less than three months, rabbit grazed plots had a foliar
304 biomass of approximately 35-40% less than the ungrazed sward, by which time AM
305 colonization levels were around 40% higher. This trend remained for the duration of the
306 experiment with seasonal fluctuations in all treatments. Moreover, colonization levels in
307 rabbit grazed treatments were not increased simply by a reduction in root mass as no
308 significant differences in root biomass between treatments was found (Wearn 2006).

309 The fertility of the soil may have been an important factor influencing the effects of
310 herbivory on AM colonization. Although factors limiting photosynthesis above-ground

311 generally cause an increased proportion of total assimilate allocated to shoots (for regrowth),
312 if factors affecting the below-ground uptake are in short supply (such as N and P in the sandy
313 loam soil at RHUL) an increased proportion of total assimilate is translocated to the roots
314 (Estes et al. 1982). Thus, if both above-ground (AG) and below-ground (BG) stresses occur
315 simultaneously there must be a trade-off between the needs of each. It is possible that this
316 trade-off was 'satisfied' by the grasses diverting sufficient resources BG to recruit AMF, thus
317 increasing colonization (Giovanetti and Sbrana 1998). This would have allowed the plants to
318 extend their BG uptake network by the use of AM hyphae. It is also probable that the loss of
319 carbon to the BG zone for AM recruitment was less than the cost for increasing root growth to
320 source nutrients (Fitter 1991). Gemma et al. (1997) showed that benefits of AM fungi were
321 only conferred when *Agrostis palustris* (L.) was grown in a low fertility substratum. Further
322 to this, Johnson et al. (1997) argued that carbon allocated to support mycorrhizas is only a
323 cost to the plant if it could have been allocated to enhance plant fitness. In addition, any
324 resources gained through the activities of a fungal symbiont are only beneficial if they are in
325 limiting supply to the plant. In the RHUL field site soil nutrients were low, and high
326 proportions of arbuscules observed in roots indicated that increased colonization levels under
327 rabbit grazing were beneficial to both partners in terms of the mutualism. It should be noted
328 that nutrient enhancements in grazed treatments (and their impacts upon mycorrhizal
329 colonization), following natural additions of faeces and urine were considered. Field
330 assessments of faecal deposition and a controlled experiment investigating impacts of total
331 excreta at field levels were carried out (Wearn 2006). The increase in mycorrhizal
332 colonization levels in grazed treatments was not found to have been attributable to excretory
333 inputs (Wearn 2006). Instead, it was a response to the above-ground defoliation.
334 Defoliation of grasses can cause greater exudation of carbon in the form of simple
335 carbohydrates (Paterson and Sim 1999) and so increases in root exudation would likely have

followed an increase in below-ground allocation of carbon. Increased colonization of roots by AMF could have been stimulated in this way as attraction of mycorrhizal hyphae to roots would have been enhanced through an increase in the exudate content of the rhizosphere and surrounding soil (Giovannetti and Sbrana 1998). Both quality and quantity of exudates seem to be important in the dynamic interaction between plant and AM fungus (Elias and Safir 1987; Jones et al. 2004).

Each of the three grass species showed similar responses in colonization levels. Although rabbits are often selective herbivores (Diaz 2000), all grass species were grazed, so colonization levels do not seem to reflect any impacts of selective feeding by the rabbit population. Spring colonization levels were low and can partly be explained in terms of root elongation. Roots were rapidly growing at this time of year and outstripping the rate of colonization by the AM fungi (Titus and Leps 2000).

Seasonality of AM colonization was very similar for each of the grass species so this was not indicated by colonization data alone. However, the level of colonization peaked earlier in all species in the absence of all herbivores (most clearly seen in *A. tenuis*). Seasonal patterns of colonization vary with host plant and mycorrhizal species (Bever et al. 2001) and abiotic factors (such as soil moisture) can have a strong influence too (Muthukumar and Udaiyan 2002).

There was no significant effect of insect herbivory on total colonization. Klironomos et al. (2004) and Titus and Leps (2000) found that defoliation can affect not just the %RLC but also the relative proportions of each structure (spores, vesicles, intraradial hyphae and arbuscules) making up the total colonization value. A further insight into the more subtle impact of insect herbivory in the absence of vertebrate grazers came from the proportions of AM structures within roots in this study. It appeared that insects could have had a stressful impact upon AMF in roots at certain times of the year (although not seasonally consistent), revealed by

high proportions of vesicles (storage structures) relative to arbuscules (Johnson 1993; Duckmanton and Widden 1994; Titus and Leps 2000). Thus, it may be that invertebrate herbivory is not consistent in effect, as published studies have found (Gange and Bower 1997; Gange et al. 2002; Kula et al. 2005) and may be non-significant in field situations where other factors have a greater influence. It is thought that rabbits masked any invertebrate effects in unfenced plots due to the larger quantities and rates of vegetation removal. It could also have been true that the experimental duration was not sufficient for insect effects to become significant. For example, Brown and Gange (1999) found that at least three years were required for insect effects on above-ground plant parameters to be seen.

At Silwood Park the effect of rabbit grazing on mycorrhizal colonization was both consistent (plots were located throughout the grasslands on the Silwood estate) and positive. These data support the findings from the RHUL field site. The mycorrhizal status of roots seems to respond quickly following initiation of or release from grazing (shown at both field sites) and then appears to remain consistent at the new levels over long periods (at least up to 19 years after exclosure erection at Silwood) as the lack of correlations between mycorrhizal colonization and exclosure age could not be explained by variations in soil parameters. These data, concerning rabbit grazing, can be compared with findings relating to other larger vertebrate grazers from the (very few) other published studies of this type. The literature shows that ungulate grazing can increase AM colonization, as in this experiment (Reece and Bonham 1978; Eom et al. 2001), induce no changes in colonization levels (Wallace 1987) or decrease mycorrhizal activity (Bethlenfalvay and Dakessian 1984).

Where defoliation is severe, it is likely that plants do not allocate as much carbon belowground due to photosynthetic reduction (Holland and Detling 1990). One would therefore expect that intense defoliation would always reduce AM colonization. However, two trends emerged in highly grazed plants. Either a decrease in total AMF colonization

386 occurred or no change in total colonization from the increased level elicited under moderate
387 grazing but with a decline in the presence of arbuscules. The question arises as to whether
388 changes in colonization were due to different fungal species compositions (numbers of AM
389 species and relative representation within roots) colonizing plants grazed at differing
390 intensities. At the extreme, a single species could have dominated following a release from
391 competition (Pinior et al. 1999; Saito et al. 2004). Another possibility is that a change in
392 species of mycorrhizas colonizing roots could result from changing carbon availability (Saito
393 et al. 2004). Less carbon-demanding species that can cope with lowered C availability may
394 colonize when a plant is releasing less exudates, and other, more demanding species may
395 colonize when there is greater assimilate availability in the root zone. At Silwood, where no
396 change in colonization level occurred even when plants were intensely grazed, mycorrhizal
397 fungi colonizing the root may have become less beneficial (Gange and Ayres 1999) and/or be
398 different (less carbon demanding) species colonizing causing species replacement
399 (Bethlenfalvay and Dakessian 1984).
400 The two different trends observed under intense grazing may therefore be dependent on the
401 species present in the mycorrhizal community itself. If there is a lack of AM species
402 available to cope with low carbon conditions or an initially colonizing species inhibits further
403 colonization (Sanders and Fitter 1992) then overall colonization will decline. Indeed, Eom et
404 al. (2001) found that root colonization by AM fungi was greater under moderate and intense
405 grazing in a tallgrass prairie when compared to ungrazed sites. They also discovered that AM
406 diversity in terms of species richness and evenness (based on spores present) decreased under
407 both moderate and high grazing intensities. Species richness showed significant reductions in
408 both years of their experiment while richness was only significantly altered by year 2,
409 possibly indicating a temporal effect for both aspects of diversity to be changed by grazing.
410 However, increases in exudation may not be required to cause colonization by certain AM

411 species. For example, Murray et al. (2004) showed that defoliation of *A. capillaris* (*A. tenuis*)
412 did not cause a significant difference in overall carbon exudation, yet this species was
413 consistently more colonized by AM fungi in grazed samples at Silwood and in the RHUL
414 field site. Therefore, increased exudation may not always be a critical factor (or it may
415 simply be an increased carbon supply is given to the fungus without further root exudation if
416 there is already a host-fungal partnership established in the root). The suggestion that
417 different species of AMF could have been colonizing grazed and ungrazed plants, or more /
418 less AM species colonizing grazed plants, requires molecular analyses of colonized roots (this
419 is a focus of our current research) but it is clear that direct observational techniques (i.e. root
420 staining and microscopy) are still a key component of assessing the responses of mycorrhizal
421 fungi.

422 The effect of grazing on mycorrhizas is important since grasslands have evolved with
423 herbivory as a dominant influence shaping the community (Estes et al. 1982; Bardgett and
424 Cook 1998). Grasslands support an active and large subterranean microbial community due
425 to high botanical and organic carbon turnover (Bardgett and Cook 1998). Grasses usually
426 form mycorrhizal associations in both agricultural and natural grasslands so their response to
427 defoliation is of high importance. Arbuscular mycorrhizas have been linked to increased
428 nutrient acquisition and contribute to competitive ability especially in low fertility
429 environments (Saint-Pierre et al. 2004) as well as conferring many other beneficial
430 characteristics upon their hosts and increasing soil stability through particle aggregation
431 (Miller and Jastrow 1990).

432 Temporal scales are an extremely important consideration when interpreting and linking
433 above-ground and below-ground facets of ecological systems (Bardgett et al. 2005). Often,
434 changes have been measured a considerable period of time after treatments were imposed
435 (e.g. 7 years in Stark and Grellman (2002) and 3-4 years in Alvey et al. (2003)). The actual

time taken for responses to have occured cannot be deduced from such experiments. By using frequent sampling events, this experiment has shown the degree and direction in which AM fungi can respond to two different types of above-ground herbivores. In addition it has demonstrated that AM fungi can respond quickly in the highly dynamic plant-soil microbe system in the field and (most importantly from a plant community and ecosystem perspective) colonization can remain at 'response levels' under a sustained above-ground influence over many years. Intermediate timescales such as this are considered the greatest for above-below-ground interactions to influence ecosystem function through inherent feedback mechanisms (Bardgett et al. 2005). At the finest temporal scales (minutes and hours), even slight changes in grazing can cause rapid changes in the quantity and quality of exudative flux and microbial responses (Jones et al. 2004; Kuzyakov and Jones 2006). Results from our study suggest that these rapid responses are translated into a consistent response in the field in a matter of weeks in terms of mycorrhizal colonization. Studies on agricultural grazers (e.g. Bardgett et al. 2001) have shown that large vertebrate herbivores can impact upon soil microbial communities; this study has demonstrated that rabbits can structure below-ground microbiota too.

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Figure Legends

Fig. 1 Changes in live foliar biomass following field manipulations at RHUL, + rabbits + insects (■); + rabbits - insects (□); - rabbits + insects (▲); - rabbits – insects (Δ).

Fig. 2

Total mycorrhizal colonization patterns over time at RHUL for each of the three grass species. Treatment symbols as in Fig. 1.

Fig. 3

Proportional representation of total mycorrhizal structures within host roots by arbuscules, for each of the three grass species over time at RHUL. Treatment symbols as in Fig. 1.

Fig. 4

Proportional representation of total mycorrhizal structures within host roots by vesicles and spores, for each of the three grass species over time at RHUL. Treatment symbols as in Fig. 1.

Fig. 5

Proportional representation of total mycorrhizal structures within host roots by hyphae, for each of the three grass species over time at RHUL. Treatment symbols as in Fig. 1.

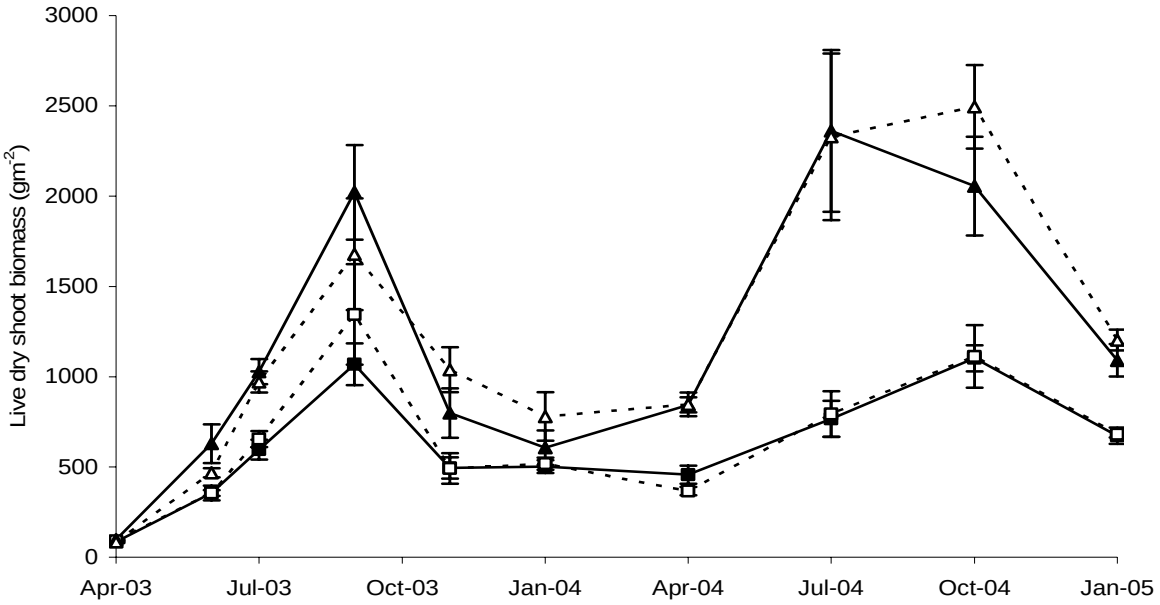
Fig. 6

Mycorrhizal colonization at Silwood Park for each of the three grass species in summer (July) and winter (December) 2004. Unshaded bars represent exclosures and shaded bars represent rabbit grazing.

Table 1

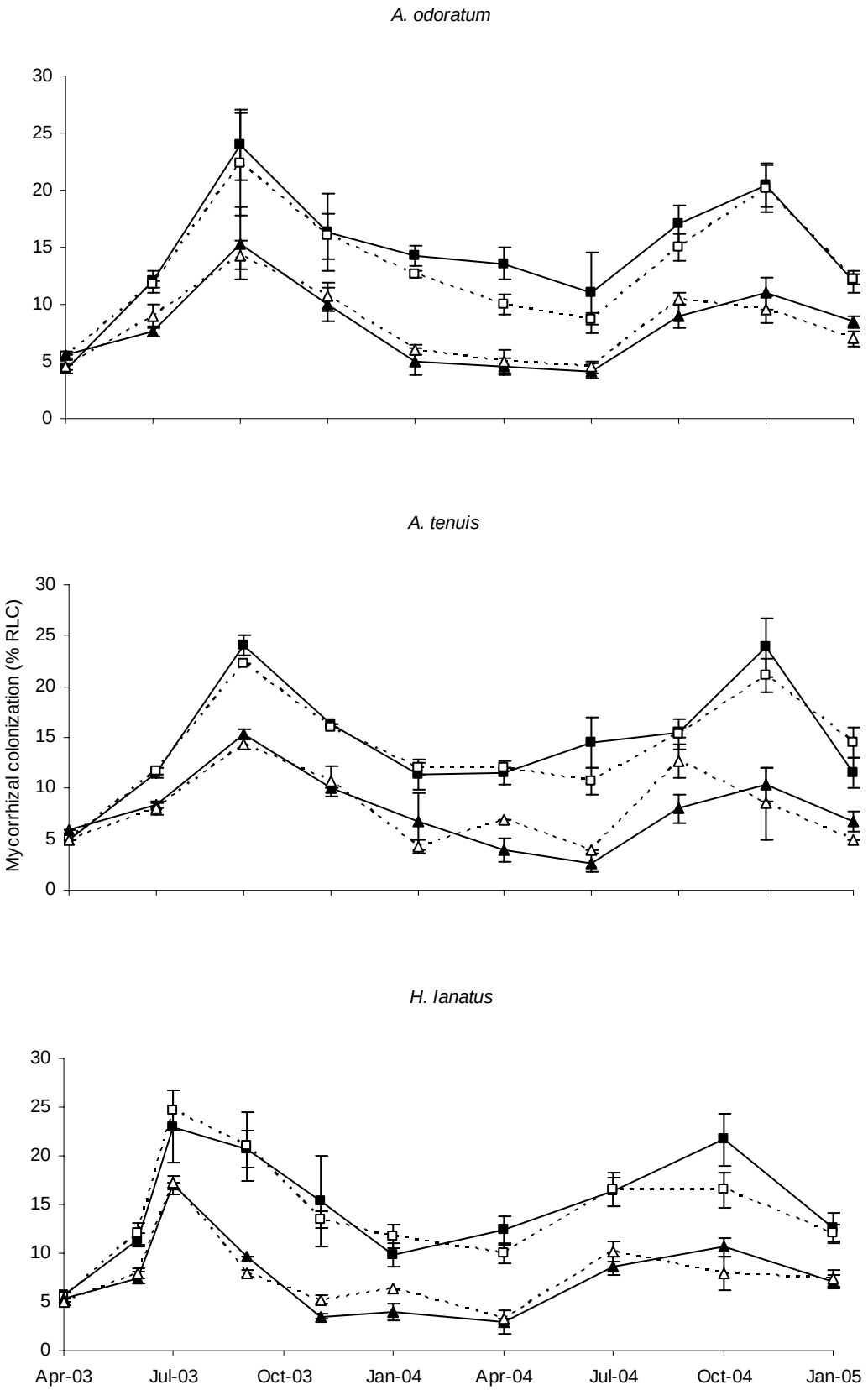
Summary of ANOVA results investigating the effects of grazing and plot age on mycorrhizal colonization at Silwood Park**.**

629 **Fig. 1**



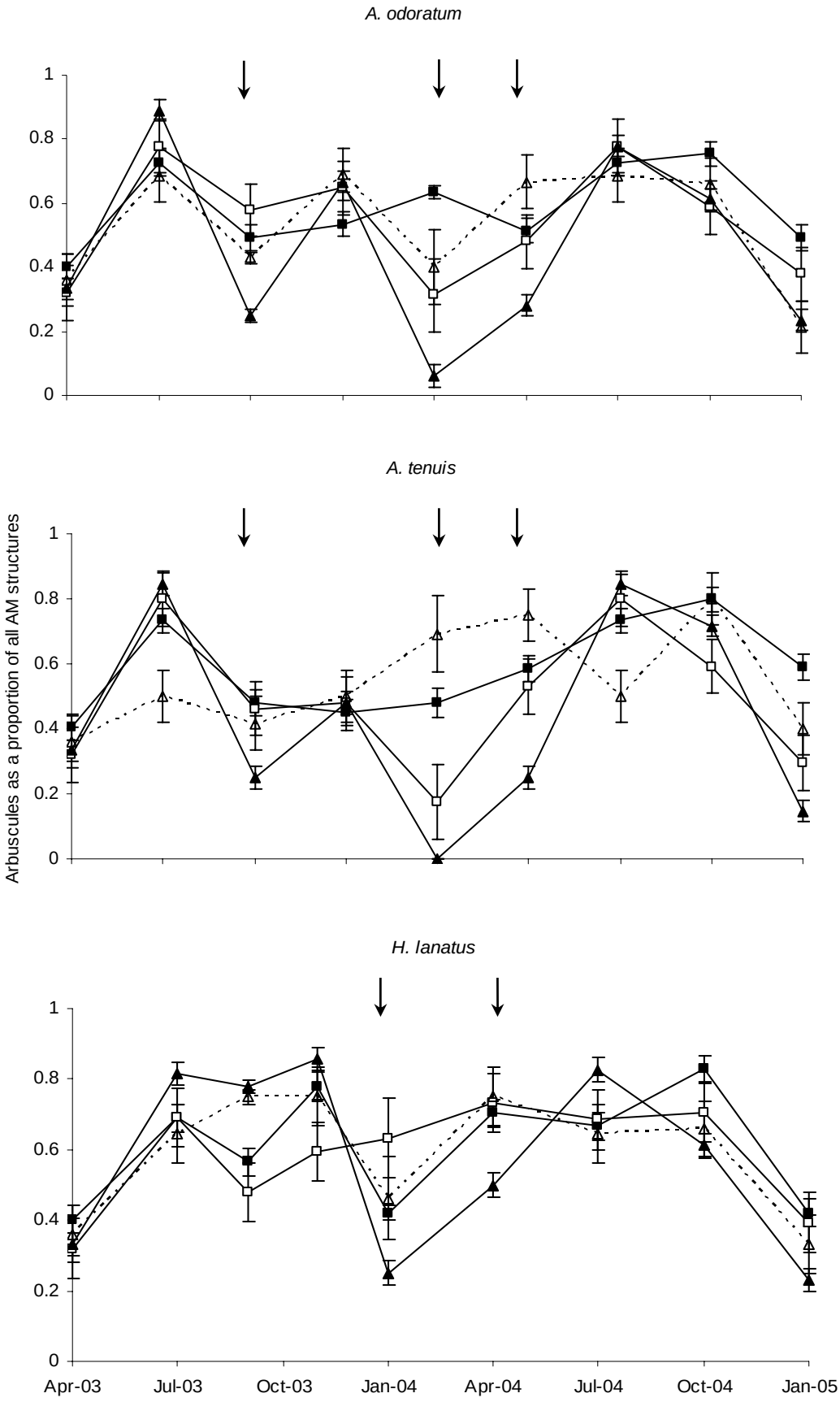
630
631

632 **Fig. 2**


A. odoratum
A. tenuis
H. lanatus
Mycorrhizal colonization (% RLC)
0
5
10
15
20
25
30
Apr-03
Jul-03
Oct-03
Jan-04
Apr-04
Jul-04
Oct-04
Jan-05

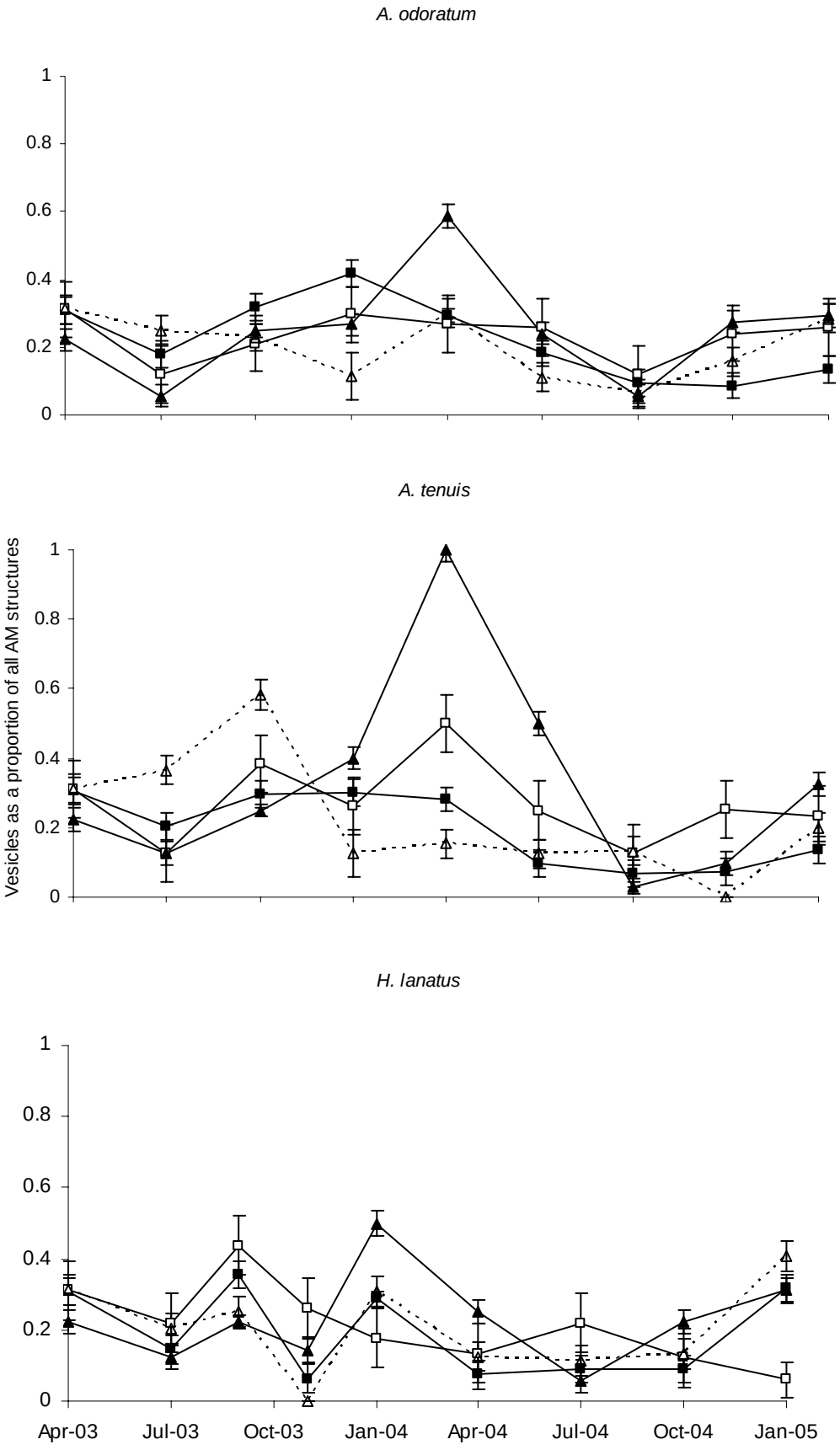
633

634 **Fig. 3**


A. odoratum
A. tenuis
H. lanatus
Arbuscules as a proportion of all AM structures
0
0.2
0.4
0.6
0.8
1
Apr-03
Jul-03
Oct-03
Jan-04
Apr-04
Jul-04
Oct-04
Jan-05

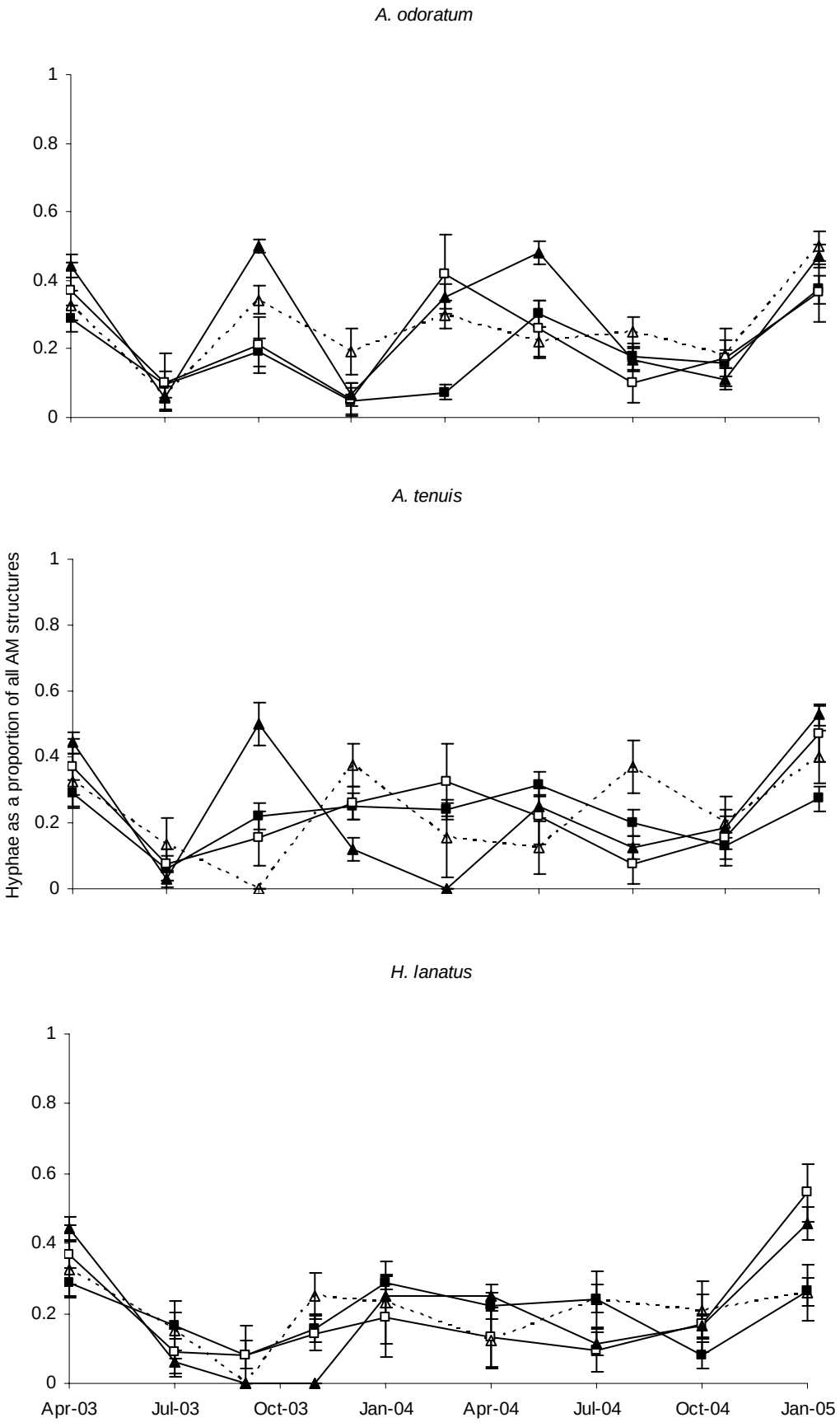
635

636 **Fig. 4**


A. odoratum
A. tenuis
H. lanatus
Vesicles as a proportion of all AM structures
0
0.2
0.4
0.6
0.8
1
Apr-03
Jul-03
Oct-03
Jan-04
Apr-04
Jul-04
Oct-04
Jan-05

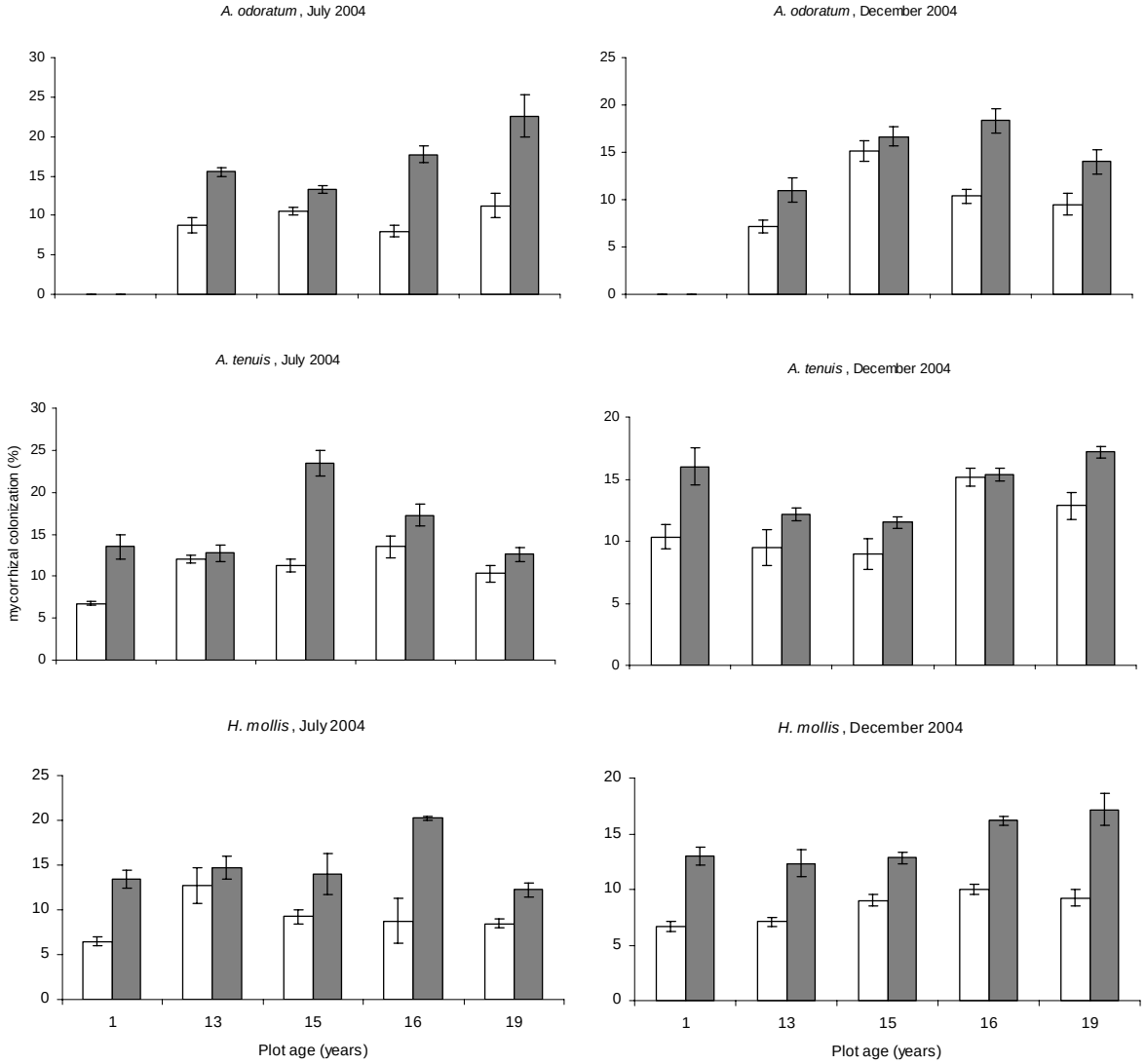
637

638 **Fig. 5**


A. odoratum
A. tenuis
H. lanatus
Hyphae as a proportion of all AM structures
1
0.8
0.6
0.4
0.2
0
Apr-03
Jul-03
Oct-03
Jan-04
Apr-04
Jul-04
Oct-04
Jan-05

639

640 **Fig. 6**


A. odoratum , July 2004
A. odoratum , December 2004
A. tenuis , July 2004
A. tenuis , December 2004
H. mollis , July 2004
H. mollis , December 2004
mycorrhizal colonization (%)
Plot age (years)
1
13
15
16
19

641

Table 1

July 2004

	Grazing		Plot age		Grazing x Plot age	
	F	*P*	*F*	*P*	*F*	*P*
A. odoratum	128.3	< 0.01	0.96	n.s.	0.62	n.s.
A. tenuis	43.22	< 0.05	1.19	n.s.	0.76	n.s.
H. mollis	6.00	n.s.	2.36	n.s.	1.74	n.s.

df = 1,2 for grazing. For *A. odoratum* df = 3,6 for plot age and the interaction term. For *A. tenuis*, *H. mollis* df = 4, 8 for plot age and the interaction term.

December 2004

	Grazing		Plot age		Grazing x Plot age	
	F	*P*	*F*	*P*	*F*	*P*
A. odoratum	17.44	< 0.05	4.06	n.s.	0.80	n.s.
A. tenuis	3.95	n.s.	3.95	n.s.	0.11	n.s.
H. mollis	2456.2	< 0.001	1.09	n.s.	0.18	n.s.

df = 1,2 for grazing. For *A. odoratum* df = 3,6 for plot age and the interaction term. For *A. tenuis*, *H. mollis* df = 4, 8 for plot age and the interaction term.