Interactions between collembola and saprotrophic basidiomycete fungi: effects of grazing on mycelial morphology and nitrogen flux

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Degree of Philosophiae Doctor

by

George Mark Tordoff, BSc. (Hons)

Cardiff School of Biosciences
University of Wales, Cardiff
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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Summary

Saprotrophic cord-forming basidiomycete fungi are important decomposition agents in woodlands; their interactions with soil invertebrates remain little understood. Collembola (springtails) are an abundant group of soil-dwelling invertebrates; many species are mycophagous. I describe several studies that investigate effects of collembola on morphology and nutrient partitioning in mycelia of cord-forming fungi. The collembola (*Folsomia candida*, *Protaphorura armata* and *Proisotoma minuta*) and fungi (*Phanerochaete velutina*, *Hypholoma fasciculare*, *Phallus impudicus* and *Resinicium bicolor*) used occur commonly in British woodlands. Each study employed two-species (one fungus: one collembola) microcosms containing compressed soil. The fungus was added in a pre-colonized wood block and allowed to grow out across the soil before collembola were added. Fungal growth and morphology were characterized using image analysis techniques. Collembola grazing occurred predominantly on the diffuse hyphae present at mycelial margins, except for *R. bicolor* where thick cords were regularly severed. Grazing reduced extension rate and hyphal coverage of mycelia; effects were more dramatic at high collembola densities. The region of mycelium attacked was consistent across collembola species, but the magnitude of grazing impacts varied. Fungal responses to grazing were species-specific: *R. bicolor* mycelia were often destroyed; *P. velutina* and *H. fasciculare* retained an intact cord system but displayed modified growth; and *P. impudicus* was little affected. Mycelial architecture was altered. In addition to changes in fungal morphology, wood decay rate of *H. fasciculare* was decreased by grazing. Mycelial nutrient distribution was explored by pulse-labelling *P. velutina* mycelia with ^{15}N. This showed that *P. armata* grazing reduced uptake and transfer of ^{15}N to outgrowth mycelium, and did not increase nitrogen release into soil. Overall, the effects of collembola on mycelial morphology and nutrient partitioning suggest that grazing may alter the ability of saprotrophic cord-forming fungi to locate, decompose and transfer nutrients between dead woody resources on the forest floor.

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Chapter 1. Introduction

Throughout much of the twentieth century, the majority of terrestrial ecological research was conducted above-ground. The discrepancy between the relative contributions of the above- and below-ground components of ecosystems to the advancement of ecological theory and knowledge is startling. The reasons for this discrepancy are manifold, the most obvious being that soils are opaque and as a result the species that inhabit them are often impossible to observe *in situ*. In addition, the study of soil organisms is fraught with taxonomic difficulties, partly because there exists a high diversity of soil species, many of which are microscopically small. It is not surprising that soils have been branded "the poor man's tropical rainforest" (Usher *et al.* 1982).

Despite this background of neglect the crucial role of soils has long been recognized and their importance in ecological processes cannot be overstated. Plants depend on soil not only for anchorage and water uptake, but also for the cycling of nutrients. The importance of soils is highlighted by the fact that the bulk of global net primary productivity is decomposed below ground or on the surface of soil (Wall *et al.* 2005). Part of the difficulty in attempting to understand the functioning of the soil ecosystem has been the lack of suitable methodologies to enable the study of soil processes. In recent years however the development of a suite of suitable methodologies, including molecular and stable isotope approaches, has allowed at least a glimpse into the inner workings of the soil environment (Bardgett *et al.* 2005).

The recent renaissance in soil ecology is timely. Terrestrial ecosystem carbon fluxes both respond to and strongly influence atmospheric carbon dioxide increase and consequent climate change (Cao & Woodward 1998). There is more carbon stored in soils than in the biological and atmospheric compartments of the biosphere combined (Hopkins & Gregorich 2005). Understanding soil carbon dynamics is therefore a key goal in global change research.

It is only relatively recently that the critical importance of below-ground diversity in the functioning of terrestrial systems has been fully realized by ecologists (Wardle

2002). For example, in a manipulative microcosm experiment soil organism diversity affected plant community composition, microbial and root biomass, decomposition rate and abundance of mycorrhizal fungi (Bradford *et al.* 2002). Soil fauna can also enhance local plant species diversity through the suppression of dominant plant species (De Deyn *et al.* 2003). Although these and other studies highlight the importance of below-ground diversity, trophic interactions within the soil are still poorly understood. Improving our knowledge of soil food webs is key to understanding the mechanisms underlying ecosystem-level processes such as decomposition and nutrient cycling.

Linkages between above- and below-ground terrestrial systems are also relatively poorly understood (Wardle 2002), despite their importance in the inter-dependent functioning of the two systems. Although the direct negative effects of root herbivores, such as Tipulidae, on plant growth have been well documented (van der Putten, 2003, Hartley & Jones 2004), the more subtle effects of saprophagous, mycophagous and bacteriophagous soil fauna on plants have received less attention. Pot experiments have demonstrated that soil detritivores can indirectly increase plant growth by mobilising soil nutrients (Setälä & Huhta 1991), and that this can have knock-on effects on higher trophic levels (Scheu *et al.* 1999, Newington *et al.* 2004). The beneficial effects of soil fauna on plant growth may be mediated mainly via mycorrhizal fungi, which supply soil nutrients to the majority of land plants. Mycorrhizal fungi may benefit from reduced competition and increased nutrient acquisition when soil fauna selectively graze on other soil microbes (Gange 2000).

Collembola, commonly known as springtails, represent one of the most ubiquitous and abundant groups of soil invertebrates. In most ecosystems they contribute relatively little to total soil animal biomass due to their small size. Despite this, collembola are thought to have important effects as 'drivers' of ecosystem processes (Hopkin 1997). Perhaps their most important effect occurs through their grazing of saprotrophic microorganisms, which indirectly influences decomposition (Hopkin 1997). Many species of collembola are mycophagous, and their interactions with fungi have been long studied (e.g. Poole 1959, Mills & Sinha 1971). The majority of these studies have investigated interactions between collembola and mycorrhizal fungi and/or saprotrophic microfungi. Only limited research has been directed

towards understanding the ecological interactions between collembola and saprotrophic basidiomycete fungi, exceptions being the studies of Visser and Whittaker (1977), Parkinson *et al.* (1979), Newell (1984a, b) and Kampichler *et al.* (2004). This relative neglect is somewhat surprising given that basidiomycete fungi are the major decomposers of wood and leaf litter in forests (Boddy & Watkinson 1995). Collembola grazing may alter fungal community structure through selective grazing (Visser 1985); if this occurs with saprotrophic basidiomycetes then collembola have the potential to affect ecosystem process rates on the forest floor.

Cord-forming fungi are a notable group of saprotrophic basidiomycetes as individual mycelia often occur over large spatial scales and attain a considerable biomass on the forest floor (Boddy 1999). They interconnect discrete resources (e.g. dead wood and litter patches) and can rapidly translocate mineral nutrients between them. As far as I am aware prior to the current studies only one published investigation has considered the interactions between collembola and saprotrophic cord-forming basidiomycetes in soil (Kampichler *et al.* 2004). This latter study employed only one fungal species and quantified grazing effects based solely on mycelial morphology; consequently many questions regarding the interactions between these organisms remain unanswered. For example, does grazing by collembola affect the way cord-forming fungi forage for new resources? Is fungal nutrient translocation and partitioning affected by grazing? Which regions of cord-former mycelium are grazed? Are the regions of grazing consistent across species? Answering these questions will help us to understand better the ecological functioning of cord-forming basidiomycete fungi.

Using laboratory microcosms the studies reported in this thesis set out to:

1. investigate the extent to which mycophagous collembola modify cord-former growth and morphology through their grazing activity, and whether the magnitude of these effects depends on grazing intensity and the maturity of cord-former mycelium;

2. investigate whether different cord-forming species are affected to different extents by collembola grazing. If so, are grazing impacts determined by fungal morphology and foraging strategy?

3. investigate whether different collembola species have different effects, in terms of region of mycelium preferentially attacked and magnitude of effects. Are effects dependent on collembola body size, as observed by Kampichler *et al.* (2004), even when biomass is standardized?

4. investigate nitrogen flux within cord-former mycelium. Compared to phosphorus and carbon, very little is known about nitrogen partitioning in cord-forming fungi, and as far as I am aware the possibility that fungal nutrient partitioning might be modified by collembola grazing has never been investigated;

5. determine whether collembola grazing affects wood decay rate by cord-forming fungi; and finally

6. determine whether collembola abundance is affected by the species of cord-forming fungus that is being grazed.

Exploring these objectives will enable a more detailed understanding of the interactions between cord-forming fungi and collembola, and of the characteristics of fungus-collembola interactions in general.

Chapter 2 reviews the published literature on the ecology of cord-forming fungi and collembola. First, decomposition processes on the forest floor, where these organisms occur abundantly, are introduced, before the biology and ecological roles of cord-forming fungi and collembola are highlighted. By reviewing current understanding of the characteristics and ecological importance of fungus-collembola interactions the gaps in existing knowledge are highlighted. Finally, the use of stable isotopes is introduced in the context of fungal and collembola ecology.

Chapter 3 describes the general methods used in the following experimental chapters. These include culturing of experimental organisms, a description of the experimental microcosms, and capture and analysis of images of mycelia.

Chapter 4 reports two experiments that investigate the effects of collembola grazer density and degree of mycelial establishment on the growth of three cord-forming fungi. Observed morphological changes are discussed in terms of the ecology of cord-forming fungi. In these experiments, which employed only one collembola species (*Folsomia candida*), responses to grazing varied across the three fungi. It was therefore considered important to determine whether grazing effects also depend on the identity of the collembola grazer species. This was investigated in **Chapter 5**, where three collembola and four fungal species were factorially combined in two species (one fungus: one collembola) microcosms. Grazing density was standardized to allow comparisons across all experimental combinations.

In **Chapter 6** the possibility of using network architecture analysis to examine grazing-induced changes in fungal mycelia is explored. This novel approach allows the quantification of mycelial integration, and, in this study, is used to gain a preliminary insight into whether collembola modify mycelial network architecture.

The mycelial responses to grazing recorded in Chapters 4-6 were quantified using image analysis techniques. In **Chapter 7** the effects of collembola on the functioning of mycelia were explored in a nitrogen flux experiment. A nitrogen stable isotope (^{15}N) label was added to mycelia and partitioning of ^{15}N in different system components of microcosms with and without collembola was determined over time. The potential consequences of grazing-induced changes are discussed.

Findings from the experimental chapters of this thesis are brought together in **Chapter 8** in an attempt to summarize what has been learnt about collembola grazing on saprotrophic basidiomycete fungi. Questions raised by the current work are discussed, and remaining gaps in the knowledge are highlighted.

Chapter 2. Literature review

2.1 The forest soil subsystem

2.1.1 Global extent

Forests and other predominantly wooded biomes such as woodlands and shrublands occupy approximately 39% of the global land surface area and contain roughly 92% of global standing crop biomass (Whittaker 1975). Forest biomes occur in tropical, temperate and boreal climatic zones, and forests may be further categorized based on whether they are wet or dry, and deciduous or evergreen. The majority of the planet's terrestrial carbon is contained in forest ecosystems (Rayner & Boddy 1988), and within forests 40-73% of plant carbon is estimated to occur below ground (Simard *et al.* 2002). It is therefore critically important to understand fully the dynamics of forest soil carbon if we are to model accurately the fluxes between terrestrial and atmospheric carbon pools. Carbon dynamics in soils, however, remain poorly understood in comparison to those of above-ground vegetation and the atmosphere, and it is still a matter of debate as to whether forest soils will act as a sink or a source of carbon under future climate scenarios (Cao & Woodward 1998).

2.1.2 Dead organic resources

Dead plant material accounts for a significant proportion of the biomass of forest ecosystems. This substrate ranges from small fragments of leaf litter through to entire trees, and occurs mainly as fallen dead litter on the forest floor or as standing dead litter. In addition to above-ground litter, wood is a major contributor to dead, below-ground biomass as lignified roots (Boddy & Watkinson 1995). Fallen dead litter is distributed heterogeneously in time and space (Rayner & Boddy 1988). Litter inputs vary across the year, with an obvious pulse of leaf litter in the autumn in deciduous systems and other, often major, pulses of larger woody material following storm events. All forms of woody litter occur discontinuously in space. The discontinuous nature of branches and tree stumps is obvious, but from a fungal hypha's perspective even leaf litter represents a discontinuous resource, with small gaps between leaves sometimes representing insurmountable barriers to growth (Boddy 1999). Woody litter is usually heavily lignified and is characterized by a very high carbon: nutrient ratio, and as such represents a recalcitrant resource (Rayner & Boddy 1988).

2.1.3 The importance of microbes in carbon and nutrient cycling

Soil and litter microbes are the main decomposers of dead organic material in forests (Rayner & Boddy 1988), although their biomass and activity is influenced to a large extent by soil invertebrates (Hopkins & Gregorich 2005). Fungi constitute a major food source not only for collembola (Section 2.3) but also for many other groups of soil animals, particularly oribatid mites, enchytraeid worms and nematodes (Harley 1971, Visser 1985). This is not surprising, given that mycelia of soil fungi usually have a much higher nitrogen content than does plant litter, and thus represent a high quality nutritional substrate (Harley 1971, Lindahl 2000).

Bacteria and fungi make up the two main groups of microbial saprotrophs. Basidiomycete and xylarious ascomycete fungi are the major agents of wood decomposition (Boddy & Watkinson 1995), as their powerful enzymatic capabilities enable them to break down the complex organic molecules found in dead wood. Most bacteria and lower fungi do not produce these enzymes, and unicellular bacteria also lack the penetrative ability possessed by fungal hyphae. This results in bacteria having limited access to dead wood until it has been substantially decayed by fungi (Rayner & Boddy 1988). As most forest soil nutrients occur in recalcitrant rather than labile forms, it is fungi, rather than bacteria, that are responsible for most of the nutrient turnover that occurs (Lindahl *et al.* 2002).

Saprotrophic fungi have traditionally been considered to be the primary agents responsible for the mineralization of complex organic molecules in forest soils. Nutrient mobilization is thought to occur when the carbon: nitrogen ratio of litter decreases as decomposition proceeds, causing the fungi to excrete excess nutrients into the soil solution to avoid toxicity (Lindahl *et al.* 2002). This relatively labile soil nutrient pool is taken up by other organisms, particularly the ectomycorrhizal (EM) fungi that constitute the most important mutualistic symbionts of temperate and boreal forest trees (Erland & Taylor 2002). EM fungi return some of these nutrients to their plant partners, completing the nutrient cycle within the forest. More recent studies have however shown that saprotrophic fungi are more conservative of nutrients than previously thought, and that they commonly translocate nutrients between different organic resources (Section 2.2.2) rather than excreting them at the point of excess.

Nevertheless a net transfer of nutrients from saprotrophic to mycorrhizal fungi must take place to allow continued primary productivity (Boddy 1993, Lindahl *et al.* 2002). Some of this transfer may occur during competitive interactions between EM fungi and saprotrophs (Leake *et al.* 2001); for example, in one study 25% of added phosphorus was transferred from the saprotroph *Hypholoma fasciculare* to two EM fungi, with little reciprocal transfer of phosphorus back to the saprotrophic fungus (Lindahl *et al.* 1999). This net transfer from saprotrophic to EM fungi, however, seems to occur only when the saprotroph occupies a relatively small resource; in a further study there was a net transfer of phosphorus in the opposite direction when the saprotroph was introduced in a larger wood resource (Lindahl *et al.* 2001b). The latter study indicates that 'resource potential' is important in deciding the competitive balance, and hence the net transfer of nutrients, between EM and saprotrophic fungi. It has also been discovered relatively recently that EM fungi can degrade some complex organic nutrients, particularly proteins and amino acids (Leake *et al.* 2002), and that this may reduce their reliance on scavenging more labile nutrient sources from saprotrophic mycelium. EM fungi do not, however, possess the same enzymatic capabilities as saprotrophic fungi for degrading the complex carbohydrates, particularly lignocellulose, that occur in plant cell walls (Colpaert & van Tichelen 1996). Saprotrophic fungi are therefore always likely to play the major role in litter decomposition. In addition, there is evidence from many studies that saprotrophs are superior competitors to plant-mycorrhizal symbioses for inorganic soil nitrogen (see Kaye & Hart 1997), even if assimilated nutrients are eventually lost to EM fungi during combat. One group of saprotrophic fungi that commonly attain a large biomass in forest systems is the cord-forming basidiomycetes, and the importance of these fungi in the decomposition of dead woody material is discussed in Section 2.2.2.

2.2 Cord-forming fungi

2.2.1 Non unit-restricted fungi

Cord-forming fungi are non unit-restricted. This means they can proliferate from and interconnect discrete organic resource units. Unit-restricted fungi, in contrast, are unable to interconnect resource units and must rely on spore dissemination to encounter new resources. Cord-forming species have specialized organs that are used to interconnect resources (Fig. 2.1). These are generally referred to as cords, although other names such as strands and syrrotia have been used (Boddy 1993). Cords are

defined as aggregations of predominantly parallel, longitudinally aligned hyphae which form behind a diffuse mycelial growth front (Boddy 1999). They are characterized by a thick rind, often encrusted with crystals, which reduces the passage of molecules between the cord and the environment. Cords also possess large-diameter apoplastic 'vessel' hyphae, which are believed to be involved in translocation of solutes around the mycelial network (Section 2.2.2), as well as narrower cytoplasmic hyphae (Cairney 1992, Boddy 1993). Most species of cord-forming fungi, for example *Phallus impudicus*, *Phanerochaete* spp., *H. fasciculare*, *Megacollybia platyphylla* and *Resinicium bicolor*, are saprotrophic basidiomycetes occurring at the soil/litter interface on the forest floor where they interconnect dead woody resources. Another important group of non unit-restricted species are the rhizomorphic fungi, typified by the genus *Armillaria*. These form root-like aggregations of hyphae that are apically-dominant (Rayner & Boddy 1988). Somewhat confusingly, the mycelial cords of true cord-forming species are sometimes referred to as rhizomorphs (e.g. by Cairney 1992, 2005), but rhizomorphic species never possess the diffuse growth front that occurs in true cord-formers.

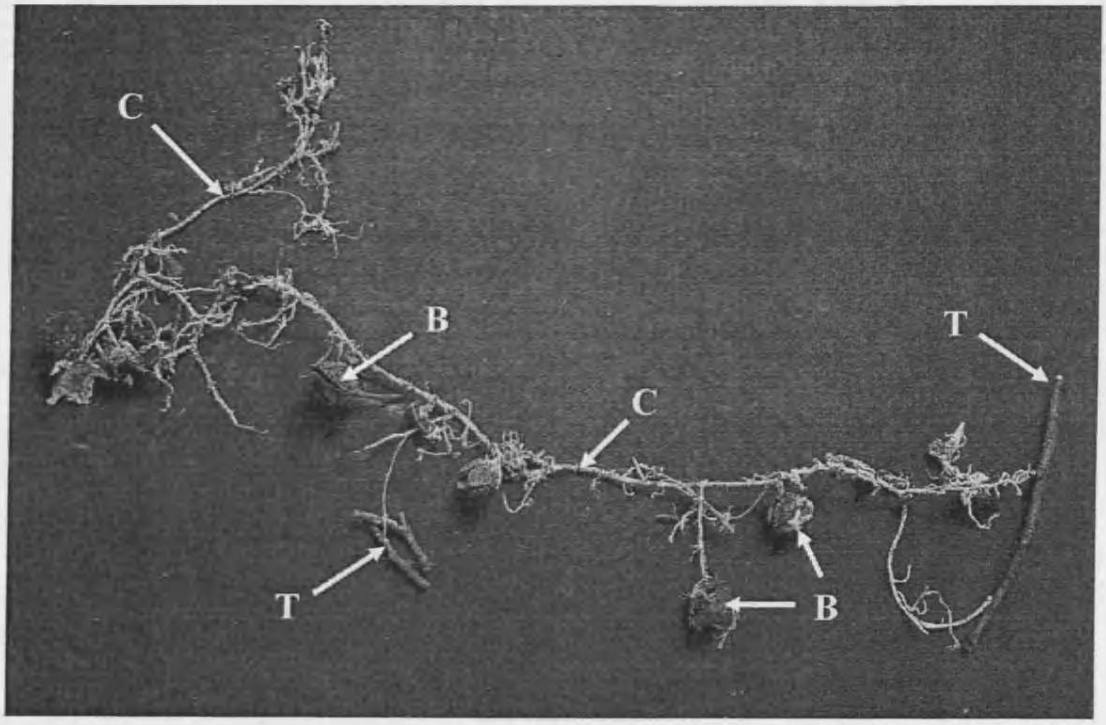


Figure 2.1. Section of a *Megacollybia platyphylla* cord system excavated from beneath beech (*Fagus sylvatica*) leaf litter. Bute Park, Cardiff, 31.07.2003. Twigs (T) and beech cupules (B) have been interconnected by cords (C).

2.2.2 Ecological importance

Cord-forming and rhizomorphic fungi are ubiquitous in boreal, temperate and tropical forest ecosystems, where they are thought to represent a large proportion of the total microbial biomass (Boddy 1993). Their success is, at least in part, due to their ability to access new resources through vegetative growth. This provides them with more 'competitive strength' than fungi that arrive at new resources as spores, and often enables cord-forming fungi to monopolize resources once they have outcompeted primary colonists (Boddy & Abdalla 1998). Cord-formers also colonize dead organic material in other ecosystems, for example grasslands and sand dunes (see Boddy 1993), and even occur in rabbit pellets (Thompson & Rayner 1983). Rhizomorphic species are among the largest and oldest organisms on earth – an individual of *Armillaria gallica* (formerly *A. bulbosa*) in a Michigan hardwood forest was shown using molecular techniques to be at least 1500 years old, occupy a minimum of 15 hectares, and was conservatively estimated to weigh 100 tonnes (Smith *et al.* 1992). Although never documented as reaching similar proportions, cord-forming fungi frequently cover large spatial areas. For example, oak stumps over 10 m apart have been found colonized by a single genotype of *Phanerochaete velutina* (Thompson & Boddy 1983), and mycelia of one genotype of *M. platyphylla* (formerly *Tricholomopsis platyphylla*) were found separated by 150 m (Thompson & Rayner 1982a). Mycelia of these large individuals are not normally connected across their entire network, but are frequently fragmented into smaller 'daughter' colonies, which nonetheless attain total cord lengths of tens of metres (Thompson & Rayner 1982b, Thompson & Rayner 1983). Cord systems are dynamic – relatively rapid growth at colony margins during the autumn and spring is often accompanied by the loss of mycelia from the centre of colonies (Thompson & Rayner 1982b, Thompson & Rayner 1983). Their persistence on the forest floor is determined by three related factors: microclimate, resource availability and incidence of competition (Dowson *et al.* 1988b). The ability of cord-forming and rhizomorphic fungi to develop a large biomass demonstrates their potential for storing significant quantities of carbon in forest ecosystems.

Saprotrophic cord-forming species play an important role in decomposition and nutrient dynamics in forests (Boddy & Watkinson 1995). Some species are relatively specialized on particular types of resources, ranging from leaf litter and twigs to fallen

and standing trunks, and dead roots (Boddy 1999). Most of the cord-forming saprotrophs are white-rotters, producing enzymes to degrade the lignin in wood and so gain access to cellulose and hemicelluloses (Rayner & Boddy 1988, Cooke & Whipps 1993, Hammel 1997), and are capable of decomposing wood more rapidly than most other fungi (Boddy 1993). White rot fungi also utilize all of the simpler molecules, for example sugars, that are present in wood. Cord-formers obtain most of their carbon from woody resources while acquiring mineral nutrients such as phosphorus directly from the soil (Morrison 1982, Wells *et al.* 1997, Donnelly & Boddy 1998), as well as from wood and leaf litter. Microcosm studies have shown that phosphorus, nitrogen and carbon are preferentially allocated to mycelium within wood resources rather than to outgrowth mycelium (Wells & Boddy 1990b, Wells *et al.* 1990, Wells *et al.* 1995, Owen 1997); this is also the case for phosphorus in the field (Wells & Boddy 1995).

Cord-formers translocate nutrients between discrete wood resources over relatively short time scales (Boddy 1999). In one of the few field studies to investigate this, radiotracer ^{32}P was added to wood inocula of a range of cord-forming fungi, and after 5 d had been translocated distances of up to 75 cm to the margins of the system and to connected litter components (Wells & Boddy 1995). Results of an earlier field study, in which limited translocation of ^{32}P was found (Clipson *et al.* 1987), are compromised by the addition of the radiotracer to thick cords rather than to sites of uptake (colonized wood and mycelial growth fronts).

The exact mechanism of nutrient translocation within cords is still a matter of debate (Wells *et al.* 1998a, Cairney 2005). The rapid movement of solutes along cords and rhizomorphs in laboratory systems has been attributed to mass flow of solution along 'vessel' hyphae, driven by solute and water uptake (Brownlee & Jennings 1982, Granlund *et al.* 1985). Cairney (1992) proposes a model in which carbon is translocated acropetally (i.e. from resource base to growing margin) via mass flow, and phosphorus is translocated basipetally (i.e. from growing margin, where it is taken up from soil, to resource base) via cytoplasmic streaming. This would account for the simultaneous acropetal and basipetal translocation of nutrients (Granlund *et al.* 1985, Lindahl *et al.* 2001a), and the independent movement of carbon and phosphorus (Wells *et al.* 1995) in cord systems, which together imply that multiple parallel or

circulating pathways are required. Recent advances in technology have allowed the imaging of radioisotope distribution within mycelia at submillimetre resolution by growing fungi across a scintillation screen and counting the photons that are emitted (Tlalka *et al.* 2002, Tlalka *et al.* 2003). These studies show that the cord-former *P. velutina* rapidly translocates amino acid to growing margins as it extends from a resource base and, rather intriguingly, that this acropetal movement occurs in pulses with a period of 11-12 h (Tlalka *et al.* 2002). The authors suggest that the rhythmic nature of translocation might indicate that *P. velutina* uses signalling information to co-ordinate the use of newly encountered resources. In any case, the pulse frequency is temperature-dependent, suggesting that pulses arise directly from a metabolic process rather than reflecting any kind of biological 'clock' (Watkinson *et al.* 2005).

The explanation for observed patterns of nutrient translocation in cord-forming and rhizomorphic fungi is still subject to debate (e.g. Cairney 1992, Wells *et al.* 1998a). Results of early laboratory studies (e.g. Wells & Boddy 1990b, Wells *et al.* 1990, Hughes & Boddy 1994) suggest that acropetal ^{32}P translocation from inocula (colonized wood) to regions of encounter with new wood resources reflect source/sink relationships and are driven by the metabolic demand for nutrients at these sites to enable the breakdown of newly-acquired wood (Cairney 1992). Recently, however, more elaborate studies have indicated that phosphorus requirements within newly-acquired wood are mainly fulfilled through acquisition from local soil patches by outgrowth mycelia rather than via translocation from previously colonized resources (Wells *et al.* 1998a). Also, ^{32}P scavenged from soil is sometimes translocated in greater amounts to wood resources that have been colonized for longer rather than those acquired more recently (Wells *et al.* 1998b). It thus appears that translocation might be driven by the need for conservation of nutrients rather than by metabolic demand (Wells *et al.* 1998a, Wells *et al.* 1998b). This explanation would account for the fact that the majority of fungal nutrients and carbon are located in 'safe' wood inocula rather than in 'unsafe' outgrowth mycelium, which is presumably more susceptible to loss of nutrients through interactions with other organisms.

The potential ecological importance of interactions with EM fungi (Section 2.1.3) and soil invertebrates (Section 2.4.2) are discussed elsewhere, but it is worth highlighting the role that inter-specific competitive interactions between cord-forming saprotrophs

might play in the release of nutrients from mycelia of these otherwise nutrient-conservative species (Boddy & Watkinson 1995). Cord-forming species that inhabit wooded ecosystems are likely to encounter each other on a regular basis, both when foraging across soil and when they co-occur within a wood resource. Laboratory studies (e.g. Dowson *et al.* 1988a) have shown relatively consistent hierarchies of competitive interactions between cord-formers in soil, wood, and on agar. *P. velutina* was the most combative species in all these substrates, and in a field-based experiment (Dowson *et al.* 1988b). Interspecific interactions between cord-forming fungi are often highly aggressive and lead to lysis of hyphae through mycelial interference and eventually the replacement of the less combative species by the superior competitor (Dowson *et al.* 1988a). It is likely that nutrient release from mycelia occurs at this time, although this nutrient pool may be largely taken up by the surviving fungus. There is some evidence for this transfer of nutrients – in a laboratory study *P. impudicus* was able to accumulate carbon from *P. velutina* and *H. fasciculare* during inter-specific interactions (Wells & Boddy 2002), and the same pattern was also observed for phosphorus (J.M. Wells, M. Harris & L. Boddy, unpublished data). In summary, it is highly likely that cord-forming species affect the spatial distribution of nutrients on the forest floor (Boddy & Watkinson 1995). It is, however, difficult to predict the magnitude of their contribution to nutrient redistribution, as the spatial scales within which individual mycelia operate in nature are largely unknown (Cairney 2005).

2.2.3 Search strategies

Cord-forming species exhibit variable foraging patterns as they grow across soil (Fig. 2.2). Some species have a relatively diffuse, broad search front with little uncolonized space between foraging hyphae. These species tend to extend relatively slowly from the base resource and it is thought that this 'exploitative' growth form is employed to maximise the chance of capturing small, homogeneously located resources (such as dead leaves and twigs) over relatively small spatial scales (Boddy 1999). *H. fasciculare* and *Stropharia* spp. are examples of species exhibiting this strategy (Boddy 1993, Donnelly & Boddy 1998). Other species have narrower search fronts and tend to extend rapidly from the base resource. This 'explorative' growth form allows foraging over relatively large areas, and is thought to be employed by species that degrade larger resources such as branches and tree stumps (Boddy 1999). These

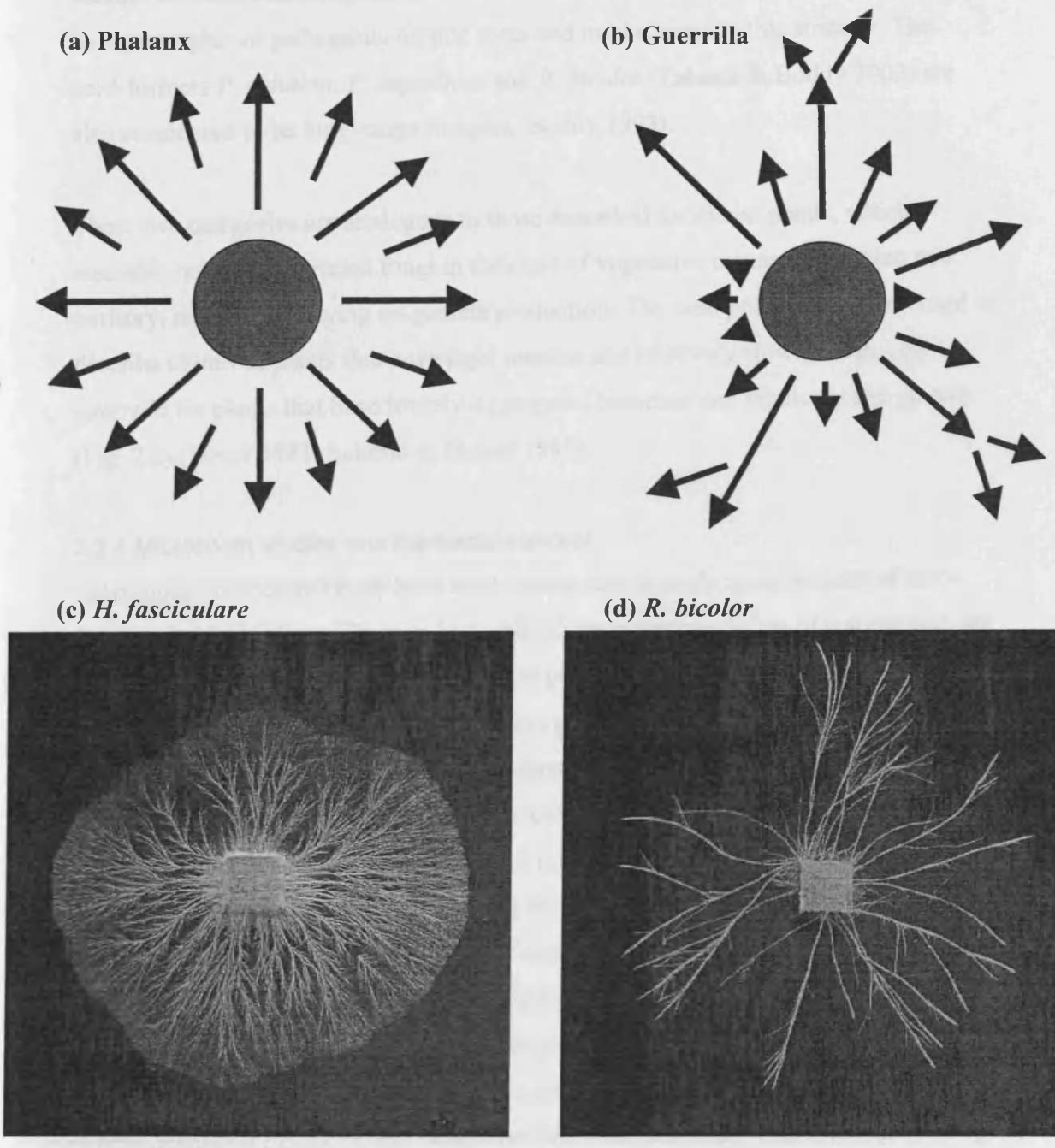


Figure 2.2. Phalanx (a) and guerrilla (b) foraging patterns of cord-forming fungi. Species that possess a phalanx growth form have broad search fronts and extend relatively slowly across soil, e.g. *Hypholoma fasciculare* (c). Species operating a guerrilla foraging strategy have narrower search fronts that usually extend more rapidly across soil, e.g. *Resinicium bicolor* (d).

resources tend to be heterogeneously distributed on the forest floor, and this strategy enables efficient searching for them. Rhizomorphic *Armillaria* species, which tend to be saprotrophic or pathogenic on tree roots and trunks, employ this strategy. The cord-formers *P. velutina*, *P. impudicus* and *R. bicolor* (Zakaria & Boddy 2002) are also considered to be long-range foragers (Boddy 1993).

These two categories are analogous to those described for clonal plants, which resemble non unit-restricted fungi in their use of vegetative means to colonize new territory, rather than relying on gamete production. The term *phalanx* has been used to describe clones of plants that have tight rosettes and relatively slow growth, and *guerrilla* for plants that have loosely-aggregated branches and relatively fast growth (Fig. 2.2) (Doust 1981, Schmid & Harper 1985).

2.2.4 Microcosm studies and the fractal concept

Laboratory microcosms have been used extensively to study many aspects of cord-forming fungal biology. The simplest microcosms comprise dishes of compressed soil to which cord-forming species are added as pre-colonized wood inocula. As subsequent fungal outgrowth is more or less restricted to the soil surface (i.e. it is two-dimensional), these systems allow the macroscopic mycelium of the fungi to be easily observed and subjected to image analysis techniques. Restricting the fungi to two-dimensional growth is not as artificial as it might at first appear – in forests cord-formers tend to extend horizontally along the interface between surface litter and mineral soil in search of new resources. Studying cord-forming fungi in microcosms has demonstrated that various biotic and abiotic factors can dramatically influence morphology and development of mycelial systems; these factors include wood inoculum size (Zakaria & Boddy 2002), wood nutrient status (Donnelly & Boddy 1997b, Harold *et al.* 2005), soil water potential (Dowson *et al.* 1989a, Donnelly & Boddy 1997a), soil nutrient status (Donnelly & Boddy 1998) and temperature (Dowson *et al.* 1989a, Donnelly & Boddy 1997a). The translocation and partitioning of nutrients, particularly using the radiotracer ^{32}P, has been extensively investigated using microcosm systems (Section 2.2.2). Microcosms have also permitted investigation of the response of cord systems to the sequential encounter of baits (i.e. new wood resources). These 'bait' studies have revealed that cord-formers vary in their responsiveness to newly-encountered resources. For example, *P. velutina*

preferentially colonizes larger resources, and only substantially modifies its subsequent foraging pattern when the new resource is considerably larger than the inoculum from which it is extending (Dowson *et al.* 1986, Hughes & Boddy 1996). *H. fasciculare*, by contrast, is more responsive, and substantially alters its mycelial morphology even when a newly-contacted wood resource is of a similar size to the inoculum (Dowson *et al.* 1986, Dowson *et al.* 1989b). These inter-specific differences may relate to the ecology of the two species on the forest floor. *P. velutina* is thought to be a longer-range forager than *H. fasciculare* (Section 2.2.3), and as such is likely to seek out larger resources (Boddy 1999). Microcosms are ideally suited to studies of combative interactions between cord-formers (Section 2.2.2) as interacting mycelia can be observed on the compressed soil surface. Caution should however be applied to the results of these studies, for although the mycelia of cord-formers tends to grow along the soil/litter interface, there is some evidence that different species occur at slightly different soil depths, which might be a mechanism of avoiding conflict (Cairney 2005). It should also be borne in mind that the soil microcosms used in studies of cord-former ecology are generally around 500 cm^2 in surface area and natural cord systems commonly attain much greater coverage (Section 2.2.2).

Early studies utilising the microcosm technique provided mainly qualitative observations, although some basic quantitative information such as measurement of the rate of mycelial extension (e.g. Dowson *et al.* 1986, Dowson *et al.* 1989b) was also gathered. Although this information provided new knowledge about the biology of cord-forming fungi, it was not until the introduction of digital image processing techniques that microcosm studies began to yield detailed quantitative information on the development and structuring of cord-former mycelia. Imaging software allows the rapid description of various measures of mycelial morphology and development, the most widely used of these being radial extension, hyphal coverage and fractal dimension (see below). Measurement of radial extension enables determination of the rate at which new substrate is colonized by a mycelial foraging front, although caution should be applied when interpreting radial extent data as rapid extension does not necessarily indicate a 'healthy' mycelium, and may in fact represent an 'escape' response (Boddy 1999). Hyphal coverage provides information on the extent to which an area occupied by mycelium is actually filled by fungal hyphae. This can be

estimated straightforwardly by counting the number of pixels making up a digital image (Boddy 1999).

Fractal analysis has become a standard tool for analysing mycelia of cord-forming fungi (Bolton & Boddy 1993, Donnelly *et al.* 1995). The fractal dimension of a system provides a measure of the way in which it permeates space, and is an extremely useful means of quantifying mycelial search patterns (Section 2.2.3). Fractal structures are not regular like Euclidian shapes, which possess whole number dimension values (i.e. straight lines = 1, flat surfaces = 2, volumes = 3), but are complex natural shapes that possess fractional dimensions. Fractal structures are self-similar; they can be broken down into smaller copies of themselves, and when a small section is magnified it appears identical to a larger part of the structure. Mycelia are approximately self-similar, albeit over a finite range of scales (Donnelly *et al.* 1995), and are thus suitable for fractal analysis. Two-dimensional mycelia possess fractal values between 1 and 2, the exact value of a system depending on its branching pattern and degree of space-filling. Fractal geometry is a sensitive measure of mycelial structure, and has revealed subtle yet significant changes in the mycelial pattern of cord-formers that were not detectable by biomass measurement (e.g. Donnelly & Boddy 1998).

The mycelia of cord-formers can be broadly divided into *surface/border fractal* or *mass fractal* types. Surface/border fractal mycelia are fractal only at exploratory search fronts and have plane-filled interiors, an example being young colonies of *H. fasciculare* (Boddy 1999). Mass fractal mycelia are fractal at growth fronts and have uncolonized spaces within the interior of the system (Obert *et al.* 1990). *R. bicolor*, for example, usually possesses a mass fractal mycelium relatively soon after emerging from wood inocula (Boddy *et al.* 1999).

Measures of radial extension and hyphal coverage of mycelium provide simpler information than does fractal geometry, but this information is nonetheless important. For example, the rate of radial extension indicates the speed at which mycelia are likely to reach new resources, and hyphal coverage quantifies the territory held by a fungus. All three measures, along with visual observation, provide different

information and should be used in conjunction with one another to achieve a detailed appreciation of mycelial growth and morphology (Boddy 1999).

2.3 Collembola

2.3.1 Introduction

Collembola are a group of microarthropods that were formerly considered by many taxonomists to be a primitive group of apterygote insects (Hopkin 1997). It is now established that their true phylogenetic position in the superfamily Hexapoda represents an early branch off the line that led to the higher insects (Fountain & Hopkin 2005), and molecular evidence suggests that this divergence occurred prior to that between insects and crustaceans (Nardi *et al.* 2003). Collembola can generally be distinguished from other endognathous hexapods by the presence of a furca (forked 'spring') on the underside of the fourth abdominal segment (Gullan & Cranston 1994). In some soil-dwelling genera, such as *Protaphorura*, the furca is reduced or absent (Hopkin 1997).

There are approximately 8,000 known species of collembola (Gullan & Cranston 1994), and many new species are described each year (Rusek 1998). Collembola are remarkable in their ubiquity, occurring in virtually all habitats from polar regions to desert and tropical ecosystems, and typically exist in abundance: 10^4–10^5 m^{-2} being the norm in most terrestrial ecosystems (Petersen & Luxton 1982). The majority of species inhabit soil and leaf litter, although arboreal species also occur. Classification of collembola is commonly based on their ecomorphology; *epedaphic* species live at the soil surface and on vegetation, *hemiedaphic* species are found in humic upper soil layers and leaf litter, and *euedaphic* species are permanent soil-dwellers. It is however difficult to fit all species into such broad categories (Hopkin 1997).

2.3.2 Feeding guilds

The composition of collembolan diets has been the subject of extensive research over the last 40 or so years. Traditional approaches involved gut content analyses of field-collected collembola and the study of food preferences in the laboratory. Using the former method it has been revealed that the proportion of fungal and plant-derived material in the guts of co-occurring collembola varies across species, which implies the existence of different feeding patterns (Anderson & Healey 1972). A wealth of

studies have employed laboratory food choice tests in an attempt to elucidate collembolan diets (e.g. Moore *et al.* 1985, Thimm & Larink 1995, Klironomos *et al.* 1999). These have used mainly fungal and algal species, and have often shown consistent hierarchies of food preference across collembola (Shaw 1988, Maraun *et al.* 2003, Section 2.4.1). Both approaches do, however, have drawbacks: gut content analyses are biased towards detecting relatively hard-to-digest materials and underestimate the proportion of readily digestible and transparent materials in the diet. Laboratory food choice studies employ only a limited range of the potential food sources that occur in the field, and, in addition, these substrates are often cultured on artificial media, which can alter their natural biochemistry.

Collembola feeding has also been investigated indirectly via assays of gut enzyme activity. A suite of different enzymes occur in most collembola species, including cellulase and chitinase to break down plant and fungal cell walls, respectively, and trehalase to digest contents of fungal hyphae (Urbášek & Rusek 1994, Berg *et al.* 2004). Results of enzyme assays imply that the majority of collembola are herbo-fungivorous, but that distinct feeding guilds do exist (Berg *et al.* 2004). It is unclear whether the gut enzymes are secreted by the animals themselves or produced by the gut microflora, which includes a range of bacteria as well as filamentous fungi (Thimm *et al.* 1998).

More recently, there have been promising developments in the use of biomarkers, such as fatty acids (Chamberlain *et al.* 2004, Ruess *et al.* 2004, Ruess *et al.* 2005), and stable isotopes of carbon and nitrogen (Section 2.5.3), to evaluate collembola diets. In one study a large proportion of forest floor collembola species were found to have nitrogen stable isotope signatures that suggested they predominantly fed on the soil microflora, particularly fungi (Chahartaghi *et al.* 2005).

Overall, it does appear that a large proportion of collembola species are mycophagous, and there is experimental evidence of feeding on saprotrophic, mycorrhizal and pathogenic fungi (Section 2.4). Predaceous, phytophagous and saprotrophic collembola species also exist. Many of the phytophagous collembola utilize algae growing on twigs and leaves as a resource (e.g. Verhoef *et al.* 1988).

Soil-dwelling predatory species feed mainly on enchytraeid worms, nematodes, rotifers and tardigrades (Hopkin 1997).

2.3.3 Ecological importance

Although collembola are abundant in most terrestrial systems their small size means they generally contribute little to total soil animal biomass and respiration. Despite this, simple microcosm experiments looking at rates of leaf litter decay with and without collembola have demonstrated stimulation of the decomposition process at densities equivalent to those found in the field (e.g. Ineson *et al.* 1982). This effect occurs in part when collembola feed saprotrophically on leaf litter. This, however, is believed to be of relatively minor importance compared to the indirect effects of collembola on the soil ecosystem, which are mediated via microorganisms. It is fungi and bacteria that perform the majority of decomposition and nutrient recycling in terrestrial ecosystems (Hopkins & Gregorich 2005); collembola can exert an important influence on microbial biomass and activity in a variety of ways. These include: comminution of litter, which increases the surface area available for microbial attack; dispersal of microbial propagules; modification of microbial community composition through selective feeding; and, perhaps most importantly, stimulation of microbial activity due to nutrient mobilization and low-intensity grazing (Section 2.4.2). Because of these indirect effects on the soil microflora collembola can act as important drivers of the decomposition of dead plant material, and hence of ecosystem nutrient cycling (Hopkin 1997). Collembola can also have major effects on the microstructure of soils; in some cases the entire humus layer may be composed of collembola faeces.

Collembola constitute an important food source for higher trophic levels in the soil food web; predatory mites in particular feed extensively on hemiedaphic and euedaphic collembola (Hopkin 1997). Many carabid and staphylinid beetles predate collembola; some species have evolved specialized mechanisms for capturing jumping species. Epedaphic collembola are also predated by vertebrates such as birds and amphibians (Hopkin 1997). In addition, collembola play host to many specialized parasites and pathogens (including fungi).

2.3.4 Study species

Folsomia candida is a euedaphic collembola and appears to be mainly mycophagous, having been shown to feed on both saprotrophic and mycorrhizal fungi (Klironomos *et al.* 1999). There is a strong bias towards the use of *F. candida* in laboratory and greenhouse experiments (Gange 2000); *F. candida* can be maintained with ease under laboratory conditions and, being parthenogenetic, attains large population sizes very rapidly. *F. candida*, which has been carried all over the world in plant pots and soil, occurs in systems with relatively high nutrient inputs, such as forests, agricultural soils, stream sides and caves, and is occasionally the dominant collembola species (Fountain & Hopkin 2005).

Protaphorura armata (formerly *Onychiurus armatus*) is a euedaphic species which, like *F. candida*, is easily-cultured and parthenogenetic, although it does not attain the same rate of population growth as *F. candida* under laboratory conditions. *P. armata* is a common collembola in forest ecosystems (Bengtsson & Rundgren 1983, Chauvat *et al.* 2003) and has been used extensively in laboratory studies with fungus as a food source (e.g. Bengtsson & Rundgren 1983, Shaw 1985, Hedlund *et al.* 1991).

Proisotoma minuta, a hemiedaphic species, is pigmented and has well-developed eyes, unlike the previous two species which are unpigmented and blind. *P. minuta* occurs commonly in a range of habitats and has been used in previous laboratory studies with EM fungi (Schultz 1991, Hiol Hiol *et al.* 1994) and saprotrophic microfungi (Draheim & Larink 1995) as food substrates.

2.4 Interactions between fungi and collembola in soil

2.4.1 Feeding preferences of mycophagous collembola

Although a large number of collembola species are predominantly mycophagous there seems to be remarkably little specialization in terms of which fungi they will actually consume. This lack of specialization is common among most soil faunal groups and forms part of what has been termed "the enigma of soil biodiversity" (Anderson 1975 cited in Maraun *et al.* 2003). The apparent paradox is that an extremely high diversity of organisms exists in the soil, and yet there does not appear to be a single decomposer organism that is specialized on just one dietary species (Maraun *et al.* 2003). Many experiments have shown that soil biota, including collembola, do exhibit

preferences under laboratory conditions, and there is growing evidence that feeding guilds exist in the field (Section 2.4.1). In laboratory choice tests, however, many species prefer the same food source (Maraun *et al.* 2003, Schneider & Maraun 2005). This is in contrast to what would be expected in a diverse ecosystem, where niche specialization is thought to allow such high diversity to persist. In above-ground terrestrial ecosystems, for example, specialization of insect herbivores on different host plants is thought to be one of the reasons for the high species diversity of this group (Strong *et al.* 1984). The lack of specialization of soil animals may be due to their movement being restricted by the complex nature of the soil habitat, which prevents access to preferred food substrates and thus favours an opportunistic lifestyle (Maraun *et al.* 2003).

Many studies of grazing on saprotrophic fungi (e.g. Poole 1959, Mills & Sinha 1971, Visser & Whittaker 1977, Whittaker 1981, Klironomos *et al.* 1992, Klironomos & Kendrick 1996) have found that collembola prefer to feed on dark pigmented fungi, a heterogeneous group (often termed Dematiacea) containing genera such as *Cladosporium*, *Curvularia* and *Alternaria*. These microfungi often make up a high proportion of fungi that are isolated from soil. This does not necessarily mean that they are the most abundant fungi in soils, as isolation using standard laboratory techniques selects for those species that produce the most spores rather than those that have the greatest biomass (Harley 1971). For example, the majority of fungi isolated in the laboratory from the bodies and faeces of the collembola *Hymenaphorura subtenuis* (formerly *Onychiurus subtenuis*) collected from woodland were species that sporulate prolifically in laboratory culture (Visser *et al.* 1987). In laboratory choice tests *H. subtenuis* fed preferentially on a range of dark-pigmented fungi rather than two unidentified basidiomycete species (Visser & Whittaker 1977); one of these basidiomycetes was subsequently found to be toxic to *H. subtenuis* (Parkinson *et al.* 1979).

It is difficult to prove which saprotrophic fungi are preferred in natural situations, and field studies of collembola food preference at the species level are scarce. Newell (1984a, b) investigated the influence of the collembola *Onychiurus latus* on *Marasmius androsaceus* and *Mycena galopus*, two basidiomycete decomposers of spruce (*Picea sitchensis*) litter, in a series of detailed laboratory and field experiments.

O. latus fed preferentially on *M. androsaceus* in laboratory choice tests, and in the field was more abundant in litter bags colonized by *M. androsaceus* than in those colonized by *M. galopus* (Newell 1984a). This caused the restriction of *M. androsaceus* to the uppermost litter horizon, where *O. latus* was not present in large numbers due to insufficient moisture content (Newell 1984b). Newell surmised that selective grazing by *O. latus* was primarily responsible for the vertical distribution of the two fungal species. These studies also demonstrate that the unpalatability of saprotrophic basidiomycetes observed by Parkinson *et al.* (1979) is not a universal phenomenon; more than 90% of fungal hyphae in the guts of field-collected *O. latus* were from basidiomycete fungi (Newell 1984a).

Preferential feeding on mycorrhizal species has also been demonstrated. Collembola feed on some arbuscular mycorrhizal (AM) fungi in the laboratory (Moore *et al.* 1985, Thimm & Larink 1995). In experiments where collembola were given saprotrophic microfungi as alternative food resources, however, these were always preferred to AM species (Klironomos & Kendrick 1996, Klironomos & Ursic 1998, Schreiner & Bethlenfalvay 2003, Gormsen *et al.* 2004, Tiunov & Scheu 2005). One laboratory study has demonstrated that there are negative reproductive consequences of feeding on AM fungi (Klironomos *et al.* 1999). In this particular study *F. candida* was offered a range of AM and saprotrophic fungi, and feeding preferences and reproductive output were investigated over two generations. *F. candida* consistently preferred the saprotrophic species *Alternaria alternata* and *Trichoderma harzianum* over six AM species, and after two generations only *A. alternata* yielded an egg production significantly higher than zero. This provides convincing evidence that saprotrophic fungi are preferred over AM species and it is questionable whether collembola graze extensively on AM fungi in the field (Gange 2000).

Food preferences of collembola for ectomycorrhizal (EM) fungi are less well understood. Laboratory choice tests have shown that *P. armata* displays a consistent hierarchy of preference for EM species when these are presented pairwise to collembola (Shaw 1988). In another study, *F. candida* and *P. minuta* preferred similar EM species (Schultz 1991). *P. minuta* preferentially feeds on the pathogenic fungus *Rhizoctonia solani* rather than a range of EM species (Hiol Hiol *et al.* 1994). When saprotrophic and EM species have been compared, preferences seem to relate to

individual fungal species rather than to a difference between saprotrophic and EM fungi *per se* (Shaw 1985, Shaw 1988). This contrasts with studies on AM fungi where saprotrophic alternatives are always preferred. Studies involving EM fungi have, however, employed basidiomycete saprotrophs rather than the short-lived saprotrophic microfungi that have been used in AM studies. This limits comparison between EM and AM systems.

In summary, it seems from laboratory and greenhouse studies that collembola grazing of EM and saprotrophic fungi is more widespread than grazing of AM fungi. Within the saprotrophic group there appears to be a preference for dark pigmented species and other soil microfungi, although experimental comparisons between these lower fungi and basidiomycetes are scarce. Selective feeding by collembola has potentially-important implications for ecosystem process rates; these are discussed in subsequent sections.

2.4.2 Effects of collembola on fungi

The most obvious effect that collembola have on soil fungal biomass is the direct removal of fungal hyphae through their grazing activities. In many situations, however, collembola actually have a stimulatory effect on fungal activity (Bengtsson & Rundgren 1983). Laboratory studies have documented that this stimulation only occurs at relatively low grazing densities; when the number of grazing animals is high then fungal activity is almost always reduced (Hanlon & Anderson 1979, Hanlon 1981). Stimulation of fungal activity has been demonstrated in response to low density collembola grazing on senescent fungal colonies (Hanlon 1981). This increased activity might occur because collembola feeding mobilizes nutrients, through faeces (Harley 1971) and urine (Verhoef *et al.* 1988) production, allowing fresh fungal growth. In addition, grazing by collembola can induce compensatory fungal growth when the fungal resource is patchily-distributed (Bengtsson *et al.* 1993).

Collembola may also aid the dispersal of fungal propagules. Spores of soil fungi often pass undigested through the collembolan gut (Poole 1959, Visser *et al.* 1987), and may then be deposited away from the site of ingestion. Spores and fragments of fungal hyphae may also be dispersed on external body parts (Visser *et al.* 1987). This

collembola-mediated dispersal is likely to benefit those species of fungi that fruit within the soil. Collembola may, however, have a negative effect on propagule dispersal in basidiomycete fungi, which usually fruit above ground, and rely on water and wind for spore dispersal. Collembola of the genus *Hypogastrura*, which are frequently found in basidiomycete fruit bodies, are capable of digesting a high proportion of ingested spores (Nakamori & Suzuki 2005) and may thus reduce the number of viable propagules that can be dispersed.

Grazing by collembola affects the morphology of fungal mycelia. In an experiment on agar plates the soil fungus *Mortierella isabellina* switched its morphology when grazed by *P. armata* (Hedlund *et al.* 1991). This switch involved the production of fast-growing fan-shaped sectors of hyphae with aerial mycelium. Interestingly, the switch occurred away from the actual site of grazing, where collembola were enclosed by a mesh barrier. This implies the presence of an 'unknown factor' that is transported from the grazed colony centre to the peripheral growth zone. The authors propose that the switch in growth form might represent an attempt by this fungus to escape the activities of grazers by penetrating pores and particles that they cannot access. In another study, grazing of the cord-forming basidiomycete *H. fasciculare* by the collembola *F. candida* in soil microcosms dramatically affected foraging morphology, although grazing by two other species (*P. minuta* and *Hypogastrura cf. tullbergi*) had little effect (Kampichler *et al.* 2004). *H. fasciculare* seemingly switched from a phalanx to a guerrilla foraging strategy when it was grazed; the authors attribute this, as do Hedlund *et al.* (1991), to a fugitive response by the fungus.

If mycophagous collembola exhibit preferential feeding in the field then they have the potential to affect not only biomass, morphology and dispersal of individual fungal species, but also to exert a selective force on fungal community composition. There is evidence from microcosm studies that collembola prefer early rather than late successional fungal species growing on leaf litter (Parkinson *et al.* 1979, Klironomos *et al.* 1992), perhaps because the latter group are more likely to possess secondary chemicals that deter feeding (Visser 1985). As late successional fungi are generally capable of more rapid degradation of substrates due to their powerful cellulolytic enzymes, collembolan enhancement of fungal succession might indirectly increase rates of decomposition (Visser 1985). In another microcosm study, however, there

was no evidence that collembola or oribatid mites increased the rapidity of fungal succession (McLean *et al.* 1996). Also, preferential feeding by *O. latus* on *M. androsaceus* over *M. galopus* (Section 2.4.1) might reduce decomposition rates, as the former species is a more rapid decomposer of spruce litter (Newell 1984b).

2.4.3 Mycorrhizas and collembola

A large number of laboratory and greenhouse studies have investigated the interactions between mycorrhizal fungi and collembola. This is presumably because: a) mycorrhizal symbioses may be one of the most important biological associations regulating community and ecosystem functioning; and b) these systems allow the indirect effects of collembola grazing on plant performance to be investigated, thus providing an obvious feedback to the above-ground system. AM and EM fungi appear to differ in their palatability to collembola (Section 2.4.1).

AM fungi colonize about 80% of land plants and are responsible for a large proportion of carbon flux into soil (Johnson *et al.* 2005). Under greenhouse conditions AM fungi usually increase primary productivity by supplying plants with nutrients, although these benefits are observed far less frequently in field situations (Klironomos & Kendrick 1993). Early grazing experiments demonstrated a bell-shaped response of plant performance, mediated via AM fungi, with increasing collembola density (Finlay 1985, Harris & Boerner 1990). The negative effects on plant growth at high collembola densities were attributed to impairment of AM functioning (Finlay 1985, Harris & Boerner 1990). In these studies, however, the AM species were the only inoculated fungi. Given that collembola prefer to feed on saprotrophic rather than AM fungi (Section 2.4.1) the results of grazing studies employing such a simplified fungal community must be called into question (Gange 2000). In similar experiments with the addition of leaf litter colonized by saprotrophic fungi, collembola effects on plant growth were always positive (Klironomos & Kendrick 1995, Schreiner & Bethlenfalvay 2003). This indirect stimulatory effect of collembola on plant growth could be because preferential feeding on saprotrophic fungi led to increased nutrient mobilization (Section 2.4.2) or because decreased competition for resources enabled AM fungi to increase their growth (Gange 2000, Tiunov & Scheu 2005). Collembola can also increase the dispersal of AM fungal hyphae which enables colonization of neighbouring non-AM plants (Klironomos & Moutoglis 1999).

Overall, it seems that collembola have positive effects on the AM-plant symbiosis when saprotrophic fungi are freely available, although some severing of AM hyphae does occur even when collembola are not extensively grazing these fungi (Klironomos & Ursic 1998). Negative effects on plants seem to be restricted to times when saprotrophic fungi are of limited availability; whether this occurs commonly in natural situations remains unknown. In fact, only one study has demonstrated collembola effects on an AM-plant symbiosis in the field (Johnson *et al.* 2005). In this study the collembola *P. armata* were added to mesh-covered soil cores that allowed penetration of fungal hyphae but not plant roots. Phospholipid fatty acid analysis revealed collembola grazing on AM fungi, and this reduced respiration (of ^{13}C) from the mycorrhizosphere. The authors propose that this demonstrates disruption of carbon flow through AM networks by collembola (Johnson *et al.* 2005). The mesh cores did not, however, include a leaf litter component, and this may have allowed grazing on preferred saprotrophic fungi rather than AM species. Collembola-mediated effects on AM functioning may therefore have been exaggerated.

Experiments investigating the indirect effects of collembola on plants forming EM symbioses are fewer than for AM fungi, but nevertheless report similar patterns. Ek *et al.* (1994) found that growth of the EM fungus *Paxillus involutus* was impeded at high collembola densities, whereas lower densities stimulated fungal growth and caused increased nitrogen uptake by pine (*Pinus contorta*) seedlings. In a microcosm experiment, using a more complex soil faunal community (which included collembola as a dominant group) and several species of EM fungi, fauna caused a significant increase in birch (*Betula pendula*) and pine (*Pinus sylvestris*) biomass, even though they dramatically reduced EM biomass compared to fauna-free controls (Setälä 1995). Setälä suggests that a relatively complex faunal assemblage enhances nutrient mineralization to such an extent that reduced mycorrhizal infection is of little consequence.

In one study, a species of EM fungus, *Laccaria bicolor*, was found to be pathogenic on collembola, passing on nitrogen gained from the animals to its plant symbiont (Klironomos & Hart 2001). In this microcosm study *L. bicolor* reduced *F. candida* populations by 95% by immobilising the animals before infecting them with hyphae.

These workers labelled *F. candida* with ^{15}N and found that up to 25% of plant (*Pinus strobus*) nitrogen was derived from collembola in the presence of *L. bicolor*, and that this caused a stimulation of plant biomass. This study presents startling evidence that forest nitrogen cycling may be more complex than had previously been imagined.

2.5 Stable isotopes in fungi and collembola

2.5.1 Introduction to stable isotope techniques

The advent of stable isotope techniques has led to a remarkable increase in our knowledge of the functioning of communities and ecosystems. In particular, soil ecology has benefited from the ability of stable isotopes to allow us to see inside the 'black box' of the opaque soil environment. Energy flows and trophic linkages are much better understood as a result. In this section the technicalities of the stable isotope approach are briefly introduced, and the potential of stable isotope techniques to further our understanding of fungus-collembola interactions is considered.

Measures of stable isotope abundance of a given element reflect the ratio of a heavy isotope to that of a much commoner light isotope. The isotope pairs most commonly used in ecological studies are $^{13}C/^{12}C$ and $^{15}N/^{14}N$. Stable isotope ratios are usually expressed in delta (δ) units as parts per thousand (‰), relative to an international standard, as the absolute abundance of the heavy isotope is typically very small. For example, the international standards for carbon (the marine fossil PeeDee Belemnite) and nitrogen (atmospheric N_2) have an absolute abundance of only 1.12% and 0.37%, respectively (Dowson & Brooks 2001).

Delta (‰) is calculated using the following equation:

$$\delta = \left(\frac{R_{sa}}{R_{std}} - 1 \right) \text{ x } 1000$$

where R is the ratio of heavy to light isotope and R_{sa} and R_{std} are the ratios in the sample and the standard, respectively.

The use of carbon and nitrogen stable isotopes in ecological studies arises from the natural variation in $\delta^{15}N$ and $\delta^{13}C$ that occurs between components of ecosystems due to discrimination, or *fractionation*, for or against the heavy isotope during biological and biogeochemical processes. When δ is positive (i.e. when a sample has more of the heavy isotope than the international standard) it is said to be *enriched.* Conversely, when δ is negative a sample is said to be *depleted* in the heavy isotope compared to the standard. For example, plants are depleted in ^{13}C compared to atmospheric CO_2, due to discrimination against the heavy isotope during CO_2 diffusion into leaves and during enzymatic reactions (Lajtha & Michener 1994).

There are two types of stable isotope experimentation: 1) natural abundance studies, which use naturally-occurring levels of $\delta^{15}N$ and $\delta^{13}C$ and rely on differences due to fractionation to elucidate differences between organisms and systems; and 2) labelling, or tracer, studies, in which an added pulse of enriched nitrogen or carbon is followed within an organism (e.g. partitioning within a fungal mycelium) or through a food web or ecosystem.

2.5.2 Stable isotopes in fungi

The use of stable isotope techniques has improved our understanding of fungal ecophysiology. Studies have shown that the uptake of carbon and nitrogen results in considerable isotopic fractionation, and that this varies across fungal species and nutrient substrates (Fernandez & Cadisch 2003, Henn & Chapela 2004, Hobbie *et al.* 2004). Recently, naturally-occurring variation in the abundance of ^{13}C and ^{15}N has been used to infer the trophic status of forest basidiomycetes (e.g. Hobbie *et al.* 1999, Högberg *et al.* 1999, Kohzu *et al.* 1999). In these studies, analysis of sporocarps (fruit bodies) of a large number of EM and saprotrophic basidiomycete species has revealed that these two groups of fungi can be separated based on their stable isotope signatures. Saprotrophs are generally enriched in ^{13}C and depleted in ^{15}N compared with EM species. These differences are thought to be due to variation in isotopic signatures in their substrates. For example, the dead wood and leaf litter used by saprotrophs are enriched in ^{13}C compared with the soluble sugars derived from plants by EM species (Kohzu *et al.* 1999). Although variation in isotopic signatures across ecosystems and geographical regions obscures these differences (Trudell *et al.* 2004), at a local scale they can be used to infer trophic status where it is unclear. In another

example, ^{15}N analysis revealed that co-existing subarctic plants colonized by EM, AM and ericoid mycorrhizal fungi use different soil nitrogen sources, demonstrating niche differentiation (Michelsen *et al.* 1996).

Studies of nutrient translocation and partitioning in fungi have generally employed radioisotopes (Section 2.2.2); these have the advantage that systems can be monitored non-destructively using photon-counting equipment. There is, however, no commonly-occurring radioisotope of nitrogen, and consequently little is known about translocation of this nutrient in comparison with phosphorus (e.g. Wells & Boddy 1990a, Lindahl *et al.* 1999) and carbon (Wells *et al.* 1995, Wells & Boddy 2002), which have readily-available radioisotopes (^{32}P, ^{33}P and ^{14}C).

2.5.3 Stable isotopes in soil food webs

Stable isotopes, particularly ^{15}N, have been especially useful in elucidating trophic positions within soil animal food webs (e.g. Caner *et al.* 2004, Schmidt *et al.* 2004). Stable isotopes of carbon and nitrogen indicate the assimilation of these nutrients into soil animals, and attempts to quantify energy flows through food webs can be made as a result. Natural abundance studies have revealed a typical shift in $\delta^{15}N$ of 3.4‰ with an increase of one trophic level (see Eggers & Jones 2000). The naturally-occurring variation in $\delta^{15}N$ between different potential food substrates is thus potentially useful in assessing collembola diet; this was investigated in the field by Chahartaghi *et al.* (2005), as outlined in Section 2.3.2. Laboratory studies have, however, demonstrated that fractionation between fungal substrates and collembola grazers varies widely depending on species identity, collembola life stage and quality (C:N ratio) of fungal diet (Ruess *et al.* 2004, Scheu & Folger 2004, Haubert *et al.* 2005). We need to understand more fully the causes of variation in ^{15}N fractionation between food substrates and their collembola grazers before this technique can be employed with confidence to assess the diet of field-collected animals.

Chapter 3. General methods

3.1 Introduction and study organisms

In this chapter the general methodologies used across the thesis are described. The more specialized methodologies used in the networking (Chapter 6) and nitrogen flux (Chapter 7) studies are described within those chapters.

The study organisms used (Table 3.1) were selected on the basis that they are common to abundant components of temperate wooded ecosystems (Chapter 2), and are therefore likely to co-occur.

Table 3.1. Species of cord-forming fungi and collembola used in experimental studies.

Species and Authority	Isolate	Source
a) Fungi		
Phanerochaete velutina (DC.: Pers.) Parmasto	KC1685	Cardiff University Culture Collection
Hypholoma fasciculare (Huds.: Fr.) Kummer	DD2	Cardiff University Culture Collection
	GTWV2	Isolated from fruit body; Trelleck Common, Tintern, UK. National Grid Ref.: SO517069. 27.08.2003
Resinicium bicolor (Abertini & Schwein.: Fr.) Parmasto	RB1	University of Aberdeen, UK
Phallus impudicus (L.) Pers.	JHY4	Isolated from cords at base of fruit body; Trelleck Common (location as above). 27.08.2003
b) Collembola		
Folsomia candida Willem	-	CEH Lancaster, UK
Protaphorura armata Tullberg	-	National Environmental Research Institute, Silkeborg, Denmark
Proisotoma minuta Tullberg	-	CEH Lancaster, UK

3.2 Preparation of fungal inocula

Fungal species (Table 3.1a) were routinely subcultured on 2% malt extract agar (MEA: 20 g^{-1} Munton & Fison spray malt, 15 g^{-1} Lab M agar no. 2) in non-vented 9 cm diam. Petri dishes, and kept at 20°C in the dark.

Wood inocula for experiments were colonized on 70 ml MEA in 14 cm diam. vented Petri dishes (20 wood blocks per dish). The agar had been preinoculated with the required fungal species and incubated at 20°C in the dark until the agar surface was covered with mycelium (10 d all species except *P. impudicus*, which required 20 d). Beech (*Fagus sylvatica*) wood blocks (2 x 2 x 1 cm unless stated otherwise) were placed on the agar, ensuring good contact with the medium, and incubated in darkness. The length and temperature of incubation was variable across studies and is detailed in individual chapters. Wood blocks had been obtained from freshly sawn planks (Wentwood Timber Centre, Wentwood, UK) and stored at -18°C until required. They were soaked overnight in de-ionized (DI) water and then autoclaved at 121°C for 30 mins in sealed autoclave bags and reautoclaved 24 h later, for sterilization. Following colonization, and in readiness for experimental use, the inoculum blocks were removed from Petri dishes and scraped free of adhering mycelium and agar using a spatula.

3.3 Collembola culturing and extraction

Collembola species (Table 3.1b) were cultured at room temperature (18-22°C) in 0.9 l plastic boxes containing a 9:1 plaster of Paris (Minerva Dental Ltd., Cardiff): activated charcoal (Sigma, Poole, UK) mixture in their bases, and with holes pierced in their lids to facilitate aeration. Culture boxes were moistened with DI water and supplied with Dried Baker's Yeast (*Saccharomyces cerevisiae*; Spice of Life, Cardiff) on a weekly basis. New culture boxes were set up and inoculated with adult collembola when old boxes became heavily contaminated with frass.

Collembola were extracted from cultures using a series of stacked metal sieves of known pore size (Nickel-Electro Ltd., Weston-super-Mare, UK). Collembola were added to the uppermost sieve and allowed to self-sort into size classes by moving through sieves of progressively smaller pore size for 5 minutes. The body width ranges used are given in individual chapters. After sieving, collembola of the desired

body width were stored in food-free culture boxes for 1 d to reduce the effect of previous food source on feeding activity. The numbers required for inoculation were then collected using an electrical Pooter (aspirator). This process resulted in negligible collembola mortality.

3.4 Preparation, inoculation and incubation of soil microcosms

Soil (loam) was collected to 20 cm depth from mixed deciduous woodland in the Coed Beddick Inclosure, Tintern, UK (National Grid Ref.: SO528018). After removing surface litter, soil was sieved through 10 mm mesh on site, and stored in lidded plastic bins. Prior to use soil was air-dried for 21 d in large plastic trays, frozen (-18°C) for 1 d to remove soil fauna, and then sieved through 4 mm and 2 mm metal mesh to remove organic material and stones. Dry soil was then thoroughly mixed with DI water to attain a soil matric potential of -0.012 MPa, which was determined by the filter paper method (Appendix I; Fawcett & Collis-George 1967). Rewetted soil was compacted to 4 mm depth in 24 x 24 cm lidded bioassay dishes (Nunc-Gibco, Paisley, UK; 200 g wet soil per dish). An inoculum wood block was pushed firmly into the centre of each dish; dishes were then sealed in polythene zip lock bags and incubated at 19°C ± 1°C in stacks in the dark. All experimental treatments were randomly allocated within stacks, and the position of each stack was altered every week to control for moisture and temperature gradients that existed within the controlled temperature (CT) room. Soil microcosms were weighed and rewetted back to the original weight with a fine mist of DI water every 7 d. Microcosms remained free of contaminating soil fauna for the duration of the experiment; although hyphae of soil fungi were periodically observed on the surface of the non-sterile soil these did not visibly impede the growth of the inoculated cord-former.

Collembola required for grazed treatment microcosms were added to uncolonized soil evenly around the margin of each microcosm. The number of collembola that were inoculated, and the size and age of mycelia at the time of this inoculation, are detailed in each chapter.

3.5 Image capture and analysis

Digital images of mycelia were captured using a Hitachi KP-MI monochrome CCD video camera with a Canon TV macro-zoom lens, connected to a computer, and

stored using a Synapse frame store (Synoptics, Cambridge, UK). Microcosms were illuminated using a circular fluorescent bulb with opalescent diffuser fitted around the camera, which was mounted 102 cm above the microcosms. A 60W tungsten spot lamp was placed beside the microcosms to enhance light levels.

SEMPER 6 for Windows was used to pre-process and analyse images, using a modified version of the method described by Boddy *et al.* (1999). Image pre-processing involved the following stages:

i) *Grading.* Each pixel in the 512 x 512 grid was graded on a scale of 0 (black) to 255 (white).

ii) *Calibration.* Each image was calibrated from the pixel length of a line drawn across the internal diameter of bioassay dish (length of line 22.6 cm). This allowed SEMPER to convert line and area measurements from pixel values into cm and cm^2, respectively.

iii) *Windowing.* Wood block inocula and dish edges were removed by manually masking out these areas of the image using a working image screen display.

iv) *Conversion to binary.* A threshold level for pixel shade values was manually determined for each image. Pixel values higher than this threshold value were converted to white (mycelium) and lower values were converted to black (soil).

v) *Particle analysis.* Particles were defined as regions of eight or more connected white pixels. Particles of 20 or fewer pixels (usually representing reflective soil or aggregations of collembola) were automatically removed. Binary images were saved in SEMPER file format at this stage.

After pre-processing, the hyphal coverage and radial extension of binary images was determined. Hyphal coverage (cm^2) was given by the number of white pixels in a binary image and radial extension (cm) was the mean length of eight lines drawn from the inoculum to the mycelial margin; lines radiated from a central point at 45° angles to each other, aligned on a grid overlaid by SEMPER.

Mass fractal dimensions (D_{BM}) were estimated from binary images in Benoit 1.31 (Trusoft International, Inc., St. Petersburg, USA) using the box count method (Obert *et al.* 1990, Donnelly *et al.* 1995). The Benoit software overlaid each image with grids

of square boxes and the number of boxes intersecting white pixels (i.e. mycelium) was recorded. For a series of boxes of side length s pixels the number of boxes (N(s)) intersected by the mycelium is related to the fractal dimension of the mycelium (D_B) by the power law

$$N(s) = cs^{-D_B}.$$

Two types of box contribute to the total number of boxes intersected (N(s)); interior boxes ($N_{interior}$) that are contained wholly within the fractal set (i.e. contain white pixels only), and border boxes (N_{border}) that contain at least one white pixel and contain or adjoin at least one black pixel. Thus,

$$N(s) = N_{border}(s) + N_{interior}(s).$$

For perfect fractal structures (i.e. structures that can be broken down into smaller copies of themselves) the power law holds for all box sizes. Natural structures tend, however, to be self-similar only over a finite range of box sizes, with departure from the power law occurring at very large box sizes (Donnelly *et al.* 1995). Hence, a restricted range of box side lengths (1-61 pixels) was used. Mass fractal dimension is estimated by regression of the linear portion of a plot of [log $N(s)$ - ½$N_{border}(s)$] against log s, giving a gradient of $-D_{BM}$.

3.6 Wood inoculum decay rate

Wood inoculum decay rate (mg cm^{-3} d^{-1}) was estimated from change in density over the duration of each experiment. Initial densities (oven dry weight / volume before drying; mg cm^{-3}) were estimated from a random subsample (5-10 replicates) of inoculated wood blocks of each fungal species, and final densities from actual inocula in microcosms. In all instances wood blocks were scraped free of adhering mycelium, frass and soil using a spatula, measured using callipers and then dried at 80°C for 7 d prior to weighing.

3.7 Statistical analyses

Radial extension was compared across treatments until the time point when mycelia in at least one treatment first made contact with the dish edge. Analysis of Covariance (ANCOVA; General Linear Model; Minitab Statistical Software, Release 13.31) was

performed, with time (days after collembola addition) as a covariate; data met assumptions of linearity, being log transformed where necessary. Significant ($P \leq 0.05$) time*treatment interaction effects were investigated further using one-way Analysis of Variance (ANOVA) and Tukey's pairwise comparisons on extension rates.

Hyphal coverage and fractal dimension data, which were non-linear, were analysed by performing Repeated-Measures ANOVA (RM ANOVA; SPSS, Release 12) with grazing treatment as main effect and time as sub-factor. Treatment data met the assumptions of RM ANOVA, being normally distributed (Kolmogorov-Smirnov Test) and equal in variance (Levene's Test). Huynh-Feldt adjusted degrees of freedom and *P*-values were used where assumptions of sphericity were violated (Mauchly's Test of Sphericity). Again, significant time*treatment interactions were investigated further using one-way ANOVA and Tukey tests on individual time points.

Wood decay rates were compared across treatments using one-way ANOVA and Tukey tests; data were normally distributed (Anderson-Darling Test) and variances were equal (Levene's Test).

Chapter 4. Effects of *Folsomia candida* grazing intensity on the mycelial morphology of *Hypholoma fasciculare*, *Phanerochaete velutina* and *Resinicium bicolor*

4.1 Introduction

Cord-forming basidiomycete fungi colonize discrete, patchily distributed woody resources and produce large, persistent but dynamic mycelial networks at the soil/litter interface of forest ecosystems (Section 2.2.2). Cord persistence indicates some resistance to grazing by soil microarthropods such as collembola and oribatid mites, which often occur at high densities in forest soil (Petersen & Luxton 1982, McLean *et al.* 1996). Indeed, it is vital for the functioning of cord-forming fungi that spatially-separated parts of mycelia remain inter-connected; cords translocate nutrients and carbon around the mycelium, which enables rapid colonization and breakdown of newly-encountered resources (Boddy 1999). If cords were severed by grazing animals on a regular basis, this would have costly implications for the functioning of the mycelium.

There are numerous studies on interactions between collembola and fungi (e.g. Booth & Anderson 1979, Klironomos *et al.* 1999, Klironomos & Hart 2001, Johnson *et al.* 2005, Section 2.4), but little on the effects of grazing on spatial changes in mycelia. In fact, as far as I am aware there is only one study in the primary literature in which the effects of invertebrate grazing on the foraging ecology of cord-forming fungi have been investigated. In that study, radial extension, hyphal coverage and fractal dimension of the cord-forming basidiomycete *Hypholoma fasciculare* were dramatically affected by *Folsomia candida* grazing, although two other collembola species had little effect (Kampichler *et al.* 2004). Effects were dependent on grazing density of *F. candida*; radial extension and hyphal coverage decreased monotonously with increasing number of grazers (Kampichler *et al.* 2004).

It is not yet known whether: (1) there are different effects of grazing on different cord-forming species; (2) effects of grazing always increase with rising grazer

density; (3) grazing occurs evenly within mycelia or at particular mycelial regions; and (4) grazing at initial stages of outgrowth has different effects to later grazing. These questions are addressed in this chapter in two experiments investigating grazing by *F. candida* on the cord-forming basidiomycetes *H. fasciculare*, *Phanerochaete velutina* and *Resinicium bicolor* in soil microcosms. Four specific hypotheses were tested: (1) since *H. fasciculare* has the least substantial cords and is the most responsive of the three fungi to encounter with new resources (Dowson *et al.* 1986, Dowson *et al.* 1989b), it will be more responsive to grazing than the longer-range foragers *P. velutina* and *R. bicolor*; (2) the impacts of grazing will increase with rising *F. candida* density (3) *F. candida* will graze preferentially on individual hyphae as they are relatively thin walled and without the protective rind of cords; and (4) mycelium newly emerging from resources will be more susceptible to grazing than established systems as it comprises a greater proportion of individual hyphae and no well developed extra-resource mycelium to rely upon.

4.2 Materials and methods

4.2.1 Experiment 1: effect of grazing intensity on young extra-resource mycelia

H. fasciculare, *R. bicolor* and *P. velutina* were subjected factorially to three grazing densities (10, 20 and 40 individuals per microcosm) plus collembola-free controls. Each of the 12 treatment combinations was replicated five times, giving a total of 60 soil microcosms. Prior to *F. candida* addition, the fungi were allowed to grow out from wood inocula until 50% of the fungal mycelia (i.e. 10 microcosms per species) had reached a diam. of 8 cm; this was after 6, 10 and 16 d for *P. velutina*, *H. fasciculare* and *R. bicolor*, respectively. Colonies used were all initially fairly radially symmetrical.

Images were captured (see below) immediately prior to collembola addition, and then after 2, 4, 10 and 15 d for *P. velutina*, 2, 5 and 10 d for *H. fasciculare*, and 4 d for *R. bicolor*. No measurements were made after 10 d as mycelia had by then made contact with dish edges.

4.2.2 Experiment 2: effect of grazing prior to and during establishment of extra-resource mycelia

F. candida (40 individuals per microcosm = constant number treatment) were added to microcosms at the same time as wood block inocula or when 50% of the mycelia of a particular species had reached 11 cm diam. This occurred 19 and 22 d after wood inocula addition for *R. bicolor* and *H. fasciculare*, respectively. Since mycelia of different species grow at different rates and fill space to different extents, collembola density was also standardized across fungal species by adding one *F. candida* per cm^2 of area that the mycelium in each microcosm occupied (for *H. fasciculare*: 80 ± 14 individuals; for *R. bicolor* 69 ± 5 individuals), and in another series of treatments by adding one *F. candida* per cm^2 of hyphal coverage (for *H. fasciculare* 83 ± 12 individuals; for *R. bicolor* 16 ± 3 individuals). Hyphal coverage was determined by analysis of images captured immediately prior to collembola addition (see below). Area covered by mycelium was determined from images by electronically joining tips of the main mycelial cords, and determining the area of the entire space enclosed by this boundary line.

There were five replicates in the treatments with no previous outgrowth, and seven in the 11 cm diam. treatments of *H. fasciculare* and *R. bicolor*. Due to erratic outgrowth of *P. velutina* collembola were only added to no previous outgrowth treatments for this species. Images were captured immediately prior to collembola addition and 1, 2, 4, 8, 12, 16 and 20 d afterwards. After 20 d images were captured every 10 d until microcosms were harvested 80 d after collembola addition.

4.2.3 Preparation of study organisms

P. velutina (KC1685), *H. fasciculare* (DD2) and *R. bicolor* (RB1) wood blocks were prepared as described in Section 3.2, with the following exceptions. Wood blocks used in Experiment 1 were 2 x 2 x 0.5 cm ($2cm^3$; i.e. half the usual volume) and were incubated at 20 ± 1°C for 90 d. Wood blocks used in Experiment 2 were incubated for 45 d, except in the case of 11 cm *R. bicolor* systems where they were incubated for 150 d due to poor colonization at 45 d.

F. candida were cultured and extracted as described in Section 3.3. Individuals of body width 250-400 μm were used in the experiments.

4.2.4 Preparation and inoculation of soil microcosms

Soil microcosms were prepared and maintained as described in Section 3.4, with the exception that microcosms in Experiment 1 consisted of 14 cm diam. Petri dishes (85 g wet soil per dish) instead of the usual 24 x 24 cm bioassay dishes.

4.2.5 Image capture and analysis

Digital images of mycelia were captured and analysed for radial extension and hyphal coverage as described in Section 3.5. The smaller size of Experiment 1 microcosms meant that the camera and associated illumination could be mounted closer to the dishes (85 cm instead of the usual 102 cm). This reduced distance meant that supplementary lighting from the spot lamp was not required. For Experiment 1 the calibration line used in image analysis was the internal diameter of a Petri dish (13.6 cm). In addition, high resolution colour images were captured periodically for all species using a Sony DSC-P71 Cyber-shot digital camera.

4.2.6 Wood inoculum decay rate

The decay rate of Experiment 2 wood inocula was determined as described in Section 3.6.

4.2.7 Statistical analyses

Mycelial radial extension, hyphal coverage and wood inoculum decay rates were compared across grazing treatments as described in Section 3.7.

4.3 Results

4.3.1 Experiment 1: effect of grazing intensity on radial extension rate and hyphal coverage of mycelia

The radial extent of mycelia increased linearly with time. With all fungal species there was a decrease in radial extension rate of colonies with increase in the number of collembola added (Fig. 4.1). With both *H. fasciculare* (Fig. 4.1a) and *P. velutina* (Fig. 4.1c) radial extension rate was significantly lower in the 40 collembola treatment than in the ungrazed treatment, with 10 and 20 collembola treatments having intermediate values (ANCOVA; *H. fasciculare*: $F_{3,72} = 5.73$, $P = 0.001$; *R. bicolor*: $F_{3,52} = 4.72$, $P = 0.005$). In the 40 collembola treatment extension rates were 62% and 45% of those of ungrazed controls for *H. fasciculare* and *P. velutina*, respectively. There was no

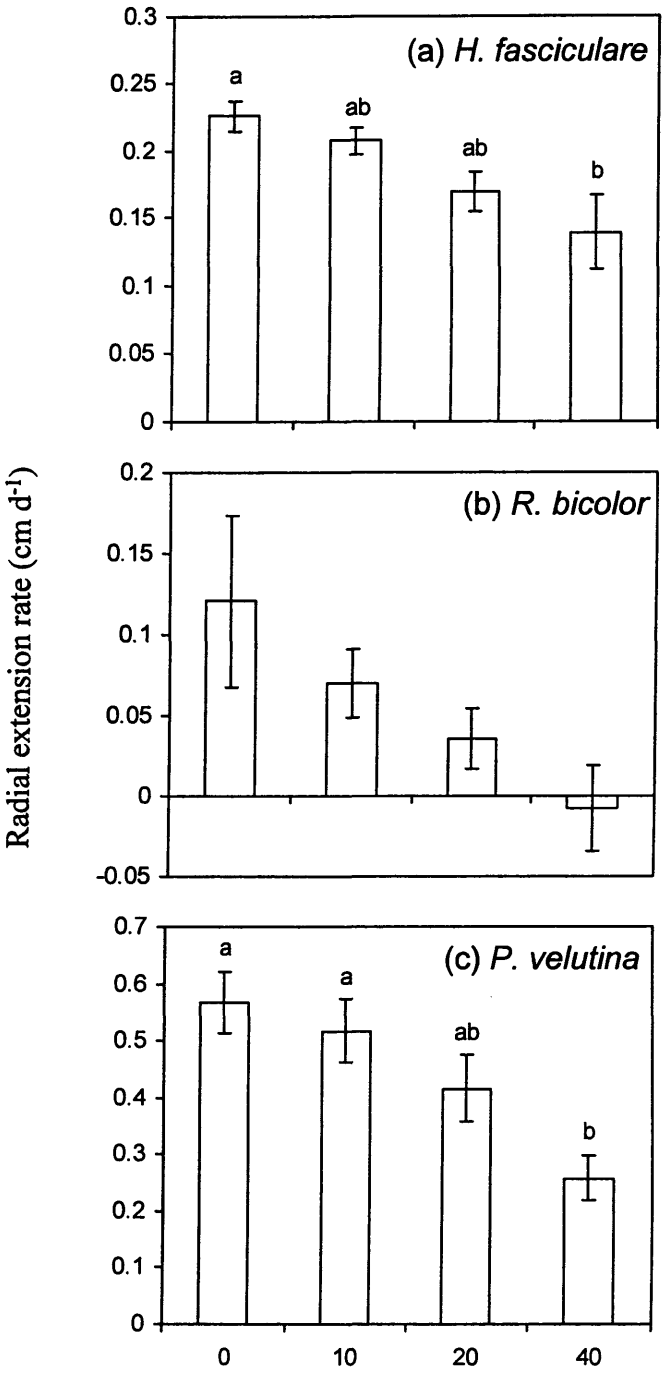


Figure 4.1. The effect of *F. candida* grazing density on mean (± s.e.) radial extension rates of *H. fasciculare* (a), *R. bicolor* (b) and *P. velutina* (c) in Experiment 1. Different letters above bars indicate significant ($P \leq 0.05$) differences between means (one-way ANOVA and Tukey). Note different y-axis scales.

significant effect of grazing on the extension rate of *R. bicolor* (Fig. 4.1b; $F_{3,32} = 0.98$, $P = 0.42$).

With all fungal species (Fig. 4.2), the greater the collembola density the lower the hyphal coverage; though differences were not significant for *P. velutina* (Fig. 4.2c; RM ANOVA; $F_{12,64} = 0.97$, $P = 0.49$). There were highly significant time*grazing treatment interaction effects on hyphal coverage of *H. fasciculare* ($F_{9,48} = 4.84$, $P < 0.001$) and *R. bicolor* ($F_{3,16} = 17.5$, $P < 0.001$). After 10 d of grazing 20 and 40 collembola treatments had lower hyphal coverage than the *H. fasciculare* ungrazed control treatment (Fig. 4.2a). With *R. bicolor* differences between the treatments were significant with 40 and 20 collembola after 4 d (Fig. 4.2b). No measurements were made after 15 d as by then the presence of juveniles confounded the original densities of collembola.

4.3.2 Experiment 1: effect of grazing intensity on mycelial morphology

The morphology of mycelia was dramatically altered by grazing. With all three fungal species effects were dependent upon collembola density; the highest densities of collembola resulted in the most striking differences (Figs 4.3, 4.4, 4.6). Mycelial margins in grazed systems of *H. fasciculare* became progressively less even than in ungrazed controls, with growth ceasing in some sectors while continuing in others (e.g. Fig. 4.3h). At collembola densities of 20 and 40 per microcosm, fanning of hyphae had occurred by 10 d at various points around mycelial margins (Fig. 4.3i, l). Diffuse white mycelium around colony margins tended to be heavily grazed, whereas yellowed cords towards the centre of the mycelia remained wholly intact.

Morphological changes were evident in *P. velutina* mycelia after 2 d of grazing: the tips of cords started fanning, and lateral cords became more branched (Fig. 4.4d, g, j). At 10 d there was evidence of cords being grazed, particularly at necrotic regions, in all collembola-grazed treatments. By this time all cord tips were well fanned in the highest density treatment (Figs 4.4l, 4.5), and aerial hyphae (in addition to those adpressed to the soil) developed towards the mycelial margins.

Discrete patches of *R. bicolor* mycelium close to the wood inoculum had been grazed 4 d after collembola addition, these patches being larger with increasing collembola

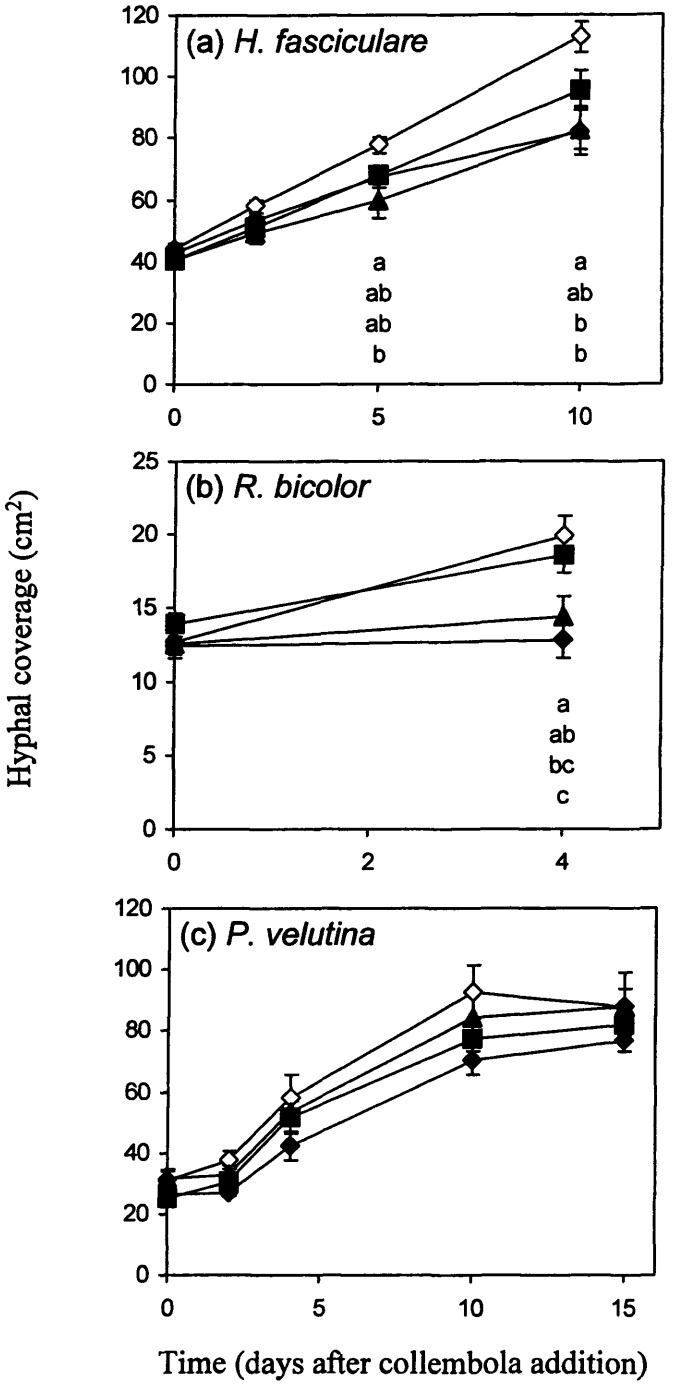


Figure 4.2. The effect of *F. candida* grazing density on mean (± s.e.) hyphal coverage of *H. fasciculare* (a), *R. bicolor* (b) and *P. velutina* (c) in Experiment 1. Number of animals added was 0 (◇), 10 (■), 20 (▲) or 40 (◆). Different letters below time points indicate significant ($P \leq 0.05$) differences between means at that time point (one-way ANOVA and Tukey). Letters are displayed in the same order as treatment means.

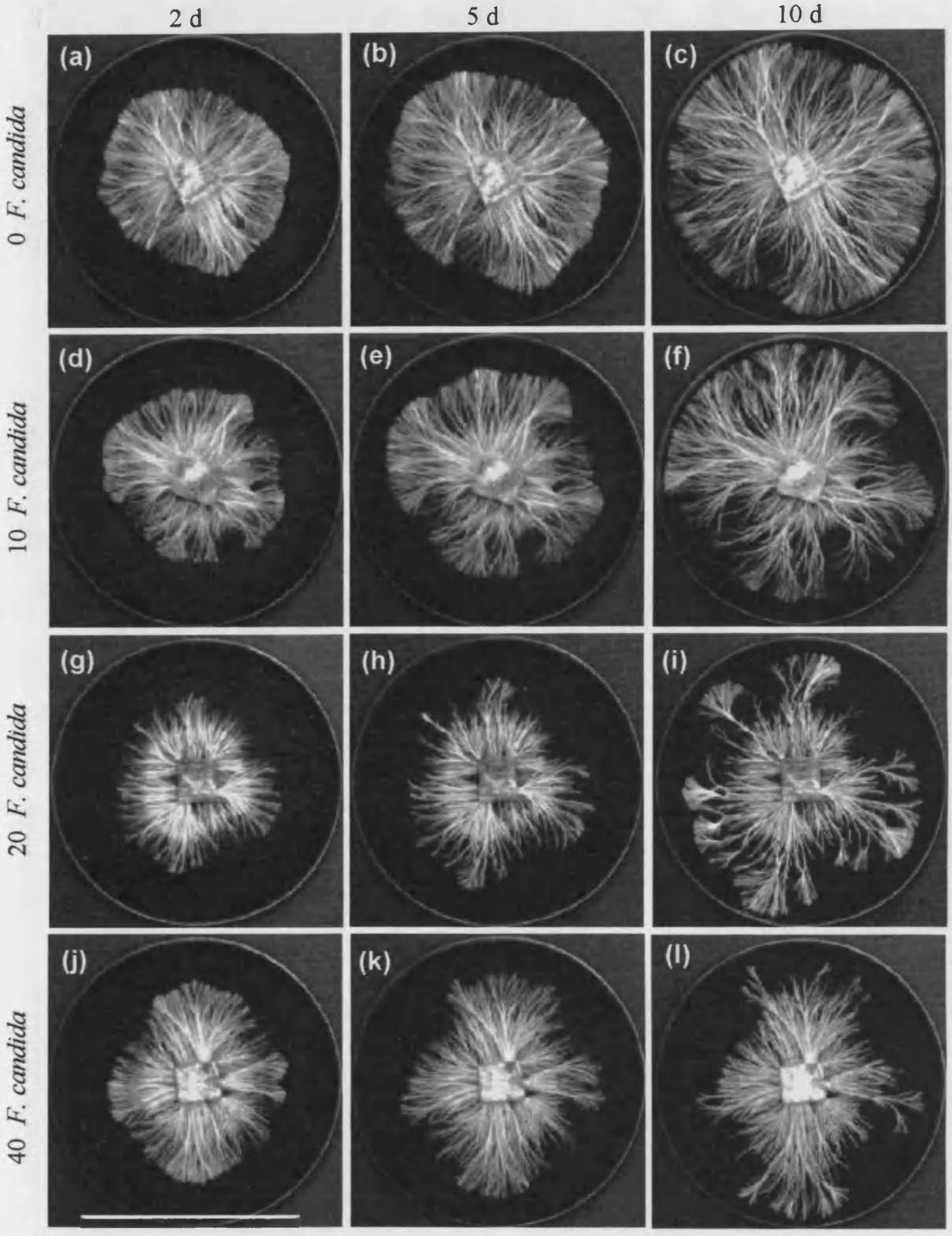


Figure 4.3. Digital images showing effects of grazing on *H. fasciculare* in Experiment 1. Columns are 2 d (a, d, g, j), 5 d (b, e, h, k) and 10 d (c, f, i, l) of collembola grazing. Rows are *F. candida* grazing density of 0 (a, b, c), 10 (d, e, f), 20 (g, h, i) or 40 (j, k, l) individuals per microcosm. Each image is representative of replicate trays in that treatment. Scale bar = 10 cm.

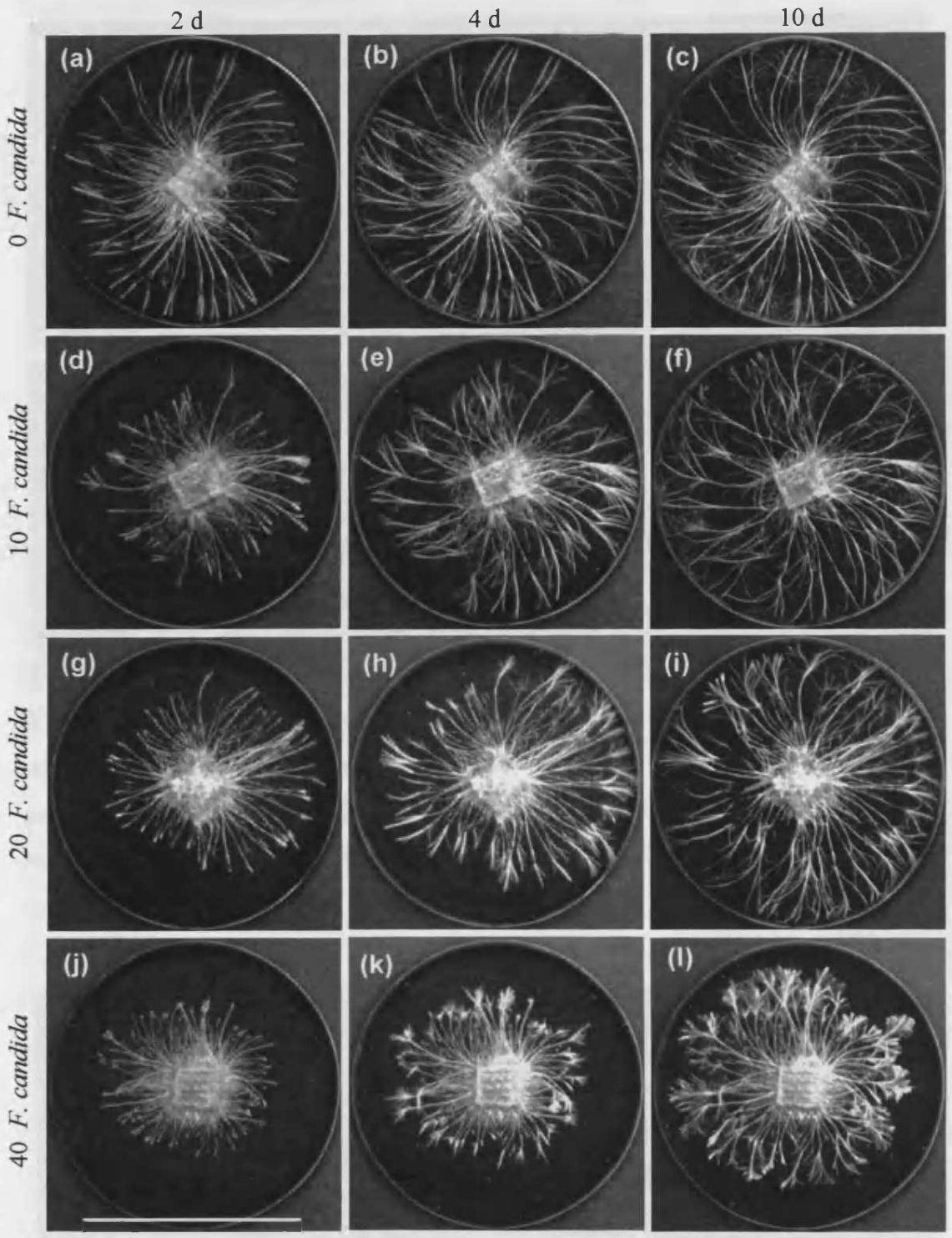


Figure 4.4. Digital images showing effects of grazing on *P. velutina* in Experiment 1. Columns are 2 d (a, d, g, j), 4 d (b, e, h, k) and 10 d (c, f, i, l) of collembola grazing. Rows are *F. candida* grazing density of 0 (a, b, c), 10 (d, e, f), 20 (g, h, i) or 40 (j, k, l) individuals per microcosm. Each image is representative of replicate trays in that treatment. Scale bar = 10 cm.

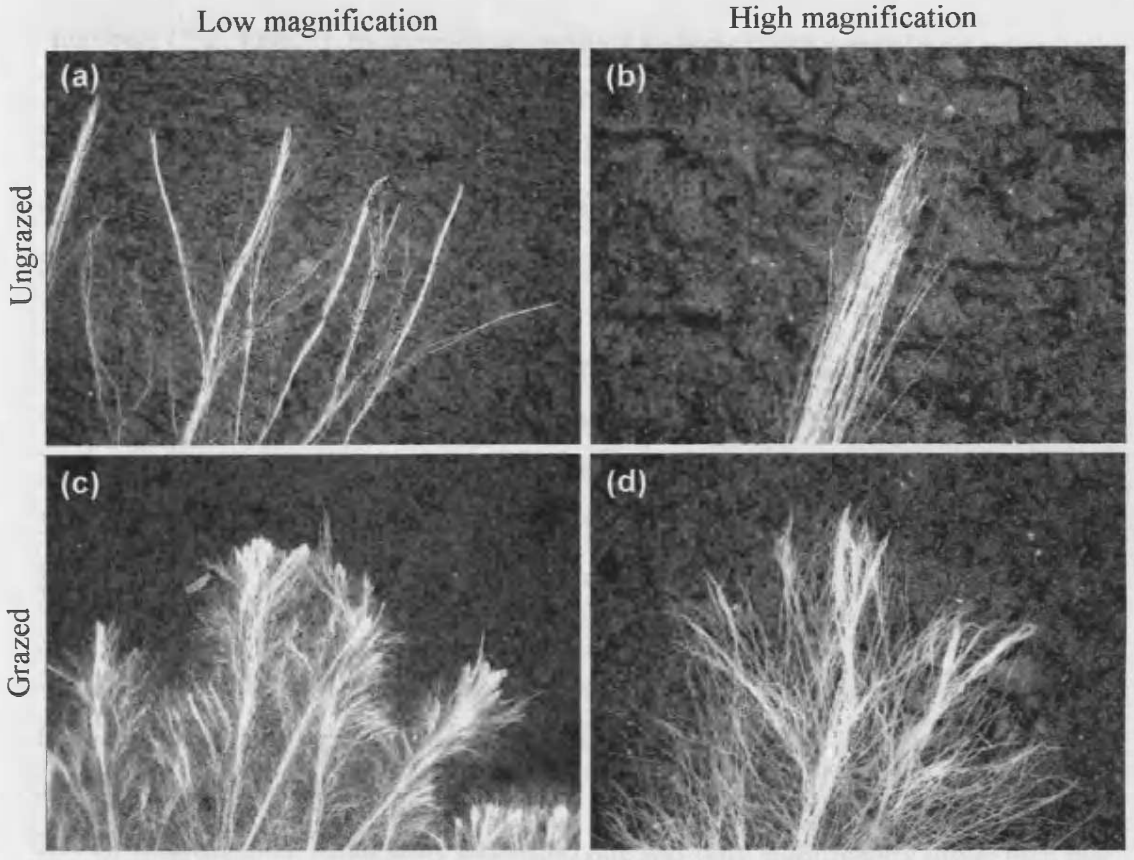


Figure 4.5. Magnified images showing effects on *P. velutina* mycelial margin after 6 d of collembola grazing at 40 *F. candida* per microcosm. Ungrazed (a, b) and grazed (c, d) mycelia shown at low (a, c) and high (b, d) magnification. Images were captured using a Leica M2 12.5 microscope coupled with a Leica DC300 digital camera.

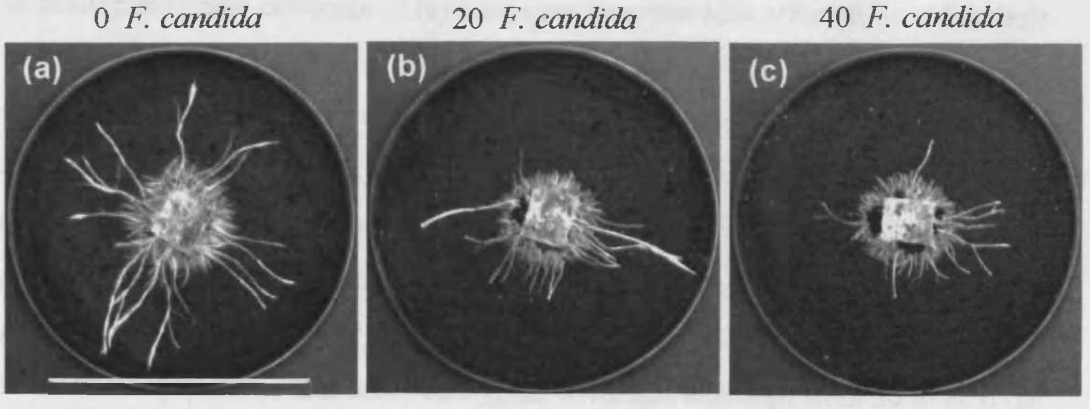


Figure 4.6. Digital images showing effects of 4 d collembola grazing on mycelial systems of *R. bicolor* in Experiment 1. *F. candida* grazing densities are 0 (a), 20 (b) or 40 (c) individuals per dish. Each image is representative of replicate trays in that treatment. Scale bar = 10 cm.

numbers (Fig. 4.6b, c). In some 40 collembola systems cords were almost completely separated from inocula. Mycelium was also grazed from the surface of the wood inoculum, an effect that was not noted in *H. fasciculare* and *P. velutina*. There was some grazing of cord tips and at intervals along cord lengths, and in some cases even thick cords were severed.

4.3.3 Experiment 2: effect of grazing on radial extension rate and hyphal coverage of mycelia 11 cm diam. or not emerged at time of collembola addition

The extremely limited and/or unusual growth of mycelia when collembola were added prior to outgrowth from wood inocula (see below) prevented analysis of measurements of radial extension and hyphal coverage.

Collembola grazing reduced the extension rate of both *H. fasciculare* and *R. bicolor* 11cm diam. mycelia (Fig. 4.7). In *R. bicolor* all grazed systems had a significantly lower extension rate than ungrazed controls (Fig. 4.7b; ANCOVA; $F_{3,160} = 9.34$, $P < 0.001$), whereas in *H. fasciculare* extension rate was only significantly lower than that of ungrazed controls when collembola density was based on hyphal coverage or mycelial area (Fig. 4.7a; $F_{3,188} = 3.83$, $P = 0.011$). In both species, the grazing treatment based on mycelial area had the lowest extension rate, being 60% and 27% of that of ungrazed controls for *H. fasciculare* and *R. bicolor*, respectively (Fig. 4.7).

The change in hyphal coverage of mycelia over time was also affected by collembola grazing treatment (Fig. 4.8), indicated by highly significant time*grazing treatment interactions in both *R. bicolor* (RM ANOVA; $F_{33,264} = 30.4$; $P < 0.001$) and *H. fasciculare* ($F_{36,288} = 7.08$; $P < 0.001$). With *R. bicolor*, there were significant ($P = 0.002$) differences between grazed and ungrazed treatments by 12 d (Fig. 4.8b; Appendix II). By 30 d the treatment with density based on mycelial area had significantly ($P < 0.001$) lower hyphal coverage than the treatment with a constant number of collembola or that based on hyphal coverage, although from 50 to 80 d the grazed treatments were not significantly different ($P > 0.05$; Fig. 4.8b; Appendix II).

Differences in hyphal coverage between grazed and ungrazed *H. fasciculare* treatments were significant from 16 d ($P = 0.002$; Fig. 4.8a; Appendix II), and by 30 d the treatment with a constant number of collembola had significantly ($P \leq 0.05$)

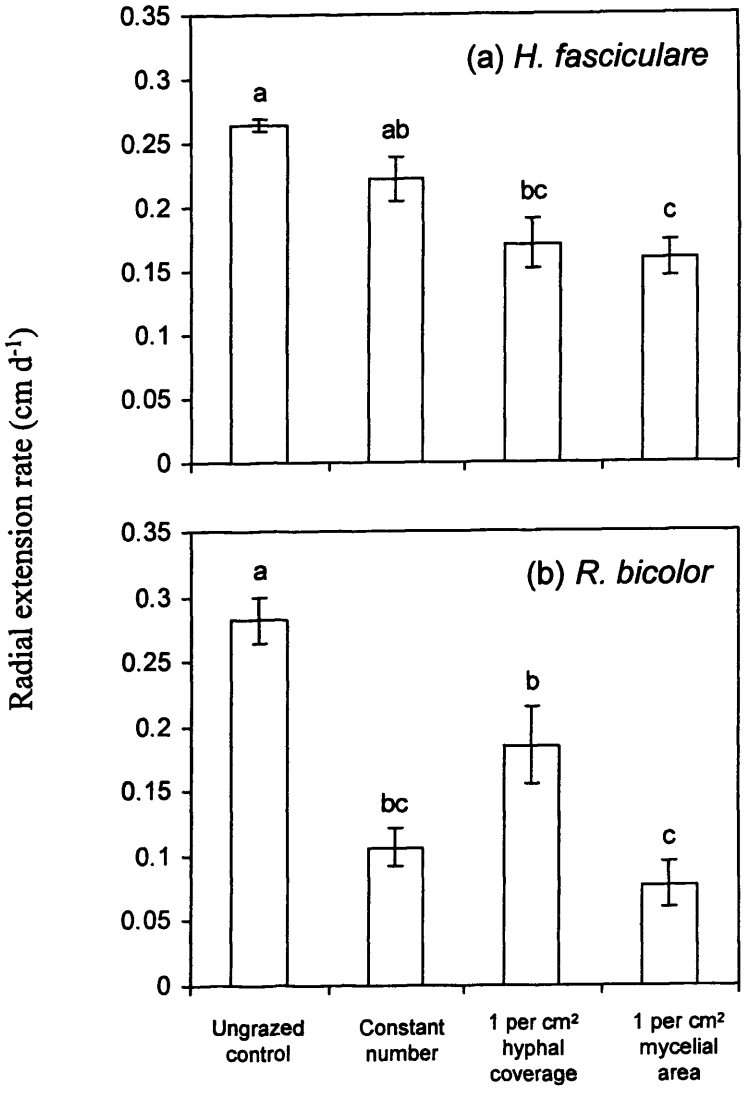


Figure 4.7. The effect of collembola grazing treatment on mean (± s.e.) radial extension rate of *H. fasciculare* (a) and *R. bicolor* (b) in Experiment 2. Different letters above bars indicate significant ($P \leq 0.05$) differences between means (one-way ANOVA and Tukey).

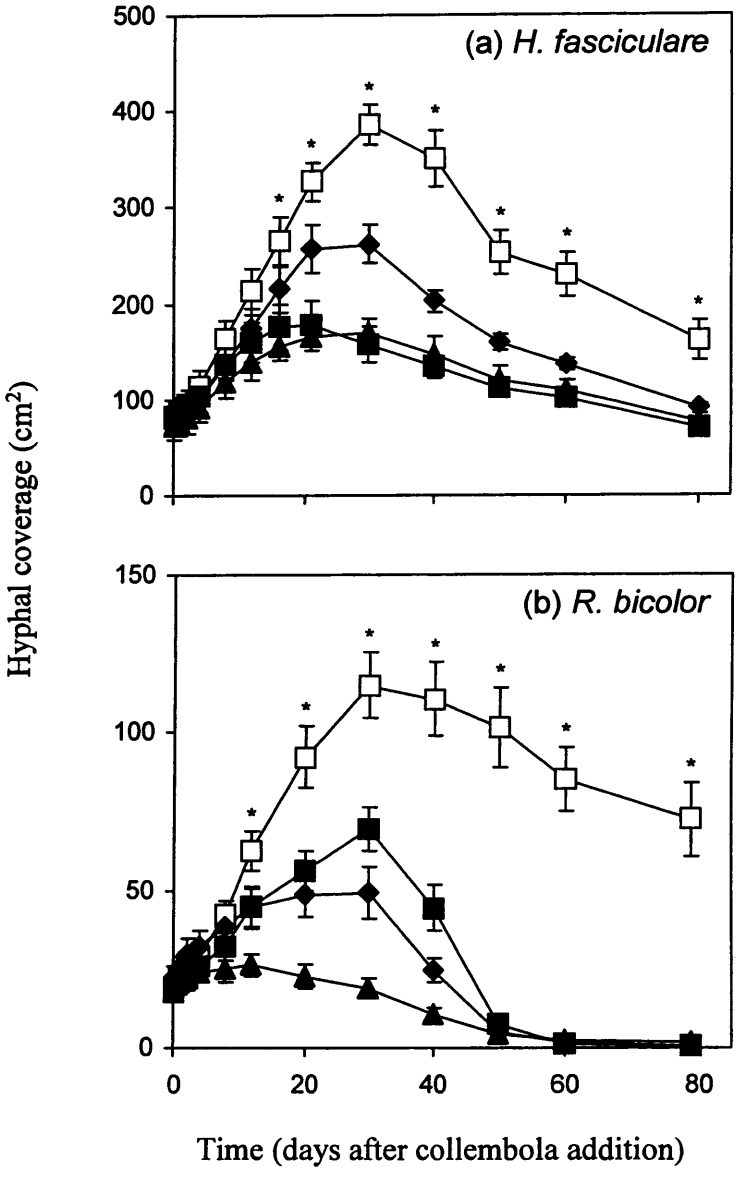


Figure 4.8. The effect of *F. candida* grazing on mean (± s.e.) hyphal coverage of *H. fasciculare* (a) and *R. bicolor* (b) in Experiment 2. Treatments are: ungrazed control (□); constant number per dish (◆); standardized as 1 per cm^2 hyphal coverage (■) or 1 per cm^2 mycelial area (▲). An asterisk above a time point indicates that there is a significant ($P \leq 0.05$) differences between means at that time point (one-way ANOVA and Tukey); details given in Appendix II. Note different y-axis scales.

higher hyphal coverage than the other two grazing regimes (Fig. 4.8a). From 40 to 80 d, however, the three grazed treatments did not differ significantly ($P > 0.05$) from each other in terms of hyphal coverage (Fig. 4.8a; Appendix II).

4.3.4 Experiment 2: effect of grazing on morphology of 11 cm diam. mycelia, and observations on collembola behaviour and reproduction

The morphology of mycelia was altered by grazing, with changes dependent upon grazing treatment in both *H. fasciculare* and *R. bicolor* (Figs 4.9, 4.10). There was evidence of grazing to the *H. fasciculare* systems 1 d after addition of *F. candida*. Fine white hyphae at the growing margin were heavily grazed in discrete patches. There was also some grazing of discrete patches in the interior of the systems adjacent to small areas of uncolonized soil. Fine diffuse mycelium and very narrow cords were grazed while thickened, yellowish cords remained wholly intact. These grazing effects became more accentuated with time from 1 to 12 d after collembola addition. In ungrazed controls the mycelium had an even margin with only a few gaps close to the inoculum (Figs 4.9b, 4.11d) whereas in grazed systems relatively large spaces appeared close to the inoculum, and the margin was often uneven with large invaginations (Fig. 4.11e). Furthermore, there were differences between the different grazing treatments. In the treatments in which grazing intensity was determined based on hyphal coverage or mycelial area (i.e. at a density > 80 collembola per microcosm) grazing effects were pronounced across the mycelium (Fig. 4.9h, k), whereas at the lower density (40 collembola per microcosm), effects were restricted more to the interior of the system, with the margin remaining relatively even (Fig. 4.9e).

After 12 d, when juvenile collembola started to increase the grazing density in all microcosms, there was a rapid increase in the magnitude of effects, with most fine white mycelium being grazed away in high *F. candida* density microcosms to leave mycelia composed of thick yellowed cords (Fig. 4.11f). From 16 to 40 d, two broad types of grazed mycelial pattern developed: (1) systems with similar extension from all sides of wood inocula, but with only thick yellowed cords remaining (e.g. Fig. 4.9i); and (2) uneven systems with little mycelium in one section of microcosm, but with the other sections relatively intact, with advancing white mycelial growth fronts still present (e.g. Fig. 4.9l). After 40 d the amount of young white mycelium

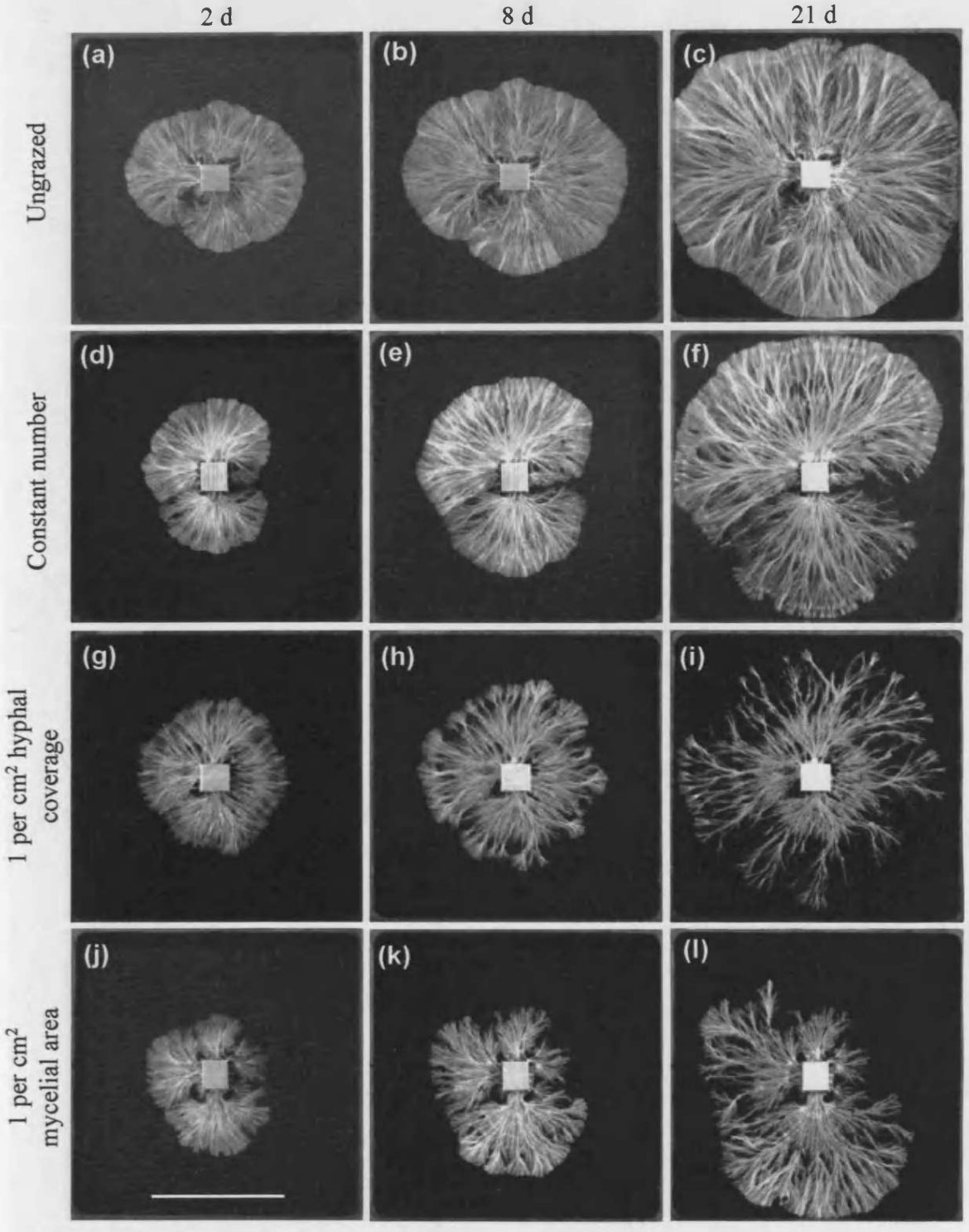


Figure 4.9. Digital images showing effects of grazing on 11 cm diam. systems of *H. fasciculare* in Experiment 2. Columns are 2 d (a, d, g, j), 8 d (b, e, h, k) and 21 d (c, f, i, l) of *F. candida* grazing. Rows are ungrazed control (a, b, c), constant number of collembola (d, e, f), or grazing density based on hyphal coverage (g, h, i) or mycelial area (j, k, l). Each image is representative of replicate trays in that treatment. Scale bar = 10 cm.

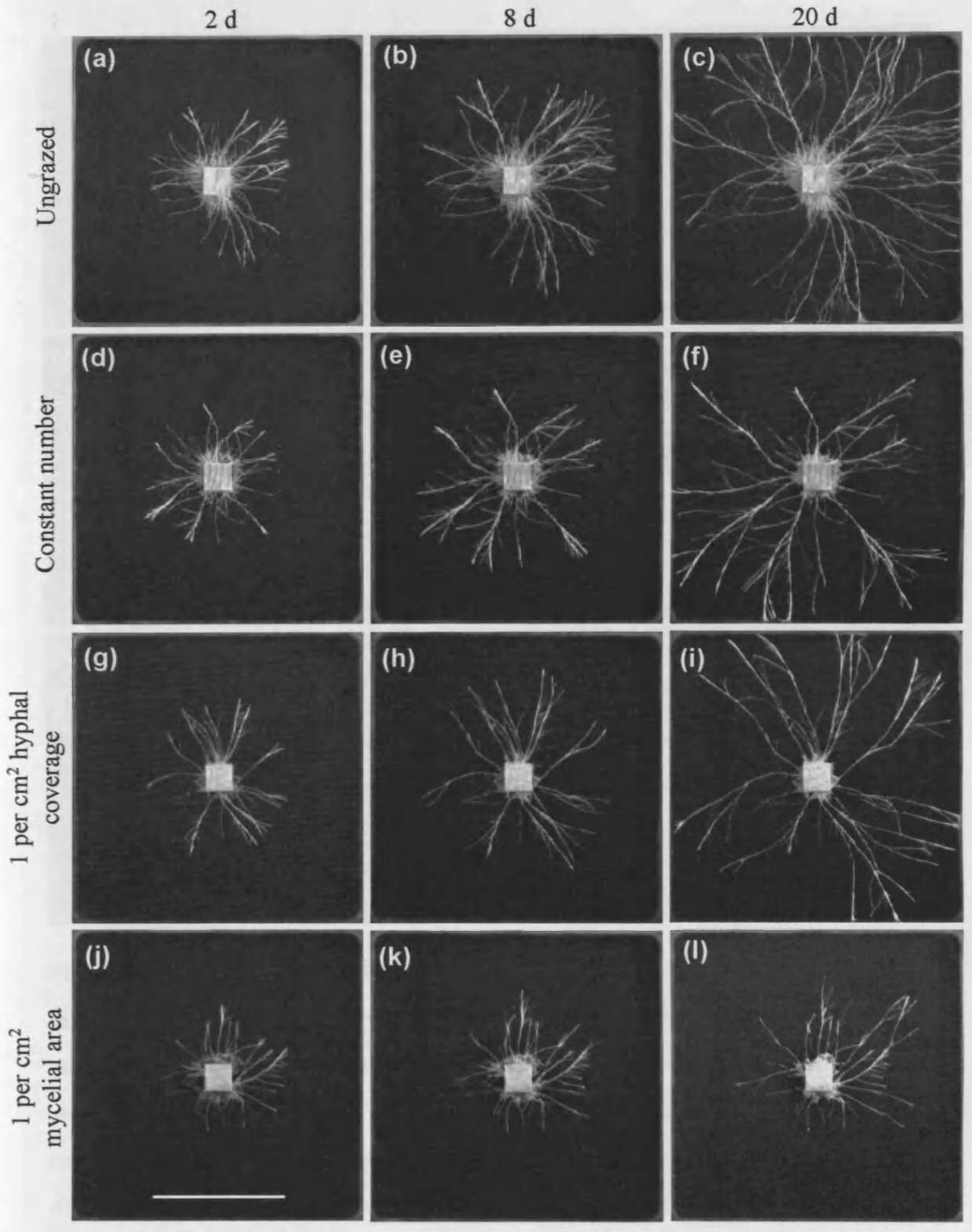


Figure 4.10. Digital images showing effects of grazing on 11 cm diam. systems of *R. bicolor* in Experiment 2. Columns are 2 d (a, d, g, j), 8 d (b, e, h, k) and 20 d (c, f, i, l) of *F. candida* grazing. Rows are ungrazed control (a, b, c), constant number of collembola (d, e, f), or grazing density based on hyphal coverage (g, h, i) or mycelial area (j, k, l). Each image is representative of replicate trays in that treatment. Scale bar = 10 cm.

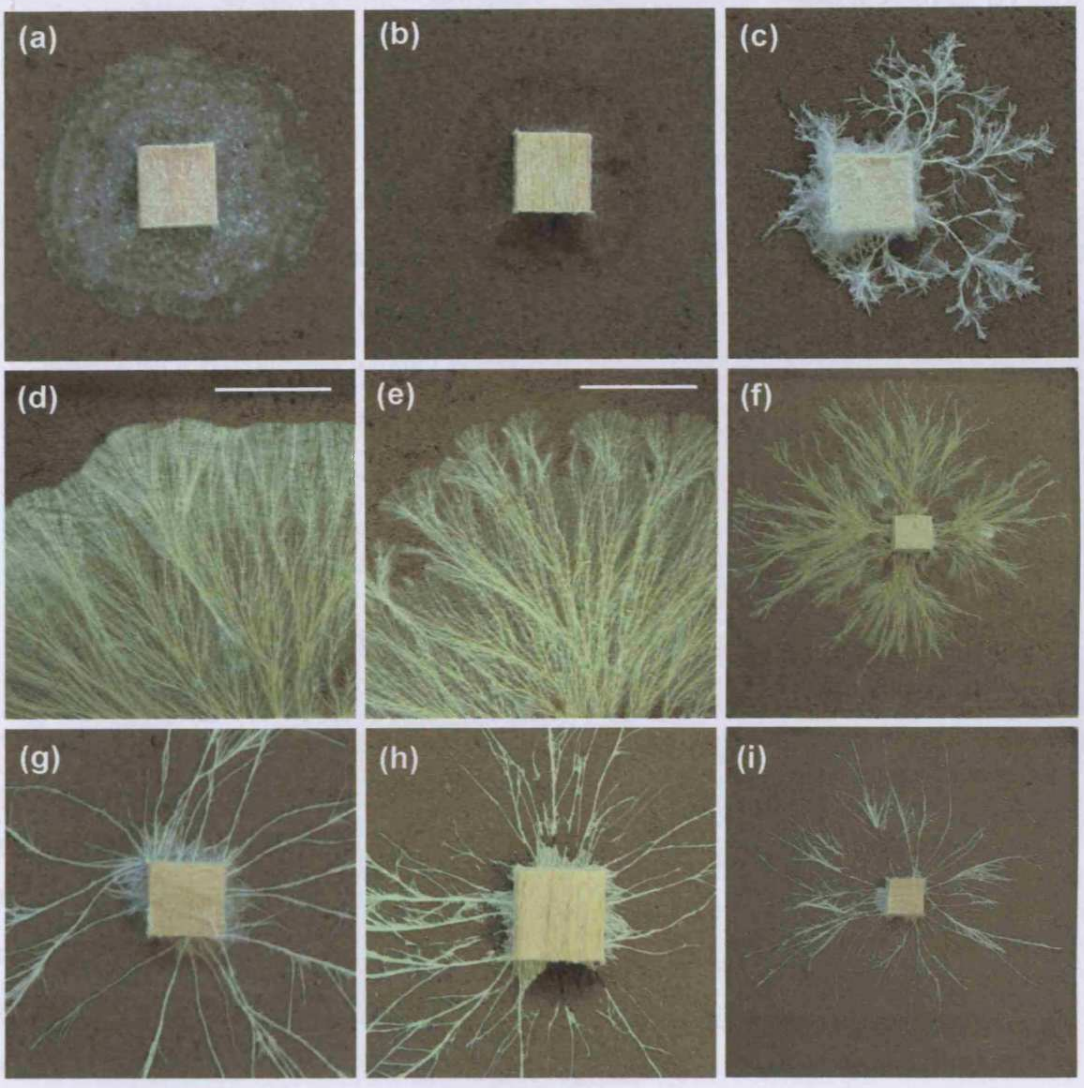


Figure 4.11. Digital images showing effects of grazing when *F. candida* were inoculated before mycelia had egressed from wood inocula (a – c) and when *F. candida* were inoculated when mycelia were of 11 cm diam. (d – i). Fungal species are *H. fasciculare* (a, d – f), *R. bicolor* (b, g – i) and *P. velutina* (c). Images were captured 8 d (d, e), 12 d (g – i), 21 d (f) or 50 d (a – c) after collembola addition. All dishes are grazed except (d) and (g) which are ungrazed controls. Wood inocula (2 cm x 2 cm) indicate scale of images, except in (d) and (e) where scale bar is 2 cm.

decreased in all grazed systems, and ungrazed controls also began to regress. By 80 d all systems were more or less reduced to thick yellowed cords only.

With *R. bicolor*, there was only slight evidence of grazing effects during the first 8 d (Fig. 4.10). This consisted chiefly of small discrete grazed patches close to the wood inocula, and occasionally cord tips were grazed and finer cords severed. By 20 d, grazing effects were much more striking and obviously dependent on grazing treatment. In the treatment where density was calculated based on hyphal coverage (mean: 16 *F. candida*; Fig. 4.10i), systems were more or less intact, resembling the ungrazed controls (Fig. 4.10c), but with a few severed cords and a more sparse mycelium. Where a constant number (40 collembola) were added irrespective of hyphal coverage or mycelial area, more damage was evident (Fig. 4.10f). In treatments where density was based on mycelial area (Fig. 4.10l; where collembola were added at a high density - mean 69 per microcosm), mycelial damage was greater still with many cords being severed (e.g. Fig. 4.11h, i). Some fanning of hyphae was noted in the mycelial area treatment, at both the cord tips and at grazed patches along the cords. Grazing damage increased slowly over time from 20 to 40 d, and was followed by a rapid increase in damage by 50 d. By this time most of the grazed systems were reduced to just a few thick cords, and these were often severed from the wood inocula. By 80 d all grazed microcosms contained very little mycelium; ungrazed control mycelia were beginning to regress.

Collembola were observed on the wood inoculum more frequently in *R. bicolor* than in *H. fasciculare* and *P. velutina*, and this resulted in more frass being present on *R. bicolor* inocula by the end of the experiments. Large egg masses were seen in treatments with all species just 1 d after adding *F. candida*. These were generally situated on soil up to 4 cm beyond the mycelial margin, or actually on the mycelium itself. Juvenile *F. candida* began to emerge from these egg masses after 12 d, and brought about a dramatic increase in the number of collembola present within microcosms. Only a few dead *F. candida* were observed during the experiment; these were consumed by surviving collembola.

4.3.5 Experiment 2: effect of grazing on morphology of mycelia that had not emerged at time of collembola addition

Collembola grazed on mycelium of all species emerging from the wood inoculum, but much more frequently on *R. bicolor* than on *H. fasciculare* and *P. velutina*. This resulted in more frass being present on *R. bicolor* inocula by the end of the experiments. The effect of grazing varied depending on fungal species. With *H. fasciculare* and *R. bicolor* the presence of collembola prevented the formation of cords. Instead, systems consisted of diffuse white mycelium which grew through a dense ring of collembola faeces around the wood inocula. In *H. fasciculare* the mycelium developed as a slowly enlarging circle, which reached a diameter of approximately 8 cm after 50 d (Fig. 4.11a) and then remained stable until 80 d. A similar pattern of growth occurred in *R. bicolor*, although the mycelium was much more sparse and attained a diameter of only 6 cm (Fig. 4.11b). In this species very little hyphal growth was visible on the wood inoculum, whereas in *H. fasciculare* the wood had a fine covering of hyphae. In *P. velutina*, by contrast, mycelial cords did form, although there was considerable fanning and aerial mycelium (Fig. 4.11c). Extension from inocula was slow but continued until harvest at 80 d. Ungrazed control systems of the three species grew rather slowly, but nevertheless all replicates produced cords and filled dishes before 80 d.

4.3.6 Experiment 2: wood inoculum decay rate

Inoculum wood decay was slower in all grazed systems than controls, except for the treatment with 40 collembola grazing on *H. fasciculare* prior to outgrowth (Table 4.1). The only significant ($P \leq 0.05$) reduction in decay rate was, however, with collembola density based on hyphal coverage of 11 cm diam. *H. fasciculare* mycelia ($F_{3,24} = 4.30$, $P = 0.015$).

Table 4.1. Decay rates (mg cm^{-3} d^{-1}; mean ± s.e.) of wood inocula when collembola were added before mycelial outgrowth or to 11 cm diam. mycelia.

	P. velutina	*H. fasciculare*		*R. bicolor*	
	Prior to mycelial outgrowth	Prior to mycelial outgrowth	11 cm diam. mycelium	Prior to mycelial outgrowth	11 cm diam. mycelium[†]
Ungrazed control	1.68 ± 0.24 [a]	1.76 ± 0.13 [a]	1.63 ± 0.12 [a]	1.85 ± 0.29 [a]	1.36 ± 0.07 [a]
Constant number	1.19 ± 0.19 [a]	2.16 ± 0.17 [a]	1.38 ± 0.06 [ab]	1.59 ± 0.33 [a]	1.26 ± 0.09 [a]
1 per cm^2 hyphal coverage	-	-	1.14 ± 0.09 [b]	-	1.34 ± 0.09 [a]
1 per cm^2 mycelial area	-	-	1.39 ± 0.10 [ab]	-	1.14 ± 0.13 [a]

Figures in the same column followed by different letters are significantly different (one-way ANOVA and Tukey; $P \leq 0.05$). Dashes indicate treatment combinations not performed; none of the *P. velutina* 11 cm diam. treatments were performed (Section 4.2.2).

[†] *R. bicolor* 11 cm diam. mycelium wood inocula were incubated for longer than other treatment inocula (Section 4.2.3).

4.4 Discussion

Mycelia morphology was affected by grazing in all fungi, and was broadly consistent across the two experiments. The greatest effects were associated with the highest density of collembola, supporting the second hypothesis. Specifically, extension rate and hyphal coverage decreased with increasing number of collembola, agreeing with the results of a previous study on *H. fasciculare* (Kampichler *et al.* 2004). In Experiment 2 the lack of significant grazing density effects on hyphal coverage beyond 40 - 50 d was probably due the fact that the collembola populations had by that time increased to such an extent that there were effectively no differences in population density. Fungal growth can be stimulated at low grazing intensities due to the positive effects of collembola on nutrient mineralization (Ineson *et al.* 1982). For example, in an earlier laboratory investigation of the effect of grazer density on microbial activity, *F. candida* was found to have a positive effect on the respiration of a wood-decay fungus, *Coriolus versicolor*, at a low grazing density, and a negative

effect at higher densities (Hanlon & Anderson 1979). The current study contrasts with these findings as the grazed fungal species were always either negatively affected or unaffected by collembola, regardless of density. It is possible that collembola would stimulate the growth of cord-forming fungi if grazing densities were lower than those used in the current study (see Chapter 8).

As far as I am aware this is the first study to show inter-specific differences between cord-formers in their response to grazing and in the zone of mycelium that is grazed. These differences were highlighted by both quantitative measurements and qualitative observations of fungal morphology. For example, while both radial extension rate and hyphal coverage were significantly reduced by grazing of *H. fasciculare*, in *P. velutina* only extension rate was reduced. *R. bicolor* was most dramatically affected, with often only a few major cords remaining. Comparing results across the two experiments should, however, be done cautiously, since adding an equal number of collembola to similarly aged systems (as in Experiment 1) does not necessarily imply equivalent grazing pressure. Adding collembola based on the area occupied by the mycelium or hyphal coverage (as in Experiment 2) provided equivalent grazing pressure to mycelia that extend at different rates and fill space to different extents. The importance of this is seen in interpreting grazing effects on *R. bicolor*, which produces a sparser mycelial system than the other two species. In Experiment 1 extension rate of *R. bicolor* mycelia was reduced to 58% of the ungrazed control with only 10 collembola added whereas that of *H. fasciculare* and *P. velutina* decreased by less than 9%. In Experiment 2 percentage reduction in extension rate of *R. bicolor* was similar to that of *H. fasciculare* when collembola addition was standardized on hyphal coverage. The addition of collembola based on coverage, to provide equivalent grazing pressure, rather than at a standard density, thus seems a necessary step to enable meaningful comparisons across fungal species.

The first hypothesis suggested that, as a short range, responsive forager, the mycelium of *H. fasciculare* would be most affected, and that of *P. velutina* and *R. bicolor*, both of which are long range and generally less responsive foragers, less affected. In fact, the morphology of *P. velutina* did change dramatically (Fig. 4.4l), and *R. bicolor* appeared to be the most palatable species in the study. In contrast, *H. fasciculare* appeared to be the least palatable species. These observations suggest that how a

mycelium responds to grazing may relate more to the sites where grazing predominates rather than to foraging strategy and general responsiveness to abiotic and biotic cues. Though the relationship between interspecific differences in hyphal/cord morphology and apparent palatability is not self-evident, intra-mycelial differences were more obvious. Thus, with *H. fasciculare*; fine white hyphae at the mycelial margin were most heavily grazed; and hyphae within the colony were sometimes grazed, in which case it was always diffuse mycelium or narrow cords that were consumed, not the yellowish thicker cords. Extensive grazing of fine cords was also evident in the other two species, as hypothesized.

There was some evidence that the more mature systems employed in Experiment 2 were more resistant to grazing, at least for *R. bicolor* though not necessarily *H. fasciculare*. Inferences must, however, be tentative as comparisons of mycelial size are being made across the two experiments, which differed in various ways (size of microcosms, volume of wood inocula, etc.). The decreased effects during early grazing on the larger *R. bicolor* systems (i.e. 11 cm diam. systems in Experiment 2) may be because the cords are less palatable, due to increase in rind thickness and/or possible production of secondary chemicals and encrusting calcium oxalate (CaC_2O_4) crystals. *R. bicolor* is known to produce these crystals (Connolly & Jellison 1995), which possibly act as a deterrent to consumption by grazing organisms. In *H. fasciculare*, only mycelial margins displayed obvious grazing effects in Experiment 1, whereas margins and interior regions of mycelium were grazed in the more mature Experiment 2 systems. This inconsistency may reflect the different mycelial sizes at the time of *F. candida* addition. *H. fasciculare* mycelia become less space-filled as they age, and the grazed patches in the interior of Experiment 2 systems originated beside small areas of uncolonized soil. The presence of uncolonized soil may thus be necessary for collembola to commence grazing. That the thick yellow cords produced by *H. fasciculare* were never grazed, even in the presence of very high collembola densities towards the end of the experiment, indicates a much more extreme unpalatability of cords in this species than in *R. bicolor*.

The morphology of a mycelium that has been grazed not only changes as a result of loss of biomass, but also by producing new growth with different characteristics. This is particularly evident at the margins of *H. fasciculare* and *P. velutina* systems grazed

by 40 collembola in Experiment 1, where cord tips were fanned compared to ungrazed systems (e.g. Figs 4.3i, 4.4l). The fanning of *P. velutina* hyphae and increased lateral branching from main cords led to the grazed systems becoming more space-filled behind the mycelial growth front than ungrazed controls. This might explain why grazing had no effect on hyphal coverage in this species, as although grazed colonies were of lesser extent (i.e. lower radial extension) they were more space-filled than ungrazed colonies. Fanned hyphae have been reported once before in response to collembola grazing (Hedlund *et al.* 1991). In this particular study the soil zygomycete *Mortierella isabellina* switched its morphology in response to grazing by *Protaphorura armata*, producing fan-shaped sectors of hyphae and extensive aerial mycelium. *P. velutina* showed a very similar grazing response in the present study although cultured on soil as opposed to the more artificial agar substrate used in the Hedlund *et al.* study. The latter authors report increased protease activity in the switched sectors of mycelium. Increased protease activity has also been reported in *P. velutina* in response to the presence of the nematode *Panagrellus redivivus* (Dyer *et al.* 1992). It is not known whether enzyme production was affected by grazing in the current study.

Grazing at initial stages of outgrowth had more dramatic effects than grazing on more mature extra-resource mycelium, supporting the fourth hypothesis. The inability of *H. fasciculare* and the ability of *P. velutina* to produce cords, albeit to a limited extent, correlates with the former's tendency to plane-fill and the latter's tendency to form more open systems with distinct mycelial cords (Boddy 1999). *R. bicolor*, however, when ungrazed formed open systems with distinct cords, but was unable to do so when grazed before an extra-resource mycelium had established, presumably reflecting its palatability. Outgrowth of all species was very slow in the presence of collembola; *H. fasciculare* and *R. bicolor* were effectively restricted to the inoculum.

The finding that more mature systems are less susceptible to grazing, and that systems with no previous outgrowth cannot easily grow out and forage for new resources in the presence of collembola, raises interesting questions regarding the establishment and persistence of cord-forming mycelia in wooded ecosystems. Taken together, these findings suggest that new cord systems might have difficulty in establishing themselves on the forest floor, but that if they can do so they may become relatively

persistent. Established systems interlink a large number of woody resources across their mycelial networks (Boddy 1993, Boddy 1999); these woody units are often much larger than the wood blocks used here. Established fungi therefore have a much greater 'inoculum potential', and are likely to be able to grow out from colonized wood much more vigorously than in small microcosm systems. This may allow established networks to swamp potential grazing effects, and possibly synthesize more toxins to deter grazers. It is important to question, however, given that collembola occur at a high density in temperate woodlands (Petersen & Luxton 1982) and that other soil invertebrate groups, particularly oribatid mites (McLean *et al.* 1996, Schneider & Maraun 2005), also graze fungal hyphae, how extensive extra-resource mycelia develop in the first place. A possible explanation is that these fungi exploit situations when grazing intensity is low. Soil faunal population densities are variable both spatially and temporally (Usher 1969, Berg *et al.* 1998), and extra-resource mycelia may be best able to develop in patches of low faunal density. It is also possible that fungi growing in forest soils are less accessible to collembola than they are in the laboratory, and that collembola may selectively feed on other fungi.

Finally, grazing may affect wood decomposition; inoculum decay of *H. fasciculare* was reduced by grazing. Interspecific fungal interactions also sometimes result in decreased decay rate, though equally there are examples of increases occurring (Boddy 2001). More generally, interspecific interactions between saprotrophic fungi and soil invertebrates are likely to have consequences for decomposition, through nutrient mobilization from leaf litter and senescent fungal material (Hanlon & Anderson 1979, Anderson *et al.* 1983). Each fungal species used in the present study appears to be palatable to collembola, and displays distinctly modified growth as a result of their grazing. It is therefore probable that collembola, and other mycophagous soil invertebrates, affect the spatial organization of these fungi and hence their ability to forage for and decompose dead organic matter. Extrapolation from microcosm experiments such as these must, however, be done cautiously; feeding by soil invertebrates on cord-forming fungi has not been investigated in the field.

Chapter 5. Effects of species identity in the interactions between collembola and cord-forming fungi

5.1 Introduction

Grazing of cord-forming fungi by collembola can dramatically affect fungal morphology (Chapter 4, Kampichler *et al.* 2004). The microcosm studies in the previous chapter employed a single collembola species, *Folsomia candida*, so it is important to investigate whether different collembola species have different effects on cord-former morphology. Only one study to date has addressed this (Kampichler *et al.* 2004). The authors found that *F. candida*, a relatively large species, caused a decrease in radial extension and hyphal coverage of *Hypholoma fasciculare* whereas two smaller collembola species, *Proisotoma minuta* and *Hypogastrura cf. tullbergi*, had little effect, despite being introduced in larger numbers than *F. candida*. It is appropriate to investigate whether the size-dependent effects observed for *H. fasciculare* by Kampichler *et al.* (2004) hold true for other species of cord-forming fungi.

In this series of experimental studies a range of fungal and collembola species were allowed to interact; effects on fungal growth parameters and collembola abundance were determined. The fungal component of the study comprised four cord-formers, three of which, *H. fasciculare*, *Phanerochaete velutina* and *Resinicium bicolor*, have been used in previous grazing studies (Chapter 4, Kampichler *et al.* 2004). The fourth species, *Phallus impudicus*, forms extensive mycelial networks in deciduous woodlands (Thompson & Rayner 1983). Three collembola species, *F. candida*, *Protaphorura armata* and *P. minuta*, were employed (Section 2.3.4); the first two are relatively large species and the latter is a smaller species.

Firstly, it is hypothesized that the morphological response of cord-formers to grazing will depend not only on the identity of the fungal species (Chapter 4) but also on the identity of the collembola grazer. This is predicted because smaller collembola species have a less marked effect on mycelial morphology than larger collembola

species, even when biomass is standardized (Kampichler *et al.* 2004). The small-sized *P. minuta* is therefore predicted to have less impact than the larger *F. candida* and *P. armata* on mycelial morphology.

Secondly, it is hypothesized that all three collembola species will respond to the fungal substrates in a similar way, attaining their highest abundance on the most palatable fungus, and have the greatest impact on the morphology of this preferred species. This is predicted because collembola show distinct feeding preferences and different species tend to prefer the same food source (Section 2.4.1). As the quality of fungal food substrate affects collembola growth (Hogervorst *et al.* 2003) and reproduction (Klironomos *et al.* 1999), population size will be affected.

5.2 Materials and methods

5.2.1 Experimental design

The experiment focussed on the factorial combination of four cord-forming basidiomycete species and three collembola species in two-species (one fungus: one collembola) soil microcosms (Table 5.1). Initially 10 replicates of each treatment were set up, but erratic fungal growth and/or insufficient numbers of the small-sized *P. minuta*, occasionally resulted in fewer replicates being used (Table 5.1).

Table 5.1. Number of replicate microcosms in each experimental treatment.

	Fungi			
	P. velutina	*H. fasciculare*	*P. impudicus*	*R. bicolor*
Collembola				
Ungrazed control	10	10	7	7
F. candida	10	10	7	7
P. armata	10	10	7	7
P. minuta	5	6	-	-

Dashes indicate treatment combinations not performed.

5.2.2 Preparation of study organisms

Wood inocula of *P. velutina* (KC1685), *H. fasciculare* (GTWV2), *R. bicolor* (RB1) and *P. impudicus* (JHY4) were prepared as described in Section 3.2, and incubated for 112 d at room temperature prior to use in microcosms.

F. candida (body width 200-400 μm), *P. armata* (200-400 μm) and *P. minuta* (140-200 μm) were cultured and extracted as described in Section 3.3.

5.2.3 Preparation and inoculation of soil microcosms

Soil microcosms were prepared and maintained as described in Section 3.4. Collembola were added when 50% of the fungal mycelia of a particular species had reached a diam. of 8 cm. This occurred 8, 14, 26 and 29 d after wood inocula addition for *P. velutina*, *H. fasciculare*, *P. impudicus* and *R. bicolor*, respectively. Microcosms were destructively harvested 60 d after adding collembola.

5.2.4 Determination of collembola inoculation density

Collembola inoculation density was based on mean body mass. To ascertain body mass ratio across the three species, 150 individuals per species of the experimental body width (Section 5.2.2) were extracted from culture, oven dried and weighed to 0.01 mg. Average weight per individual was: *F. candida* 28.7 μg; *P. armata* 24.7 μg; *P. minuta* 4.2 μg. The number of collembola added was based on the soil area colonized by mycelium (hyphal coverage) in each microcosm. Thus grazing intensity was standardized across fungal species. Collembola were added at 2.0, 2.3 and 13.7 individuals per cm^2 mycelium for *F. candida*, *P. armata* and *P. minuta*, respectively.

5.2.5 Image capture and analysis

Digital images of mycelia were captured and analysed as described in Section 3.5. Images were captured immediately prior to collembola addition and 1, 2, 4, 6, 8, 12, 16, 20, 30, 40, 50 and 60 d after addition. All time points apart from 1, 2 and 6 d were subjected to image analysis, except for *H. fasciculare* where 1, 2 and 6 d images were also analysed because the 4 d image files became corrupted. High resolution colour images were captured periodically using a Nikon COOLPIX E4500 digital camera.

5.2.6 Wood inoculum decay rate

The decay rate of wood inocula was determined as described in Section 3.6.

5.2.7 Collembola abundance

At the time of destructive harvest (60 d after collembola addition) microcosm soil was removed from dishes using a large spatula and transferred to a Tullgren funnel (Burkard Agronomics, Uxbridge, UK); collembola were extracted into 100% ethanol over 48 h. To test extraction efficiency 100 individuals of each collembola species were added from cultures to ungrazed microcosms (n = 5), left for 15 mins to allow collembola to disperse across the soil, and then harvested and extracted as above. Mean percentage extraction efficiencies (± s.e.) were: *P. armata* 73.4% ± 0.9%; *P. minuta* 69.6% ± 0.9%; *F. candida* 63.8% ± 3.0%. With such low levels of variability these values were applied as correction factors to the counts of experimental individuals harvested.

5.2.8 Statistical analyses

For each fungal species, radial extension, hyphal coverage and mass fractal dimension data were compared across grazing treatments using the analyses described in Section 3.7. Wood decay rates and collembola abundances (normalized by square root transformation) were compared across species using one-way ANOVA. Significant ($P \leq 0.05$) results were investigated using Tukey's pairwise comparisons.

5.3 Results

5.3.1 Effect of grazing on radial extension rate, hyphal coverage and fractal dimension of mycelia

Collembola significantly ($P \leq 0.05$) reduced radial extension in all species, except when *P. armata* grazed *R. bicolor* and *P. impudicus* (Fig. 5.1). Different collembola species had differential effects. In *P. velutina* systems, *F. candida* and *P. minuta* caused similar reductions in extension rate while *P. armata* had less of an effect (Fig. 5.1a; ANCOVA; $F_{3,132} = 60.7$, $P < 0.001$). With *H. fasciculare* all three collembola species resulted in a similar reduction (Fig. 5.1c; $F_{2,234} = 182$, $P < 0.001$). *F. candida* had a greater effect than *P. armata* with *R. bicolor* (Fig. 5.1b; $F_{2,99} = 24.8$, $P < 0.001$), but not with *P. impudicus* (Fig. 5.1d; $F_{2,120} = 3.22$, $P = 0.043$).

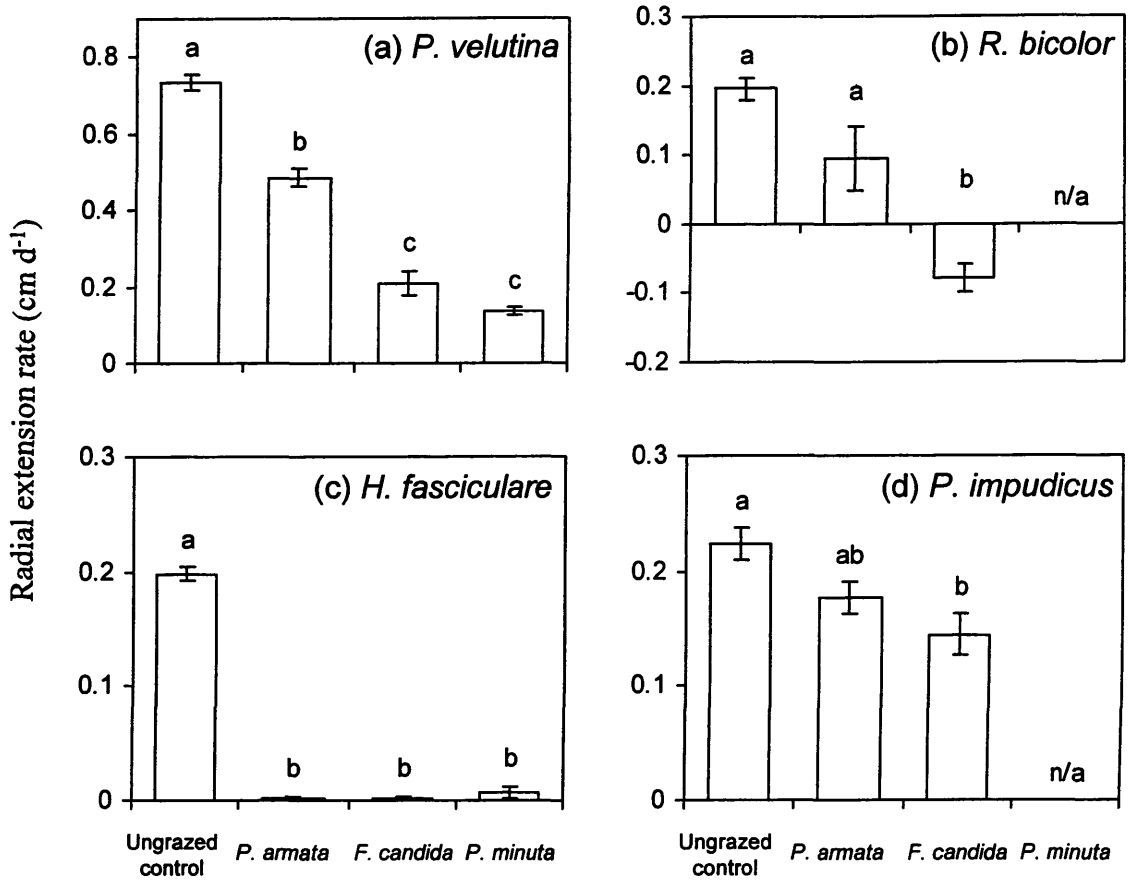


Figure 5.1. Effects of different collembola species on radial extension rate (mean ± s.e.) of *P. velutina* (a), *R. bicolor* (b), *H. fasciculare* (c) and *P. impudicus* (d).
Different letters above bars indicate significant ($P \leq 0.05$) differences in extension rate between grazing treatments (one-way ANOVA and Tukey). n/a indicates treatment combination not performed. Note different y-axis scales.

Grazing reduced hyphal coverage of *P. velutina*, *H. fasciculare* and *R. bicolor*. There were differential effects depending on both grazer and grazed species (Fig. 5.2). All three collembola species allowed no increase in hyphal coverage of *H. fasciculare* once grazing commenced (Fig. 5.2c; RM ANOVA; $F_{3,44} = 147$, $P < 0.001$). *F. candida* reduced coverage almost to zero in *R. bicolor* while *P. armata* only slightly reduced coverage compared to ungrazed controls (Fig. 5.2b; $F_{7,59} = 18.7$, $P < 0.001$). With *P. velutina*, though by 60 d all grazers reduced coverage compared to controls, trends varied over time (Fig. 5.2a; $F_{16,165} = 28.4$, $P < 0.001$). Hyphal coverage of *P. impudicus* was not affected by grazing ($F_{7,60} = 2.06$, $P = 0.066$); means for systems grazed by *F. candida* were, however, lower than those of controls at most time points (Fig. 5.2d).

As with hyphal coverage, mycelial fractal dimension of *P. velutina*, *H. fasciculare* and *R. bicolor* was affected by grazing (Fig. 5.3). Again there were differential effects depending on species. *F. candida* dramatically reduced space-filling in *R. bicolor* (Fig. 5.3b; $F_{11,90} = 10.8$, $P < 0.001$), reduced it to a lesser extent in *H. fasciculare* (Fig. 5.3c; $F_{10,121} = 32.2$, $P < 0.001$), but at some time points increased that of *P. velutina* relative to ungrazed controls (Fig. 5.3a; $F_{11,145} = 15.7$, $P < 0.001$). In contrast, *P. armata* significantly reduced fractal dimension of *P. velutina* and *H. fasciculare* (Fig. 5.3a, c) but not *R. bicolor* (Fig. 5.3b). Fractal dimension of *P. impudicus* was unaffected by grazing (Fig. 5.3d; $F_{5,42} = 0.85$, $P = 0.52$).

5.3.2 Effect of grazing on mycelial morphology

In ungrazed *P. velutina* systems relatively dense young mycelia became more open as growth fronts extended rapidly towards dish margins, and diffuse hyphae and fine cords regressed (Fig. 5.4a-c). All three collembola species caused fanning of *P. velutina* cord tips within 24 h of addition. Two to 10 d after collembola addition *F. candida* (Fig. 5.4d, e) and *P. minuta* (Fig. 5.4j, k) grazing resulted in smaller mycelia with more fanning than did *P. armata* grazing (Fig. 5.4g, h). At 12 d differences between *F. candida* and *P. minuta* grazing became apparent, with less aerial mycelium and less fanned cord tips in the latter case. After 20 d *P. armata* had grazed away small discrete patches of fine mycelium distal to the margin (Fig. 5.4i); *P. minuta*-grazed systems had fanned but fast-growing margins (Fig. 5.4l), whereas *F. candida*-grazed systems displayed little new growth (Fig. 5.4f). From 40 - 60 d

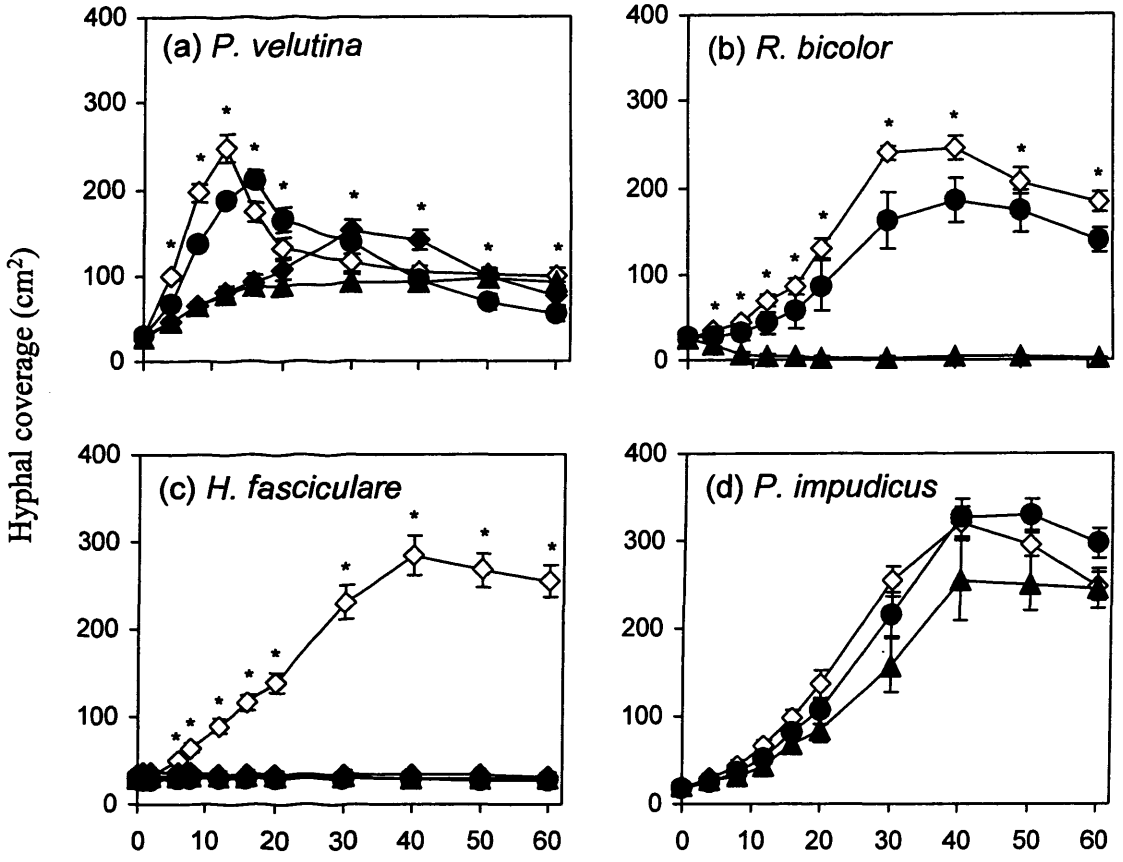


Figure 5.2. Effects of different collembola species on hyphal coverage (mean ± s.e.) of *P. velutina* (a), *R. bicolor* (b), *H. fasciculare* (c) and *P. impudicus* (d). Grazing treatments are *P. armata* (●), *F. candida* (▲) and *P. minuta* (◆), or ungrazed controls (◇). An asterisk above a time point indicates that there is a significant ($P \leq 0.05$) differences between means at that time point (one-way ANOVA and Tukey); details given in Appendix III. Where error bars are not shown they fall within the size of the symbol.

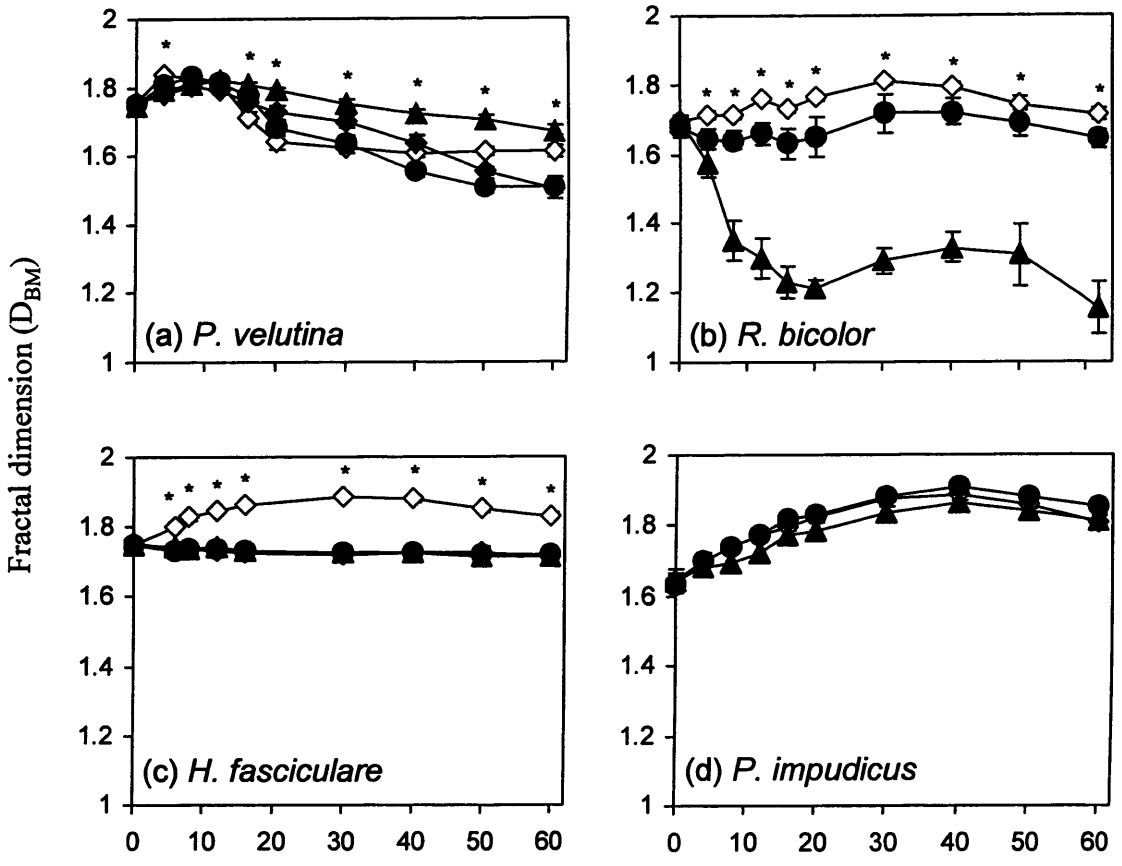


Figure 5.3. Effects of different collembola species on mass fractal dimension (mean ± s.e.) of *P. velutina* (a), *R. bicolor* (b), *H. fasciculare* (c) and *P. impudicus* (d). Grazing treatments are *P. armata* (●), *F. candida* (▲) and *P. minuta* (◆), or ungrazed controls (◇). An asterisk above a time point indicates that there is a significant ($P \leq 0.05$) differences between means at that time point (one-way ANOVA and Tukey); details given in Appendix IV. Where error bars are not shown they fall within the size of the symbol.

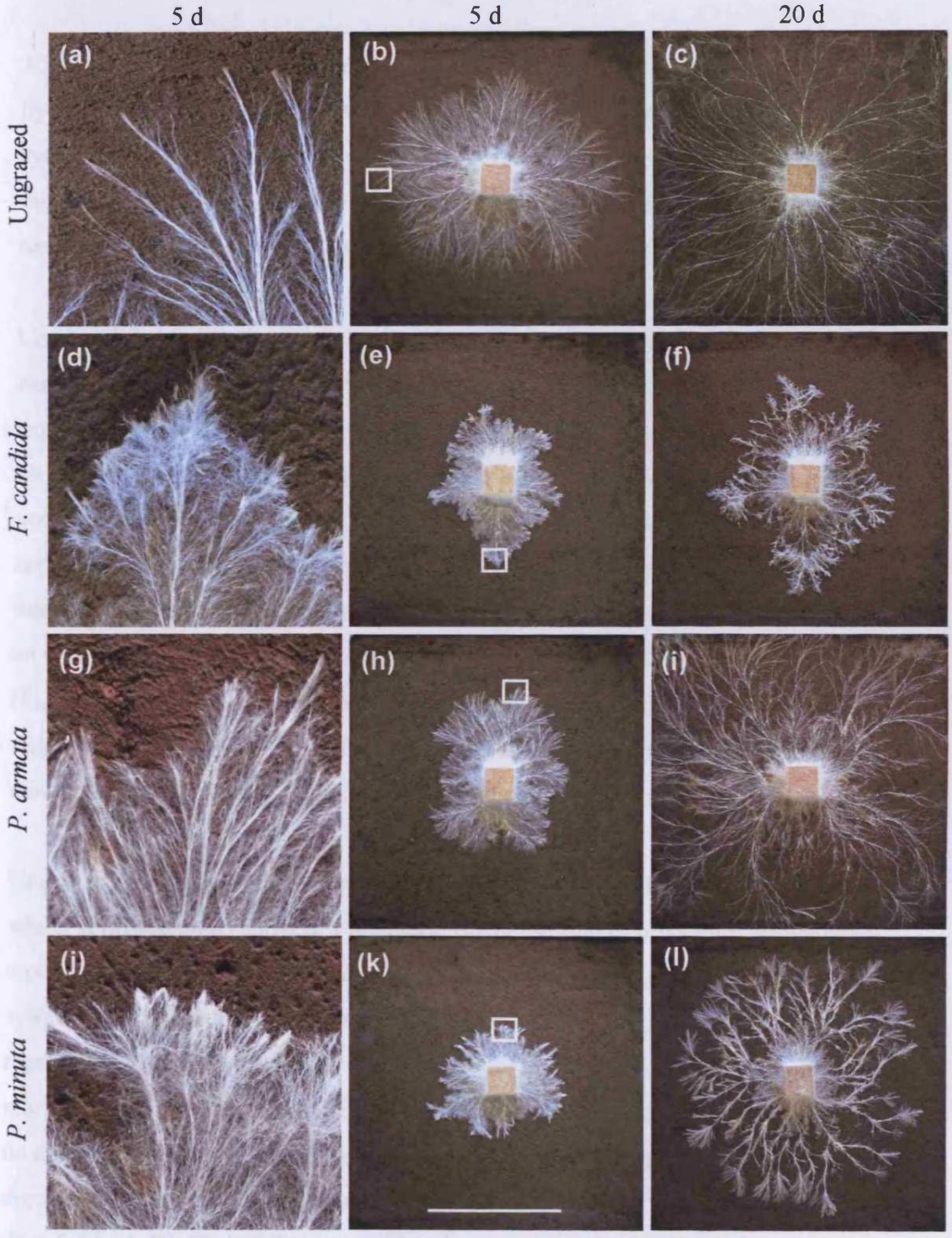


Figure 5.4. Digital images of mycelial systems of *P. velutina* after 5 d (a, b, d, e, g, h, j, k) and 20 d (c, f, i, l) of collembola grazing. Treatments are ungrazed controls (a-c), *F. candida* (d-f), *P. armata* (g-i) and *P. minuta* (j-l). Each image is representative of the replicate trays in that treatment. Scale bar (10 cm) applies to centre and right columns; magnified images (left column) are taken from marked regions in centre column.

F. candida-grazed systems continued to extend slowly towards dish edges with finer hyphae behind the growth front being grazed. *P. armata*- and *P. minuta*-grazed systems both produced new mycelial fans from points along major cords. By 60 d most ungrazed controls, and *P. armata*- and *P. minuta*-grazed mycelium had regressed towards the wood inoculum.

Ungrazed *R. bicolor* systems were dense with many fine as well as thicker cords in early stages of outgrowth (Fig. 5.5a, b). More thick cords developed as systems expanded (Fig. 5.5c) and by 60 d some cords had begun to regress. Grazed patches were evident 24 h after *F. candida* and *P. armata* addition. These patches mainly occurred close to wood inocula, with both cords and fine mycelium grazed away in an apparently indiscriminate fashion (e.g. Fig. 5.5d). With *F. candida*, grazing effects intensified over time, whereas with *P. armata* mycelia remained relatively intact after an initial burst of grazing. *F. candida* almost completely destroyed systems by 30 d (Fig. 5.5f). With *P. armata* most mycelia remained intact, although less uniform than ungrazed systems (Fig. 5.5i). After 40 d *P. armata*-grazed mycelia consisted of a dense outer ring linked to the inoculum by just a few thick cords.

Ungrazed *H. fasciculare* developed dense growth fronts of white mycelium behind which yellowed cords formed (Fig. 5.6a). The dense ring of mycelium immediately around the wood inoculum remained white throughout the experiment. Ungrazed systems reached dish margins by 30 d, and fine mycelium and minor cords began regressing. *P. minuta*, *P. armata* and *F. candida* grazed small patches around the margin within 24 h of addition. Eight days later mycelia had extended no further and no diffuse, white mycelium remained at the margins; mycelium consisted only of a dense white ring around the wood inoculum outside of which were thick yellow cords (Fig. 5.6d, g). By 60 d a white 'crust' had formed on the surface of grazed mycelia. The only difference noted in the response of *H. fasciculare* to the three collembola was the presence of small grazed patches in the interior of some *F. candida*-grazed systems from 10 d, although by 30 d these had recovered with mycelium. These patches did not occur in *P. armata*- or *P. minuta*-grazed microcosms.

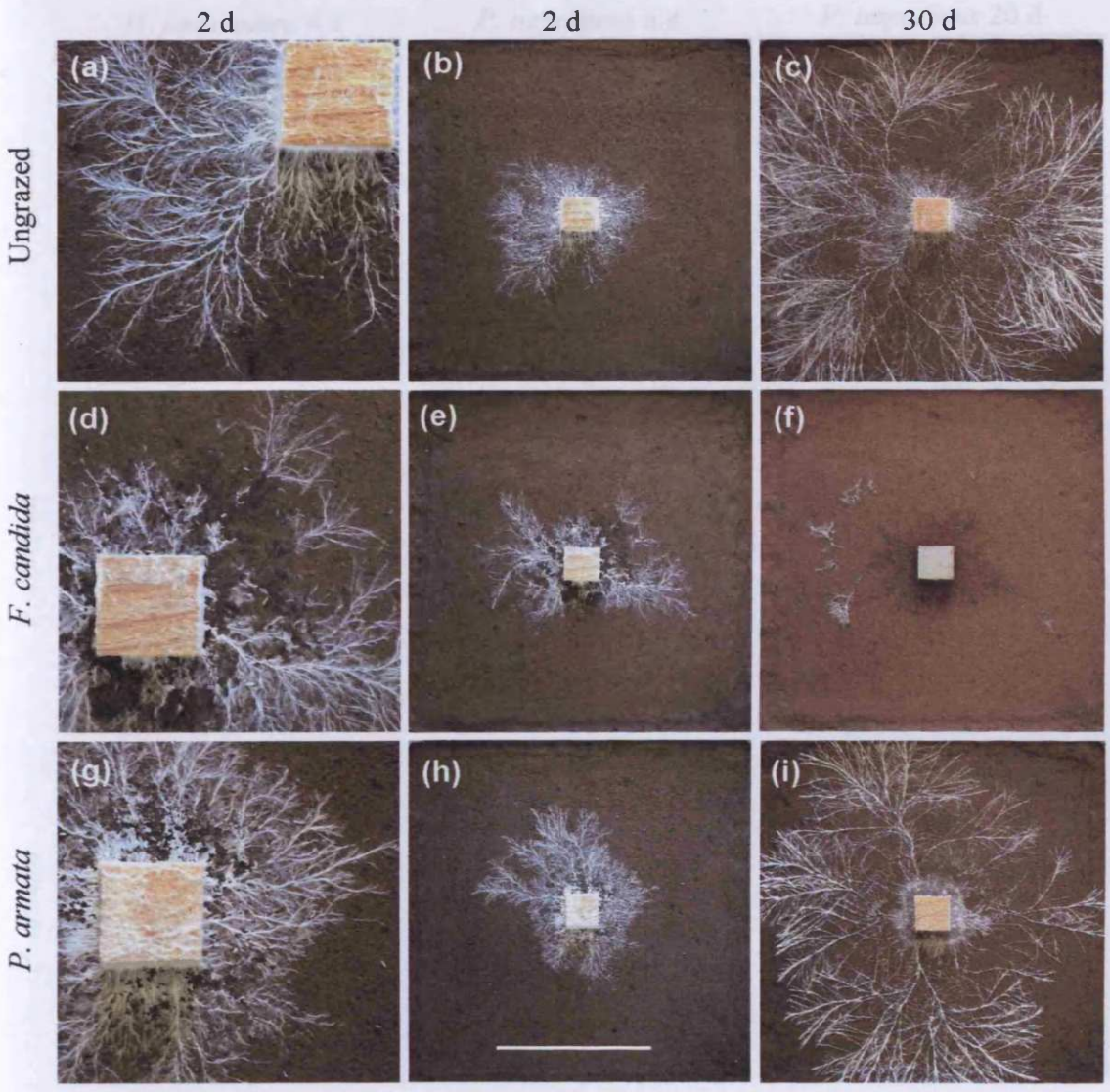


Figure 5.5. Digital images of mycelial systems of *R. bicolor* after 2 d (a, b, d, e, g, h) and 30 d (c, f, i) of collembola grazing. Treatments are ungrazed controls (a-c), *F. candida* (d-f) and *P. armata* (g-i). Each image is representative of the replicate trays in that treatment. Scale bar (10 cm) applies to centre and right-hand columns; magnified images in left-hand column are taken from mycelia in centre column.

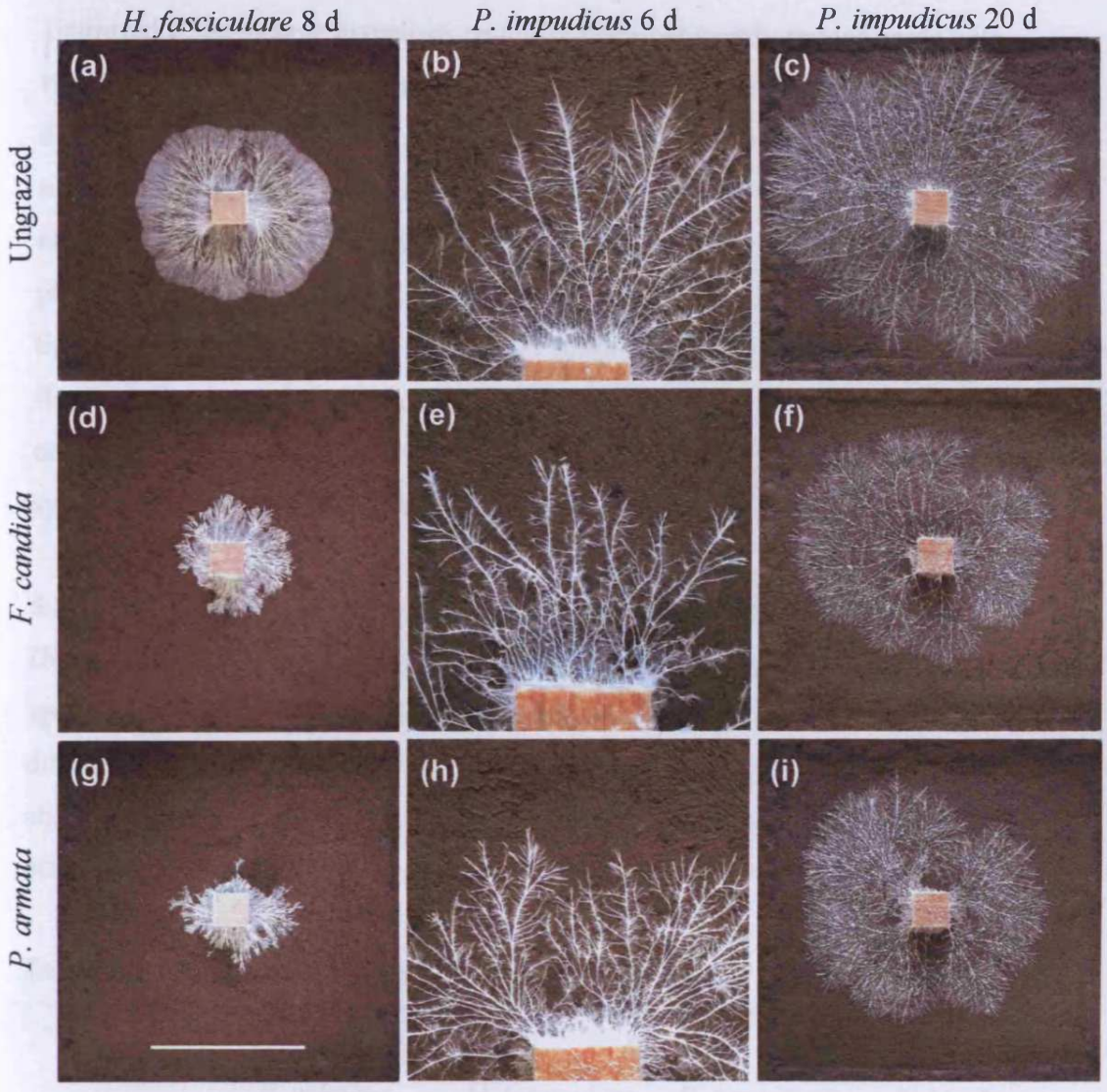


Figure 5.6. Digital images of mycelial systems of *H. fasciculare* after 8 d (a, d, g) and *P. impudicus* after 6 d (b, e, h) and 20 d (c, f, i) of collembola grazing.

Treatments are ungrazed controls (a-c), *F. candida* (d-f) and *P. armata* (g-i). Each image is representative of the replicate trays in that treatment. Scale bar (10 cm) applies to left- and right-hand columns; wood inocula (2 x 2 cm) indicate scale in centre column.

Ungrazed *P. impudicus* mycelium comprised straight evenly-spaced cords with regular lateral branches forming a dense reticulate network (Fig. 5.6c). Mycelia filled dishes by about 40 d and retained a relatively dense structure across the whole mycelium until harvest at 60 d. No distinct grazing effects were observed until 6 d after collembola addition; even these were relatively subtle, consisting of small grazed patches between cords close to the inoculum, and increased branching of some cord tips (compare Fig. 5.6b with Fig. 5.6e). *F. candida*, but not *P. armata*, grazed away fine mycelium between cords close to the wood inoculum (Fig. 5.6f, i), often leaving only a few thick cords linking the wood inoculum with the outer zone by 50 d. Grazed systems did, however, remain intact and continued to extend across the study period.

5.3.3 Effect of different collembola species on wood inoculum decay rate

Decay rate of *H. fasciculare*-colonized wood differed ($P < 0.001$) depending on which species grazed the mycelium (Table 5.2). With *P. minuta* there was no significant difference from ungrazed controls, but decay was significantly slower with *F. candida* and *P. armata*. Collembola did not significantly ($P > 0.05$) affect the decay rate of other fungi (Table 5.2).

Table 5.2. Decay rates (mg cm^{-3} d^{-1}; mean ± s.e.) of wood inocula.

	Fungi			
	P. velutina	*H. fasciculare*	*P. impudicus*	*R. bicolor*
Collembola				
Ungrazed control	2.18 ± 0.16 ^{a}	1.83 ± 0.05 ^{a}	1.81 ± 0.09 ^{a}	1.89 ± 0.21 ^{a}
F. candida	2.25 ± 0.15 ^{a}	1.46 ± 0.10 ^{b}	1.94 ± 0.12 ^{a}	1.65 ± 0.12 ^{a}
P. armata	2.05 ± 0.14 ^{a}	1.41 ± 0.10 ^{b}	1.68 ± 0.19 ^{a}	2.17 ± 0.08 ^{a}
P. minuta	2.27 ± 0.19 ^{a}	1.75 ± 0.08 ^{ab}	-	-

Values in the same column followed by different letters are significantly different (one-way ANOVA and Tukey; $P \leq 0.05$). Grazing treatment altered decay rate of *H. fasciculare* ($F_{3,38} = 7.77$, $P < 0.001$), but not *P. velutina* ($F_{3,31} = 0.39$, $P = 0.76$), *P. impudicus* ($F_{2,18} = 0.84$, $P = 0.45$) or *R. bicolor* ($F_{2,18} = 3.26$, $P = 0.062$). Dashes indicate treatment combinations not performed.

5.3.4 Effect of different fungal species on collembola abundance

Egg masses were observed in microcosms soon after collembola addition. *F. candida* deposited egg masses either on uncolonized soil close to the mycelial margin or on the mycelium itself. *P. armata* deposited egg masses adjacent to mycelial cords. Juvenile collembola began to emerge from eggs laid in microcosms after 12 d in *F. candida* and *P. minuta*, and after 20 d in *P. armata*, irrespective of fungal species. Standardized grazing densities were compromised after these times. More eggs (unquantified) were laid by *F. candida* than by *P. armata* and *P. minuta*; differences were reflected in final collembola abundance (note Fig. 5.7 y-axes values). Only a small number of dead collembola were observed during the experiment; these were consumed by surviving collembola.

After 60 d, collembola abundance varied depending on the fungal species being grazed (Fig. 5.7; *F. candida*: $F_{3,30} = 14.1$, $P < 0.001$; *P. armata*: $F_{3,29} = 14.6$, $P < 0.001$; *P. minuta*: $F_{1,9} = 300$, $P < 0.001$). Both *F. candida* (Fig. 5.7a) and *P. armata* (Fig. 5.7b) were more abundant on *P. velutina* than on *H. fasciculare* and *P. impudicus*. The two collembola species differed, however, with respect to *R. bicolor*. *F. candida* attained the same abundance on this species as on *P. velutina*, whereas *P. armata* was only as abundant on *R. bicolor* as on *H. fasciculare* and *P. impudicus*. *P. minuta* was more abundant on *P. velutina* than on *H. fasciculare* (Fig. 5.7c).

5.4 Discussion

The four cord-forming fungi were affected differently by collembola grazing, both in terms of the zone of mycelium that was attacked and the intensity of effects on mycelial structure and growth parameters. This finding broadly agrees with results from Chapter 4, where *F. candida* was the only grazer species used. In the *F. candida* component of the current study effects on *R. bicolor* and *P. velutina* morphology were similar to what was observed in Chapter 4, namely that interior mycelium was preferentially grazed and systems were eventually destroyed in the former species, while in the latter species mycelial margins were most affected and systems persisted until harvest. *H. fasciculare*, in contrast, was much more affected by *F. candida* in the current study, where extension of mycelia ceased once collembola were inoculated, than in Chapter 4, where systems continued to develop even in the presence of high collembola densities. Despite this, there was at least one similarity between the

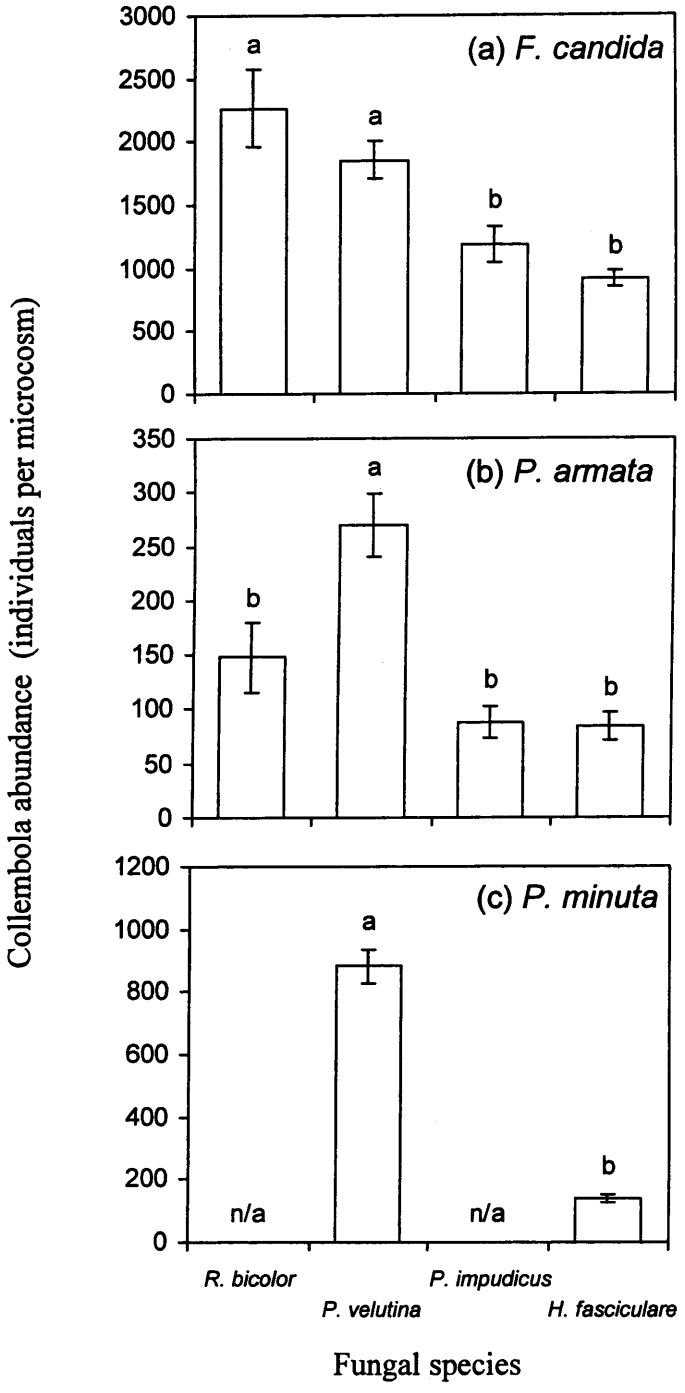


Figure 5.7. Effect of different fungi on the mean abundance (± s.e.) of *F. candida* (a), *P. armata* (b) and *P. minuta* (c) after 60 d grazing. Different letters above bars indicate significant ($P \leq 0.05$) differences within a collembola species (one-way ANOVA and Tukey). n/a indicates treatment combination not performed.

studies, namely that thick yellow *H. fasciculare* cords were never removed by grazing. The inconsistency in the response of *H. fasciculare* to grazing may be due to fungal genotype; a different isolate was used in the current study to that in Chapter 4. There is anecdotal support for this suggestion in the fact that the same isolate of *R. bicolor* and *P. velutina* was used in each of the studies, and results for these species were similar. If this is the real reason for the observed inconsistencies, then this strengthens the argument for not making far-reaching conclusions based on single isolate studies. The results for *R. bicolor* and *P. velutina*, on the other hand, demonstrate the repeatability of simple microcosm studies when the same isolates of the component species are used. The current study reports for the first time the effects of collembola grazing on *P. impudicus*, which was found to be more resistant than the other species tested and retained an intact mycelium even in the presence of very large numbers of *F. candida*. Nevertheless, fungal morphology was heavily modified by grazing in a few of the *F. candida* replicates, where the growing margins became much more densely-networked than in controls.

Although the zone of mycelium that was grazed was relatively consistent within a fungal species, the intensity of the grazing effect differed markedly across collembola. *F. candida* had a more dramatic impact on mycelia than *P. armata* in all species except *H. fasciculare*, where all three collembola had similar effects on mycelial parameters. In addition, the effect of *P. minuta* on *P. velutina* systems was intermediate between that of *F. candida* and *P. armata*. Taken together, these observations suggest substantial variation in the magnitude of collembola effects across fungal species, despite collembola biomass being standardized. This may reflect a genuine difference in collembola species preference for different fungi as a food source: *P. armata* may prefer types of fungi other than cord-forming basidiomycetes, given that it had a lesser effect on mycelia than *F. candida*. An alternative possibility for the observed species-specific effects is that the collembola have different metabolic rates: *F. candida*, which had the greatest impact, is a mobile and highly fecund species, and presumably consumes resources rapidly to meet metabolic demands. *P. armata* laid far fewer eggs, is less active than *F. candida* (Bengtsson & Rundgren 1983), and may have had less impact on mycelial morphology by consuming less fungal material.

The results broadly support the first hypothesis that the three collembola species would have different effects on fungal morphology, although it was predicted that the similar-sized *F. candida* and *P. armata* would have greater impacts than the smaller *P. minuta*. In fact, there was no obvious relationship between grazing effects and collembola body size. These findings contradict the Kampichler *et al.* (2004) study in which only *F. candida* affected *H. fasciculare* mycelial morphology, whereas two smaller species, one of which was *P. minuta*, had negligible effects. This was attributed by Kampichler *et al.* to the fungus compensating for grazing by small collembola but not by larger ones. The lack of an obvious size-dependent effect in the present study is perhaps not surprising, given that the three species have similar feeding apparatus, consisting of endognathic mouthparts with mandible tips that can be extruded and used for rasping food substrates (Hopkin 1997). If fungal hyphae and cords are simply rasped and then ingested there is no obvious basis for the supposition that a small species such as *P. minuta* would have less of an effect on mycelium than a larger species such as *F. candida* or *P. armata*, as all species are likely to be equally capable of rasping a given type of mycelium (e.g. individual hyphae or cords). In addition, mycophagous collembola share similar gut physiology (Berg *et al.* 2004), and all three species are therefore likely to be equally capable of assimilating ingested material of a given fungal species. It seems more likely that effects are dependent on metabolic rate, as discussed above, than on collembola body size.

Mass fractal dimension (D_{BM}) of mycelium was affected by collembola grazing in all species except *P. impudicus*. D_{BM} was not, however, found to be any more sensitive than the other mycelial growth parameters in detecting grazing effects; in fact it was less so in some instances (Figs 5.1 - 5.3). This finding contradicts results of a previous microcosm study in which D_{BM} was often found to be more sensitive than other measures of mycelial growth to soil nutrient enrichment (Donnelly & Boddy 1998). Nevertheless, D_{BM} did provide information that was not obtained from measurement of hyphal coverage and extension rate alone. For example, D_{BM} of *P. velutina* was often increased by *F. candida* (Fig. 5.3a), implying that grazed systems possessed more densely space-filled mycelia.

Mycophagous soil invertebrates are regarded as generalist feeders (Maraun *et al.* 2003) but are known to survive and reproduce more on certain fungal species

(Klironomos *et al.* 1999, Hogervorst *et al.* 2003). This was the case in the present study; the collembola species responded differently to the four fungal species that they were grazing. There are two potential reasons for the observed differences: 1) the fungal species employed may differ in their palatability to collembola, and this variation may have affected the fecundity and/or survivorship of collembola on different substrates; and 2) collembola may have found certain species palatable to the extent that all fungal mycelium was removed long before experimental harvest, and lack of food then led to a decrease in abundance. The latter is perhaps less likely, given that *F. candida* grazed away virtually all the mycelium of *R. bicolor* after 30 d, but still persisted at very high density for a further 30 d.

All species were expected to be most abundant on the same fungal substrate. In general the results support this hypothesis, although *F. candida* was more abundant than *P. armata* on *R. bicolor*. This correlates well with observed impacts on *R. bicolor*; *F. candida* destroyed the mycelium whereas *P. armata* had relatively little effect. These findings support other studies on collembola (Jørgensen *et al.* 2003) and oribatid mites (Schneider & Maraun 2005) that have demonstrated limited interspecific variation in food selection, but an overall preference for the same fungal species.

Grazing by both *P. armata* and *F. candida* caused a decrease in the decay rate of *H. fasciculare* wood inocula, substantiating findings in Chapter 4 where *F. candida* also decreased decay rate of *H. fasciculare*. Mycelia of cord-forming fungi scavenge nutrients from soil during outgrowth (Wells *et al.* 1990, Wells *et al.* 1997), and it may be because collembola prevented further mycelial extension onto soil that *H. fasciculare* was unable to obtain sufficient nutrients for the continued synthesis of wood decay enzymes. No effects on wood decay rate were noted in the other fungal species, which all continued to extend onto soil, at least for a time, after collembola addition.

In Chapter 4 the variable impacts of a single collembola species on different cord-forming fungi were highlighted. The present study has shown that the nature of interactions between collembola and cord-forming fungi also depend on the identity of the collembola species. This finding has potential importance: if some collembola

species have a greater impact than others on cord-forming fungi then it may be that the composition of collembolan species assemblages, which vary across space and time (Berg *et al.* 1998), affect the spatial distribution of cord-forming species. The potential importance of collembola on the spatial extent of cord-formers could be investigated in microcosm systems in which two or more cord-forming species are established, and the impact of collembola on the outcome of interactions between the fungi determined.

Chapter 6. The effects of collembola grazing on mycelial network architecture of *Resinicium bicolor*

6.1 Introduction

Communication networks, of one kind or another, pervade both nature and society. It is therefore unsurprising that their organization has been studied in fields as diverse as sociology, information technology and biology. Examples of networks that have attracted considerable interest are the World Wide Web, social interactions of humans, neural networks of animals, the epidemiology of diseases and ecological food webs. In addition to the study of 'real-world' networks, graph theory has been used to investigate the properties of regular and randomly-generated theoretical networks (for reviews see Albert & Barabási 2002, Newman 2003). In recent years the investigation of complex networks has been made possible by advances in computer processing power (Strogatz 2001), and this has revealed that many real-world networks differ considerably in their properties from randomly-generated networks (Newman 2003, Amaral & Ottino 2004). For example, real-world networks are generally much more integrated than random networks; they have short paths (Section 6.2.3) which "could provide high-speed communication channels between distant parts of the system, thereby facilitating any dynamical process that requires global coordination and information flow" (Strogatz 2001).

Fungal mycelia represent obvious examples of complex networks, many species forming highly branched systems with tangential anastomoses (hyphal fusions). The Mycology Research Groups at Oxford and Cardiff Universities have relatively recently begun to investigate the network architecture of cord-forming fungi growing in soil microcosms (Bebber *et al.* 2006, Fricker *et al.* 2006). These systems are particularly amenable to network mapping as their macroscopic two-dimensional mycelia (Section 2.2.4) approximate a planar spatial network. This can be represented as a graph comprising a set of nodes and links (Section 6.2.2).

Studying the architecture of cord-forming mycelia is not a trivial exercise. Architecture will inevitably influence the functioning of these systems. Cord-forming fungi move nutrients between different resource units within their mycelia (Section

2.2.2); the speed and efficiency at which nutrient redeployment occurs is likely to depend on mycelial topology. Network architecture will also affect the resistance and resilience of fungal mycelia to disturbance. Systems of cord-forming fungi growing on the forest floor are likely to be subject to frequent damage from invertebrate grazing and soil disturbance by larger animals. Cord-formers require an interconnected mycelium linking discrete resource units if they are to translocate nutrients between regions of supply and demand (Cairney 1992), and so it is vital to their functioning that these connections are robust to system disturbance. Otherwise, the mycelia may frequently become broken up into separate regions.

Redundancy in their 'wiring' makes many real and randomly-generated theoretical networks robust to damage (Albert & Barabási 2002, Proulx *et al.* 2005). For example, in computer simulations, the functioning of the Internet is unaffected by the random removal of 60% of its nodes, and the random deletion of species from ecological food webs results in very low rates of secondary extinction (see Albert & Barabási 2002). Oxford and Cardiff University Mycology Research Groups are currently developing computer software that will electronically remove randomly-selected nodes from networked images of mycelia and test how well these 'damaged' systems retain function (defined in terms of various topological measures – see Section 6.2.3).

Collembola grazing of cord-forming fungi growing in soil microcosms provides a less artificial means of mycelial disturbance, as damage is not random but instead targeted at particular mycelial regions (Chapters 4 and 5). For example, when *Resinicium bicolor* is grazed by *Folsomia candida* all areas of the mycelium, including thick cords, are damaged, but grazing effects are more intense close to the wood inocula. These, and other, grazing-induced changes were often readily observable by eye, and also affected radial extension rate, hyphal coverage and mass fractal dimension of mycelia (Chapters 4 and 5). These quantitative parameters are, however, relatively crude, giving information only on growth rate and space-filling capacity of mycelia. Analysis of network architecture permits investigation of potentially more subtle effects of collembola grazing on mycelia.

Analysis of complex networks is a relatively recent phenomenon; there are probably many potentially-useful topological measures that have yet to be developed (Albert & Barabási 2002). In particular, the study of the topology of mycelial networks is still very much in its infancy. This chapter reports on a preliminary investigation, using currently available tools, that sets out to gain some insight into the effects of mycophagous collembola on fungal network architecture. It is predicted that the relatively indiscriminate damage observed when *F. candida* grazes *R. bicolor* will cause decreased mycelial integration, and that this effect will depend on collembola density.

6.2 Methods

6.2.1 Selection of mycelia for network analysis

Initially it was intended to analyse the network architecture of mycelia from a range of fungus-collembola species combinations, selected from stored images of the Chapter 4 and 5 experiments. Unfortunately, although it was possible to map the mycelia of *P. impudicus* and *P. velutina*, computer software limitations prevented statistical analysis of the densely-networked mycelia of these species. Analysis was therefore restricted to the *R. bicolor-F. candida* species combination.

The mycelia of *R. bicolor* were usually rapidly destroyed when grazed by *F. candida*; network analysis of this species combination was therefore limited to the 11 cm diam. experiment in Chapter 4, where grazing was less destructive. The effects of grazing on the morphology of these *R. bicolor* systems are summarized in Section 4.3.4 (and see Fig. 4.10). Three replicates were selected at random from each of the four grazing treatments. The treatments (with mean collembola number added to each microcosm given in parentheses) were: ungrazed control microcosms (0); standard number of *F. candida* per microcosm (40); density standardized as one per cm^2 hyphal coverage (16); and density standardized as one per cm^2 mycelial area (72). Five time points (0, 8, 12, 30 and 40 d after collembola addition) were selected from the 12 that were analysed for hyphal coverage (Chapter 4), on the basis that mycelial morphology altered markedly at these times.

6.2.2 Plotting networks using MatLab

Networks of the selected images were electronically mapped using a version of MatLab 7.01 (The Mathworks Inc., Natick, USA) that was customized for mycelial network plotting by M.D. Fricker (Oxford University). First, images of *R. bicolor* were exported from SEMPER as bitmap files. The time series of images for each replicate (e.g. Fig. 6.1a-e) were then aligned by selecting points which remained fixed across the time period (e.g. soil features; mycelial anastomoses). Nodes (representing branch points and junctions) and links (cords that join branch points) were then manually plotted on the digital photographs of mycelia. The wood inoculum was represented as a central node with many links (Fig. 6.1f).

6.2.3 Output variables and data analysis

Each mapped network was calibrated as described for SEMPER in Section 3.5. Network architecture data were then extracted from MatLab and analysed according to a series of protocols designed by Oxford Mycology Research Group. This analysis employed the statistical package R (Release 2.2.0) to calculate automatically basic information (*number of nodes*, *number of links*, *total link length*) about each network, as well as the following topological measures (Fig. 6.2).

1) *Clustering coefficient*. The proportion connectivity of all nodes in a network. Connectivity is the probability that if a focal node is connected to two other nodes, they will also be connected to each other.

2) *Mean degree*. *Degree* is the number of links that connect a focal node to other nodes. The mean value of this for every node in a network is the *mean degree*.

3) *Diameter*. The *shortest path* between two nodes in a network is the path that involves the fewest links. When the shortest path is determined from every node to every other node, the longest of these shortest paths is termed the *diameter* of the network. Diameter is a measure of network integration; an increase in diameter indicates that the network is becoming less well integrated. This measure is, however, affected by the size of the network, irrespective of its integration. Diameter tends to increase as networks become larger. In an attempt to control for the potentially confounding effect of network size, *scaled diameter* was also calculated.

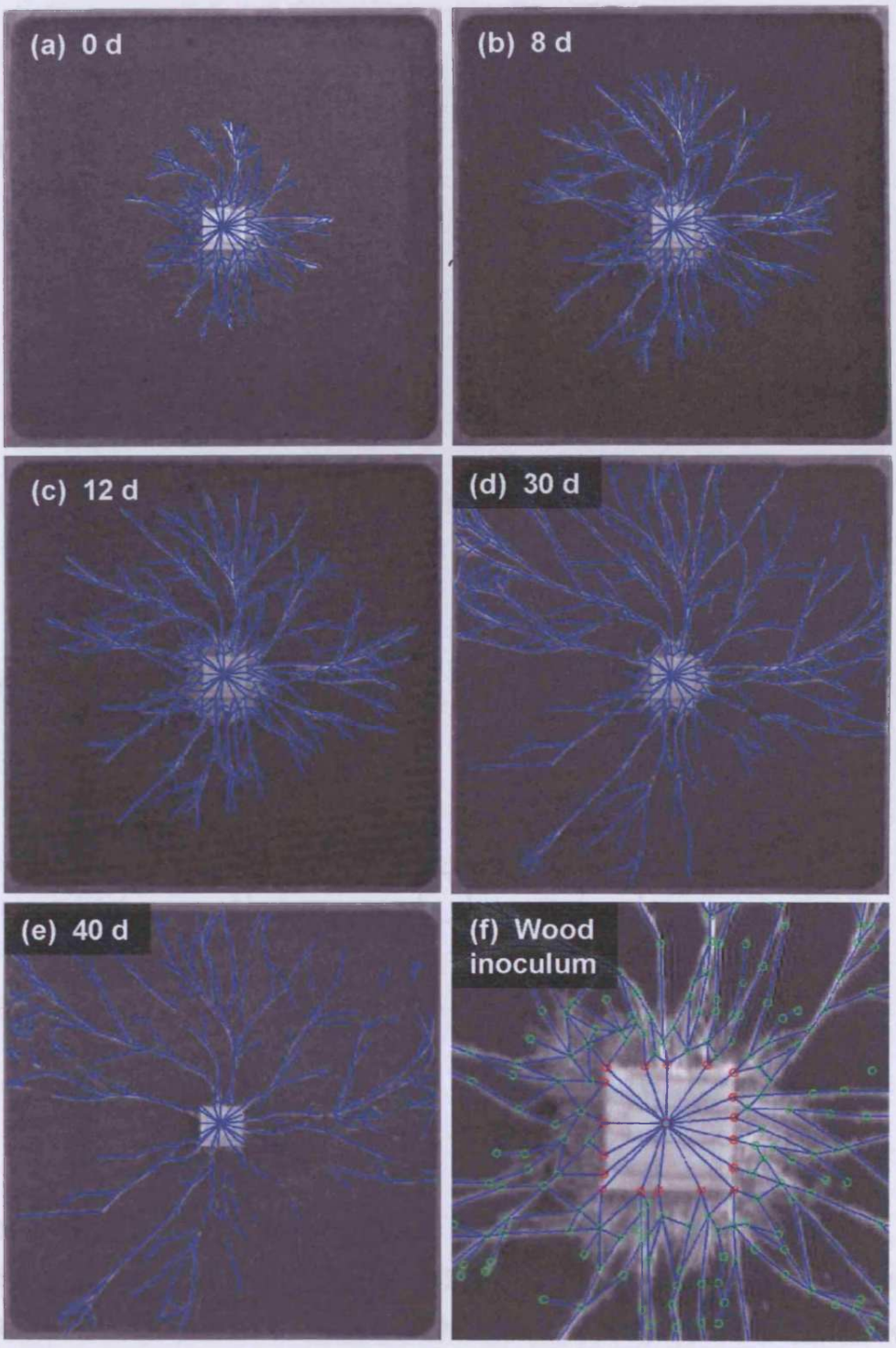


Figure 6.1. Example of mycelial networks plotted using MatLab. Images are of *R. bicolor* grazed by a standard number (40) of *F. candida*. In the time series (a-e), where number of days after collembola addition is indicated, links are displayed as blue lines; nodes are omitted for clarity. (f) illustrates the wood inoculum represented as a central node connected by many links to points of cord outgrowth. Inoculum (red) and outgrowth (green) nodes are distinguished.

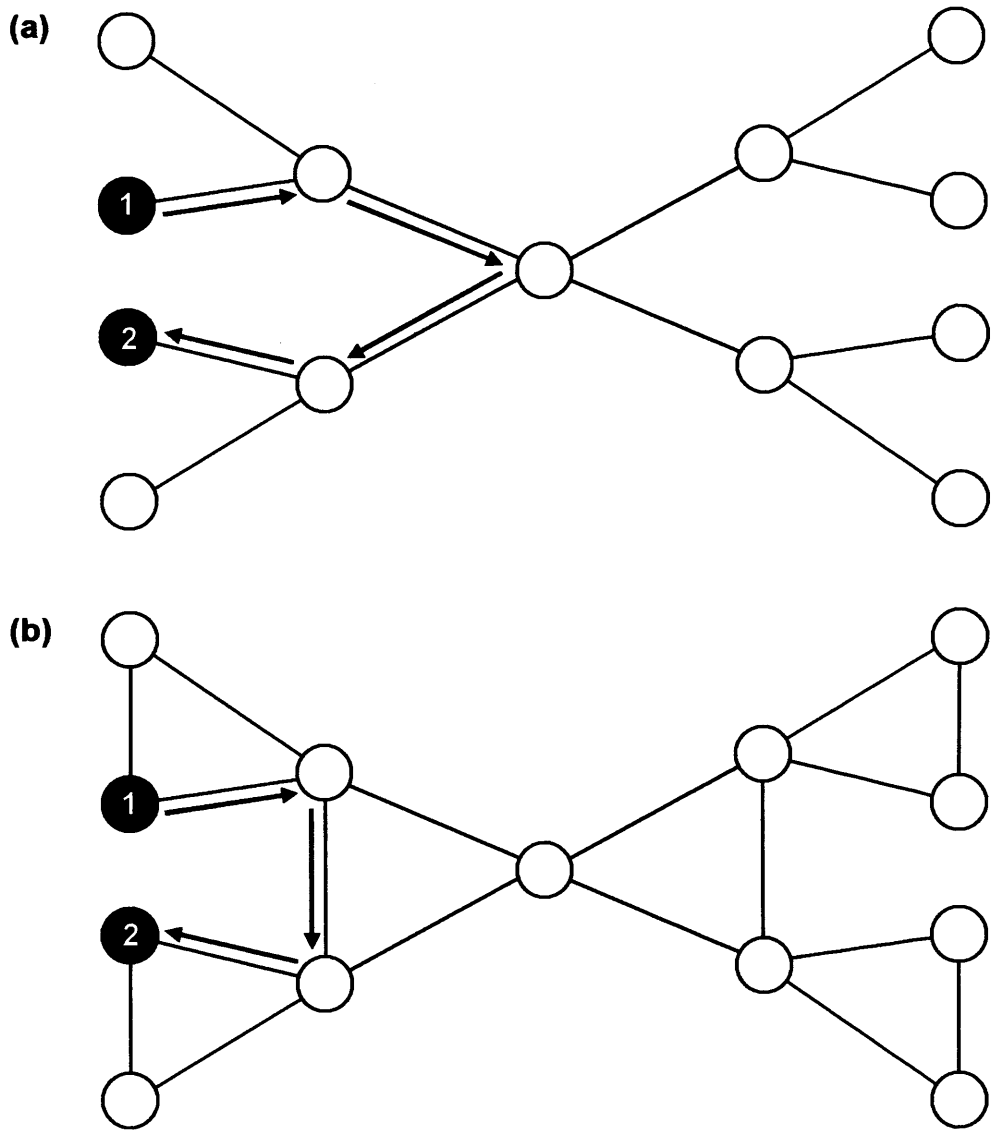


Figure 6.2. Schematic representation of two networks. Both networks have 13 nodes, but (b) has 18 links compared to (a)'s 12 . The *shortest path* between Nodes 1 and 2, illustrated by arrows, is shorter in (b) than in (a). The network *diameter* is equal in (a) and (b), the 'longest shortest path' being 4 links in each case. The *mean degree* is greater in (b) than in (a), as each node has on average more links. The *clustering coefficient* is also greater in (b) than in (a), as any two nodes connected to a focal node are more likely to be connected to each other.

4) *Scaled diameter*. Network diameter divided by total link length. The latter measure gives an approximation of the total length of the cord system, and can therefore be used in scaling for network size.

The effect of grazing treatment on each topological measure of *R. bicolor* was compared using Repeated Measures Analysis of Variance (RM ANOVA; Section 3.7). Significant time*treatment interactions were investigated further using one-way ANOVA and Tukey tests on individual time points (Section 3.7).

6.3 Results

6.3.1 Basic parameters

The number of nodes (Fig. 6.3a), number of links (Fig. 6.3b) and total link length (Fig. 6.3c) of *R. bicolor* networks showed very similar temporal patterns. Each of the three measures was reduced by *F. candida* grazing (RM ANOVA time*treatment interaction: number of nodes $F_{7,20} = 9.78$, $P < 0.001$; number of links $F_{8,22} = 10.6$, $P < 0.001$; total link length $F_{7,18} = 15.0$, $P < 0.001$). Post hoc analysis (one-way ANOVA and Tukey tests) revealed the effects of grazing to be significant ($P \leq 0.05$) only at 30 and 40 d after collembola addition, for each of the three measures. The impact of collembola was dependent on inoculation density; all three measures were reduced most in the treatment with the highest grazing pressure (collembola density determined by mycelial area; Fig. 6.3).

6.3.2 Topological measures

The clustering coefficient of *R. bicolor* mycelia was reduced by grazing (Fig. 6.4a; RM ANOVA; $F_{8,20} = 3.93$, $P = 0.006$). This reduction was most pronounced in the systems with the highest grazing density, where the clustering coefficient was reduced almost to zero by 40 d, but was also significantly ($P \leq 0.05$) different from the control in the other grazing treatments at 40 d (Fig. 6.4a). Mean degree was similarly affected (Fig. 6.4b; $F_{8,21} = 12.9$, $P < 0.001$). Again, effects became more pronounced across time and were dependent on grazing density; after 40 d the mean (± s.e.) number of links attached to each node was 2.67 ± 0.08 in ungrazed controls and 1.56 ± 0.03 when *F. candida* density was determined by mycelial area (Fig. 6.4b).

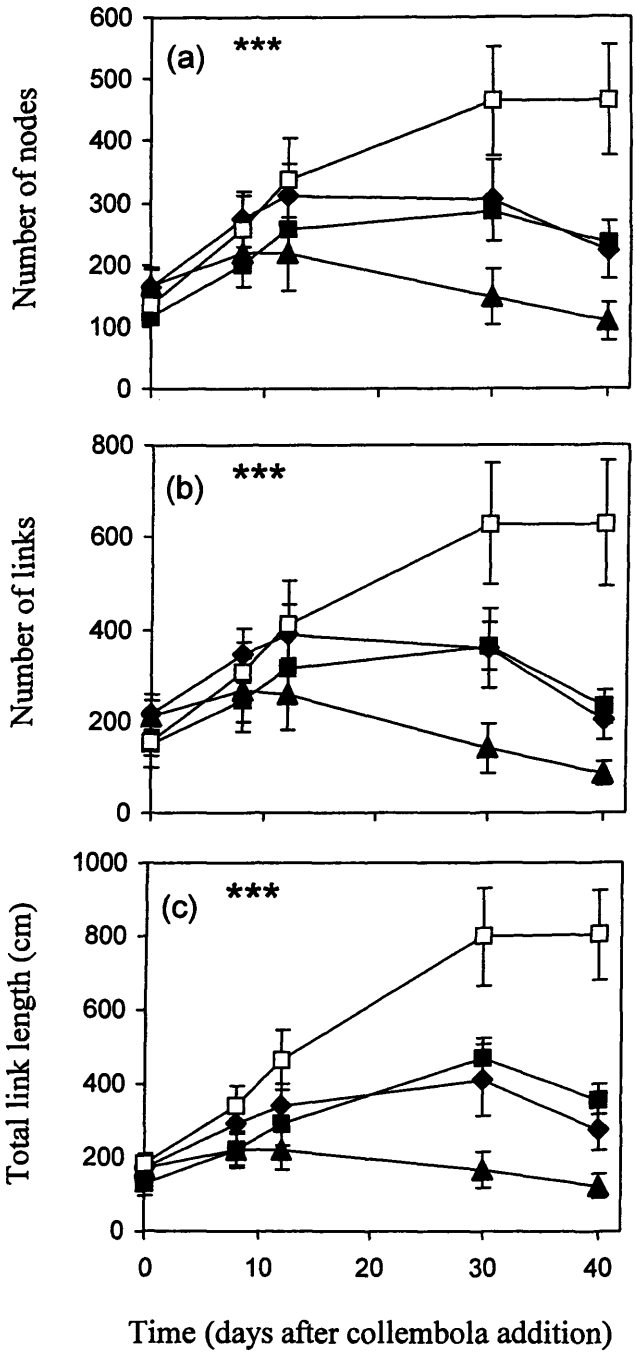


Figure 6.3. The effect of *F. candida* grazing treatment on basic parameters (mean ± s.e.) of *R. bicolor* mycelial networks; number of nodes (a), number of links (b) and total link length (c). Collembola treatments are: ungrazed control (□); constant number per dish (◆); standardized as 1 per cm^2 hyphal coverage (■) or 1 per cm^2 mycelial area (▲). Significant time*grazing treatment interactions (RM ANOVA) are indicated; *** $P < 0.001$.

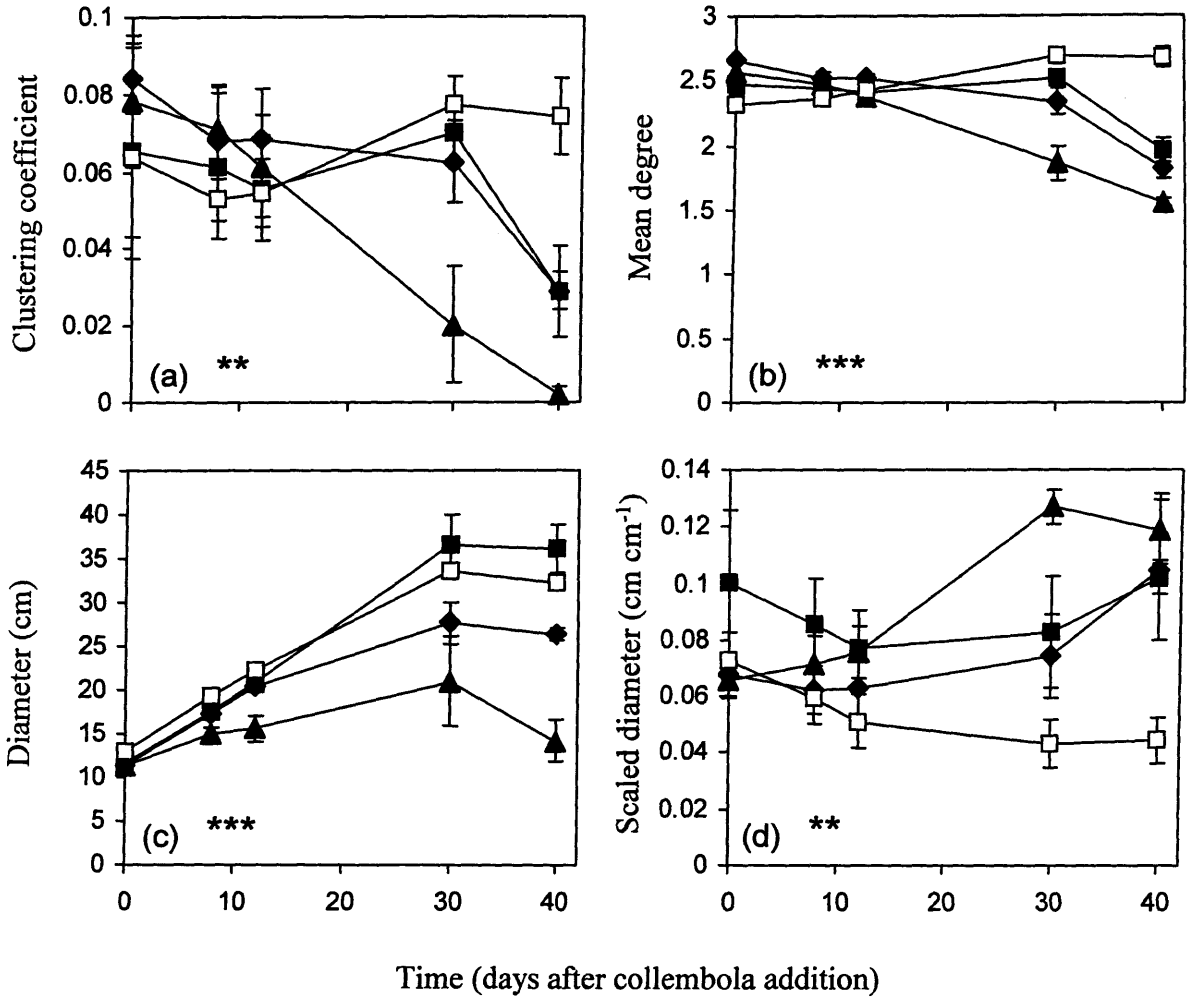


Figure 6.4. The effect of *F. candida* grazing treatment on topological measures (mean ± s.e.) of *R. bicolor* mycelial networks; clustering coefficient (a), mean degree (b), diameter (c) and scaled diameter (d). Collembola treatments are: ungrazed control (□); constant number per dish (◆); standardized as 1 per cm^2 hyphal coverage (■) or 1 per cm^2 mycelial area (▲). Significant time*grazing treatment interactions (RM ANOVA) are indicated; ** $P < 0.01$; *** $P < 0.001$.

Network diameter was reduced by grazing (Fig. 6.4c; $F_{9,23} = 6.29$, $P < 0.001$); post hoc analysis revealed that this effect was only significant ($P \leq 0.05$) in the treatment with the highest collembola density (one-way ANOVA and Tukey). When system size was controlled for, the result was reversed: scaled diameter was increased in grazed systems compared to ungrazed controls (Fig. 6.4d; $F_{8,21} = 4.65$, $P = 0.002$). This effect was significant ($P \leq 0.05$) in the treatment with the highest collembola density after 30 d and 40 d of grazing (one-way ANOVA and Tukey).

6.4 Discussion

The network architecture of *R. bicolor* mycelia was altered by *F. candida* grazing; each of the calculated topological measures was significantly affected (Fig. 6.4). In the experimental images selected for networking, basic measures of mycelial growth (radial extension and hyphal coverage in Chapter 4; total link length in the current study) were also affected by grazing. The present study demonstrates that the potentially more subtle effects of grazing on network architecture might also be important in determining the impact of collembola on the functioning of mycelial cord systems.

Network diameter is a global measure of how integrated the network is (Proulx *et al.* 2005). The fact that the network diameter of *R. bicolor* decreased when this species was grazed suggests that mycelia of this fungus become more integrated in the presence of *F. candida*. The interpretation of this measure is, however, confounded by differences in the size of mycelia. In the treatment with the highest grazing density, total link length (which gives an approximation of total mycelial cord length) was reduced to 15% of that in ungrazed controls after 40 d. This reduction in mycelial size is likely to reduce diameter irrespective of network integration, making accurate interpretation of the results difficult. When, in an attempt to control for variation in mycelial size, diameter was scaled by total link length the result was reversed: ungrazed controls had a smaller diameter than mycelia that were heavily grazed (Fig. 6.4d). Where mycelia vary greatly in size, scaled diameter, rather than diameter, is likely to be a good indicator of network integration. If this is the case, then the apparently less integrated nature of grazed networks might have important functional consequences. For example, nutrient translocation between two woody resource units might have to follow a longer path in poorly integrated grazed systems.

Unlike diameter which is a global network measure, clustering coefficient and mean degree are local properties that are averaged for the whole network (Proulx *et al.* 2005). Clustering coefficient and mean degree are therefore less informative as indicators of network integration. Nevertheless, the changes in these measures that result from the presence of *F. candida* suggest that fundamental properties of *R. bicolor* networks are affected by collembola grazing. For example, the decrease in mean degree that occurred in all collembola treatments shows that nodes have, on average, fewer attached links when systems are grazed. The collembola-mediated reduction in clustering coefficient suggests that, at a local level, grazed systems are less well connected than ungrazed systems.

The mycelia employed in the current study allow investigation of the resistance and resilience of fungal networks to disturbance (in this case collembola grazing). The reduced integration of grazed mycelia, highlighted by changes in all of the topological measures that were calculated, suggests that networks of *R. bicolor* are not particularly resistant to collembola-induced perturbation. Investigation of network resilience (i.e. how quickly a network recovers from disturbance) would be better suited to topological analysis of mycelial images from the associated experiment of S. Bretherton *et al.* (unpublished data), in which collembola were allowed to graze for a short time period and then removed (results discussed in Chapter 8). The continual presence of collembola in the current study probably prevented extensive system 'rewiring'; re-connection of severed cords occurred only occasionally. It should be remembered, however, that the *R. bicolor-F. candida* combination produced the most dramatic grazing effects of any pairing of fungus and collembola in Chapters 4 and 5. It may be more appropriate to investigate fungal resistance and resilience in terms of the less dramatic grazing effects that were observed on the relatively densely-networked mycelia of *P. velutina*, *P. impudicus* and *Hypholoma fasciculare*. In these systems thick cords were rarely severed, effects being confined mainly to peripheral regions of new mycelial growth. It is disappointing that software limitations prevented analysis of topological parameters of *P. velutina* and *P. impudicus*.

The findings reported in this chapter suggest that network analysis may be another useful tool for investigating the grazing effects of mycophagous invertebrates on mycelia. It is tempting to think that the more subtle effects of collembola grazing, not

picked up by measurements of mycelial growth, would become apparent if network architecture were analysed. For example, grazing does not alter the hyphal coverage or fractal dimension of *P. impudicus* cord systems, despite some relatively subtle changes in mycelial morphology being observed (Chapter 5). If the network topology of species such as *P. impudicus*, that appear relatively resistant to collembola, were altered by grazing then this might have important implications for fungal nutrient translocation.

Chapter 7. Nitrogen partitioning in mycelia of *Phanerochaete velutina*: an investigation into the influence of collembola grazing using stable isotopes

7.1 Introduction

Saprotrophic fungi rapidly translocate mineral nutrients and carbon within their mycelia and allocate them differentially among discrete resource units (Wells *et al.* 1990, Wells *et al.* 1995, Owen 1997). The large and persistent mycelial networks formed by forest-floor cord-forming fungi potentially enable them to influence the spatial distribution of nutrients within forest soils (Boddy & Watkinson 1995).

Despite the plethora of studies undertaken in laboratory microcosms (Section 2.2.2), as far as I am aware, only two studies have investigated the translocation of nutrients by saprotrophic fungi in natural ecosystems. Both studies employed the radioisotope ^{32}P to investigate translocation in cord-forming fungi. The first study (Clipson *et al.* 1987) revealed an almost total lack of translocation in *Mutinus caninus*. This was attributed by the authors to immobilization of phosphorus at the point of uptake. Whether this is a reliable conclusion is, however, questionable as the phosphorus was added to lengths of mature cord rather than to actively growing mycelial margins where nutrient uptake is known to occur (Boddy 1993). The second study was more revealing, demonstrating significant translocation of phosphorus in a range of cord-forming species from sites of labelling to organic resources that were inter-connected by mycelium (Wells & Boddy 1995).

A relatively large number of studies have helped to elucidate how nutrients are acquired, translocated and partitioned within the mycelia of saprotrophic fungi (Section 2.2.2). Little, if any, attention has been paid to investigating the role that mycophagous soil invertebrates play in modifying these processes. This is surprising, given that mycophagous invertebrates, including collembola, are ubiquitous and abundant in forest ecosystems (Petersen & Luxton 1982). Collembola grazing modifies the growth rate and foraging pattern of a range of saprotrophic cord-forming species in laboratory microcosms (Chapters 4 and 5, Kampichler *et al.* 2004) and, as a

consequence, may alter nutrient partitioning in these fungi. This could potentially affect nutrient distribution on the forest floor over relatively large spatial scales.

Mycophagous invertebrates might also affect nutrient release from fungal mycelia. Cord-forming saprotrophs are known to be conservative of acquired nutrients (Boddy & Watkinson 1995), which they translocate to newly-arising regions of demand or to sites of storage within their mycelia, rather than releasing into the bulk soil (Section 2.2.2). The circumstances that cause nutrients within cord-formers' biomass to become available to other organisms remain unknown. One possibility is that nutrients are lost to other fungi during competitive interactions. This is supported by a study in which 25% of a ^{32}P label was transferred from the cord-former *Hypholoma fasciculare* to two ectomycorrhizal fungi, with little reciprocal transfer of ^{32}P back to the cord-former (Lindahl *et al.* 1999). A second possibility is that grazing by soil invertebrates causes nutrient loss from mycelia. Invertebrate grazing can mobilize nutrients through consumption of fungal hyphae and excretion of faecal pellets (Hanlon & Anderson 1979), and might also make mycelia more 'leaky' by damaging hyphae during feeding activity.

In the present study a ^{15}N stable isotope label is used to investigate whether grazing by the collembola *Protaphorura armata* affects nitrogen (N) partitioning and release in the cord-forming fungus *Phanerochaete velutina* growing in soil microcosms. It is hypothesized that: 1) as *P. armata* graze outgrowth mycelium of *P. velutina* and assimilate fungal material into their body tissues they will become enriched in ^{15}N across the duration of the experiment; 2) grazing by *P. armata* will alter the partitioning of ^{15}N within mycelia, with relatively more N being stored in 'safe' wood inocula relative to outgrowth mycelium; and 3) more ^{15}N will be released to the bulk soil in grazed systems as mycelia become more 'leaky'.

7.2 Materials and methods

7.2.1 Experimental design

The study involved a one-off application of ^{15}N-labelled ammonium nitrate ($^{15}NH_4NO_3$) to allow investigation of the partitioning and release of N in collembola-grazed and ungrazed mycelia. Study microcosms comprised soil dishes with a central compartment to which wood inocula and subsequently ^{15}N were added (Fig. 7.1). The

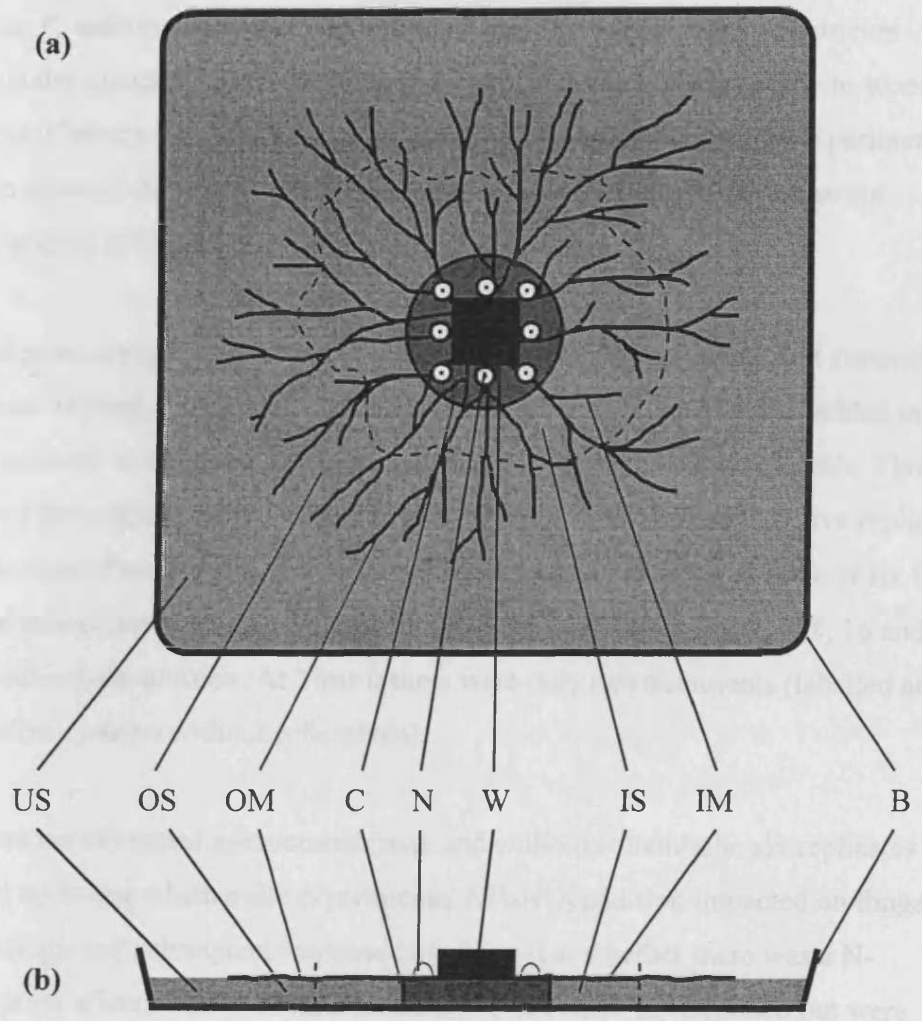


Figure 7.1. Diagram of an experimental microcosm, a) from above, and b) in side profile. The position of the central compartment (C) and wood inoculum (W) within the 24 x 24 cm bioassay dish (B) are shown. The eight sites of NH_4NO_3 addition (N) are indicated. Dashed lines show the size of the mycelium at the time of collembola addition; this was used to separate inner zone mycelium (IM) and soil (IS) from outer zone mycelium (OM) and soil (OM). Uncolonised soil (US) was not harvested.

compartmentalized design prevented movement of the ^{15}N label to other regions of the microcosm through the bulk soil; ^{15}N label found post addition in mycelium outside the central compartment must, therefore, have been translocated there by the fungus. *P. velutina* and other cord-formers are known to translocate nutrients acropetally to rapidly-extending mycelial growth fronts and basipetally to wood inocula (Cairney 1992, Lindahl *et al.* 2001a, Tlalka *et al.* 2002). The experimental design allowed the effect of collembola (*P. armata*) grazing on the expected translocation of N within mycelia to be investigated.

The experiment comprised four treatments: ^{15}N-labelled and unlabelled systems, both with and without collembola. Unlabelled systems, which had NH_4NO_3 added in the same quantity as in labelled systems, served as natural abundance controls. This allowed determination of ^{15}N excess in labelled systems (see below). Five replicate microcosms of each treatment combination were destructively harvested at six time points: immediately prior to collembola addition (Time 0), and at 2, 4, 8, 16 and 32 d after collembola addition. At Time 0 there were only two treatments (labelled and unlabelled systems without collembola).

An extra set of control microcosms (with and without collembola; six replicates each) was set up to test whether the experimental NH_4NO_3 addition impacted on fungal morphology and subsequent response to grazing (i.e. whether there was a N-fertilization effect). These 12 microcosms (N-) had no NH_4NO_3 added but were identical to the main experiment microcosms (N+) in every other respect. Although N- microcosms were not destructively harvested, images were captured and analysed to determine mycelial growth parameters, specifically hyphal coverage and radial extension (see below), for comparison with N+ microcosms.

7.2.2 Preparation of study organisms

P. velutina (KC1685) wood blocks were prepared as described in Section 3.2, and incubated in darkness at 15 ± 1°C for 330 d. *P. armata* were cultured and extracted as described in Section 3.3. Collembola of body width 200-400 μm were used in the experiment.

7.2.3 Preparation and inoculation of soil microcosms

Soil microcosms were prepared and maintained as described in Section 3.4, with the exception that the 24 x 24 cm bioassay dishes contained a central compartment. This consisted of a 5 cm diam. Petri dish lid that had been glued centrally with aquarium silicone sealant (Geocel Ltd., Plymouth, UK). As the soil level in the central compartment (approx. 7 mm) needed to be flush with that in the rest of the dish, the amount of wet soil required was 400 g, rather than the usual 200 g, per microcosm.

7.2.4 Isotopic labelling and collembola addition

Microcosms were isotopically labelled 8 d after fungal inoculation. At this time the average mycelial diam. was 10 cm. 0.4 ml NH_4NO_3 solution (^{15}N-labelled or unlabelled) or de-ionized (DI) water (to N- controls to assess fertilization effects) was added in 50 µl aliquots at eight locations immediately around the wood inoculum (i.e. on the surface of the mycelium within the bounds of the central compartment; Fig. 7.1) using a P100 Gilson Pipette. Ammonium nitrate solution was labelled by adding 0.6042 g/l of 98 atom% single-labelled $^{15}NH_4NO_3$ to 92.8945 g/l unlabelled NH_4NO_3. The amount of added NH_4NO_3 doubled the concentration of N in the central compartment (original total N in soil was 0.26% by weight), and the ^{15}N concentration (44.7 µg ^{15}N excess per microcosm) was sufficient to create a detectable label. Unlabelled NH_4NO_3 solution (natural abundance; $\delta^{15}N$ 0.33 ‰) was at equivalent concentration (93.4987 g/l unlabelled NH_4NO_3).

Sixty *P. armata* were added evenly to uncolonized soil in microcosms 2 d after isotopic labelling; droplets of NH_4NO_3 solution initially remained on the hydrophobic mycelium but were absorbed within 2 d, preventing transfer around microcosms by collembola movement.

7.2.5 Experimental harvesting

At destructive harvest all system components collected for isotopic analysis were immediately frozen at -18°C. Collembola were extracted from microcosms using an electric pooter, enumerated and housed in food-free culture pots for 4 h prior to freezing, to allow excretion of gut contents. A small number of juvenile *P. armata* were observed at 32 d; these were not harvested. Mycelia were divided into two zones (Fig. 7.1): inner (hyphae present at the time of collembola addition) and outer (hyphal

growth since collembola addition). Photographs captured with a Nikon Coolpix E4500 digital camera at the time of collembola addition were used to determine zone limitations, and the two areas were sectioned using a scalpel. Mycelium was collected from each zone using lightweight forceps to avoid contamination with soil particles. Soil from beneath each of the two mycelial zones was collected using a spatula. Wood inocula were scraped free of adhering mycelium, frass and soil.

7.2.6 Image capture and analysis

Digital images of mycelia were captured and analysed for radial extension and hyphal coverage as described in Section 3.5. Images were captured immediately prior to each harvest time point; in each case six grazed and six ungrazed microcosms were selected at random from the microcosms to be harvested. The six replicate grazed and ungrazed N- control microcosms were also photographed.

7.2.7 Isotope sample preparation and analysis

Collembola and mycelium samples were freeze-dried (Modulyo Pirani 10 Freeze Dryer, BOC Edwards, Tonawanda, USA) from frozen for 24 h. Mycelium was cut into fragments using a scalpel and a sub-sample taken for analysis. Wood inocula and soil were oven-dried from frozen at 80°C for 2 d. Soil was homogenized by hand and sub-sampled for analysis. Wood inocula were liquid nitrogen ball-milled (Spex 6700 Freezer Mill, Glen Creston Ltd, Stanmore, UK) and sub-sampled. All system components were weighed prior to sub-sampling. Samples were transferred into individual 6 x 4 mm tin capsules (Elemental Microanalysis Ltd, Okehampton, UK) in readiness for isotope ratio analysis.

Prepared samples were loaded into the autosampler of a Eurovector Elemental Analyser (Eurovector, Milan, Italy), which was coupled in-line to an Isoprime Stable Isotope Mass Spectrometer (GV Instruments, Manchester, UK). Each sample was dropped into a combustion reactor at 1020°C at the same time as a pulse of oxygen was introduced. Products from the resulting flash combustion were carried by a flow of helium through a reduction reactor containing copper wires at 650°C; this removed any remaining oxygen and reduced NO_xs back to N_2. Water vapour and carbon dioxide were removed by traps containing granular magnesium perchlorate and Carbosorb (both Elemental Microanalysis Ltd), respectively. The helium carrier gas

containing N_2 from the test sample was then delivered, via an 'open-split', to the Isotope Ratio Mass Spectrometer (IRMS). The isotope ratio ($\delta^{15}N$; Section 2.5.1) of the N_2 peak was compared directly to that in pulses of N_2 reference gas of known isotopic composition. Quality control working standards of solid materials of known isotopic and elemental composition were included after every tenth sample. Percentage total N content of samples was also determined from IRMS analysis. From these data the total ^{15}N excess (μg) in each ^{15}N-labelled system component was estimated.

Firstly, the ratio of ^{15}N:^{14}N in each sample (R_{sa}) was calculated from the $\delta^{15}N$ value:

$$\text{(A)} \quad R_{sa} = R_{std}\left(\frac{\delta^{15}N}{1000}+1\right)$$

where R_{std} is the ratio of ^{15}N in the standard, which in this case is atmospheric nitrogen (N_2) where the ratio is 0.0036765. The proportion of ^{15}N in each sample (as a proportion of all N, i.e. ^{14}N and ^{15}N) was then calculated:

$$\text{(B)} \quad Proportion\ ^{15}N = \frac{R_{sa}}{R_{sa}+1}.$$

The mean proportion ^{15}N was calculated for each unlabelled system component and grazing treatment, at each time point (e.g. wood inocula, grazed, 2 d after collembola addition). This was necessary, rather than assuming all unlabelled samples to be of the same value, as fractionation and differential use of N sources would mean that $\delta^{15}N$ would vary across system components and time. Mean unlabelled values were then used to estimate the proportion ^{15}N excess of the equivalent labelled sample:

$$\text{(C)} \quad Proportion\ ^{15}N\ excess = (B)_{labelled} - (B)_{unlabelled}.$$

Finally, the value given by (C) was converted to an absolute ^{15}N excess value:

$$\text{(D)} \quad Total\ ^{15}N\ excess = (Proportion\ ^{15}N\ excess)(sample\ N)(sample\ mass)$$

where *sample N* is the proportion of N (μg/g) in a sample and *sample mass* is the biomass (g) of a system component. By taking biomass into consideration the ^{15}N excess data indicate the total amount of the added ^{15}N in each component, and thus the distribution of the added ^{15}N across the biotic components of the microcosm system. This information is not provided by δ values; although these are a sensitive

measure of the ^{15}N:^{14}N ratio they do not account for the relative biomass of each component.

7.2.8 Statistical analyses

^{15}N-labelling was successfully achieved, δ ^{15}N values for labelled and unlabelled treatments being clearly different in magnitude. Consequently, δ ^{15}N data for labelled and unlabelled systems components were analysed separately. Mycelium, wood inoculum and soil δ ^{15}N and excess ^{15}N values were compared using one-way Repeated Measures Analysis of Variance (RM ANOVA) as described in Section 3.7.

Percentage N of labelled and unlabelled system components were included in the same model; values were expected to be similar as the amount of NH_4NO_3 added to each of these treatments was equivalent. Mycelium, wood inoculum and soil data were analysed using two-way RM ANOVA with grazing treatment and labelling treatment as the main effects, and time (days after collembola addition) as a sub-factor. Collembola values in labelled and unlabelled treatments were compared using one-way RM ANOVA. Collembola survivorship (proportion of inoculated number surviving at each time point) across labelled and unlabelled systems was also investigated using one-way RM ANOVA.

In grazed systems there was only a small amount of new mycelial growth during the first few days after collembola addition; consequently many mycelial and soil samples in the outer zone were too small for IRMS analysis across 2 to 4 d. These data are retained in the figures but the number of replicate samples was too few for RM ANOVA analysis; hence outer zone soil and mycelial analyses are for 8-32 d only. Radial extension and hyphal coverage data were analysed as described in Section 3.7. Mycelial biomass was analysed by performing two-way RM ANOVA with grazing and labelling treatments as the main effects.

In most cases treatment data met the necessary assumptions; log transformation improved normality and equality of variance on the few occasions where these assumptions were violated for untransformed data. Unless otherwise stated, data are presented with standard error of the mean.

7.3 Results

7.3.1 Mycelial growth characteristics and collembola survivorship

Ungrazed mycelia initially grew in the manner characteristic of *P. velutina* (Chapters 4 and 5); relatively thick cords formed behind more diffuse growth fronts and an area of diffuse mycelium, with aerial hyphae, was present around the wood inoculum. Mycelia became asymmetrical as they extended further, with zones of rapid extension and other zones where extension ceased completely (Fig. 7.2a). During the later stages of the study mycelia became more even; dishes were more or less filled with mycelium by 16 d (Fig. 7.2c) and by 32 d diffuse mycelium and thin cords had begun to regress. Systems that were grazed by *P. armata* were less symmetrical than those that were ungrazed; new growth was more fanned and cords were usually thicker and more densely-aggregated behind the growing margin (Fig. 7.2b, d). Only in a few replicates had mycelia made contact with the dish margin by 32 d and there was less regression in grazed than in ungrazed systems. The morphology of N- control mycelia was superficially similar to that of main experiment (N+) systems (Fig. 7.2e, f).

The radial extension rate of mycelia was unaffected by grazing or by N addition (Fig. 7.3a; Table 7.1), although it should be noted that measurements were only made to 4 d after collembola addition as some mycelia made contact with dish margins at this time. Hyphal coverage was significantly ($P \leq 0.05$) reduced by grazing irrespective of whether N was added or not (Fig. 7.3b; Table 7.2). There were trends towards a N treatment effect and towards an interaction between N and grazing treatments, with collembola grazing having a more negative influence on hyphal coverage in the presence of added N (Fig. 7.3b; Table 7.2).

Mycelial biomass data were not collected for N- controls; the same set of control microcosms was used across the study and this precluded destructive harvest. Biomass data are compared across grazing and labelling treatments, although the latter treatment was not expected to affect biomass as the amount of N added to ^{15}N-labelled and unlabelled mycelia was equivalent. There was no significant ($P > 0.05$) time*treatment interaction of grazing or labelling treatment on mycelial biomass in the inner zone, nor was there any interaction between the two treatments (Fig. 7.3c; Table 7.3); collembola grazing, however, did have a significant ($P \leq 0.05$) negative impact on biomass, irrespective of time (Fig. 7.3c; Table 7.3). Biomass in the outer

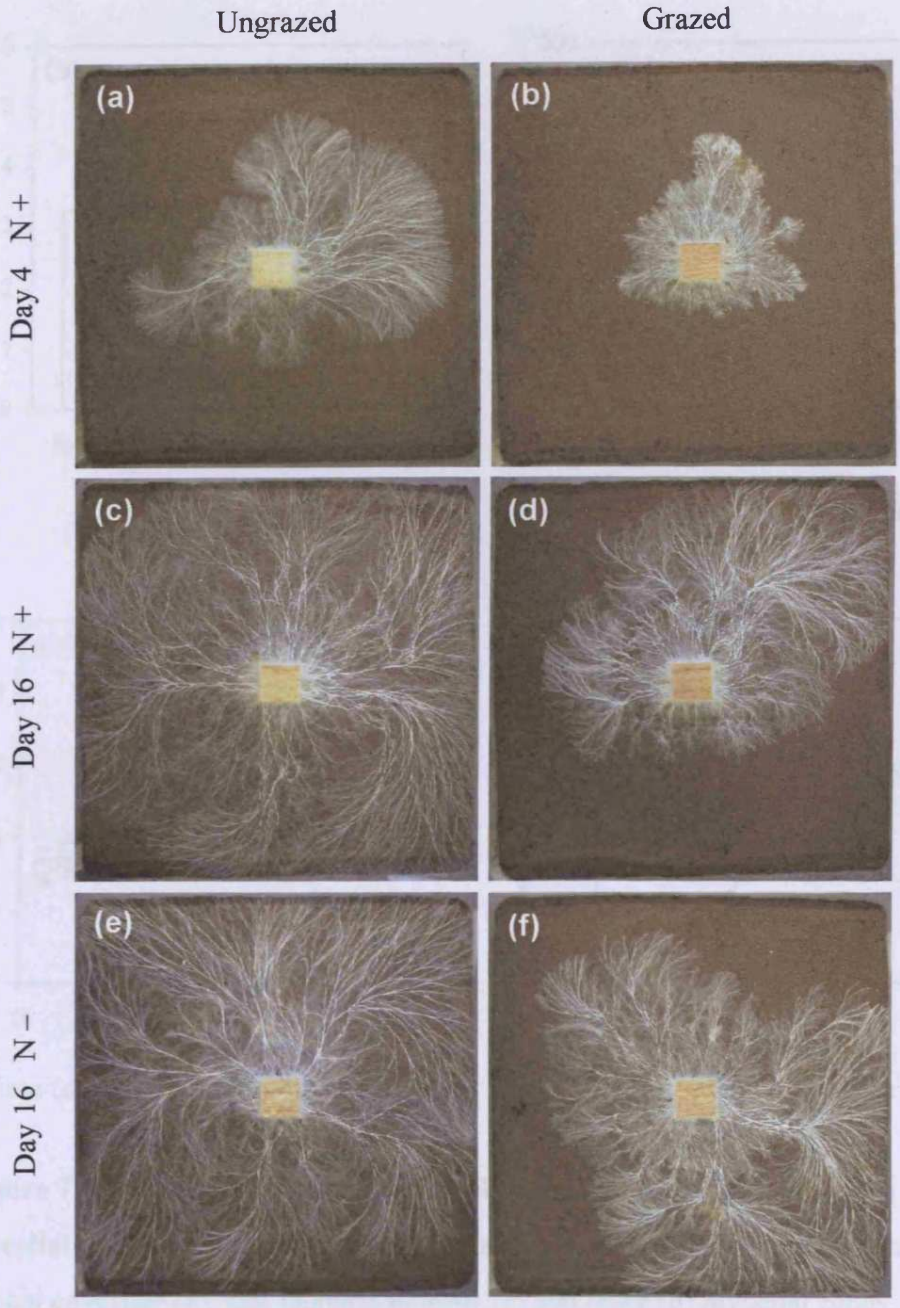


Figure 7.2. Digital images of mycelial systems of *P. velutina* after 4 d (a, b) and 16 d (c-f) of collembola grazing. Grazing treatments are: ungrazed controls (a, c, e); *P. armata*-grazed systems (b, d, f). Labelling treatments are: NH_4NO_3 (N+) added (a-d); DI water (N-) controls (e, f). Each image is representative of replicate trays in that treatment.

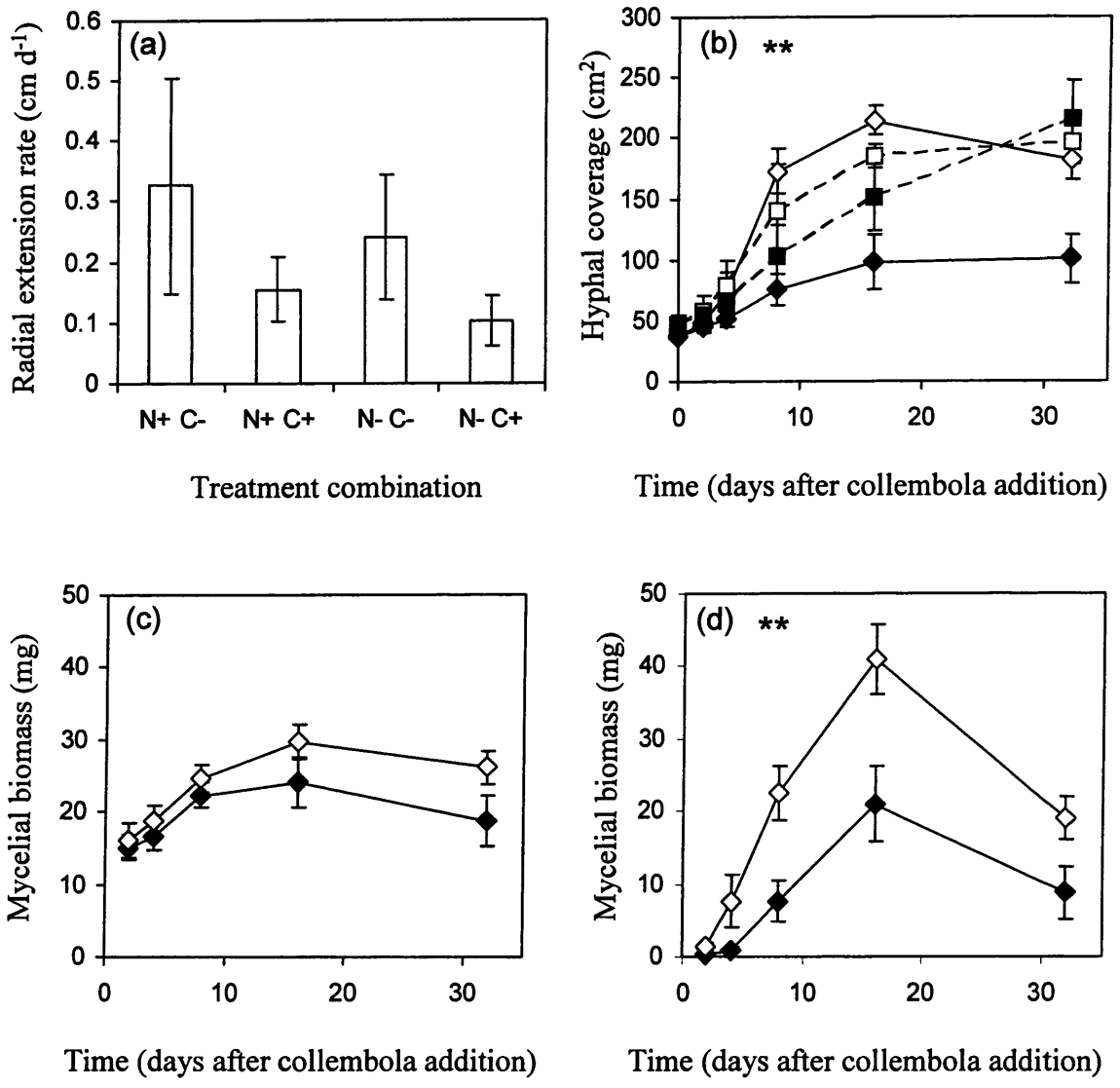


Figure 7.3. The effects of nitrogen, labelling and grazing treatments on mycelial growth parameters (mean ± s.e.). Figures are: radial extension rate (a), hyphal coverage (b), and biomass in inner (c) and outer (d) mycelial zones. In (a) and (b) treatments are with (N+) and without (N-) added nitrogen and with (C+) and without (C-) collembola. Symbols and lines are:—◇— N+ C-; —◆— N+ C+; --□-- N- C-; --■-- N- C+. In (c) and (d) all data are N+; ^{15}N-labelled and unlabelled treatments have been pooled for clarity as there were no significant ($P >$ 0.05) differences between them. Significant time*grazing treatment interactions (RM ANOVA) are indicated; ** $P < 0.01$.

Table 7.1. Results of ANCOVA analysis of radial extension rates of mycelia.

Factor	*d.f.*	SS	MS	*F*	*P*
Time	1	8.17	8.17	8.83	**0.004**
Grazing	1	0.65	0.65	0.71	0.403
Nitrogen	1	0.41	0.41	0.44	0.510
Grazing*Nitrogen	1	0.17	0.17	0.18	0.671
Error	67	61.96	0.92		
Total	71	71.35			

Table 7.2. Results of RM ANOVA analysis of hyphal coverage.

Factor	*d.f.*	SS	MS	*F*	*P*
Within-subjects effects					
Time	3	396911	124867	59.29	**<0.001**
Time*Grazing	3	30574	9618	4.57	**0.005**
Time*Nitrogen	3	16993	5346	2.54	0.061
Time*Grazing*Nitrogen	3	15030	4729	2.25	0.088
Error (time)	64	133896	2106		
Between-subjects effects					
Intercept	1	1519859	1519859	339.76	**<0.001**
Grazing	1	36694	36694	8.20	**0.010**
Nitrogen	1	10468	10468	2.34	0.142
Grazing*Nitrogen	1	15491	15491	3.46	0.078
Error	20	89467	4473		

Table 7.3. Results of RM ANOVA analysis of mycelial biomass.

Factor	*d.f.*	SS	MS	*F*	*P*
a) Inner zone					
Within-subjects effects					
Time	4	1668.98	417.24	13.86	**<0.001**
Time*Grazing	4	140.89	35.22	1.17	0.332
Time*Labelling	4	93.00	23.25	0.77	0.547
Time*Grazing*Labelling	4	83.82	20.96	0.70	0.597
Error (time)	64	1926.60	30.10		
Between-subjects effects					
Intercept	1	44973.68	44973.68	1645.58	**<0.001**
Grazing	1	338.19	338.19	12.37	**0.003**
Labelling	1	91.20	91.20	3.34	0.086
Grazing*Labelling	1	12.60	12.60	0.46	0.507
Error	16	437.28	27.33		
b) Outer zone					
Within-subjects effects					
Time	4	11013.28	2753.32	56.49	**<0.001**
Time*Grazing	4	1036.62	259.16	5.32	**0.001**
Time*Labelling	4	250.66	62.67	1.29	0.285
Time*Grazing*Labelling	4	178.05	44.51	0.91	0.462
Error (time)	64	3119.30	48.74		
Between-subjects effects					
Intercept	1	16827.28	16827.28	268.26	**<0.001**
Grazing	1	2823.86	2823.86	45.02	**<0.001**
Labelling	1	234.09	234.09	3.73	0.071
Grazing*Labelling	1	78.15	78.15	1.25	0.281
Error	16	1003.65	62.73		

mycelial zone was reduced by grazing; this effect was indicated by a significant ($P <$ 0.01) time*treatment interaction and was also significant ($P < 0.001$) irrespective of time (Fig. 7.3d; Table 7.3). Labelling treatment did not affect outer zone biomass, nor were there any interactions between labelling and grazing treatments (Table 7.3).

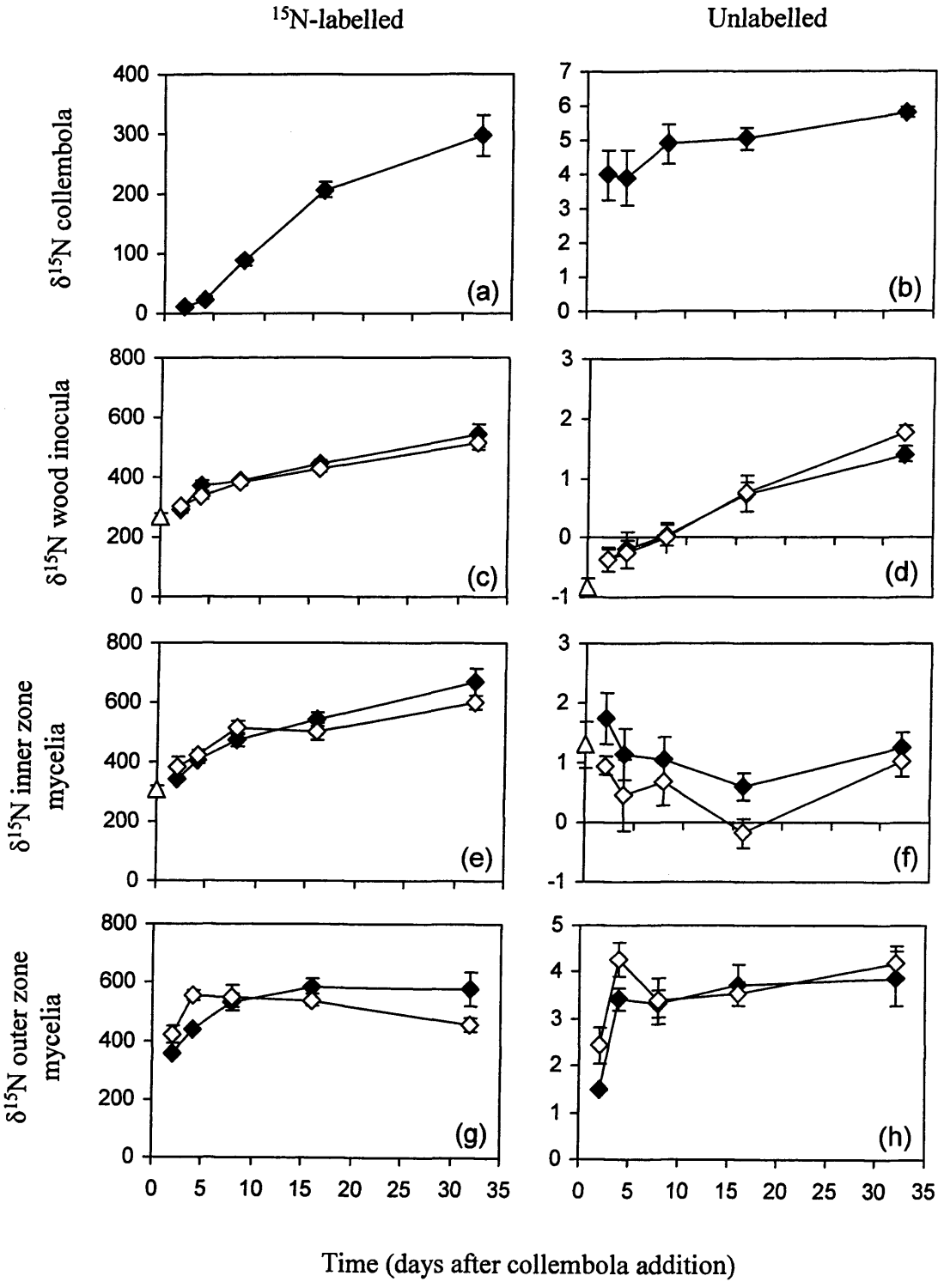
The number of collembola retrieved from the harvest microcosms declined linearly across the 32 d study period (RM ANOVA effect of time: $F_{4,32} = 17.1$, $P < 0.001$). The percentage of the original 60 *P. armata* retrieved at 2 and 32 d was 93% ± 1.8% and 61% ± 4.3%, respectively. There was no effect of labelling treatment on the survivorship of collembola (time*treatment interaction: $F_{4,32} = 0.36$, $P = 0.84$).

7.3.2 $\delta^{15}N$ of system components

The $\delta^{15}N$ signature of *P. armata* in labelled systems increased from 13 ± 1.5 ‰ at 2 d to 297 ± 35.2 ‰ at 32 d (Fig. 7.4a). The rate of increase was approximately linear across the early to middle time points of the experiment, and then slowed to 32 d. The $\delta^{15}N$ of collembola in unlabelled systems also increased over time, albeit much more steadily, from 4.0 ± 0.72 ‰ at 2 d to 5.8 ± 0.15 ‰ at 32 d (Fig. 7.4b).

Wood inocula in both labelled and unlabelled systems increased in $\delta^{15}N$ across the experiment (Fig. 7.4c, d; RM ANOVA effect of time: $F_{4,32} = 45.8$, $P < 0.001$; $F_{4,32} =$ 24.7, $P < 0.001$, for labelled and unlabelled systems, respectively). Collembola grazing did not affect the change in wood inocula $\delta^{15}N$ over time in either labelled ($F_{4,32} = 0.49$, $P = 0.74$) or unlabelled ($F_{4,32} = 0.59$, $P = 0.67$) systems (Fig. 7.4c, d).

Inner zone mycelial $\delta^{15}N$ of both labelled and unlabelled systems altered significantly over time (Fig. 7.4e, f; labelled $F_{4,32} = 35.1$, $P < 0.001$; unlabelled $F_{4,32} = 3.66$, $P =$ 0.015). For labelled systems the pattern was of a more or less linear increase over time, whereas in unlabelled systems $\delta^{15}N$ decreased to 16 d and then increased again at 32 d (Fig. 7.4e, f). Collembola grazing did not affect these patterns of $\delta^{15}N$ change over time (labelled $F_{4,32} = 1.95$, $P = 0.13$; unlabelled $F_{4,32} = 0.32$, $P = 0.87$). There were no time*grazing treatment effects on $\delta^{15}N$ of outer zone mycelia (Fig. 7.4g, h; labelled $F_{2,16} = 2.03$, $P = 0.16$; unlabelled $F_{2,14} = 0.44$, $P = 0.65$), nor was there any significant ($P > 0.05$) change in $\delta^{15}N$ over time. It is, however, cautionary to note that analyses for outer zone mycelia were only performed across 8 to 32 d.



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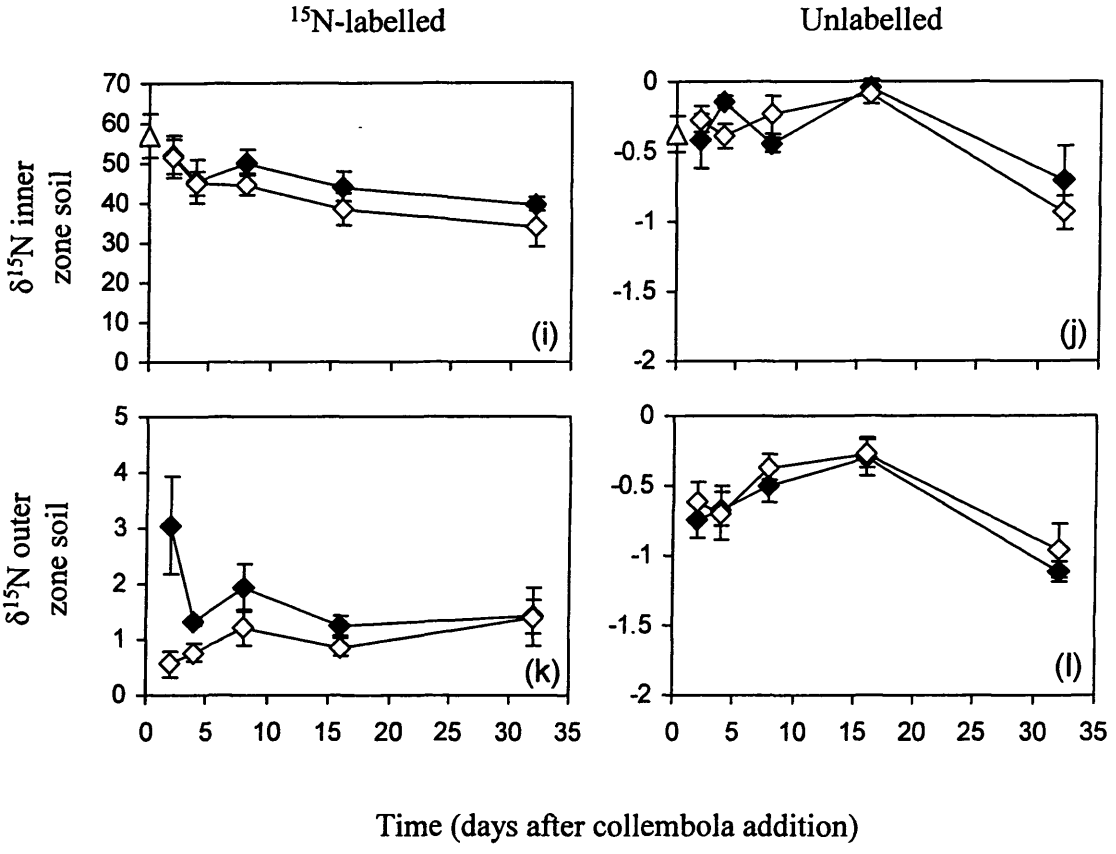


Figure 7.4. $\delta^{15}N$ ‰ (mean ± s.e.) of collembola (a, b), wood inocula (c, d), inner (e, f) and outer (g, h) zone mycelia, and inner (i, j) and outer (k, l) zone soil across the 32 d study period. ^{15}N-labelled systems are in the left-hand column and unlabelled systems are in the right-hand column. Treatments are ungrazed (◇) and grazed (◆) systems. $\delta^{15}N$ values at Time 0 (△) are immediately before grazing commenced. There were no significant time*treatment effects of collembola grazing on $\delta^{15}N$ of any of the system components (RM ANOVA; $P > 0.05$). Note different y-axis scales.

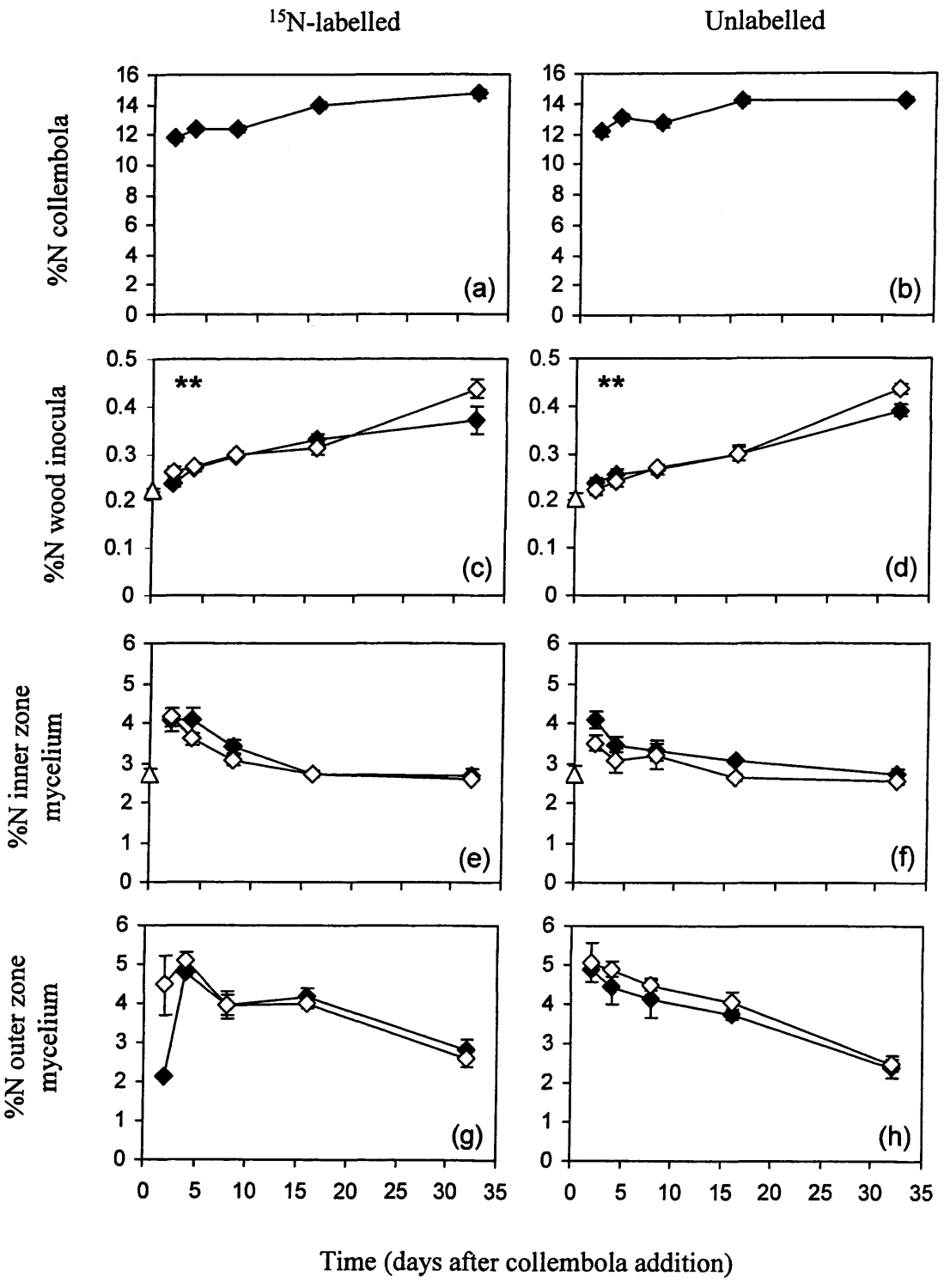
$\delta^{15}N$ of inner zone soil decreased across the 32 d study period in labelled systems (Fig. 7.4i; $F_{4,32} = 5.41$, $P = 0.002$). In unlabelled systems $\delta^{15}N$ also changed over time (Fig. 7.4j; $F_{3,27} = 8.42$, $P < 0.001$), although the magnitude of this variation was small (< 1 ‰). Collembola grazing did not affect these patterns of $\delta^{15}N$ change over time (Fig. 7.4i, j; labelled $F_{4,32} = 0.35$, $P = 0.84$; unlabelled $F_{3,27} = 1.27$, $P = 0.31$). $\delta^{15}N$ of unlabelled outer zone soil increased to 16 d and then decreased by 32 d (Fig. 7.4l; $F_{2,16} = 23.7$, $P < 0.001$); the magnitude of this change was small but significant. In labelled systems time did not affect $\delta^{15}N$ of soil in the outer zone (Fig. 7.4k; $F_{2,16} = 1.43$, $P = 0.27$) and collembola grazing did not affect soil $\delta^{15}N$ across 8-32 d (Fig. 7.4k, l; labelled $F_{2,16} = 0.67$, $P = 0.53$; unlabelled $F_{3,27} = 0.47$, $P = 0.63$). $\delta^{15}N$ of labelled soil was considerably higher in grazed than ungrazed systems at 2 and 4 d, although there were too few samples for statistical analysis (Fig. 7.4k).

7.3.3 Percentage total N of system components

The %N content of *P. armata* was more than double that of mycelia, which in turn was approximately an order of magnitude higher than that of wood inocula and soil (note Fig. 7.5 y-axes). *P. armata* %N increased over time in both ^{15}N-labelled and unlabelled systems (Fig. 7.5a, b; RM ANOVA effect of time: $F_{4,32} = 82.1$, $P < 0.001$). There was a significant time*labelling treatment interaction effect on *P. armata* %N ($F_{4,32} = 3.08$, $P = 0.03$).

Collembola grazing significantly affected the %N of wood inocula across time (RM ANOVA time*grazing treatment interaction: $F_{4,64} = 4.23$, $P = 0.004$); at 32 d wood %N was higher in ungrazed than grazed microcosms of both labelled and unlabelled systems (Fig. 7.5c, d). Wood %N increased over time irrespective of grazing and labelling treatments (Fig. 7.5c, d; $F_{4,64} = 105.1$, $P < 0.001$). Overall, wood %N was higher in labelled than unlabelled systems ($F_{1,16} = 9.28$; $P = 0.008$), although there was no time*labelling treatment interaction ($F_{4,64} = 1.41$, $P = 0.24$).

Mycelial %N decreased over time in both the inner and outer zones (inner $F_{4,64} = 33.0$, $P < 0.001$; outer $F_{2,30} = 52.4$, $P < 0.001$). In the outer zone the pattern was of continual decline in %N across the experimental time period (Fig. 7.5g, h); inner zone



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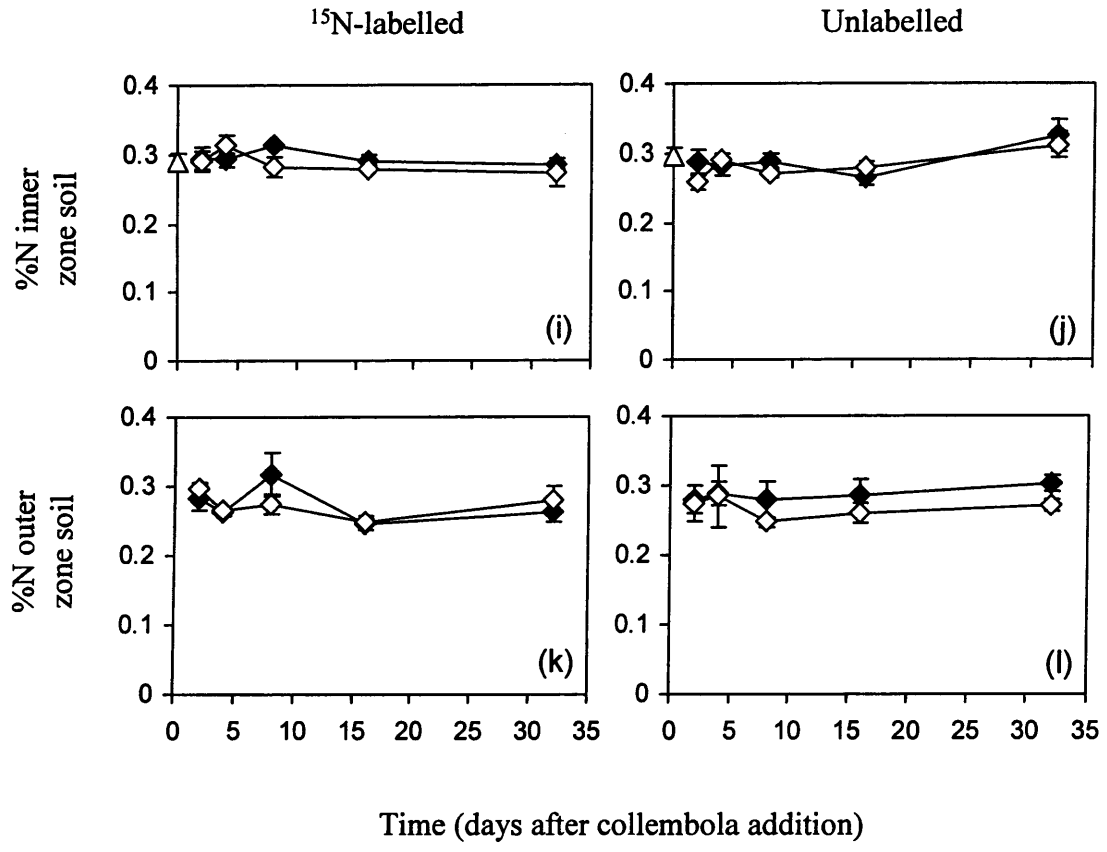

Figure 7.5. %N (mean ± s.e.) of collembola (a, b), wood inocula (c, d), inner (e, f) and outer (g, h) zone mycelia, and inner (i, j) and outer (k, l) zone soil across the 32 d study period. ^{15}N-labelled systems are in the left-hand column and unlabelled systems are in the right-hand column. Treatments are ungrazed (◇) and grazed (◆) systems. %N values at Time 0 (△) are immediately before grazing commenced. Significant time*grazing treatment interactions (RM ANOVA) are indicated; ** $P < 0.01$. Note different y-axis scales.

%N increased rapidly to 2 d, then declined to 16 d before finally stabilising by 32 d (Fig. 7.5e, f). There were no significant ($P > 0.05$) time*treatment effects of grazing and labelling on %N of inner zone mycelia. There was, however, an overall significant effect of grazing treatment; %N was higher in grazed than ungrazed mycelia (Fig. 7.5e, f; $F_{1,16} = 7.18$, $P = 0.016$). Outer zone mycelial %N was not significantly ($P > 0.05$) affected by grazing or labelling treatments, nor were there any significant time*treatment interactions (Fig. 7.5g, h).

Soil %N altered little over time and, irrespective of labelling treatment, was not affected by collembola grazing in either soil zone (Fig. 7.5i-l; $P > 0.05$ for all time*grazing treatment interactions).

7.3.4 Total ^{15}N excess of system components

The amount of added ^{15}N present in *P. armata* biomass increased over time; this increase was rapid across 4-16 d but then slowed to 32 d (Fig. 7.6a). Total ^{15}N excess in wood inocula also increased over time (Fig. 7.6b; RM ANOVA effect of time: $F_{4,32} = 43.3$, $P < 0.001$), but was not influenced by grazing (time*treatment interaction: $F_{4,32} = 0.68$, $P = 0.62$).

Total ^{15}N excess present in inner zone mycelia increased steadily across the study (Fig. 7.6c); that in outer zone mycelia increased markedly to 16 d but then decreased at a similarly rapid rate to 32 d (Fig. 7.6d). Collembola grazing reduced ^{15}N excess in both mycelial zones. In the inner zone this negative grazing effect was not influenced by time (Fig. 7.6c; time*treatment interaction: $F_{4,32} = 0.19$, $P = 0.94$), but was significant overall ($F_{1,8} = 7.28$, $P = 0.027$). In the outer mycelial zone the grazing effect was time-dependent (Fig. 7.6d; time*treatment interaction: $F_{4,32} = 3.67$, $P = 0.049$) and was also highly significant overall ($F_{1,8} = 13.8$, $P = 0.006$).

The ^{15}N excess of inner zone soil decreased across the 32 d study period, whereas that in the outer zone increased over time (Fig. 7.6e, f). Grazing by *P. armata* resulted in significantly higher ^{15}N excess in the inner soil zone (Fig. 7.6e; $F_{1,8} = 9.42$, $P = 0.015$); this effect did not alter significantly across time (time*treatment interaction: $F_{4,32} = 2.00$, $P = 0.12$). Grazing also had a significant effect in the outer soil zone; in this case ^{15}N excess was lower than in controls overall (Fig. 7.6f; $F_{1,8} = 28.1$, $P =$

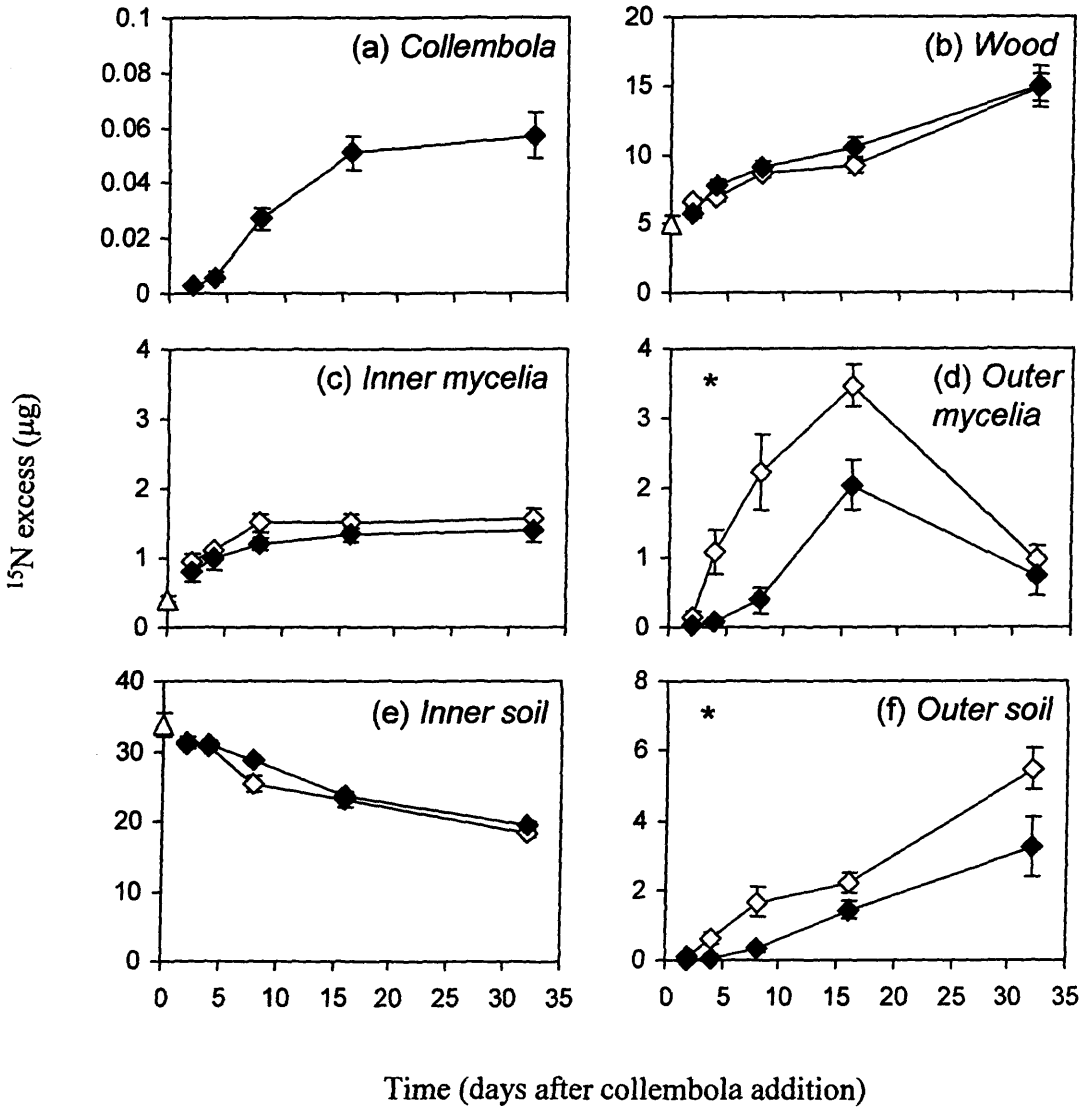

Figure 7.6. ^{15}N excess (mean ± s.e.) of collembola (a), wood inocula (b), inner (c) and outer (d) zone mycelia, and inner (e) and outer (f) zone soil across the 32 d study period. Treatments are ungrazed (◇) and grazed (◆) systems. ^{15}N excess values at Time 0 (△) are immediately before grazing commenced. Significant time*grazing treatment interactions (RM ANOVA) are indicated; * $P \leq$ 0.05. Note different y-axis scales.

0.001) and the pattern of differences was also influenced by time (time*treatment interaction: $F_{3,24} = 4.39$, $P = 0.013$).

Some clear patterns emerge when comparing the distribution of added ^{15}N across the whole system (Fig. 7.7). The amount of added ^{15}N in the inner soil zone decreased with increasing time; N which was lost was located mainly in wood inocula, and outer zone mycelia and soil. Although this general pattern was consistent across grazed and ungrazed systems (Fig. 7.7) some differences were apparent. There was a smaller loss of ^{15}N from the inner soil zone in grazed systems, where there was less ^{15}N located in outer zone mycelia and soil.

7.4 Discussion

The effects of *P. armata* grazing on *P. velutina* were similar to those observed in Chapter 5; grazed colonies filled dishes more slowly than ungrazed colonies, and became less radially-symmetrical with fanned margins. These observations, in combination with the reduction in hyphal coverage and mycelial biomass that occurred in the presence of *P. armata*, point towards a significant level of grazing on *P. velutina* in the current study. The isotopic data provide supporting evidence; $\delta^{15}N$ and ^{15}N excess values for *P. armata* increased across the duration of the study. This supports the first hypothesis and shows, for the first time, that collembola not only graze on cord-forming fungi but also assimilate into their biomass the N that they gain from them. Ungrazed mycelia extended relatively quickly across soil, although growth was less vigorous and more asymmetrical than in previous experiments (Chapters 4 and 5), despite the same fungal isolate being used. Why this is so remains unclear, and is surprising given that mycelia grew more vigorously in a trial run, using the same source of wood inocula and identical soil and abiotic conditions, conducted immediately prior to the main experiment (data not presented). Nevertheless, growth became more vigorous as systems matured, and there is no reason to suppose that the patterns of N partitioning that were observed after isotopic labelling should be regarded as atypical for this species.

The microcosms to which no NH_4NO_3 was added (N- controls) enabled the fertilization effect of the N that was added to main experiment microcosms to be assessed. While there was little influence of the added N on hyphal coverage in

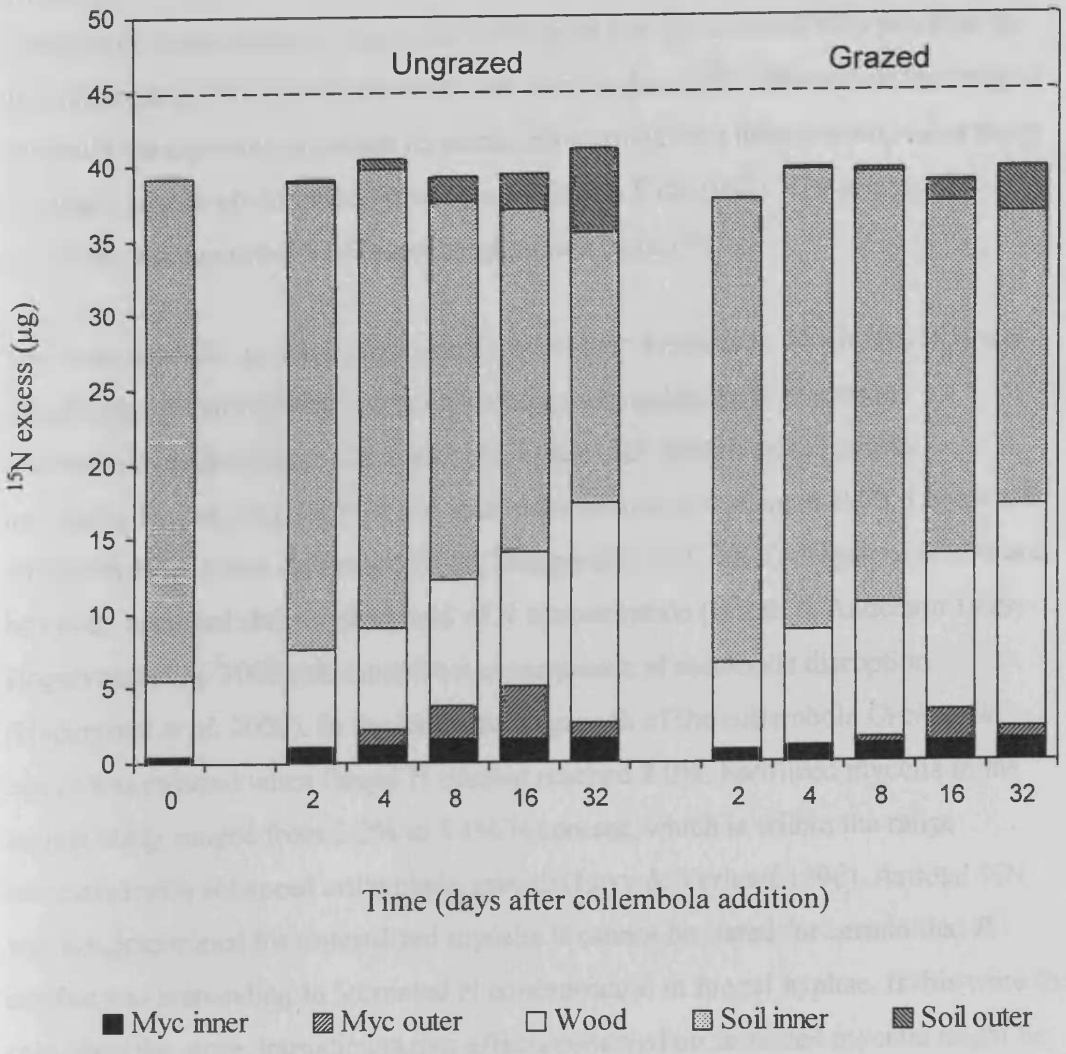

Figure 7.7. Effect of collembola grazing on the amount (^{15}N excess) of added N present in inner and outer zone mycelium (myc), wood inocula, and inner and outer zone soil across the 32 d study period. Collembola portions of grazed bars are too small to be visible. The dashed line indicates the amount of ^{15}N excess added to each microcosm 2 d before collembola addition.

ungrazed systems, in grazed systems hyphal coverage was reduced in the presence of added N when compared to unfertilized systems (Fig. 7.3b). The fact that ungrazed mycelia of *P. velutina* do not exhibit any clear morphological response to fertilization agrees with observations in Dutch forests subject to high levels of N-deposition. In these ecosystems the species diversity and fruiting frequency of wood-decay fungi is generally unresponsive to added N, unlike mycorrhizal and litter-decomposing fungi which are negatively affected (van Breemen & van Dijk 1988). The effects of N-deposition on the mycelia of forest fungi remain unknown.

The more dramatic grazing effects on *P. velutina* in systems to which NH_4NO_3 was added suggest that fertilized mycelia become more palatable to *P. armata*. Collembola generally respond positively, in terms of growth and fecundity, to increasing N content of their fungal substrates (Booth & Anderson 1979, Leonard & Anderson 1991, Lavy & Verhoef 1996, Hogervorst *et al.* 2003). Negative effects are, however, apparent above a threshold of N concentration (Booth & Anderson 1979, Hogervorst *et al.* 2003); this may be a consequence of metabolic disruption (Hogervorst *et al.* 2003). In the latter study, growth of the collembola *Orchesella cincta* was reduced when fungal N content reached 8.0%. Fertilized mycelia in the current study ranged from 2.2% to 5.1% N content, which is within the range associated with enhanced collembola growth (Lavy & Verhoef 1996). As total %N was not determined for unfertilized mycelia it cannot be stated for certain that *P. armata* was responding to increased N concentration in fungal hyphae. If this were the case, then the more dramatic grazing effects observed on fertilized mycelia might be due to increased consumption of hyphae to meet growth requirements; this has been reported in a previous study (Leonard & Anderson 1991). Although the magnitude of observed grazing effects of *P. armata* was increased due to N-fertilization, they remained less dramatic than when *Folsomia candida* grazed *P. velutina* (Chapters 4 and 5). This was advantageous in the context of the current study; intense grazing might have resulted in cessation of fungal growth, which would have hampered investigation of the influence of collembola on mycelial N flux.

Within the main experiment (N+) microcosms the method of ^{15}N-labelling proved highly effective; $\delta^{15}N$ ‰ values of all system components were markedly higher than their unlabelled (natural abundance) counterparts (Fig. 7.4). As an equivalent amount

of NH_4NO_3 was added to labelled and unlabelled microcosms, the %N content of system components was not expected to differ between the two labelling treatments. For some system components, however, differences did exist. There was a significant ($P \leq 0.05$) time*labelling treatment interaction effect for %N content of *P. armata*, and %N of wood inocula was greater in labelled than unlabelled systems overall. It is not obvious why these differences should occur; there is no evidence that *P. velutina* discriminates between ^{14}N and ^{15}N when taking up N (Owen 1997) and, in any case, the absolute amount of ^{15}N was only a fraction of that of ^{14}N even in labelled systems.

The current study was not explicitly designed to examine the variation in natural abundance $\delta^{15}N$ values between different component of the microcosm system. The systems to which unlabelled NH_4NO_3 was added do, however, permit some investigation of $\delta^{15}N$ variation at natural abundance, both across system components and over time. Outgrowth mycelium was more enriched in ^{15}N than all its potential N sources (i.e. added NH_4NO_3, wood and soil). This is somewhat surprising given that saprotrophic basidiomycetes generally reflect the $\delta^{15}N$ of their sources (Kohzu *et al.* 1999, Hobbie *et al.* 2004), and become depleted in ^{15}N during uptake of NH_4-N from agar (Henn & Chapela 2004). In addition, $\delta^{15}N$ values varied within the mycelium: that from the outer zone was substantially more enriched in ^{15}N than that from the inner zone (Fig. 7.4f, h). Fractionation of $\delta^{15}N$ in fungi is determined by three processes: substrate preference, uptake discrimination and internal processing (Henn & Chapela 2004). It is not clear which of these are driving the patterns observed in the current study; understanding the mechanisms underlying fractionation of ^{15}N is difficult even in simple agar experiments with a single N source (Henn & Chapela 2004). In the current study there are numerous potential N sources that the fungus might be using, in addition to the added NH_4NO_3. Even within NH_4NO_3 the fungus may be using NO_3^- as well as NH_4^+, although basidiomycete fungi are often known preferentially to take up and utilize the latter (Rayner & Boddy 1988, Cooke & Whipps 1993). Despite this complexity, the observed patterns of mycelial $\delta^{15}N$ might be more fully understood by considering other components of the microcosm. Soil became slightly depleted in ^{15}N when colonized by *P. velutina* (Fig. 7.4j, l; uncolonized soil $\delta^{15}N$ was -0.05 ‰), which suggests that the enrichment observed in outer zone mycelium might be due at least in part to discrimination in favour of ^{15}N during scavenging of N from soil. Also, the basipetal movement of ^{15}N-enriched N

from outgrowth mycelium might explain the fact that wood inocula became enriched across the study period (Fig. 7.4d). Collembola were more enriched in $\delta^{15}N$ than any other system component; the amount of enrichment relative to outer zone mycelium, where collembola grazing was most evident, increased across the study and by 32 d was 2.01 ± 0.43 ‰. This is less than the 3.4 ‰ shift which has been reported to occur with increasing trophic level (Section 2.5.3), although ^{15}N fractionation in mycophagous collembola varies markedly depending on a range of factors (Section 2.5.3).

^{15}N excess data provide the best insight into the partitioning of the added ^{15}N between different system components, as values are absolute. $\delta^{15}N$ values are proportional, and although useful indicators of the ratio of ^{15}N:^{14}N they do not reveal the total amount of added ^{15}N in a given component. When considering partitioning within the mycelium of *P. velutina*, only outgrowth mycelium and wood inoculum data are of concern, as any ^{15}N excess present in soil and collembola has been lost from the fungus, or else was never taken up in the first place. The majority of this mycelial ^{15}N was located in the wood resource; in ungrazed systems the percentage of mycelial ^{15}N present in wood varied from a maximum of 86% at 2 d to a minimum of 65% at 16 d, when outgrowth mycelium was at its most extensive. The storage of the majority of N in wood inocula agrees with existing knowledge of nutrient partitioning within mycelia of cord-forming fungi. In a previous microcosm study of N partitioning, although a percentage breakdown of N distribution is not given, ^{15}N was located mainly within wood resources rather than outgrowth mycelium of the cord-formers *P. velutina* and *Coprinus picaceus* (Owen 1997). Isotopic labelling studies of carbon (Wells *et al.* 1995) and phosphorus (e.g. Wells & Boddy 1990b, Wells *et al.* 1990, Hughes & Boddy 1994) partitioning in cord-formers have typically found more than 80% of added nutrients to be located in wood resources. This agrees broadly with what was observed at most time points in the current study, but is substantially greater than the 65% in wood inocula at 16 d. In previous studies extra wood blocks ('baits') were also present in the systems. These proved to be a sink for added nutrients, so perhaps the lower proportion of nutrients allocated to wood in the current study can be explained by the lack of extra wood resources, meaning more ^{15}N was present in outgrowth mycelium.

The total amount of ^{15}N excess detected in microcosms was consistent over time (Fig. 7.7), and on average 89% of added ^{15}N was accounted for. This indicates that, while the distribution of N varied over time, relatively little N was being lost from the microcosm systems. Nonetheless, most of the added ^{15}N remained in the inner soil zone, indicating that it was never taken up by *P. velutina*. This suggests that N was provided in excess of what was required by the fungus, although by 32 d the amount of N in wood had almost equalled that in the inner soil zone. Given a longer experimental period and the addition of new wood resources the excess N might have been utilized. The temporal increase in added ^{15}N in the outer soil zone, which reached 13% of ^{15}N excess in ungrazed systems after 32 d, points towards considerable leakiness of *P. velutina* mycelia. This is perhaps surprising given that cord-formers have been suggested to be highly conservative of acquired nutrients (Wells *et al.* 1998b, Boddy 1999). This strategy may, however, depend on the fungus occupying sufficient wood resources to which it can translocate nutrients from points of excess (Lindahl *et al.* 2002); perhaps if new wood resources had been provided then more N would have been translocated to them rather than released into the soil.

Grazing by *P. armata* had no effect on $\delta^{15}N$ values in any of the labelled system components (Fig. 7.4); the same ^{15}N:^{14}N ratio existed in all components of both grazed and ungrazed systems. The ^{14}N and ^{15}N in these systems comes from two sources: that already present within the systems (introduced in soil, wood inocula and collembola) and that added with the ^{15}N-labelled NH_4NO_3. As the ^{15}N already present within the systems was at natural abundance levels, the high $\delta^{15}N$ values in labelled systems must have been due to the added NH_4NO_3, but the relative proportion of this in each component was clearly not affected by grazing. Differences did, however, exist between the total %N content of some system components. For example, the %N of wood inocula was higher in ungrazed than grazed systems at 32 d. This extra N in ungrazed wood cannot have come from the added NH_4NO_3 alone, as $\delta^{15}N$ values in grazed and ungrazed wood were not significantly different. The extra N may have been translocated back to wood inocula from outgrowth mycelia as systems regressed; there was less regression observed in grazed microcosms and so perhaps less movement of N back to the wood inocula.

The total amount of added ^{15}N was similar in grazed and ungrazed systems, and a negligible amount of this N was present in collembola biomass (Fig. 7.7). Hence, any differences in ^{15}N excess of components of grazed and ungrazed systems must have been due to the influence of collembola on N distribution, irrespective of the ^{15}N present in their own biomass. Some clear differences in N distribution between grazed and ungrazed mycelia are apparent. The amount of added ^{15}N present in outgrowth mycelium was reduced in grazed systems, presumably because grazing reduced mycelial biomass. Conversely, ^{15}N excess was higher in the inner soil zone in grazed microcosms, the obvious inference from this being that less of the added ^{15}N is taken up when mycelia are grazed. Despite this, the amount of added ^{15}N present in wood inocula was the same in both grazed and ungrazed systems. As outgrowth mycelium contained less ^{15}N in grazed microcosms, the proportion of mycelial ^{15}N housed in the wood inoculum was higher in grazed than ungrazed systems, lending support to the second hypothesis. This adds further weight to the general concept that cord-formers operate a nutrient-conservative strategy. Collembola cannot penetrate wood inocula until late stages of wood decay, and so fungal nutrients are 'safer' there than in outgrowth mycelium, which can be subject to intense grazing activity. An alternative explanation might be that the lower proportion of fungal nutrients in outgrowth mycelium is simply a function of the reduced growth in grazed systems; lower fungal biomass would require less N for structural use and enzyme production.

No support was found for the third hypothesis, that more ^{15}N would be released to the bulk soil in grazed systems as mycelia become more 'leaky'. In fact, there was less added ^{15}N present in the outer zone soil when systems were grazed. On a unit area basis, nutrient release appeared to be similar in grazed and ungrazed mycelia, as $\delta^{15}N$ and %N values in the outer soil zone were unaffected by grazing. It could be that 'leakiness' was not enhanced by collembola grazing because any highly labile nutrients released from damaged hyphae and in collembola faeces were very rapidly reabsorbed by the mycelium, leading to no net increase in nutrients in the bulk soil. If, however, grazing genuinely does not increase nutrient release from cord-forming fungi, then this has implications for nutrient recycling in forest soils. It has been postulated that release from these nutrient-conservative fungi may be mediated via interactions with other organisms (Boddy & Watkinson 1995), and there is empirical support for this idea from studies of interactions between cord-forming species (Wells

& Boddy 2002) and with ectomycorrhizal fungi (Lindahl *et al.* 1999). The current results do not provide any evidence that collembola increase nutrient loss from cord-formers. This result contrasts with other microcosm studies which demonstrate increased nutrient mineralization, particularly of NH_4-N, from deciduous leaf litter in the presence of collembola (Ineson *et al.* 1982, Anderson *et al.* 1983). The observed increase in nutrient mobilization coincided with a decrease in fungal standing crop, and was attributed to fungal grazing (Ineson *et al.* 1982). In a more complex microcosm study using coniferous forest soil, fauna caused no increase in nutrient release to soil (Setälä & Huhta 1991). Microcosms in the latter study did, however, contain birch (*Betula pendula*) seedlings, which showed increased growth and tissue N concentrations in the presence of fauna. Collectively, these studies indicate an important role of collembola and other soil fauna in nutrient release from fungal biomass. The fact that this did not occur in the present study could be due to the use of *P. velutina* as a study species. The previous studies did not, to my knowledge, contain any cord-formers among the assemblage of soil fungi that were inoculated using a soil-water suspension. Cord-forming fungi, which are relatively persistent on the forest floor (Boddy 1999), probably need to be more nutrient-conservative than more ephemeral soil fungi, and so they might be less likely to release nutrients when they are grazed.

In summary, *P. armata* appears to influence the uptake, partitioning and release of nutrients by *P. velutina*. This suggests that collembola have the potential to affect the functioning of cord-forming fungi as well as modifying their growth patterns (Chapters 4 and 5). As stressed in previous chapters, it is precautionary to emphasize that these results should not be interpreted too widely; what is now required is an investigation into the possible influence of invertebrate grazing on nutrient fluxes in cord-forming fungi growing in more natural situations. The current study could easily be scaled up to larger and more complex microcosms, with a greater number of wood resources and a longer experimental time frame. Also, the potential to investigate nutrient movements in naturally-occurring cord-forming fungi has been demonstrated (Wells & Boddy 1995), and a similar study could be conducted in which the density of grazing organisms were manipulated through the use of mesh bags. This would allow the true impact of collembola grazing on mycelial nutrient flux to be quantified; at present the magnitude of this impact can only be guessed at.

Chapter 8. General discussion

8.1 Experimental studies

Interactions between mycophagous invertebrates and saprotrophic cord-forming fungi have been little studied. The first experiments reported in this thesis (Chapters 4 and 5) were designed primarily to investigate the potential of collembola grazing to affect fundamentally the development of these cord-forming fungus colonies. Grazing affected the growth and overall morphology of each of the cord-forming species that was investigated; the magnitude of these impacts was highly variable across fungal species.

Collembola had a negative, or occasionally neutral, effect on fungal growth – there was no evidence that grazing stimulated extension rate or increased hyphal coverage. In an associated experiment (S. Bretherton *et al.*, unpublished data), however, collembola grazing did stimulate the growth of *Phanerochaete velutina* in similar experimental microcosms to those used in the present studies. In the Bretherton *et al.* study, an inoculation density of 20 *Folsomia candida* per microcosm increased hyphal coverage by 16% compared to ungrazed systems, although collembola did have detrimental effects on fungal growth at higher densities. The stimulated growth observed by Bretherton *et al.* contrasts markedly with results presented in Chapter 4, where *F. candida* had a negative effect on the growth of *P. velutina* at a density of 10 and 20 animals per microcosm. Results from the two studies are not, however, directly comparable, as there were potentially important differences in the experimental methodologies. For example, wood inocula were 2 cm^3 in the study reported in Chapter 4 but 4 cm^3 in the Bretherton *et al.* study. Results from a second associated experiment (Harold *et al.* 2005) show that increasing the volume of the wood inoculum reduces the impacts of collembola grazing. The larger inocula used by Bretherton *et al.* might therefore have allowed *P. velutina* to grow more vigorously in response to grazing. Bretherton *et al.* also employed a set of treatments in which collembola were allowed to graze *P. velutina* for 2 d and were then removed. Collembola grazing also stimulated growth in these systems, compared to ungrazed controls, but only when inoculation density was high (80 *F. candida* per microcosm). Taken together, the Bretherton *et al.* results suggest that collembola effects on cord-

forming fungi may be more complex than those reported in the present studies, where negative effects were attributed simply to the removal of fungal standing crop and disruption of hyphal growth.

Grazing damage was not random but instead targeted at particular mycelial regions; the region that was most affected varied across fungal species. For example, *Resinicium bicolor* was predominantly grazed proximal to the wood inoculum, although other regions of the mycelium, including thick cords, were also damaged. Conversely, with *P. velutina*, *Hypholoma fasciculare* and *Phallus impudicus*, grazing occurred mainly at mycelial margins; this resulted in morphological changes in the mycelium that developed subsequent to collembola addition. Thick cords were rarely (*P. velutina*) or never (*H. fasciculare*, *P. impudicus*) severed during grazing on these species. Klironomos and Kendrick (1996), in a laboratory study of grazing on arbuscular mycorrhizal (AM) fungi, reported that collembola graze on the fine hyphae that occur away from the mycorrhizal plant root in preference to the thicker hyphae closer to the plant. The findings of these authors are analogous to those in the present studies, where, in all species except *R. bicolor*, diffuse hyphae were grazed in preference to cords. The more dramatic impacts on *R. bicolor* led to changes in mycelial architecture, with cord systems seemingly becoming less well connected as a result of grazing (Chapter 6). It would be interesting to determine whether network architecture was affected by the less severe grazing impacts that occurred with the other fungal species.

The fact that the thick yellow cords of *H. fasciculare* were never grazed, even towards the end of the experiments when collembola densities had increased due to reproduction, suggests that cords of this species are extremely unpalatable to collembola. As far as I am aware, the chemical basis of this unpalatability has not been investigated. One possibility is that calcium oxalate crystals, which encrust the cords of most cord-forming fungi (Rayner & Boddy 1988), deter grazing. Cromack *et al.* (1975) suggest that, because calcium is used to dispose of the oxalic acid that is a waste product of fungal biodegradation, oxalate formation represents a small energy loss to the fungus. If cord-forming fungi can produce calcium oxalate inexpensively, in metabolic terms, then these crystals would be suitable candidates for helping defend extensive naturally-occurring cord systems from invertebrate grazing. Calcium

oxalate has negative effects on the rate of larval fungus gnat (Diptera: Sciaridae) development (Binns 1980), and its production by lichens might also deter grazers (Seaward *et al.* 1998). The palatable nature of *R. bicolor* cords, however, suggests that calcium oxalate is not solely responsible as a deterrent to collembola grazing; calcium oxalate crystals are produced in abundance on the cords of this species (Connolly & Jellison 1995). The frequency with which cords of *R. bicolor* were severed by collembola raises the question of how this species can persist in nature in the presence of high densities of invertebrate grazers. One possibility is that *R. bicolor*, which occurs mainly in coniferous forests, requires chemicals that are present in conifer wood, but lacking in angiosperm wood, for the synthesis of defensive compounds. In the present studies *R. bicolor* was inoculated into beech wood, which might lack the basic chemicals necessary for defence. It would be appropriate to investigate whether *R. bicolor* mycelia are more resistant to grazing when growing out from conifer wood inocula.

The magnitude of grazing effects was expected to relate to fungal foraging strategy. Long-range foragers, including *R. bicolor* and *P. velutina*, generally possess thicker cords than do short-range foragers such as *H. fasciculare*. Short-range foragers were expected to be most affected by collembola grazing due to their less substantial cords; in fact the relatively narrow yellow cords of *H. fasciculare* were never severed by grazing while *R. bicolor*, which has thicker cords than the other experimental fungi, appeared to be the most palatable. Growth of the long-range forager *P. impudicus* was little affected by collembola. The lack of association between foraging strategy and grazing impact agrees with Hiol Hiol *et al.* (1994) that hyphal chemistry, rather than morphology *per se*, is responsible for determining grazing effects.

Where cord-forming species co-occur, selective grazing on more palatable species may favour growth and enhanced foraging ability of less palatable species. As collembola populations are both spatially and temporally dynamic (Berg *et al.* 1998), the impacts of grazing on fungi are complex. Selective grazing by collembola on saprotrophic fungi in preference to arbuscular mycorrhizal species can affect fungal community composition (Tiunov & Scheu 2005); selective grazing also alters the vertical distribution of saprotrophic basidiomycete species in forest soil (Newell

1984b). Collembola might also modify the composition of saprotrophic cord-forming communities and bring about changes in fungal distribution across the forest floor.

Fungal growth patterns are not generated simply by removal of hyphae but also by fungal response to grazing. Cord-forming basidiomycetes show morphological plasticity in response to changing environmental conditions such as temperature (Donnelly & Boddy 1997a) and soil nutrient status (Donnelly & Boddy 1998). Altered fungal morphology in response to collembola grazing has been reported previously by Hedlund *et al.* (1991) and Kampichler *et al.* (2004). In these studies, fast-growing sectors of mycelium occurred as a result of grazing; both sets of authors interpret this change as a possible attempt by the fungus to 'escape' from localized regions of intensive grazing pressure. Kampichler *et al.* (2004) suggested that the fungus switched its growth strategy as a result of grazing. It is difficult, however, to determine whether altered mycelial foraging pattern results from a change in growth strategy or is imposed by disruption of hyphal growth by collembola. For example, the fanned mycelial margins of *P. velutina* that result from grazing could be due to collembola disrupting the otherwise longitudinally-aligned hyphae that occur at the growth fronts of this species. In addition, the suggestion that fungi show a fugitive response to collembola grazing seems a little far-fetched. Collembola can travel much more quickly than fungal hyphae, and so it seems unlikely that fungi would be capable of escaping from sites of grazing by increasing their rate of extension.

There are attractive comparisons between observed collembola-mediated changes in fungal growth pattern and other situations where invertebrates influence morphology through their grazing. In Australia, for example, the larval feeding of sawflies on *Eucalyptus* causes the development of multiple leading shoots after the removal of terminal buds (Carne 1969). A more direct comparison with the fungus-collembola system comes from a study in which the morphological effects of invertebrates grazing on lichens were quantified. A range of invertebrate species, including some collembola, graze the fruticose lichen *Evernia prunastri* growing on tree trunks; intensive grazing causes the lichen to become more irregularly-branched than ungrazed lichens (Prinzing 1999). This finding bears close resemblance to the altered branching pattern of *P. velutina* and *P. impudicus* in response to collembola grazing.

The morphological changes to mycelia that resulted from collembola grazing were often striking. Such changes undoubtedly affect mycelial function; for example, it is intuitive that a slowing of extension rate will decrease the frequency with which new resources are encountered. Nevertheless, altered fungal growth pattern does not indicate the possible influence of mycophagy in the wider context of process rates occurring on the forest floor. The influence of collembola on fungal decomposition of wood is of more direct relevance from an ecosystem-wide perspective. In the present studies collembola consistently reduced the decay rate of *H. fasciculare* inocula. As collembola could not access the interior of wood inocula, effects on decay rate were probably indirectly-mediated through grazing on outgrowth mycelium. If extensive grazing of outgrowth mycelium occurs in the field, then collembola might alter the rate of wood decomposition brought about by cord-forming fungi. This would contrast with the general perception that collembola stimulate decomposition processes through their interactions with soil microorganisms (Hopkin 1997). Collembola grazing of outgrowth mycelium also modified fungal nitrogen partitioning (Chapter 7). By reducing the outgrowth of *P. velutina* colonies, *Protaphorura armata* decreased the total amount of added nitrogen (^{15}N) in the mycelial biomass, with more ^{15}N remaining in the soil region to which it was added in grazed systems. Less ^{15}N 'leaked' from outgrowth mycelium into the microcosm soil in grazed systems, as a result of the reduced fungal biomass that was present. This was in contrast to the expected increase in release of nitrogen to soil from collembola-grazed mycelia. Invertebrate mycophagy has been predicted to be one of the routes through which the otherwise nutrient-conservative cord-forming fungi might 'leak' nutrients from their biomass, making them available to other soil microbes and ultimately plants (Boddy & Watkinson 1995). Numerous microcosm studies have demonstrated that collembola and other soil invertebrates cause the mobilization of nutrients from fungal biomass (Ineson *et al.* 1982, Anderson *et al.* 1983, Setälä & Huhta 1991). The fact that increased nutrient release did not occur as a result of grazing (Chapter 7) suggests that cord-formers may be more resistant to nutrient loss than the fungal groups used in other microcosm studies, although the possibility that mineralized nutrients were rapidly reabsorbed by *P. velutina* cannot be ruled out.

8.2 Microcosm studies

The microcosm environments used in the studies reported in this thesis were relatively uniform; by compacting the microcosm soil, collembola and fungi were effectively restricted to the soil surface. In a previous microcosm experiment, collembola grazing had a much more dramatic impact on fungal biomass in two-dimensional compared to three-dimensional systems (Leonard & Anderson 1991). The authors report that small-scale heterogeneity greatly increased the stability of the fungus-collembola interaction. Other studies have found that collembola have positive effects on fungal growth only when the fungi are patchily-distributed within microcosms (Bengtsson & Rundgren 1983, Bengtsson *et al.* 1993). It would be appropriate to investigate the collembola grazing of cord-forming fungi in three-dimensional soil microcosms, where the fungi would perhaps be better able to avoid intensive grazing due to the increased complexity of the habitat. The opaque soil medium would, however, preclude the investigation of fungal morphology and network architecture. Studies that employ more complex two-dimension microcosms would also be valuable. One such experiment was carried out recently (J. Wood *et al.*, unpublished data), and demonstrates that collembola have a smaller impact on fungal morphology when grazing more mature mycelial systems with extra wood resources. Studying nutrient flux using these larger, more complex, microcosms would be potentially rewarding.

The soil microcosms used in the present studies were also simplified in another potentially-important way: collembola were not offered any food source other than the cord-forming fungus that was inoculated. Visser and Whittaker (1977) report that collembola preferred a range of dark pigmented microfungi over two basidiomycete species in laboratory choice tests; one of these basidiomycetes was subsequently found to be toxic to the collembola (Parkinson *et al.* 1979). The cord-forming species used in the present studies do not appear to be particularly toxic, as collembola usually increased in abundance on each of the fungal substrates. In addition, Newell (1984a) presents convincing evidence that collembola do graze on basidiomycetes in the field. In her study, 90% of fungal hyphae in the guts of *Onychiurus latus* from a spruce forest were of basidiomycete origin. Although this latter study demonstrates that grazing on basidiomycetes is by no means unlikely, the current studies do have some parallels with examples of early research on AM fungi. In several greenhouse experiments, in which non-mycorrhizal fungi were not inoculated into the soil,

collembola had a negative impact on plant growth, mediated by grazing on AM fungi (Finlay 1985, Harris & Boerner 1990). When leaf litter was inoculated into similar greenhouse microcosms, collembola had positive effects on plants (Klironomos & Kendrick 1995), presumably because they grazed on saprotrophic litter microfungi in preference to AM fungi, thus mobilising nutrients and indirectly stimulating plant growth (Gange 2000). Several experiments have subsequently demonstrated that soil microfungi are always preferred over AM species in laboratory food choice tests (Section 2.4.1), and it is questionable whether extensive grazing of AM fungi occurs in the field (Gange 2000). Klironomos and Kendrick (1995) highlight the fact that AM fungi rely on having relatively long-lived exploratory mycelia to form effective symbioses with plants. Grazing, especially the severing of thick connecting hyphae, is probably very costly to AM fungi, and as a result they are likely to have evolved to be unpalatable to soil invertebrates (Klironomos & Kendrick 1995). Soil microfungi, on the contrary, are relatively ephemeral; grazing may even benefit these fungi as collembola can aid dispersal of their spores (Visser *et al.* 1987). Cord-forming basidiomycetes, like AM fungi, possess long-lived mycelial networks. The severing of cords, which are required for nutrient translocation and exploratory growth, is likely to be extremely costly to saprotrophic basidiomycetes, and so it is probable that this group of fungi will have evolved to be unpalatable to grazing invertebrates. It is therefore unlikely that mature cord systems are extensively damaged by collembola and other invertebrates in the field. Grazing of mycelial growth fronts, whose hyphae do not possess the thick rind present in mature cords, is more likely to occur, as was observed in the current experiments with *P. velutina*, *H. fasciculare* and *P. impudicus*.

8.3 Future work

There is clearly a lot to learn about collembola grazing of cord-forming fungi, and this thesis, partly due to its 'mycocentric' nature, has generated more questions than it has answered. It is possible that decreased palatability and the availability of alternative food sources result in naturally-occurring cord systems being less grazed than those in laboratory microcosms. As the extent of field interactions between cord-forming fungi and invertebrates remain unknown, they should be investigated as a matter of urgency. Stable isotopic labelling of cord systems, in conjunction with litter bags to contain invertebrates, would be a suitable system for investigating the degree to which forest floor cord systems are grazed by invertebrates. Laboratory food choice

experiments, involving the pairwise combination of cord-forming versus non-cord-forming fungal species, could be used to provide supporting information on whether or not cord-formers are preferentially grazed. The possible physical and chemical mechanisms of cord unpalatability to collembola could be investigated using a combination of Scanning Electron Microscopy (SEM) and High-Pressure Liquid Chromatography (HPLC). Grazed and ungrazed cord systems could be compared to determine whether changes in cord sheath morphology (using SEM), the abundance of calcium oxalate crystals on the cord surface (using SEM), and the production of specific chemicals (using HPLC) occurred in response to grazing.

8.4 Conclusions

1. Mycophagous collembola can modify the growth, morphology and network architecture of cord-forming fungi through their grazing activity. The magnitude of these effects increases with increasing collembola density. Mature, corded mycelium is generally less susceptible to grazing damage than young, diffuse mycelium.

2. Different cord-forming species are affected to different extents, although grazing impacts do not appear to be determined by fungal foraging strategy.

3. The magnitude of grazing impacts on fungal morphology depend on the species of collembola. The zone of mycelium preferentially attacked is, however, consistent across collembola species. Grazing effects are not dependent on collembola body size, even when biomass is standardized.

4. Fungal nutrient partitioning is modified by collembola grazing. Grazed systems take up less nitrogen (^{15}N), have less ^{15}N in their biomass, and are less 'leaky' than ungrazed systems.

5. Collembola grazing reduces the wood decay rate of *H. fasciculare* but not that of other experimental cord-forming fungi.

6. Collembola abundance is affected by the species of cord-forming fungus that is being grazed, with all collembola species responding in a similar way.

Taken together, these results have potentially important consequences not only for interactions between cord-forming fungi and collembola on the forest floor, but also for the ecosystem processes on which these organisms impact. Saprotrophic fungi are the major agents of decomposition in forest soils (Boddy & Watkinson 1995) and this recycling role could be modified by grazing effects on fungal physiology, morphology and nutrient movement. While microcosm experiments should always be interpreted with caution (see above), the fundamental fact that wood decay rate and nitrogen distribution was affected by the presence of grazers suggests that the interrelations between cord-forming fungi and collembola may impact on decomposition and nutrient cycling processes.

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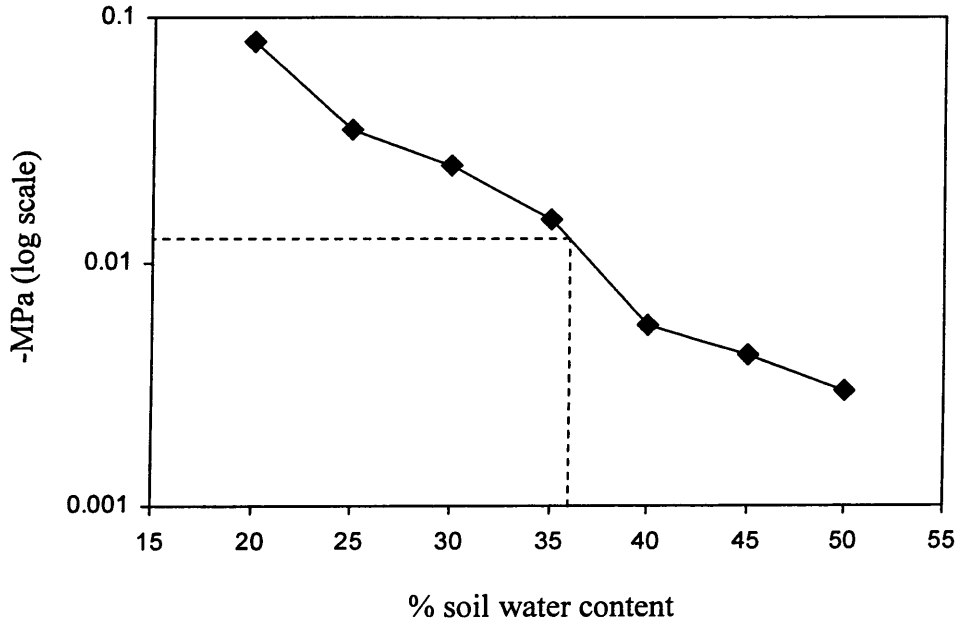
Appendix I. Calculation of soil water potential

Soil water potential was calculated using the filter paper method (Fawcett & Collis-George 1967). Known volumes of DI water (0.2 – 0.5 $l kg^{-1}$ at 0.05 $l kg^{-1}$ intervals) were added to 100 g subsamples of air dried soil. For each moisture level, 30 g wet soil was compacted into a glass Petri dish with three Whatman 42 filter papers in the middle of the soil layer. The central filter paper had been cut to a small square (approx. 1 cm^2) to avoid contact with the soil. There were three replicate dishes at each moisture level. Dishes were sealed and incubated for 7 d at 20°C. After incubation, filter paper squares were weighed immediately to 0.1 mg accuracy, oven-dried at 80°C for 2 d, and reweighed. Water potential (MPa) at each moisture level was estimated from percentage moisture content of filter papers using an existing water potential curve (Fawcett & Collis-George 1967). A graph of percentage soil moisture versus water potential was plotted (see below), and from this the amount of water required to give the desired soil water potential (-0.012 Mpa) was determined.



Percentage moisture versus water potential for soil used in experiments. Note log scale on y-axis.

Appendix II. One-way ANOVA results for hyphal coverage data presented in Fig. 4.8

Analyses are for 11 cm diam. mycelia (Section 4.3.3). Within a row, letters indicate differences between means (one-way ANOVA and Tukey). ** $P < 0.01$; *** $P < 0.001$.

	Days	*F*	Ungrazed control	Constant number	1 per cm^2 hyphal coverage	1 per cm^2 mycelial area
H. fasciculare	0	0.27				
	1	0.27				
	2	0.32				
	4	0.43				
	8	1.12				
	12	2.72				
	16	6.43**	a	ab	b	b
	21	16.9***	a	a	b	b
	30	33.3***	a	b	c	c
	40	27.4***	a	b	b	b
	50	19.2***	a	b	b	b
	60	19.0***	a	b	b	b
	80	12.4***	a	b	b	b
R. bicolor	0	0.49				
	1	0.49				
	2	0.72				
	4	0.78				
	8	3.00				
	12	6.66**	a	ab	ab	b
	20	16.9***	a	bc	b	c
	30	28.3***	a	b	b	c
	40	37.5***	a	bc	b	c
	50	56.8***	a	b	b	b
	60	67.9***	a	b	b	b
	79	36.4***	a	b	b	b

Appendix III. One-way ANOVA results for hyphal coverage data presented in Fig. 5.2

Analyses are for Chapter 5 experiments (Section 5.3.1). Within a row, letters indicate differences between means (one-way ANOVA and Tukey). * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$. *P. impudicus* not included as RM ANOVA time*treatment interaction was not significant ($P > 0.05$). Dashes indicate treatment combinations not performed.

	Days	*F*	Ungrazed control	*F. candida*	*P. armata*	*P. minuta*
P. velutina	0	1.19				
	4	82.9***	a	c	b	c
	8	76.6***	a	c	b	c
	12	62.4***	a	c	b	c
	16	42.2***	b	c	a	c
	20	8.90***	ab	c	a	bc
	30	5.20**	ab	b	a	a
	40	4.23 *	ab	b	b	a
	50	3.57 *	a	ab	b	ab
	60	6.53**	a	a	b	ab
H. fasciculare	0	0.52				
	1	0.16				
	2	0.44				
	6	10.1**	a	b	b	b
	8	26.0***	a	b	b	b
	12	56.6***	a	b	b	b
	16	83.9***	a	b	b	b
	20	87.5***	a	b	b	b
	30	107***	a	b	b	b
	40	123***	a	b	b	b
	50	159***	a	b	b	b
	60	162***	a	b	b	b
R. bicolor	0	0.13				-
	4	4.26*	a	b	ab	-
	8	14.0***	a	b	a	-
	12	15.4***	a	b	a	-
	16	10.9***	a	b	a	-
	20	12.6***	a	b	a	-
	30	37.6***	a	c	b	-
	40	57.3***	a	c	b	-
	50	44.5***	a	b	a	-
	60	84.0***	a	c	b	-

Appendix IV. One-way ANOVA results for mass fractal dimension (D_{BM}) data presented in Fig. 5.3

Analyses are for Chapter 5 experiments (Section 5.3.1). Within a row, letters indicate differences between means (one-way ANOVA and Tukey). ** $P < 0.01$; *** $P < 0.001$. *P. impudicus* not included as RM ANOVA time*treatment interaction was not significant ($P > 0.05$). Dashes indicate treatment combinations not performed.

	Days	*F*	Ungrazed control	*F. candida*	*P. armata*	*P. minuta*
P. velutina	0	0.76				
	4	19.4***	a	c	b	c
	8	2.37				
	12	1.19				
	16	12.5***	b	a	a	ab
	20	12.3***	b	a	b	ab
	30	10.0***	b	a	b	ab
	40	17.7***	bc	a	c	b
	50	25.1***	b	a	c	bc
	60	19.1***	a	a	b	b
H. fasciculare	0	0.03				
	6	12.3***	a	b	b	b
	8	27.2***	a	b	b	b
	12	45.0***	a	b	b	b
	16	73.4***	a	b	b	b
	30	80.2***	a	b	b	b
	40	66.8***	a	b	b	b
	50	51.5***	a	b	b	b
	60	36.0***	a	b	b	b
R. bicolor	0	0.20				-
	4	6.17**	a	b	ab	-
	8	25.1***	a	b	a	-
	12	39.4***	a	b	a	-
	16	48.6***	a	b	a	-
	20	61.9***	a	b	a	-
	30	54.9***	a	b	a	-
	40	54.3***	a	b	a	-
	50	18.7***	a	b	a	-
	62	47.0***	a	b	a	-

Appendix V. Publications associated with thesis

Harold, S., **Tordoff, G.M.**, Jones, T.H. & Boddy, L. (2005) Mycelial responses of *Hypholoma fasciculare* to collembola grazing: effect of inoculum age, nutrient status and resource quality. *Mycological Research*, **109**, 927-935.

Tordoff, G.M. (2005) If you go down to the woods today... *Planet Earth*, **Spring Issue**, 14. http://www.nerc.ac.uk/publications/documents/pe-spr05/woodstoday.pdf

Tordoff, G.M., Boddy, L. & Jones, T.H. (2006) Grazing by *Folsomia candida* (Collembola) has differential effects on mycelial morphology of the cord-forming basidiomycetes *Hypholoma fasciculare*, *Phanerochaete velutina* and *Resinicium bicolor*. *Mycological Research*, in press.

Bretherton, S., **Tordoff, G.M.**, Jones, T.H. & Boddy, L. (2006) Stimulatory and over-compensatory growth of *Phanerochaete velutina* mycelial systems grazed by *Folsomia candida* (Collembola). *FEMS Microbiology Ecology*, in press.

Tordoff, G.M., Jones, T.H. & Boddy, L. Species-specific impacts of collembola on functional ecology of saprotrophic fungi. Submitted *Ecology Letters*.

Wood, J., **Tordoff, G.M.**, Jones, T.H. & Boddy, L. Reorganization of mycelial networks of *Phanerochaete velutina* in response to new woody resources and collembola grazing. Submitted *Mycological Research*.