Invertebrate grazing

during mycelial interactions

Thesis presented for the

Degree of Philosiphiae Doctor

by

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Summary

Saprotrophic cord-forming basidiomycete fungi are major agents of wood decomposition in woodland and support the decomposer food-web. Limited resource availability and the abundance of mycelium in soil leads to competition between fungi. These fungal interactions are aggressive involving reallocation of mycelial biomass, pigment formation, changes in gene expression and enzyme synthesis. Collembola are abundant mycophagous invertebrates in woodlands and affect fungal morphology and growth. Experiments investigated the effects of collembola grazing on fungal interaction progression and the effects of these interactions on collembola behaviour and mortality. In British woodlands, the collembola *Folsomia candida* and *Protaphorura armata* are common as are the cord-forming fungi *Hypholoma fasciculare*, *Phallus impudicus*, *Phanerochaete velutina* and *Resinicium bicolor*. Pairwise interactions between these fungi were investigated in agar and compressed soil microcosms. Multiple genetic isolates of two of the fungi studied were also used. Fungal morphology was affected by collembola grazing in soil- but less so in agar-microcosms. In particular, when interacting with *H. fasciculare*, grazing of *P. velutina* mycelia accelerated growth over the opposing mycelium but reduced extension over soil. This was associated with an increased ability to colonise the wood resource of *H. fasciculare*. Grazing did not reduce the transport efficiency of *P. velutina* but the estimated cost of biomass production rose more steeply with increasing area than in ungrazed systems. Despite changes in progression, interaction outcome was not generally substantially altered by grazing. Collembola exhibited strong preferences for certain mycelia during interactions but showed a change in preference in others. Collembola mortality on fungal interactions in agar microcosms also varied with the species interacting. There was limited evidence of attraction of collembola to the fungal interaction zone. Overall, the results suggest that collembola grazing may have important impacts on fungal species assemblage and their ability to extend in search of new resources.

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1.0 Introduction

Soils are key to terrestrial life providing the habitat for primary producers. Plant roots are anchored within the soil matrix and also access water and nutrients for uptake. As well as supporting primary productivity, soils also host a high diversity of decomposer organisms. The decomposition processes that occur in soil are crucial to the continued cycling of nutrients such as carbon, nitrogen and phosphorus. The central importance of soils was highlighted in the dust bowl of America in the 1930s in which poor agricultural practices and drought turned fertile land into an uninhabitable desert.

Despite awareness of the fundamental importance of soils, their ecology and organisms have been little studied when compared with above-ground research (Bardgett, 2005). Such neglect is unsurprising since the opaque nature of soil makes both the organisms and processes within difficult to observe. In addition, the high diversity, small size, complex taxonomy and lack of appeal with the public compared with striking above-ground organisms have all served to limit soil research.

More recently, however, interest in soils has increased dramatically due in part to the development of new technologies such as powerful microscopic imaging and molecular techniques (Sugden *et al.*, 2004; Young & Crawford, 2004). The research has highlighted the close linkages that exist between above-ground and below-ground processes (Wardle *et al.*, 2004), and raised interesting questions about how soil invertebrate diversity is maintained despite an apparently limited amount of niche exploitation (Mauran *et al.*, 2003). Such a resurgence in soil research is apt given the important role soils play within the carbon cycle and the increasing awareness of climate change. Soils store approximately three times the amount of carbon found in the atmosphere (Schimel, 1995), and both respond to, and affect, increases in atmospheric CO_2 concentrations (Cao & Woodward, 1998).

Decomposition processes in woodland are dominated by the saprotrophic fungi whose combined array of enzymes can completely degrade wood to CO_2, water and mycelium (Rayner & Boddy, 1998). The white-rot saprotrophic cord-forming basidiomycetes are of particular interest due to their abundance on the temperate

woodland floor. In addition to completely decomposing wood to water, CO_2 and mycelium, these fungi form linear aggregations of hyphae known as cords which can extend for many metres across the forest floor (Cairney, 1992). Persistent networks of these cords form at the soil litter interface and link the heterogeneously distributed woody resources. Limited space and resources inevitably lead to competition between the fungi. Such competition is manifest in dramatic aggressive interactions across the entire mycelial front, resulting either in deadlock, where neither fungus is able to wrest resource from the other, or some degree of replacement (Boddy, 2000). These interactions are associated with dramatic changes in mycelial morphology and physiology, and probable leakage of what are normally closely-guarded nutrients (Wells & Boddy, 2002).

Through degrading complex polymers and leaking nutrients during interactions, fungi make recalcitrant nutrients available to mycophagous soil invertebrates. Among these invertebrates are the collembola, an abundant group of soil invertebrates found in all the world's major terrestrial biomes (Petersen & Luxton, 1982). Collembola feed predominantly on fungi and plant material and, despite their small size and low contribution to soil biomass, are considered important drivers of decomposition processes (Hopkin, 1997). Studies of collembola grazing and preferences are widespread but there has been only limited work looking at their interactions with cord-forming decay fungi. These studies have shown that collembola grazing can have substantial effects on fungal morphology and physiology (Bretherton *et al.*, 2006; Kampichler *et al.*, 2004; Tordoff *et al.*, 2006, 2008). As far as the author is aware, however, no studies have considered the effects of fungal interactions on collembola grazing behaviour and the effects of collembola grazing on fungal interactions. This is surprising given the ubiquitous nature of both fungal interactions and collembola especially in woodland soils.

Using a combination of agar and soil laboratory microcosms, the experiments here presented set out to investigate:

1. whether collembola grazing alters the progression or outcome of fungal interactions in agar microcosms;

2. the extent to which fungal isolate is important both during interspecific interactions and in response to collembola grazing;

3. the effect of species and isolate in fungal interactions on collembola movement and mortality;

4. whether collembola respond to odours emitted from fungal interactions through a change in movement behaviour;

5. how collembola grazing effects fungal growth and combativeness during interactions in soil microcosms; and

6. the effect of fungal grazing on the network architecture on fungi during aggressive interspecific interactions.

Through these aims the role of collembola during fungal interactions will be explored to provide an insight into how these two groups of organisms influence one another.

Chapter 2 is a review of the published literature on wood-decay fungi and collembola. Initially, soils are introduced with a focus on temperate woodlands and the saprotrophic decay fungi found there. Aggressive fungal interactions, associated changes in the mycelia and the factors influencing them are then considered. Fungal grazers and, in particular the collembola, are then reviewed, especially their feeding preferences and ecological significance. The effects of collembola on mycelial morphology and functioning are also addressed. Finally, the possible importance of fungal interactions for collembola are considered.

Chapter 3 investigates the effects the interactions of four wood-decay cord-forming basidiomycete species and some of their genetically distinct isolates in simplified systems. The effects of collembola grazing on fungal interaction progression, outcome and morphology are studied.

Chapter 4 records the response of collembola to fungi interacting in simplified systems. Changes in preference over time and collembola mortality are investigated.

The results from the experiment in Chapters 3 and 4 suggested that collembola were preferentially attracted to particular regions within certain fungal interactions. To investigate this further and to explore the role of volatile chemicals during interactions, collembola movement behaviour when exposed to only the volatile organic chemicals emitted from fungal interactions were investigated in **Chapter 5.**

A soil microcosm approach was adopted in **Chapter 6** in which the effects of collembola grazing in interspecific interactions of four wood-decay fungi were studied. In particular, the effect of mycelial extension and combative ability were investigated.

Chapter 7 introduces a novel methodology for studying the effect of both collembola grazing and fungal interactions on the architecture of the mycelial network. By translating the image of the mycelial network into a digital representation of that image, changes in network structure can be both visualised quantitatively and analysed quantitatively.

Chapter 8 seeks to draw together the salient points from the experimental chapters and discusses questions raised by the studies presented. Further avenues of research to help fill remaining gaps in the knowledge are suggested.

2.0 Literature Review

2.1 The subterranean soil ecosystem

2.1.1 Soil functions

Soils are a central, but often neglected part of the biosphere. They provide a source of nutrients for plants, a habitat for a high diversity of organisms and store large reserves of organic carbon as well as other nutrients such as nitrogen and phosphorus. It has been proposed that the majority of global diversity is found in soils (Wardle, 2002) and this has led to them being described as the 'poor man's tropical forest' (Usher *et al.*, 1982). As major stores of organic carbon, soils contain an estimated three times more carbon than the atmosphere (Schimel, 1995). Soils are also highly variable, their development being controlled by five major factors: parent material, climate, biota, relief and time (Jenny, 1941).

Growing awareness of climate change has also highlighted the important role of soils in the carbon cycle. In certain systems, such as arctic tundra and ombrotrophic bogs, inputs of organic carbon from plant production have traditionally exceeded soil decomposition rates, leading to carbon accumulation and the production of a carbon sink (Lal, 2004). This trend is particularly pronounced in the northern latitudes where an estimated 24% of the global soil carbon pool is located in boreal forests alone (Moore, 1996; cited in Lindahl *et al.*, 2002). A combination of increasing CO_2 concentration and temperature may, however, reverse the trend for soils to act as carbon sinks through the following mechanism: increasing temperature leading to increased soil respiration at levels over and above the predicted increases in net primary productivity though plant photosynthesis (Cao & Woodward, 1998). Greater nutrient inputs through anthropogenic activity can also lead to soils becoming carbon emitters (Mack *et al.*, 2004). The role of soils in the maintenance of a habitable global climate is becoming increasingly clear and has helped to spark a renaissance in soil research (Bardgett *et al.*, 2005; Ellis & Mellor, 1995; Schlesinger, 1997).

2.1.2 Woodland soils

Woodlands cover an estimated 39% of the global terrestrial surface (Whittaker, 1975) with woody tissues accounting for 80% of the total global organic carbon pool (Rayner & Boddy, 1988). Woodlands can be divided into two broad groups: deciduous and evergreen. Deciduous forest soils are characterised by annual pulse nutrient inputs through leaf fall in which relatively accessible (labile) nutrients are deposited and rapidly degraded. In evergreen woodland, such as boreal forest, leaf litter input is steady throughout the year. In addition, leaf litter nutrients, due in part to the presence of polyphenols, are highly inaccessible (recalcitrant) to decomposer organisms (Bardgett, 2005; Lindhal *et al.*, 2002). Aside from leaf fall, all woodland soils are subject to spatially and temporally heterogeneous nutrient inputs through fallen branches and tree stumps (Boddy, 1999). In addition, substantial inputs of woody litter often occur following windy conditions. Woody litter accounts for large quantities of recalcitrant complex nutrients which can only be degraded by a limited number of specialised organisms. In systems where there are continual inputs of labile nutrients, a relatively homogeneous soil with a high rate of nutrient turnover develops, and bacteria dominate the decomposition processes (Bardgett, 2005). In woodland soils, however, it is the saprotrophic fungi, with a wide array of lignolytic enzymes, which are responsible for the majority of decomposition (Rayner & Boddy, 1998). There are a range of bacteria capable of degrading wood but these account for a very small proportion of total decomposition when saprotrophic fungi are present (de Boer and van der Wal, 2008). The one exception to this is in very wet situations where bacteria can account for a substantial amount of wood decomposition (de Boer & van der Wal, 2008).

Even though leaf litter on the forest floor is often plentiful, in unmanaged deciduous woodland, most of the organic carbon is bound in the wood litter, principally comprising fallen branches and trunks (Christensen *et al.*, 2005). Fungal propagules, such as spores, are often latently present in functional sap wood but these only develop into conspicuous mycelium following an improvement in the abiotic conditions resulting from damage or death of the tree or tree section (Boddy & Rayner, 1983). Even when dead, the environmental conditions in standing trunks remain extreme; high temperatures and

strongly negative water potentials dominate (Rayner & Boddy, 1988). It is, therefore, only when standing wood falls to the ground, ameliorating abiotic conditions, that more rapid decay commences (Rayner & Boddy, 1988). Indeed, fungi found in standing dead wood are highly specialised and tolerant of extreme abiotic conditions; the rare oak polypore, *Piptoporus quercinus*, was able to continue to grow at pH 1.8 and at over 30°C, enabling it to colonise the low pH environment of its habitat, oak (*Quercus)* heartwood (Wald *et al.*, 2004a).

2.2 Saprotrophic fungi

2.2.1 Fungal arrival at a resource

The heterogeneous nature of coarse woody debris and the progressive decomposition of a resource from the point at which it becomes available, obligates fungi to engage in a continual search for fresh resources (Jonsson *et al.*, 2005). Unit resource-restricted fungi arrive at new resources as propagules (spores or hyphal fragments) which subsequently develop into mycelium in the wood. Non-unit resource-restricted fungi are, however, capable of arriving either as propagules or as mycelium extending across the soil between resource patches.

2.2.2 Cord-forming basidiomycetes

When growing as extra-resource mycelium, fungi form linear aggregations of parallel hyphae known as cords (Rayner & Boddy, 1988). Cords form behind a diffuse growing mycelial front and are characterised by differentiated thick-walled hyphae at the exterior and large diameter apoplastic hyphae in the centre, thought to be important in nutrient translocation (Cairney, 1992). Other fungi, especially those in the genus *Armillaria*, also form highly melanised linear aggregations termed rhizomorphs. Important for nutrient transport, rhizomorphs are distinct from cords as they do not form behind a diffuse mycelial front rather they show apical dominance of the whole organ (Rayner & Boddy, 1988).

Many cord-formers are saprotrophic basidiomycetes which grow at the soil-litter interface (Rayner & Boddy, 1988) and exhibit a spectrum of search strategies across the

forest floor. Some, such as *Hypholoma fasciculare*, forage as densely packed mycelium. This is energy-intensive to produce, but maximises encounters of all available resources. Others, such as *Resinicium bicolor*, grow out from a resource as sparse cords, efficient for long range foraging and searching for more heterogeneously distributed resources (Boddy, 1993). A study of fungi directly inoculated in the field revealed the short range forager *H. fasciculare* to have colonised leaves as well as twigs and beech cupules, whereas longer-range foragers such as *Phanerochaete velutina* and *Phallus impudicus* were found to have colonised twigs alone reflecting the different foraging strategies (Dowson, *et al.*, 1988b). The slow mycelial extension and exploitation of multiple small resources typified by *Stropharia* spp. and *H. fasciculare* has been termed exploitative growth (Donnelly & Boddy, 1998). Explorative growth, in which a larger area is covered with a lower density of cords, is typified by *Resinicium bicolor* as it forms cords with limited branching (Boddy, 1993).

2.2.3 The ecological significance of cord-forming decay fungi

Cord-forming fungi extend across the woodland floor forming a network connecting discrete resources (Dowson, *et al.*, 1988b), which can be tens of meters long (Thompson & Rayner, 1982). Rhizomorphic systems are even bigger with one fungal isolate covering several hectares (Smith *et al.*, 1992). As cord systems are persistent over time, they not only extend in search of new resources, but also employ a sit-and-wait strategy in which new litter falling onto the network can be colonised immediately (Boddy, 1999). A key advantage of cord-forming fungi is that on encountering new resources they can rapidly translocate nutrients from one part of the network to the mycelium at the new resource (Cairney, 1992). The wood-decay fungus *Phanerochaete velutina* moves phosphorus to new resources, presumably to facilitate the initial decay process (Wells *et al.*, 1990). Similarly, in boreal forests, nitrogen is translocated to new resources and then, as decay progresses, phosphorus is moved away probably towards other new resources (Lindahl & Boberg, 2008). Cord systems do not only move nutrients to areas of new colonisation; *Hypholoma fasciculare*, for example, can move phosphorus bidirectionally between two resources (Lindahl *et al.* 2001a). Nutrient translocation has been principally studied using radio-labelled phosphorus (^{32}P), but work studying the nitrogen flow

around basidiomycete mycelia indicates that it too is actively translocated, principally toward the growing hyphal tips (Tlalka *et al.*, 2002). This transport is examined in more recent work showing that, when extending from a large wood resources, the carbon contained in the resource is the energy source for extension, but the morphology and extension rate of the fungus are modified by the nitrogen content of the surrounding soil. In such instances the ability to translocate nutrients is key to effective foraging (Tlalka *et al.*, 2008a)

The ability to translocate nutrients surmounts the problem of the heterogeneity of woodland floors as fungi can effectively allocate nutrients to areas of need by sequestering them from other areas in contact with the network (Boddy, 1999). The exact process by which nutrients are moved around the network is uncertain, but mass flow toward the growing tips (acropetal), through non-septate cords may account for the rapid rates of movement seen within mycelia (Cairney, 1992). Movement of nutrients away from newly colonised resources is, however, thought to occur by cytoplasmic streaming (Cairney, 1992). The ability of cord-formers to move nutrients in this way is not only important from the fungal perspective; with nutrient translocation, the woodland floor becomes a highly dynamic environment in which nutrient inputs do not necessarily remain concentrated at the location where they are first added to the system. In addition, nutrient movement by fungi appears to be conservative with fungi evaluating the quality of new resources before committing high concentrations of valuable nutrients (Wells *et al.*, 1990). The drive to conserve nutrients may be more important than originally thought; *P .velutina* conserves phosphorus in an established resource to the extent that when colonising a new resource, phosphorus in the soil surrounding the new resource is preferentially utilised over that already sequestered by the mycelium (Wells *et al.*, 1998a). This conservation of nutrients by fungi may serve to act as a buffer against nutrient lack in the soil environment (Boddy, 1993).

The presence of saprotrophic cord-formers at the soil-litter interface, along with latent propagules in fallen wood and the arrival at new resources of spores of unit resource-restricted fungi, leads to inevitable competition between fungi. Competition is often for

occupation of space within a resource from which nutrients can then be extracted over time (Boddy, 2000). Another factor in competition is the changing nature of wood during the decomposition process. Community progression in wood has been widely studied and three main strategies known as competitive (C), stress-selected (S) and ruderal (R) have been identified (Rayner & Boddy, 1988). In standing dead wood the challenging abiotic conditions favour fungi exhibiting stress-selected characteristics, whereas on first falling to the floor many ruderal species rapidly colonise. As fungi employing ruderal strategies are normally unable to degrade the complex polymers in wood and are generally poor competitors, they are rapidly replaced by more competitive fungi. Fungi with ruderal growth strategies are commonly prolific spore producers with a high dispersal capability (Boddy & Heilmann-Clausen, 2008). More competitive fungi are characterised by a strong combative ability and long residence times in the substrate with a wide spectrum of enzymes to degrade more complex polymers such as lignins (Boddy, 2001). It is important to note, however, that a species will often display more than one given strategy with changing environmental conditions (Boddy, 2001). Fungi are therefore described as displaying a given characteristic under a given set of environmental conditions. Unless a highly disturbed environment, woodland decomposer communities will tend to be dominated by competitive-selected organisms and these will be responsible for the bulk of wood decay (Boddy, 2001). In terms of nutrient cycling in established systems, therefore, the competitive selected species are of particular interest.

2.2.4 Fungal interactions

Competitive interactions between fungi fall into two broad groups: interactions mediated at a distance and interactions following hyphal or mycelial contact (Boddy, 2000). Antagonism mediated at a distance arises through the production of volatile or diffusible organic compounds (VOC/DOCs). The most well known example of antagonism at a distance is the antibiotic activity of *Penicillium* spp. which inhibits the growth of microbes through VOC production (Flemming, 1929). Similar responses are seen in wood-decay fungi, where growth of different species in close proximity to one another leads to the production of stress compounds and low molecular weight metabolites (Woodward & Boddy, 2008). In addition, some wood-decay species emit VOCs when

growing on woody substrates which may have antibiotic effects (Woodward *et al.*, 1993). For example, the mycelial extension of *Piptoporous quercinus* was inhibited when growing in the presence of some basidiomycetes, but stimulated in the presence of others (Wald *et al.*, 2004a).

Whilst interactions do occur at a distance they do not necessarily prevent fungi from meeting. Fungal interactions following contact occur at the level of the individual hyphae and the entire mycelium. There are two groups of hyphal interaction: mycoparasitism and hyphal interference. During mycoparasitism the attacker physically attaches to the host (e.g. *Pseudotrametes gibbosa* on *Bjerkandera* spp.) and obtains nutrients either necrotrophically or biotrophically (Boddy, 2000). In hyphal interference, the attacking fungus makes contact with a hypha of an opponent and vacuolation and death of the hyphal compartment follows. The most widely studied example of mycoparasitism is where hyphae of *Phlebiopsis gigantea* destroy compartments of the tree pathogen *Heterobasidion annosum* creating a highly effective biocontrol agent (Rayner & Boddy, 1988).

The most important interaction type among wood-decay basidiomycetes occurs across the entire mycelial front (gross mycelial contact) and can be highly aggressive (Boddy, 2000). These interactions occur both in wood resources and at the soil-litter interface, and have been widely studied (e.g. Donnelly & Boddy, 2001; Griffith *et al.*, 1994; Iakovlev *et al.*, 2004; Wald *et al.*, 2004a, b). During the early stages of an interaction, immediately following contact, mycelial morphology often changes producing dense mycelia at the interaction site with reallocation of mycelial biomass away from the non-interacting colony centre (Boddy, 2000). Changes in resource allocation are dynamic; the addition of precolonised wood blocks onto the established mycelium of *Phanerochaete velutina* led to increased biomass toward the precolonised resource but not increased allocation of ^{32}P. This suggests that the nutrient was allocated to the "safest" resource, the original *Phanerochaete velutina* wood block (Harris & Boddy, 2005). Aside from morphological changes, fungal biochemical activity also increases during interactions, with the upregulation of genes (Iakovlev *et al.*, 2004) and increased enzyme synthesis.

For example, the white rot basidiomycete *Hypholoma fasciculare* increased laccase production when growing in the presence of other soil organisms, but no such increase was recorded when growing across sterilised soil (Baldrian, 2004). Fungal interactions are also characterised by pigment production and changes in VOC emissions. The interaction of *Resinicium bicolor* with *Hypholoma fasciculare* showed increased emission of some VOCs compared to when the species were growing alone, and the production of 10 novel VOCs (Hynes *et al.*, 2007).

2.2.5 The role of biotic and abiotic factors during interactions

The outcome of interactions between basidiomycete fungi can be characterised as deadlock, where neither species gains territory or resource, or replacement where one species gains over the other (Boddy, 2000). In some interactions, fungi only partially replace the opponent whilst in others each replaces the other in different areas of the mycelium (mutual replacement; Boddy, 2000). When not growing in a wood, mycelia may sometimes overgrow each other without one fungus replacing the other. When multiple species are interacted pairwise, interaction hierarchies can be established, and these are frequently broadly repeatable (Chapela *et al.*, 1988; Dowson *et al.*, 1986). Often, however, the combative ability of one fungus is not superior against all opponents and this serves to maintain fungal diversity (Boddy, 2000). This situation is complicated further as interaction outcome also depends on other factors. Inoculum size plays a key role with species occupying larger resources generally having a competitive advantage (Holmer & Stenlid, 1993, 1997). The interaction of *Hypholoma fasciculare* with the ectomycorrhizal fungi *Suillus variegatus* or *Paxillus involutus* favoured the sparotroph when it grew out from the larger 1.6 cm^3 inocula. This was reversed when *H. fasciculare* grew from the smaller 0.44 cm^3 resource. This study was of particular interest as the inoculum size also determined which fungus scavenged ^{32}P from the other hence determining whether the nutrients were returned to the plant system via the mycorrhizal symbiont or remained in the soil within the saprotroph (Lindahl *et al.*, 2001b). In another study, the highly combative *Resinicium bicolor* was interacted with opponents using an experimental design permitting a change in fungal inoculum size whilst retaining the same interaction area. *Resinicium bicolor* could not defeat any opponent when it

occupied only 8% of the total available inoculums, but with a larger share of the resource successfully replaced all opponents (Holmer & Stenlid, 1993). Interestingly, a further study not only confirmed the importance of inoculum size for competitiveness but also revealed that species which were rapid to fruit were poorer competitors, whereas those which took longer to fruit were more competitive, supporting the R-, C- and S- selected theory discussed earlier (Holmer & Stenlid, 1997).

The state of decay of a resource is also thought to be important in determining combative ability (Boddy, 1993). As resources are decomposed, their nutrient content decreases and a loss of combative ability over time may, therefore, reflect a reduction in resource quality (Boddy, 1993). Abiotic variables, such as water potential, temperature and gaseous regime, can also alter fungal interaction progression and outcome (Chapela & Boddy, 1988). When growing on beech (*Fagus sylvatica*), the cord-forming fungi *Phallus impudicus* and *Phanerochaete velutina* were effective at replacing *Trametes versicolor* in atmospheric gaseous conditions but, under low O_2 / high CO_2 concentrations comparable to those found in wood, the interaction outcomes were sometimes completely reversed (Chapela *et al.*, 1988). In another example, *Hypholoma fasciculare* was less combative when water potential was reduced and at low O_2/high CO_2 levels when interacting with rare tooth fungi (Wald *et al.*, 2004b).

Often fungal interactions have been studied in pairwise combinations on sterile media or defaunated soil but, in the field, interactions take place in the presence of a wide range of other soil flora and fauna. The importance of considering the natural environment was demonstrated by White *et al.* (1998); the outcomes of tripartite fungal interactions were not predictable by extrapolating from the outcomes of pairwise interactions involving the same species. In another study, variability in the outcomes of pairwise basidiomycete interactions was caused by the presence of diffusible metabolites of the ascomycete *Trichoderma harzianum* (Schoeman *et al.*, 1996). Substrate type can also alter fungal competitive ability. In a study comparing combative interactions in agar, in wood and across soil, *Steccherinum fimbriatum* was highly effective at replacing opponents in wood but not in soil or on agar (Dowson *et al.*, 1988a). Such combative variability may be due

to morphological and physiological differences when growing on homogeneous media such as agar compared to soil; *Hypholoma fasciculare* rarely produces cords on agar but does in soil systems (Boddy, 1993). Another example of the effects of resource quality was shown in *Phanerochaete sanguinea* which, in interactions with other wood-decay basidiomycetes, was able to retain its resource 80% of the time when extending over clay but only 60% of the time when extending over sand (Holmer & Stenlid, 1996).
The wide variety of factors playing a role in determining the combative ability, and outcome of fungal interactions, probably serves to maintain fungal diversity within woodland ecosystems. In addition, the changes seen in fungal mycelia during aggressive interactions such as alteration of enzyme production, emissions of volatile and diffusible chemicals, and the movement of nutrients are likely to have an effect on other soil organisms. For example, fungi are major agents of nutrient mineralisation but, as discussed, they appear to be more conservative of nutrients than has been previously thought. During interactions, however, leakage of nutrients at the point of interaction has been suggested as a potential route for nutrient mineralisation (Wells & Boddy, 2002).

2.3 Fungal mycelia as a food resource

2.3.1 Fungal feeding by invertebrates

Once fungi have successfully degraded the nutrients in soil and litter they become a food source in the decomposer food web (Bardgett, 2005; Rayner & Boddy, 1988). The diversity of soil invertebrates is very high and they are regularly classified either by size (Petersen & Luxton, 1982) or by the role they play in the ecosystem (termed their functional group; Rusek, 1998). For example, a study in temperate forests in Vancouver Island analysed collembola gut contents and assigned them to four distinct functional groups based on their feeding habits (Addison *et al.*, 2003). The collembola in three groups fed on different types of fungi and the fourth consisted of collembola consuming particulate organic matter (Addison *et al.*, 2003). Species of nematodes, enchytraeid worms (Oligochaeta, known as potworms), mites (Acari) and collembola are known to be mycophagous. Like many nematodes, the mycophagous *Aphelenchus avenae* has specialised mouthparts (the stylet) for penetrating fungal hyphae (Bakhtiar *et al.*, 2001). Nematodes are also numerically important with densities of 30 million m^{-2} having been

recorded (Volz, 1951; cited in Bardgett, 2005). In boreal forests with acidic soils, enchytraeid worms can make up a substantial part of the biomass and are considered 'keystone' species (Didden, 1993). In temperate woodland soils, however, collembola are highly abundant with typical densities of 10^4 to 10^5 m^{-2} (Petersen & Luxton, 1982). Their small body size, up to a maximum of about 10 mm but more commonly 1-2 mm (Hopkin, 1997), means that collembola account for only a small biomass in the soil with an estimated 110 mg dry mass collembola m^{-2} in deciduous woodland (Petersen & Luxton, 1982). The reported collembola biomass figure in temperate deciduous woodland was second only to tundra ecosystems, indicating the high populations compared to other ecosystems (Petersen & Luxton, 1982; Rusek, 1998).

2.3.2 Collembola

Collembola are microarthropods, apterous and have six abdominal segments. Traditionally considered to be primitive insects (Hopkin, 1997), analysis of collembola mitochondrial DNA suggests that they are neither in the insect nor crustacean clades with divergence occurring before that of crustaceans and insects (Nardi *et al.*, 2003). The total number of collembola species is unknown with many new species being described each year (Rusek, 1998), but over 8000 species have already been classified (Gullan & Cranston, 1994). Collembola are traditionally divided into three main groups based on their habitat. *Epidaphic* species are found above-ground and on vegetation, *hemiedaphic* species are found in the upper layers of the soil and in the leaf litter. The widely studied collembola *Folsomia candida* is a *hemiedaphic* species. *Euedaphic* species are soil dwellers and often have no pigmentation and reduced antennae (Hopkin, 1997). Other systems for collembola classification have been attempted but, as with all broad definitions, it is difficult to account for all species within such categories (Hopkin, 1997).

There have been many studies investigating the diets of collembola (e.g. Bardgett *et al.*, 1993b; Hedlund *et al.*, 1995; Jensen *et al.*, 2006; Nakamori & Suzuki, 2005b; Ruess *et al.*, 2000; Scheu & Simmerling, 2004; Visser & Whittaker, 1977). Often gut content analysis has been used (e.g. Addison *et al.*, 2003) but this has limitations in that substrates that are totally degraded within the gut may be under represented or not

represented at all (Poole, 1959). In addition, undigested particles may be unrecognisable having undergone mastication and enzymic attack. If the collembola diet consists largely of fungi, distinguishing fungal species from hyphal fragments is particularly challenging. Furthermore, some collembola appear to be generalists ingesting whatever falls in their path (Maraun *et al.*, 2003). In such a situation it is impossible to determine whether the collembola are deriving nutritional benefit from a given item present in the gut. Laboratory studies have shown collembola to exhibit distinct preferences (Klironomos *et al.*, 1992; Maraun *et al.*, 2003; Shaw, 1988) although caution is required when extrapolating laboratory-derived preferences to actual field behaviour. Despite the difficulties, collembola diets have been elucidated and collembola shown to feed predominantly on detritus and fungi (Jørgensen *et al.*, 2005; Newell, 1984a, b; Hopkin, 1997). There are also incidences of carnivory such as nematode predation (Lee & Widden, 1996) and herbivory (Rusek, 1998; Scheu & Folger, 2004).

As an alternative to basic gut content analysis, enzyme gut assays reveal what can be degraded in the gut and provide a guide to possible food sources. Cellulase, chitinase, to degrade fungal cell walls, and trehalase, to digest hyphal contents, have all been found (Berg *et al.*, 2004; Urbášek & Rusek, 1994), but it is not clear whether these are produced by gut microflora or by the collembola themselves (Thimm *et al.*, 1998). Either way, the presence of these enzymes indicates that collembola eat both plants and fungi although, whether these are preferentially consumed as living or dead material in the field is indeterminable. More recent advances in the use of biomarkers such as phospholipid fatty acids (Haubert *et al.*, 2008), stable isotope analysis (Jonas *et al.*, 2007) and molecular analysis (Jørgensen *et al.*, 2005) have permitted a greater understanding of collembola feeding habits. Nematodes are often rapidly digested within the collembola gut, but may form an important part of their diet (Lee & Widden, 1996). By labelling fungi and nematodes with different stable isotopes of carbon and utilising the different fatty acid profiles exhibited by each species, the dietary preferences of the collembola *Proisotoma minuta* and *Folsomia candida* were studied. Both species of collembola fed almost exclusively on nematodes (Chamberlain *et al.*, 2006). These techniques remove

many of the problems associated with traditional dietary analysis although use in the field can be limited.

2.3.3 The ecological significance of collembola

Despite their small size and low contribution to soil organic biomass, collembola form an important part of ecosystem functioning. The comminution of leaf litter (breaking it down into smaller particles) increases its surface area to volume ratio and, therefore, the ability of bacteria and fungi to degrade them. Collembola also mineralise nutrients releasing nutritional faecal pellets, potentially accelerating the rate of decomposition (Hopkin, 1997). To quantify the contribution to decomposition, some experimental designs have compared systems with collembola absent and present (Teuben & Verhoef, 1992 Teuben & Roelofsma, 1990). The general trend is for collembola to accelerate the rate of decomposition, soil respiration (either oxygen consumption or carbon dioxide release), the rate of nutrient release and enzymic activity (Teuben & Verhoef, 1992). In terms of total ecosystem functioning, the importance of collembola presence will depend on the field populations. The effect of removal of collembola in grassland for example, is likely to be lower than in arctic tundra where collembola are central to soil decomposition processes (Hopkin, 1997).

Aside from direct effects on decomposition, collembola also effect fungal growth and distribution through grazing. By feeding in one area and defecating in another, collembola may act as a dispersal mechanism for bacteria and fungi provided that they can survive the digestive process. On the other hand, propagules damaged through digestion may be selected against when ingested. For example, the spores of cultivated fungus *Hypsizygus marmoreus* were damaged, to different degrees, by collembola of the genus *Hypogastura*, possibly inhibiting the ability of *H. marmoreus* to disperse within the soil (Nakamori & Suzuki 2005 a,b). Selective digestion may also affect bacteria; the gut of the collembola *Folsomia candida* was found to reduce populations of some bacteria by 60 000 fold and others by only 500 fold, substantially altering the bacterial faecal community compared to that which was ingested (Thimm *et al.*, 1998). These indirect effects on decomposition may, therefore, be as important as direct feeding. Indeed,

Petersen (1994; cited in Hopkin, 1997) concluded that the main functional role of collembola in decomposition processes was through the stimulation and inhibition of microorgranisms.

2.3.4 Collembola as prey

Collembola also form the food source for a wide range of soil organisms. Whilst there are examples of vertebrates including lizards, frogs and birds consuming collembola, the majority fall prey to other arthropods (Hopkin, 1997). Predators include harvestmen (Opiliones), beetles (Coleptera) ants (Formicidae) and mites (Acari). The main method of predation avoidance in collembola is the furca. This springing organ derives from a fused pair of appendages on the fourth body segment and is everted away from the body causing the collembola to leap, in some cases considerable distances, avoiding predation (Hopkin, 1997). It is the spring that gives collembola the English name springtails. Some ground beetles (Carabidae) and rove beetles (Staphylinidae) have highly specialised organs for trapping collembola before they can leap to safety (Hopkin, 1997). The compact nature of the soil environment renders the furca ineffective and many soil dwelling species have a reduced or absent furca and these prey species are of particular importance to the mites (Hopkin, 1997).

2.3.5 Collembola interactions with fungi

Collembola are fungal grazers and this grazing activity alters fungal species abundance, mycelial morphology and function (Bretherton *et al.,* 2006; Hedlund *et al.,* 1991; Kampichler *et al.,* 2004; Newell, 1984a, b; Tordoff *et al.,* 2006, 2008). As is the case with most soil animals, collembola do not appear to have highly specialised preferences for particular resources (Mauran *et al.*, 2003; Ponge, 2000). This is surprising as, in above-ground systems, it is thought that niche specialisation permits high diversity among insect herbivores (Strong *et al.,* 1984, Visser, 1986). In addition, the wide variety of complex secondary chemicals emitted by fungi would normally be expected to facilitate the development of specialist feeding guilds (see earlier, Scheu and Folger, 2004). Adding further to this paradox is that, in laboratory tests, collembola tend to demonstrate a hierarchy of preferences (Jørgensen *et al.*, 2003; Klironomos *et al.*, 1992;

Shaw, 1988; Visser & Whittaker, 1977). The collembola *Protaphorura armata* fed predominantly on one of 33 different fungal taxa present in soil samples (Jørgensen *et al.*, 2005). Not only do feeding preferences exist but collembola fitness also varies with fungal resource (Chen *et al.*, 1995). Reproduction of the collembola *Heteromuras nitidus* was significantly lower when fed on *Aspergillus fumigatus* than when fed on the ectomycorrhizal fungus *Laccaria laccata* (Scheu & Folger, 2004). When fed on mixed diets collembola performance improved further. The dense soil environment may limit collembola movement thus preventing the expression of the preferences often observed in laboratory conditions where freedom of movement is often part of the experimental setup.

Most studies of saprotrophic fungi indicate a general preference for dark pigmented (dematiaceous) microfungi, including the genera *Cladosporium*, *Alternaria* and *Phoma* (Jørgensen *et al.*, 2003; Klironomos *et al.*, 1992; Maraun *et al.*, 2003; Poole, 1959). Whilst these fungi do contain high levels of nutrients compared to other fungi, they are also highly melanised rendering them difficult to digest and, therefore, other explanations for such preference are needed. Their abundance in soil, as evidenced through isolation onto sterile media, has been suggested as one possibility although soil isolations tend to favour these highly sporulating species (Harley, 1971). The pigmented microfungi may also indicate a particular stage of substrate decay and may, therefore, be ingested coincidentally (Mauran *et al.*, 2003). The link between collembola preference and resultant fitness, however, challenges this interpretation (Klironomos *et al.*, 1992). A striking example of collembola preference was shown by Newell (1984a, b) working in plantation forests in the English Lake District. *Onychiurus latus* significantly preferred *Marasmius androsaceus* over *Mycena galopus* grazing the former much more extensively than the latter (Newell, 1984a). In the absence of grazing, however, *M. androsaceus* dominated the litter at the expense of *M. galopus* (Newell, 1984b). The study linked the presence of collembola with the distribution of fungi in the field. *M. androsaceus* was restricted to the upper litter layer, an area too dry for the *O. latus*, making the fungus safe from high grazing pressure.

Collembola preferences have also been demonstrated in mycorrhizal fungi, but the results have been mixed. The collembola *Proisotoma minuta* grazed heavily on a variety of ectomycorrhizal (EM) species exhibiting a hierarchy of preferences within the study species (Klironomos & Kendrick, 1996). When presented with a choice between EM fungi and a pathogen (*Rhizoctonia solani*), however, the pathogen was preferred (Hiol Hiol *et al.*, 1994). Kaneda and Kaneko (2004) found that collembola preferred to graze on hyphae of the ectomycorrhiza *Pisolithus tinctorius* when they had been severed from the rest of the mycelium indicating a preference for low vitality hyphae. In another study, Schultz (1991) investigated collembola grazing of ectomycorrhizal fungi and concluded that observed preferences were probably due to avoidance of toxic species as opposed to nutritional benefit of the preferred fungi. Together these studies suggest that EM fungi may be consumed by collembola primarily due to their abundance and not their palatability.

Whilst collembola have been demonstrated to feed on, and exhibit preferences for, arbuscular mycorrhizal (AM) fungi (e.g. Shaw, 1985; Thimm, 1993), in studies with a choice between AM and saprotrophic fungi, the saprotrophs were consistently preferentially grazed (Klironomos *et al.*, 1999; Klironomos & Kendrick, 1996). Indeed, a reduction in reproductive output was seen in *Folsomia candida* when grazing exclusively on AM fungi (Klironomos *et al.*, 1999).

Collembola do, therefore, appear to exhibit feeding preferences, preferring saprotrophic microfungi and some EM species over AM fungi. Preferences for EM fungi over AM species may not be surprising as EM fungi are generally associated with trees (Smith & Read, 1997) and therefore woodland, where collembola are often highly abundant. Preference studies are limited, however, almost exclusively to microcosm and *in vitro* experimental designs. There is also a limited amount of information regarding the grazing preferences of wood-decay basidiomycetes. Despite these shortcomings, it is likely that collembola grazing does alter soil ecosystem function and this is explored below.

2.3.6 The effects of collembola on ecosystem functioning

Many studies into the effects of collembola on ecosystem functioning have concentrated on mycorrhizal associations (Gange, 2000 although see Bardgett *et al.*, 1993a, b, c; Newell, 1984a, b). This is probably because of the importance of mycorrhizae in plant growth and development, and below-ground effects can be easily measured above-ground such as change in plant biomass. Collembola tend to have a negative impact on fungal biomass or extension but the effect on plant growth is generally positive at medium grazing densities (Finlay, 1985; Harris & Boerner, 1990). When birch (*Betula pendula*) and Scots pine (*Pinus sylvestris*) seedlings were grown in a complex (high soil fauna diversity and abundance) and simple (nematodes alone) system, a high diversity of soil fauna led to increased carbon and nitrogen concentration in the foliage and a greater above-ground biomass (Setälä, 1995). This was despite a reduction in the amount of EM fungi in the complex systems (Setälä, 1995). Despite collembola grazing on fungi being beneficial to plants, grazing itself was also advantageous to the plant. The lack of a negative effect implies that collembola did not reduce the capability of the EM fungi and this may be due to grazing of weak or dead hyphae. In fact, preference has been shown for low vitality hyphae of EM fungi (Kaneda & Kaneko, 2004). Indeed grazing appears to be beneficial to the plant, and collembola grazing of older and senescing hyphae may improve fungal performance (Bardgett *et al.*, 1993a). Disruption of ecosystem functioning through collembola grazing has also been reported. For example, a field study involved adding *P. armata* to cores with a mesh permitting the passage of AM hyphae but not roots showed that collembola grazed AM hyphae and this decreased mycorhizosphere respiration (Johnson *et al.*, 2005). The study demonstrated the disruption of the hyphal network by collembola and was interpreted as a negative effect. It should be noted, however, that the absence of a resource for saprotrophic fungi in this study may have obliged collembola to feed on the AM mycelium. In studies where a saprotrophic component is present collembola grazing does not appear to have a negative effect even at high densities (Klironomos & Kendrick, 1995; Schreiner & Bethlenfalvay 2003).

Collembola have also been shown to alter processes in saprotrophic fungi. At greater than typical field densities *Onychiurus procampatus* had a negative impact on fungal biomass and respiration (Bardgett *et al.*, 1993c). Due to the above normal levels of hyphae in the microcosms and the tendency of collembola to aggregate in the field the results may be more realistic that at first appears (Bardgett *et al.*, 1993c). The studies by Newell (1984a, b) also showed collembola regulating field distribution of basidiomycete fungi (Section 2.3.5). In another study the mycelium of the cord-forming wood-decay basidiomycete, *Resinicium bicolor* was completely removed through grazing by *Folsomia candida* and *Proisotoma minuta* whilst the less active *Protaphorura armata* had a limited effect on fungal morphology (Tordoff *et al.*, 2006). In other studies, the cord-forming fungus *Hypholoma fasciculare* changed growth rate and morphology in response to grazing by *Folsomia candida* (Harold *et al.*, 2005; Kampichler *et al.*, 2004). In small systems, the mycelial extension of *Phanerochaete velutina* has been shown to be substantially reduced through collembola grazing activity at normal densities (Tordoff *et al.*, 2006, 2008), accelerated or over compensated at low densities (Bretherton *et al.*, 2006), and unaltered when grown in large microcosms (Wood *et al.*, 2006). In these larger systems it was thought that collembola grazed senescing hyphae hence not adversely affecting the fungal growth.

As in fungal interactions, resource size and quality also affects fungal responses to collembola grazing. For example, the mycelium of *Hypholoma fasciculare* when extending out from both older and larger inocula was more luxuriant than from younger and smaller wood blocks, and showed a greater tolerance of collembola grazing (Harold *et al.*, 2005). There are clearly a wide number of factors that come into play in determining fungal response to collembola grazing. These differences could be important for determining the composition of species assemblages. For example, a highly competitive species such as *Resinicium bicolor* (Holmer & Stenlid, 1996) may be less competitive if substantially negatively affected by grazing (Tordoff *et al.*, 2006). Despite the wealth of research on the occurrence and importance of aggressive fungal interactions (Boddy, 2000), there are apparently no studies investigating the effect of invertebrate grazing on the outcome of fungal interactions. The study by Newell (1984a, b) showed

that collembola altered fungal community structure, but the fungi were not necessarily interacting aggressively; rather the collembola grazing partitioned the two fungal species. One reason for the possible value of studies into invertebrate grazing during fungal interactions is that when fungi interact they increase biochemical activity and may leak what are normally closely guarded nutrients (Wells & Boddy, 2002). These sites of interaction are, therefore potential sources of valuable nutrients for fungal feeders. Recent work has highlighted changes in the VOC bouquet of fungi during interactions and this appears to be linked with DOCs as well (Evans *et al.*, 2008; Hynes *et al.*, 2007; Su, 2005). A range of soil and ground-dwelling invertebrates are known to detect and respond to fungal odours. Ciid beetles, for example, were able to discriminate between fungal bracket species and even bracket age using odour as a method for resource partitioning (Guevara *et al.*, 2000a). Termites are particularly well known for being attracted to timber decomposed by certain brown rot species (Swift and Boddy, 1984 and references therein). More specifically, collembola can detect and respond to mycelial odours (Bengtsson *et al.*, 1988) and other chemicals, such as odours emitted by dead conspecifics (Nilsson & Bengtsson, 2004).

2.3.7 Conclusions

This literature review has highlighted the vital role of soils in not only supporting a high faunal biodiversity but also hosting a wide array of complex decomposition processes. These, in turn, lead to cycling of essential nutrients within the ecosystem. In particular, the key roles of wood-decay cord-forming fungi and collembola have been examined; fungi degrading complex polymers into labile compounds and collembola activity potentially accelerating soil processes. The review has highlighted the dearth of knowledge concerning the interaction of these two taxonomic groups, despite their abundance and acknowledged importance. A greater understanding of the interactions that occur between these organisms and how they affect their functioning will help unravel the complexities of soil decomposition processes.

3.0 Fungal interaction progression and outcomes: The effect of collembola grazing and distinct fungal isolates.

3.1 Introduction

As many species of saprotrophic cord-forming fungi occupy similar spatial niches at the soil-litter interface, interactions are inevitable (Boddy, 2000). Interspecific fungal interactions are highly aggressive and generally occur following gross mycelial contact (Boddy, 2000). Interactions result in either deadlock or some form of replacement; these outcomes are variable and can be substantially affected by both biotic and abiotic variables (Chapela *et al.*, 1988; Woods *et al.*, 2005). Intraspecific genetic variability may also be important in determining fungal interaction progression and eventual outcome. Most studies investigating interactions of higher fungi have tended to concentrate on one genetic isolate of each species studied. A few studies with *Trichoderma* species (Ascomycota) interacting with wood rot fungi have involved multiple isolates (Bruce *et al.*, 2000 but see Phillip *et al.*, 1995; Wheatley *et al.*, 1997), but most of these have focussed on strains of medical or biocontrol value (Vainio *et al.*, 2001; Walker *et al.*, 1995). Despite possible implications for both species abundance and diversity, the role played by fungal genetic variability in fungal fitness remains unknown.

As well as encountering other fungi, non-unit resource restricted fungi growing out from resources are also more accessible to grazers. Various soil invertebrate taxa, including nematodes, collembola and earthworms, are known to graze on fungal mycelia and can substantially alter fungal morphology and physiology (Boddy & Jones, 2008; Harold *et al.*, 2005; Ruess *et al.*, 2000; Tordoff *et al.*, 2006). Such effects are likely to alter fungal fitness and, therefore, their combativeness in interactions with other soil microorganisms, including fungi.

The visible changes in morphology and physiology during fungal interactions are associated with biochemical change, such as increased enzymic activity and production of diffusible (DOC) and volatile (VOC) organic compounds (Baldrian, 2004; Griffith *et al.*, 1994; Hynes *et al.*, 2007). This enhanced activity may also lead to compartment lysis and nutrient leakage into the surrounding environment (Wells &

Boddy, 2002). Numerous plant species use chemicals emitted as result of insect-herbivore action to attract parasitoids to the vicinity (Dicke, 1994; Tentelier & Fauvergue, 2007); a similar process may occur with fungi. Mycophagous grazers may use chemical cues arising from interaction activity to locate high quality resource patches while fungi may recruit grazers to provide a competitive advantage over an opponent. Orientation to fungal odours by invertebrates has been well documented (Guevara *et al.*, 2000b; Hedlund *et al.*, 1995; Swift & Boddy, 1984) and anecdotal evidence exists of attraction of fungus gnats to fungal interaction zones (Boddy *et al.*, 1983).

This study aims to: (i) elucidate the effects of invertebrate grazing on the morphological and competitive abilities of basidiomycete fungi when interacting interspecifically; (ii) determine if there is evidence of preferential invertebrate grazing at the interaction zone and (iii) whether fungal isolate variability affects fungal combativeness and invertebrate grazing.

3.2 Materials and methods

3.2.1 Collembola culturing

Folsomia candida (obtained from Centre for Ecology and Hydrology Lancaster, UK; Appendix I) were cultured in 0.6 l plastic tubs with pierced lids for aeration. Each tub contained 9:1 plaster of Paris (Minerva Dental Ltd., Cardiff, UK): activated charcoal (Sigma, UK). Collembola were supplied with dried baker's yeast (*Saccharomyces cerevisiae*, Spice of Life Ltd., Cardiff) weekly. Tub moisture was maintained with deionised water (DI).

Experimental *F. candida* were selected using a stacked sieving system with sieves of known pore size, the larger sieves being uppermost (Nickel-Electro Ltd., Weston-super-Mare, UK). Collembola were added to the top sieve and allowed to self-sort by moving through the sieves for 5 minutes. Those of body diameter 250-400 μm were placed in fresh culture pots and left without food for 24 h to evacuate gut contents. Collembola were transferred to experimental plates using an electrical entomological suction pump or 'pooter'.

3.2.2 Fungal isolates

Hypholoma fasciculare (four isolates labelled 1-4; see Appendix I), *Phallus impudicus*, *Phanerochaete velutina* and *Resinicium bicolor* (two isolates; labelled 1, 2) were cultured on 2% malt extract agar (MEA, 20g L^{-1} malt, Munton and Fison, UK, Lab M agar no. 2, Fisher Scientific, UK) and maintained in the dark at 20°C.

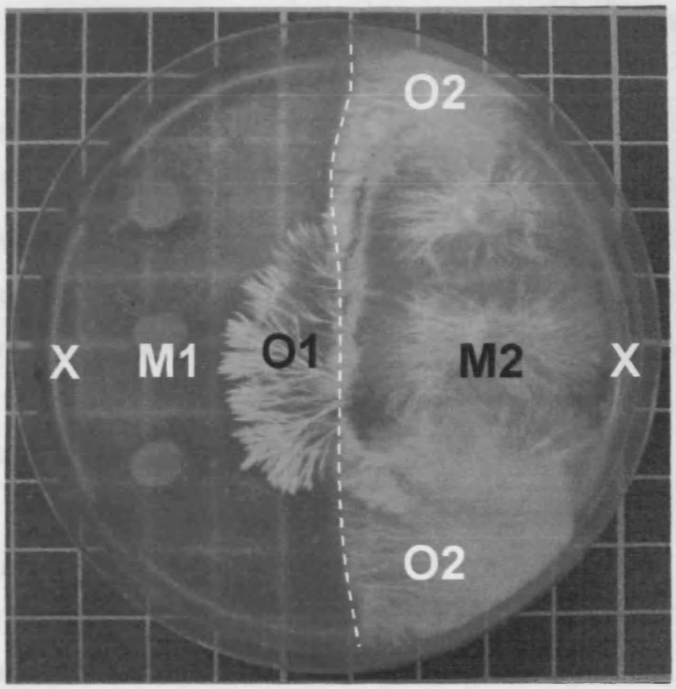


Fig 3.1: Interaction of *Phanerochaete velutina* (growing left to right, white lettering) and *Hypholoma fasciculare* (black lettering) marked with the five possible areas of the interaction. M1/M2 = original mycelium, O1/O2 = overgrowth and the dashed line the original interaction zone. The original interaction zone was defined as being 2 mm either side of this line. X indicates points of collembola addition.

3.2.3 Agar plate inoculation

Fungi were paired in all possible (36) combinations (10 replicates of each) and inoculation was timed to ensure that when the two mycelia met each individual had colonised an equivalent area of agar. Three, 5 mm inoculum plugs were spaced equidistantly along a chord 2 cm from the edge of 2% MEA plates (Fig 3.1). Plates were incubated at 20°C in the dark and maintained in cardboard boxes within black polythene bags to reduce water loss. Twenty collembola were added at two points, 10 on each mycelium (see Fig. 3.1) to each of five replicates per combination when the

interacting fungi in a minimum of 50% of the replicates had made contact for 2 d or more (Fig. 3.1).

3.2.4 Data collection

Digital photography of inoculated plates using a Nikon© Cooolpix ™ 5700 mounted on a Kaiser RA1 camera stand (Kaiser, Germany) set at 36.7 cm started on the day of collembola addition (t_0) and then every 2 d until 14 d, followed by every 4 d to 26 d.

3.2.5 Reisolations

To identify which fungi replaced the other in the agar resource, reisolations were carried out. Twelve weeks following collembola addition all plates not contaminated by bacteria or fungi were inverted and agar cut from the media in the centre of each fungal interaction area (as defined) and plated onto fresh 2% MEA under aseptic conditions. The mycelium growing out from each reisolation was identified. Contaminated plates were discarded.

3.2.6 Visual outcome of interactions

Twenty-six days following collembola addition, interaction outcomes were classified by visual assessment as follows:

- Deadlock: neither mycelium progressed into the territory of the other
- Partial replacement: mycelium of one species overgrew the other but did not reach the opposite side of the Petri dish from where it was inoculated. Overgrowth deemed to have occurred when at least 5 mm mycelial progression was observed beyond the original interaction line.
- Total replacement: as with partial replacement but the mycelium reached the far side of the Petri dish
- Mutual replacement: each mycelium overgrew in part and was overgrown in part by the opposing mycelium.

3.2.7 Competitive analysis

To analyse quantitatively the competitiveness of the different fungal isolates the interaction outcome for each replicate of all interactions was attributed a score. Scoring was based on the proportion of replicates displaying each outcome for any

given interaction. For each replicate scores were: total replacement of opponent 2; partial replacement of opponent 1; deadlock 0; partial replacement by opponent -1; total replacement by opponent -2 (after Crockatt *et al.*, 2008). A species showing total replacement in all replicates, therefore, scored 10, while a species totally replaced in all replicates -10. A cumulative score was attributed to each species providing a competitiveness index. This was repeated for all species but the wide range of interactions masked important competitive differences; only comparisons of all species interacting with *H. fasciculare* are shown.

3.3 Results

For clarity, results are considered per species, focussing in each case on combative ability in both overgrowth and through-medium replacement (as tested using reisolations), morphological changes during interactions, pigment production and responses to grazing including holes grazed in the mycelia and morphological change not seen in ungrazed plates.

3.3.1 *Hypholoma fasciculare*

H. fasciculare was strongly combative. Only *P. velutina* was successful in completely overgrowing it. *H. fasciculare* 1 was the most combative of the four isolates, *H. fasciculare* 2 and 3 were also strongly combative (Tables 3.1, 3.2) while, of the four isolates used, *H. fasciculare* 4 was the weakest competitor at both overgrowth and within-medium combat (Tables 3.1, 3.3). *H. fasciculare* was effective at wresting resource from opponents (Table 3.3). There were, however, marked differences between isolates. *H. fasciculare* 3, for example, replaced *R. bicolor* 1 whereas *H. fasciculare* 4 did not (Tables 3.2 and 3.3). All *H. fasciculare* isolates were unable to replace *P. impudicus* within the medium. Furthermore, *H. fasciculare* 3 partially overgrew *R. bicolor* 1 but *R. bicolor* 1 replaced *H. fasciculare* 3 within the agar in the majority of replicates. In contrast *H. fasciculare* 2 overgrew opponent species to only a limited extent, yet it always partially replaced *R. bicolor* 1 through the substratum. *H. fasciculare* grew as dense mycelium from the inoculum and overgrew with the formation of cords (Table 3.4), occasionally emerging through the interaction zone from several discrete points (Fig. 3.2a). *H. fasciculare* 4 often produced non-linear, apparently disordered, cords when overgrowing other species (Fig. 3.2 i). While there were no visible morphological changes in *H. fasciculare* attributable to grazing,

combativeness was altered; grazing increased combativeness in two isolates (*H. fasciculare* 2 and *H. fasciculare* 3) and reduced it in the others (Table 3.1). All *H. fasciculare* isolates produced yellow pigment although *H. fasciculare* 1 only produced pigmentation (Fig. 3.2 a, b) when overgrowing another species. With the exception of the cords of *H. fasciculare* 4 when interacting with *P. impudicus*, which developed a yellow colour (Fig. 3.2 i), all new growth of *H. fasciculare* isolates was white.

3.3.2 *Phallus impudicus*

Although it was overgrown to varying degrees by all *H. fasciculare* isolates, except *H. fasciculare* 4, *P. impudicus* was strongly combative (Tables 3.2, 3.3). Uniquely among the fungi in the study, when in the presence of heterospecifics, *P. impudicus* morphology changed before contact occurred. This change was in the form of dense aerial cords originating from up to 1 cm behind the growing front (Fig. 3.2 k, Table 3.3). This morphological response also occurred when *P. impudicus* was paired against itself, but has not been seen when growing alone (T.D. Rotheray unpublished data). *P. impudicus* mycelium often proliferated around the original inoculum plugs leading to dense aerial hyphae (Fig. 3.2 g). In interactions where *P. impudicus* was substantially overgrown it occasionally broke through the opposing mycelium at the interaction zone, producing fast growing attack plumes (Fig. 3.2 g).

P. impudicus mycelia darkened in some interactions but did not produce strong pigmentation (Table 3.4). Occasionally, *P. impudicus* produced exudate when overgrowing other species and this usually occurred at the initial point of contact (Fig. 3.2 o). During interactions with *R. bicolor*, a zone of lysis, where the interaction enzymes lead to a zone of clearing between the two species, was produced at the interaction zone as *P. impudicus* replaced *R. bicolor* (Table 3.3, Fig. 3.2 k, o). In these interactions with both isolates of *R. bicolor*, there was distinct burrowing by collembola along the lytic zones (Fig. 3.2 n). Grazing had no effect on the combativeness of *P. impudicus* which ranked fourth both when grazed and when ungrazed (Table 3.1).

3.3.3 *Phanerochaete velutina*

P. velutina was frequently successful at gaining a 'foothold' in the territory of the opposing mycelium of all species and growing across, albeit not replacing, it (Table

3.3). When overgrowing another mycelium, *P. velutina* formed finely branched cords often emerging from a narrow point of the interaction zone (Fig. 3.2 j). When paired against itself, and when overgrown by *R. bicolor* 1, *P. velutina* formed cords between inocula, and aerial mycelium.

P. velutina did not benefit from grazing pressure, being ranked sixth most combative compared to fifth when ungrazed (Table 3.1). Grazing increased the extension rate over an opposing mycelium (Table 3.3, Fig. 3.2 c, d; see Chapter. 6). In the interaction with *P. impudicus*, *P. velutina* was always partially replaced in the substrate in grazed but not ungrazed, interactions (Table 3.3). At the surface *P. velutina* did not overgrow *P. impudicus* in the absence of collembola (Table 3.2) but, when grazed both species overgrew the other. *P. velutina*, produced limited pigmentation except if overgrown, when a dark pigment permeated the medium below it. This pigment was also seen when *P. velutina* interacted with itself.

3.3.4 *Resinicium bicolor*

R. bicolor 1 was the more combative of the two *R. bicolor* isolates. *R. bicolor* 2 partly replaced only one isolate (*H. fasciculare* 4) whereas only *H. fasciculare* 3 and *P. impudicus* were consistently able to overgrow *R. bicolor* 1. The combative ability of *R. bicolor* 1 through the medium was greater than surface overgrowth (Table 3.3). Following contact with mycelium of a different species, *R. bicolor* 1 rapidly produced dense aerial hyphae up to about 1 cm behind the interacting front (Fig. 3.2 l), but this was either less marked or absent in *R. bicolor* 2 (Table 4). *R. bicolor* overgrew as dense, tightly packed, unbranched linear cords (e.g. Fig. 3.2 l). Uniquely, *R. bicolor* did not produce cords behind the interaction zone. When *R. bicolor* self-interacted, areas of low density mycelium were produced where the individuals met.

Table 3.1: Combative scores of *H. fasciculare* isolates against other species

		R. bicolor 2		*R. bicolor* 1		*P. velutina*		*P. impudicus*			
		Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	**Total row score**	**Combative rank**
H. fasciculare 1	Ungrazed	10		1		10		4		**25**	1
	Grazed		10		0		10		3	**23**	**1**
H. fasciculare 2	Ungrazed	10		4		-2.5		0		**11.5**	2
	Grazed		10		3		2.5		1	**16.5**	**2**
H. fasciculare 3	Ungrazed	3		5		-1.1		0		**6.9**	3
	Grazed		3		5		5.5		0	**13.5**	**3**
H. fasciculare 4	Ungrazed	-5		-3.5		0		-5		**-13.5**	7
	Grazed		-1		-3		-10		-5	**-19**	**7**
Inverse column score		**-22**	**-22**	**-6.5**	**-5**	**-6.4**	**-8**	**1**	**1**		
Combative rank		8	**8**	6	**5**	5	**6**	4	**4**		

Total replacement in all replicates scored 10 points, partial replacement, 5 points, and deadlock or mutual replacement 0 points. Overall interaction score is based on the outcome of all replicates. Score in the right column is for the isolates listed in the corresponding row. Scores in the bottom row relate to the species listed in the columns. The combative rank is based on the scores for either grazed (in bold type) or ungrazed (in normal type) the highest cores being ranked 1 and the lowest 8. Inverse column score is the negative value of the sum of scores in that column.

Table 3.2: Surface outcome of interactions after 24-26 d.

	R. bicolor 2		*R. bicolor* 1		*P. velutina*		*P. impudicus*		*H. fasciculare* 4		*H. fasciculare* 3		*H. fasciculare* 2	
	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed
H. fasciculare 1	**o** 100	**o** 100	**M** 80 **P** 20	**M** 60 **P** 20 **p** 20	**O** 100	**O** 100	**M** 20 **p** 80	**M** 40 **p** 60	**D** 100	**D** 100	**D** 100	**D** 100	**D** 100	**D** 100
H. fasciculare 2	**p** 100	**p** 100	**M** 20 **p** 80	**M** 40 **p** 60	**M** 75 **O** 25	**M** 75 **o** 25	**M** 100	**M** 80 **p** 20	**D** 100	**D** 100	**D** 100	**D** 100		
H. fasciculare 3	**p** 60 **D** 40	**p** 60 **D** 40	**p** 100	**p** 100	**M** 22 **o** 33 **O** 44	**o** 77 **O** 22	**M** 100	**M** 100	**D** 100	**D** 100				
H. fasciculare 4	**P** 100	**M** 80 **P** 20	**M** 30 **P** 70	**M** 30 **P** 60 **D** 10	**M** 20 **D** 80	**O** 100	**P** 100	**P** 100						
P. impudicus	**p** 100	**p** 100	**p** 100	**p** 100	**M** 40 **o** 60	**M** 100								
P. velutina	**M** 80 **D** 20	**M** 80 **p** 20	**p** 20 **o** 80	**M** 20 **p** 40 **o** 40										
R. bicolor 1	**D** 100	**D** 100												

Outcomes were mutual overgrowth (M), partial overgrowth (P), total overgrowth (O) and deadlock (D). For outcomes designated P and R, uppercase signifies overgrowth was by the fungus listed in the column whereas lowercase signifies overgrowth by the fungus listed in the row. Values are percentage of replicates exhibiting a given response.

Table 3.3: Outcome of fungal interactions from reisolations taken from the underside of agar substrate.

	R. bicolor 2		*R. bicolor* 1		*P. velutina*		*P. impudicus*		*H. fasciculare* 4		*H. fasciculare* 3		*H. fasciculare* 2	
	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed	Ungrazed	Grazed
H. fasciculare 1	-	-	-	-	-	-	**P** 100	**P** 100	**D** 100	**D** 100	**D** 100	**D** 100	**D** 100	**D** 100
H. fasciculare 2	**p** 100	**D** 20 **p** 80	**p** 100	**P** 100	-	-	**M** 100	**M** 40 **P** 60	**D** 100	**D** 100	**D** 100	**D** 100		
H. fasciculare 3	**p** 60 **D** 40	**p** 50 **D** 50	**r** 11 **P** 89	**r** 22 **P** 78	-	**r** 80 **R** 20	**D** 100	**P** 33 **D** 67	**D** 100	**D** 100				
H. fasciculare 4	**P** 100	**P** 100	**p** 10 **P** 40 **R** 50	**d** 11 **M** 11 **P** 22 **R** 56	-	-	**D** 100	**D** 100						
P. impudicus	**p** 100	**p** 100	**p** 100	**p** 100	**D** 25 **P** 50 **p** 25	**p** 100								
P. velutina	-	-	-	-										
R. bicolor 1	**D** 100	**D** 100												

Outcomes were mutual replacement (M), partial replacement (P), total replacement (R), and deadlock (D). For outcomes designated P and R uppercase signifies replacement was by the fungus listed in the column whereas lower case signifies replacement by the fungus listed in the row. Values are a percentage of replicates exhibiting a given response. Hyphen (-) indicates contamination in three or more replicates hence insufficient data. Reisolations taken 12 weeks following collembola addition.

Table 3.4: Changes in fungal morphology and pigmentation during interactions

	Morphology				Pigmentation			
	Change	Time	Change	Time	Change	Time	Change	Time
***H.fasciculare* 1 vs.**								
H.fasciculare 1	C	>0 d	C	>0 d	WY	14 d	WY	14 d
H.fasciculare 2	C	10 d	C	10 d	MY	10 d	MY	10 d
H.fasciculare 3	CSB	14 d	-	-	MY	14 d	MY	10 d
H.fasciculare 4	CS	14 d	CS	08 d	SY	02 d	MY	08 d
P.impudicus	CA	04 d	AeCSB	>0 d	WY	06 d	-	-
P.velutina	-	-	CA	02 d	-	-	-	-
R.bicolor 1	CA	06 d	AeM	>0 d	SY	10 d	SR	04 d
R.bicolor 2	CA	04 d	AeM	>0 d	SY	10 d	WR	04 d
***H.fasciculare* 2 vs.**								
H.fasciculare 2	-	-	-		-	-	-	-
H.fasciculare 3	CSB	14 d	CSB	14 d	MY	10 d	MY	10 d
H.fasciculare 4	-	-	-	-	WY	10 d	SY	04 d
P.impudicus	CB	00 d	AeCS	>0 d	MY	02 d	-	
P.velutina	CDA	06 d	CDA	02 d	MY	06 d	Wda	06 d
R.bicolor 1	CDA	04 d	AeM	>0 d	MY	06 d	SR	04 d
R.bicolor 2	CDA	04 d	AeM	>0 d	SY	08 d	WR	06 d
***H.fasciculare* 3 vs.**								
H.fasciculare 3	-	-	-	-	WY	10 d	WY	10 d
H.fasciculare 4	AeM	06 d	AeM	10 d	SY	02 d	SY	06 d
P.impudicus	AeC	>0 d	AeC	>0 d	MY	02 d	-	-
P.velutina	CDB	04 d	CDB	02 d	WY	06 d	WD	10 d
R.bicolor 1	CDA	02 d	-	-	SY	02 d	SR	02 d
R.bicolor 2	CA		-	-	SY	12 d	-	-
***H.fasciculare* 4 vs.**								
H.fasciculare 4	AeM	06 d	AeM	06 d	-	-	-	-
P.impudicus	CDB	04 d	AeC	>0 d	MY	04 d	-	-
P.velutina	-	-	CD	04 d	WY	06 d	-	-
R.bicolor 1	-	-	CDA	10 d	SY	04 d	few	WR
R.bicolor 2	CDA	04 d	-	-	WY	02 d	-	-
***P.impudicus* vs.**								
P.impudicus	CD	>0 d	CD	>0 d	-	-	-	-
P.velutina	CB	>0 d	CS	10 d	MD	08 d		
R.bicolor 1	CD	>0 d	-	-	MD	08 d	-	-
R.bicolor 2	CD	>0 d	-	-	MD	08 d	-	-
***P.velutina* vs.**								
P.velutina	AeM	04 d	AeM	04 d	WD	06 d	WD	06 d
R.bicolor 1	-	-	DC	06 d	MD	06 d	-	-
R.bicolor 2	-	-	CS	06 d	MD	10 d	-	-
***R.bicolor* 1 vs.**								
R.bicolor 1	-	-	-	-	-	-	-	-
R.bicolor 2	-	-	-	-	WR	22 d	-	-
***R.bicolor* 2 vs**								
R.bicolor 2	-	-	-	-	-	-	-	-

Time indicates when change first observed. In morphology columns C=cord formation, A=aggregated, Ae=aerial, B=branched, D=dense, M=mycelium and S=sparse. In pigmentation columns D=darkened, M=medium, R=red, S=strong, W=weak and Y=yellow. Changes are an overall summary; some replicates did not confirm exactly to what is described here. For each descriptor (morphology, pigmentation) the two left columns describe the species listed in bold, the two right columns describe the species listed in that row.

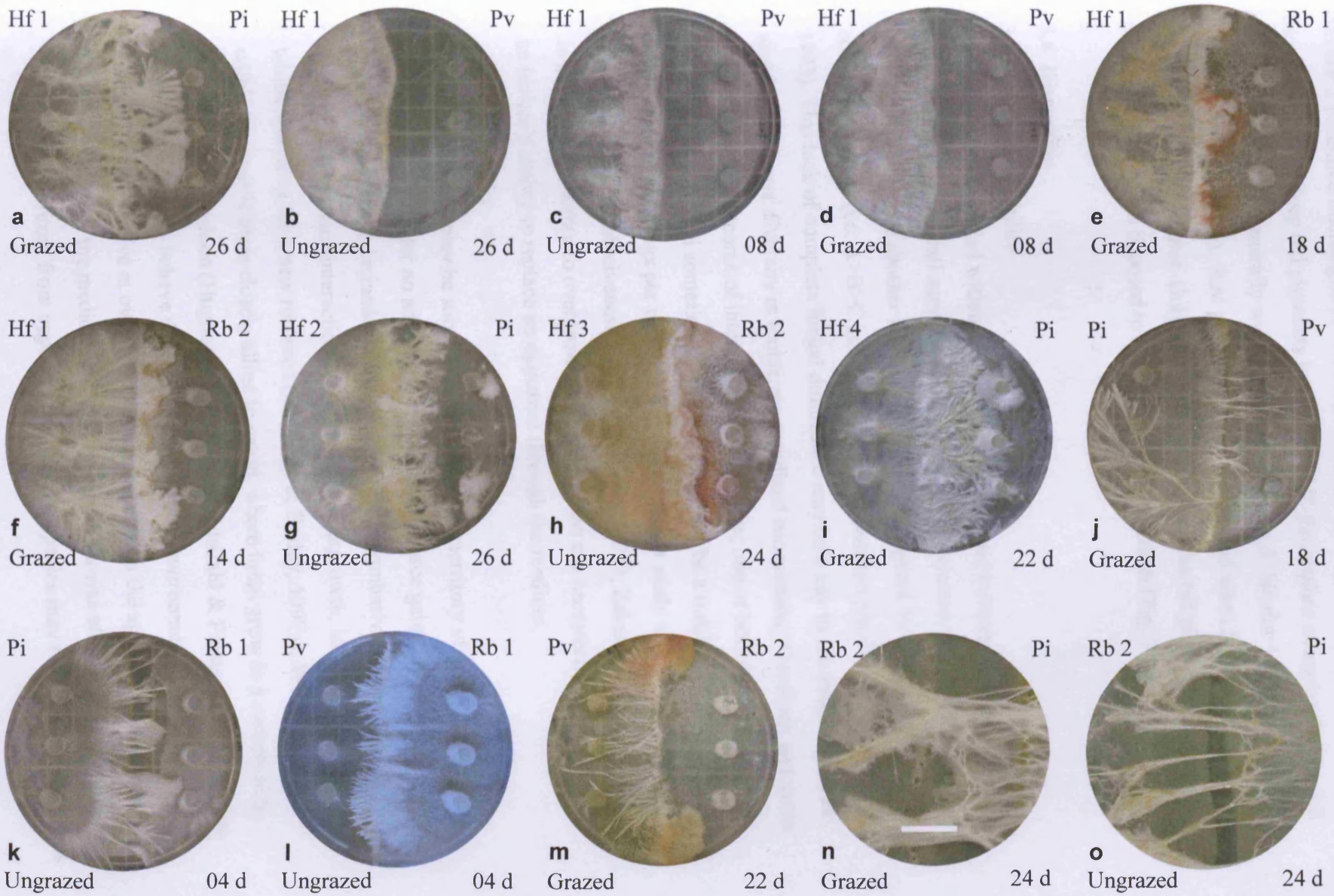


Fig 3.2: Morphological and pigment changes during interactions. Hf: *Hypholoma fasciculare,* Pi: *Phallus impudicus*, Pv: *Phanerochaete velutina,* Rb: *Resinicium bicolor.* Images a-m are 9 cm diameter. Images n, o (scale bar 5 mm) are interaction zone close-ups.

The combative score of *R. bicolor* 1 improved when grazed whereas the score of *R. bicolor* 2 remained unchanged (Table 3.1). When interacting with *H. fasciculare*, *R. bicolor* produced deep red pigments in regions of *H. fasciculare* overgrowth (Fig. 3.2 e, f, h); this pigment generally was more pronounced in *R. bicolor* 1 than in *R. bicolor* 2 (Fig. 3.2 e, f but see h). Red pigment was also produced when *R. bicolor* 2 interacted with *P. velutina*. Only in this interaction was the red pigment seen in the overgrowing cords as opposed to the 'defending' mycelium (Fig. 3.2 m).

3.4 Discussion

3.4.1 Combative ability

Combative ability varied within and between species, and between replicates, with no strain emerging as overall superior. Such transitive hierarchies, in which one fungus is able to out-compete another which is in turn out-competed by a third individual out-competes the first (i.e. A>B>C but C>A) has been observed previously (Boddy, 1993). This lack of complete fungal dominance may be a key to the maintenance of wood decomposer diversity in temperate woodland ecosystems. Deadlock was never the predominant outcome of interspecific interactions; one or both isolates always partially overgrew and sometimes replaced. This may be a result of the large initial inoculum (3, 5mm plugs per isolate) used in the present study as inoculum size is central to fungal combativeness (Holmer & Stenlid, 1993; Zakaria & Boddy, 2002). In addition, the ability to overgrow the opponent was not necessarily an indicator of an isolate's ability to replace an opponent through the medium.

Whilst one species may be successful at invading the territory of another, such overgrowth may confer no advantage in terms of resource gained. Surface outcomes alone may, therefore, be inadequate to determine the combative ability of a species in the field. The surface interaction is similar to soil outgrowth, in which fungi forage whilst searching for a new resource (Donnelly & Boddy, 1997a). What happens within the agar is more closely allied to wood, where fungi grow in a carbon-rich, poorly-aerated medium (Hughes & Boddy, 1994; Lindahl & Finlay, 2006). It is not surprising that fungi behave differently in these two environments. *P. velutina*, for example, was effective at overgrowing other species on the agar surface but poor at replacement within the medium, whereas *P. impudicus* was effective at both retaining and wresting territory from opponents. These two species may represent extreme ends

of a combative spectrum. *P. velutina* grows rapidly, reaching and quickly colonising new resources, but is out-competed by slower 'late-comers'. In contrast, the slower-growing *P. impudicus* may arrive at a resource and successfully take territory from other species. *P. velutina* could, therefore, be considered as demonstrating r-selected behaviour relative to the more K-selected behaviour of *P. impudicus*. When compared among the guild of wood-rotting fungi, however, white-rot cord forming basidiomycetes are highly combative; strategies observed in this present study are, therefore, a spectrum of strategies within the more combative end of the guild of wood-rotting fungi (Boddy, 1993, 2000).

3.4.2 Morphological change and pigment production during interactions

Fungal combat was often associated with substantial morphological change (Fig. 3.2) and was normally manifested as cord formation in the overgrowing mycelium. Mycelial cords are thick-walled organs, resistant to the passage of compounds in or out of the cytoplasm within (Boddy, 1999). Thick cord walls may provide protection against the inhospitable environment created by a combative opposing mycelium (Donnelly & Boddy, 1997a). Secondly, as fungal interactions are sites of increased metabolic activity (Iakovlev *et al.*, 2004; Wells & Boddy, 2002), the development of transport organs would allow rapid, efficient movement of nutrients to the interaction area, increasing the fungus's 'firepower' as it attacks its opponent. The production of cords before contact with an opponent in *P. impudicus* may be a precautionary response affording increased protection from opponents. This behaviour also was seen when *P. impudicus* was paired against itself. The possible reasons for aerial mycelium production, upon contact and during interactions, remain unclear but increased allocation of fungal biomass towards interaction zones is typical of combative basidiomycetes (Boddy, 2000).

In addition to morphological change, pigment development during interactions was widespread (Table 3.4). Production of pigment was especially pronounced in *H. fasciculare* and *R. bicolor* through the production of yellow cords and red exudates, respectively. Pigment production during basidiomycete fungal interactions has been documented (Donnelly & Boddy, 2001; Hynes *et al.*, 2007). Pigment production is correlated with volatile organic compound production with several of these chemicals having putative roles as defence against attack of fungi and invertebrates (Hynes *et*

al., 2007). During combative interactions, pigmentation was observed either only when an isolate was attacking (e.g. some *H. fasciculare* isolates) or only when defending (e.g. *R. bicolor*). Pigment production appears to show that these fungi alter their response based on their combative ability against a given opponent. This may allow the fungus to optimise a response either shunting valuable nutrients to a site where they are either best protected (in the case of defence), or most useful in combat (in the case of attack). Fungal networks are known for their ability to move nutrients around and, with interactions being ubiquitous, such behaviour would be advantageous (Hughes & Boddy, 1994; Wells & Boddy, 1995).

3.4.3 The importance of genetically distinct fungal isolates

The use of multiple isolates demonstrated a wide range of combative, morphological and biochemical (e.g. pigmentation) responses. There is a dearth of ecological research concerning fungal isolates. Differences in combative ability and responses to abiotic variables have been seen between genetically distinct individuals of rare fungi (Wald *et al.*, 2004a, b) although, similarities between isolates were also observed when tested for enzyme production and volatile emissions (Dyer *et al.*, 1992; Philip *et al.*, 1995). The present study indicates a wide variety of differences among isolates and, therefore, fungal isolate, as well as species assemblage, may be crucial in determining decomposer community development.

3.4.4 The impact of collembola

In addition to the effects of varying isolate, this study also examined the effects of collembola on fungal interactions. Collembola did not appear to have any affect on overall interaction outcome but did alter interaction progression. For example, when growing alone, *P. velutina* growth was inhibited by *F. candida* (Tordoff *et al.*, 2006) but in this study collembola interactions accelerated *P. velutina* growth over the opponent. This has also been seen in soil microcosms (see Ch. 6). Despite some burrowing at the interaction zone in the interaction of both isolates of *R. bicolor* with *P. impudicus*, there was generally no evidence of preferential occupation of the interaction zone, where nutrients may be expected to leak out. Such a preference might be expected as it has been seen in fungus gnats (Mycetophilidae; Boddy *et al.*, 1983); large numbers of fungus gnats were found on agar plates in which combative interactions between fungi were occurring but the gnats were not found on plates

where fungi were growing alone. There was, however, extensive burrowing in interactions *R. bicolor* against *P. impudicus* which may indicate attraction.

3.4.5 Conclusions

The basidiomycete fungal interactions studied were highly aggressive but no clear hierarchy of combativeness emerged. This absence of hierarchy contrasts with previous studies (Donnelly & Boddy, 2001; Dowson *et al.*, 1988a). The pigmentation changes associated with combativeness may hold clues as to differential physiological responses of fungi to aggressive encounters, with possible effects on nutrient allocation on the forest floor. Although the presence of invertebrate grazers does not appear to alter outcome, grazing did alter interaction progression. In the longer term, grazers may be involved in affecting species assemblages. The variety of interaction outcomes from both species and isolates highlights the need for a thorough understanding of these organisms. Such an understanding will provide a fuller picture of how these systems develop and stabilise.

4.0 Collembola foraging responses to interacting fungi.[1]

4.1 Introduction

Collembola are an extremely abundant (10^4-10^5 m^{-2} or more) group of invertebrates that feed on fungal hyphae and plant debris (Hopkin, 1997), and are a primary consumer group in decomposer systems (Scheu & Simmerling, 2004). Fungi, especially basidiomycetes, are the dominant component of soil microbial biomass and the major agents of ligno-cellulose decomposition in forest ecosystems (Boddy, 2001). Fungal mycelia are a highly nutritious food source to the many invertebrates that either graze directly on mycelia or fruit bodies, or indirectly by ingesting mycelium within decomposing organic matter (Swift & Boddy, 1984; Maraun *et al.*, 2003; Boddy & Jones, 2008).

Collembola grazing induces a variety of responses in fungi including changes in extracellular enzyme production and mycelial morphology, increases and decreases in growth rate and biomass production (e.g. Hedlund *et al.*, 1991; Scheu & Simmerling, 2004; Tordoff *et al.*, 2006). Some basidiomycetes have evolved defence mechanisms against invertebrate grazing of fruit bodies and mycelia, for example, aerial stalks of *Pleurotus* species produce droplets of toxin (Barron & Thorn, 1987; Hibbett & Thorn, 1994); adhesive secretory cells on hyphae or conidia of *Hohenbuehelia* species (Thorn & Barron, 1984); and stephanocysts producing adhesive chemicals in some *Hyphoderma* species (Tzean & Liou, 1993).

Chemicals deposited on or within the fungal cell wall may also act as deterrents to invertebrate feeding. These chemicals include calcium oxalate (CaC_2O_4) crystals (Horner *et al.*, 1995; Connolly *et al.*, 1999) and melanin (Rayner & Boddy, 1988; Scheu & Simmerling, 2004). Basidiomycete fruit bodies, mycelium and colonised organic matter produce a wide range of volatile (VOC) as well as dissolved (DOC) organic compounds (Faldt *et al.*, 1999; Rosecke *et al.*, 2000; Xu *et al.*, 2004; Hynes *et al.*, 2007). Some invertebrates show attraction, repulsion, arresting activity and antifeeding responses to these fungal chemicals (Boddy & Jones, 2008) with many invertebrates, including collembola, exhibiting fungal preferences (Bardgett *et al.*,

[1] A version of this Chapter has been accepted for publication in Ecological Entomology (Appendix II)

1993b; Jørgensen *et al.*, 2003; Klironomos *et al.*, 1999; Sadaka-Laulan *et al.*, 1998; Scheu & Simmerling, 2004).

During interspecific mycelial interactions, VOCs and DOCs often increase in quantity and quality when fungi are physically damaged, as, for example, when grazed (Faldt *et al.*, 1999; Hynes *et al.*, 2007; Stadler & Sterner, 1998; Woodward & Boddy, 2008). Gene expression, morphology and physiology are also altered, and nutrients are released (Baldrian, 2004; Donnelly & Boddy, 2001; Iakovlev *et al.*, 2004; Adomas *et al.*, 2006; Woodward & Boddy, 2008). There is limited circumstantial evidence that invertebrates are attracted to areas where different mycelia meet and interact (Boddy *et al.*, 1983). Interactions between basidiomycete fungi may also cause the release of nutrients often closely guarded by fungi (Boddy & Watkinson, 1995) and not freely available in soil (Bardgett 2005). Further, different fungal individuals/strains exhibit differences in physiology (Clausen *et al.*, 2000), and may therefore have differential effects on invertebrates.

This study: (i) investigates the response of collembola to basidiomycete mycelial interaction zones; (ii) tests the hypothesis that collembola exhibit consistent preferences for a given fungus during an interaction; and (iii) investigates the importance of genetic variability of fungi on invertebrate behaviour using several different isolates.

4.2 Materials and methods

4.2.1 Experimental design

Collembola culturing, experimental design and image recording are as described in Section 3.3. The location of each collembola was recorded according to the one of five possible areas of each interaction on which it was found (Fig 4.1). Recording started on the day of addition (t_0) and continued every 2 d until 14 d, followed by every 4 d until 26 d. The locations of dead collembola were recorded at the same time by marking positions on the lid of each dish. This permanent record of all collembola ensured that any individuals subsequently eaten by conspecifics were not discounted at future time points.

4.2.2 Recording collembola movement

Interacting mycelia were divided into a maximum five possible areas (Fig. 4.1; described in detail in Section 3.3). Image J 1.33u (National Institute of Health, USA) was used to determine the area of each of the five regions (as defined in Fig. 4.1) on grazed interaction plates at the time of addition (t_0), 2 d, 6 d, 10 d, 18 d and 26 d. Data were subsequently combined with the collembola counts to determine collembola density on the different areas of each dish.

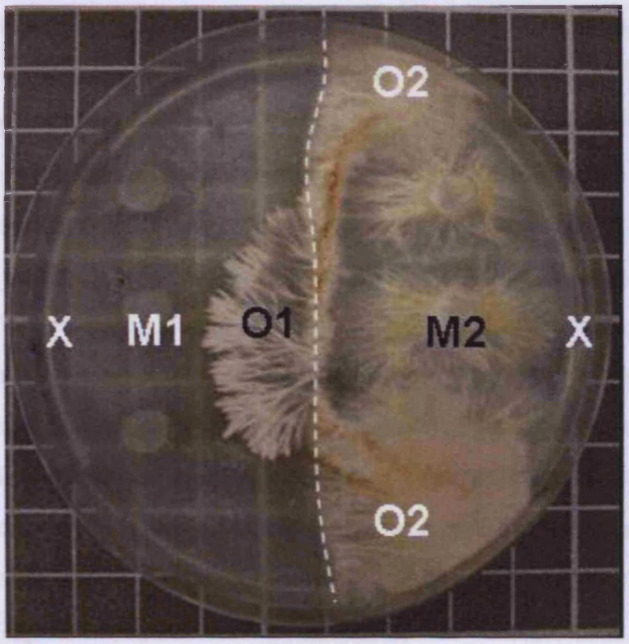


Fig 4.1: Repeated from Chapter 3 for clarity. Interaction of *Phanerochaete velutina* (growing left to right, white lettering) and *Hypholoma fasciculare* (black lettering) marked with the five possible areas of the interaction. M1/M2 = original mycelium, O1/O2 = overgrowth and the dashed line the original interaction zone. The original interaction zone was defined as being 2 mm either side of this line. X indicates points of collembola addition.

4.2.3 Statistical analysis

As collembola position was, in the majority of cases, confined to the initial mycelium of each fungus, with very few collembola being identified on the overgrowth or interaction areas, statistical comparisons were only carried out on the collembola densities on these former areas. Data were normalised using log transformation where applicable. Differences between the collembola density on the two mycelia in each

interaction both overall and over time, were tested using repeated measures Analysis of Variance (ANOVA) using SPSS 12 statistical software. In one instance, data could not be normalised and the non-parametric equivalent of repeated measures ANOVA, the Scheirer-Ray-Hare test, was employed (Minitab 14).

4.3 Results

4.3.1 Collembola preferences

Contrary to expectation, collembola were not attracted to the interaction zone. In half (18) of the 36 interactions, collembola showed significant preference (Table 4.1; treatment effect) for one mycelium over the other. This analysis, however, does not enable determination of whether collembola changed preference over time; exploring the time*treatment interaction provides this information (Table 4.1). There was a persistent significant preference over time by collembola for one of the fungal mycelia in 12 of the 36 interactions (Table 4.1). Nine of these 12 interactions included *H. fasciculare* (Table 4.1, Fig. 4.2 b, d, e) and in most of these it was the least preferred mycelium that harboured greater collembola mortality, although this was not always significant (e.g. Figs 4.2 b, d, e and 4.3 b, d, e). Although *H. fasciculare* was consistently the least preferred species, no species was consistently preferred above all others, irrespective of fungal opponent. There was generally low final total collembola mortality in these 'clear preference' interactions (e.g. *P. velutina* against *H. fasciculare* 1 Fig. 4.3 b, e).

4.3.2 Collembola preference switch during interactions

In some fungal interactions collembola showed switching behaviour, moving from one mycelium to the other during the experiment. On the basis of the results, this switching was defined to have occurred by a change after 2 d or more following collembola addition. Switching occurred in 11 interactions (Table 4.1) and involved all eight isolates (e.g. Fig. 4.2 c, f, h, j, k). When collembola switching occurred movement was toward the species on which, at the end of the experiment, most dead collembola were found (e.g. Fig. 4.3 j). An exception was during the interaction of *H. fasciculare* 3 against *R. bicolor* 2 when there was a switching away from *R. bicolor* 2 to *H. fasciculare* 3, but the greatest mortality occurred on *R. bicolor* (Fig. 4.2 f, 4.3 f). The greater mortality on the mycelium to which collembola switched was significant in all but two cases (e.g. Fig. 4.3 c). In *P. impudicus* against *R. bicolor* 1 (Fig 4.2. j),

collembola movement from *R. bicolor* 1 to *P. impudicus* occurred at 5 d but no death was recorded on *R. bicolor* for the duration of the experiment. During the same interaction, there were dead collembola from 6 d on *P. impudicus* with the number of dead individuals rising to an average of seven on the *P. impudicus* mycelium by 26 d (Fig. 4.3 j).

4.3.3 Collembola mortality

Collembola died on both mycelia during all interactions (Fig. 4.3) and in the majority of interactions collembola death was significantly greater on one mycelium than the other (Fig. 4.3). An exception was the interaction between *P. impudicus* and *R. bicolor* 1 where collembola only died on *P. impudicus* (Fig. 4.3 j). Generally, if one mycelium had higher initial mortality, there was a greater cumulative number of deaths on that mycelium throughout the interaction (e.g. Fig. 4.3 d, g but see 4.3 i).

4.3.4 The effect of fungal isolate

Except for *H. fasciculare* 2, collembola showed no significant mycelial preference on interactions between genetically identical isolates or in interactions between *R. bicolor* 1 against *H. fasciculare* 2, and *P. velutina* against *H. fasciculare* 2 (Table 4.1). Collembola preferred one mycelium over another in four (of seven) interactions between genetically different individuals of the same species (Table 4.1, Fig. 4.2 a, d). Collembola never switched preference during any conspecific interaction (Table 4.1). With interactions involving different isolates of a given species against the same third heterospecific, such as *H. fasciculare* 1 or *H. fasciculare* 2 against *P. velutina*, collembola generally followed similar patterns of movement behaviour irrespective of isolate. There were, however, some exceptions. For example, the general pattern in *R. bicolor* against *H. fasciculare* interactions was for collembola to switch from *R. bicolor* to *H. fasciculare* after 5-15 d (e.g. Fig. 4.2 c, k). In these cases, most deaths occurred on *H. fasciculare* (e.g. Fig. 4.3 c, k). In *H. fasciculare* 4 against *R. bicolor* 1, however, no switching occurred and the greatest collembola mortality was on *R. bicolor*, the most preferred species (Fig. 4.2 g, 4.3 g). Similarly with *P. velutina* and *R. bicolor* (Fig. 4.2 h, i), while there was no significant difference in final collembola mortality in *P. velutina* against *R. bicolor* 2 (Fig. 4.3 i), in *P. velutina* against *R. bicolor* 1, mortality was significantly greater on *R. bicolor* (Fig. 4.3 h). Collembola mortality on *P. velutina* was lower when interacting with *R. bicolor* 1 than when interacting with *R. bicolor* 2 (Fig. 4.3 h, i).

Table 4.1: Collembola preference during interactions.

	R. bicolor 2	*R. bicolor* 1	*P. velutina*	*P. impudicus*	*H. fasciculare* 4	*H. fasciculare* 3	*H. fasciculare* 2	*H. fasciculare* 1
H. fasciculare 1	**$F_{1,8}$= 16.166** M ($F_{5,40}$=13.587)	**$F_{1,8}$= 18.200** M ($F_{5,40}$=19.335)	**$F_{1,8}$= 372.393** P ($F_{5,40}$=24.503)	**$F_{1,8}$= 6.398** P ($F_{3,23}$=4.371)*	**$F_{1,8}$= 47.954** P ($F_{5,40}$=10.185)	$F_{1,8}$= 2.989 NS ($F_{5,40}$=1.657)	$F_{1,8}$= 1.303 NS ($F_{3,23}$=3.029)*	**$F_{1,8}$= 13.681** NS ($F_{2,16}$=2.685)*
H. fasciculare 2	$F_{1,8}$= 0.053 M ($F_{5,40}$=4.445)	**$F_{1,8}$= 6.156** NS ($F_{2,18}$=1.782)*	**$F_{1,8}$=23.271** NS ($F_{2,17}$=1.602)*	**$F_{1,8}$= 18.995** P ($F_{5,40}$=9.957)	$F_{1,8}$= 0.008 NS ($F_{5,40}$=1.041)	**$F_{1,8}$= 28.805** P ($F_{5,40}$=4.110)	$F_{1,8}$= 4.189 P ($F_{2,19}$=3.481)*	
H. fasciculare 3	**$F_{1,8}$= 7.340** M ($F_{3,22}$=28.017)*	$F_{1,16}$= 9.387 M ($F_{3,50}$=37.549)*	**($\chi^2_{1,48}$=0.999)** P ($\chi^2_{5,48}$=0.997)	**$F_{1,6}$= 32.875** P ($F_{2,14}$=6.308)*	**$F_{1,8}$= 30.732** P ($F_{5,40}$=4.198)	$F_{1,8}$= 0.603 NS ($F_{3,26}$=0.211)*		
H. fasciculare 4	$F_{1,8}$= 1.544 M ($F_{2,19}$=8.223)*	**$F_{1,18}$= 69.704** P ($F_{4,67}$=56.315)	**$F_{1,8}$= 86.777** P ($F_{4,38}$=19.524)*	**$F_{1,10}$= 9.206** M ($F_{5,50}$=34.521)	$F_{1,8}$= 1.272 NS ($F_{5,40}$=0.654)			
P. impudicus	$F_{1,6}$= 0.815 NS ($F_{2,12}$=1.986)	$F_{1,8}$=1.545 M ($F_{5,40}$=10.095)	$F_{1,8}$= 0.063 M ($F_{5,30}$=34.643)	$F_{1,6}$= 3.344 NS ($F_{5,30}$=1.123)				
P. velutina	$F_{1,8}$= 0.114 NS ($F_{2,17}$=1.908)*	$F_{1,8}$= 4.506 **M** ($F_{5,40}$=9.678)	$F_{1,8}$= 2.786 NS ($F_{5,40}$=2.297)					
R. bicolor 1	**$F_{1,8}$= 7.468** P ($F_{3,16}$=3.980)*	$F_{1,8}$= 3.295 NS ($F_{5,40}$=1.037)						
R. bicolor 2	$F_{1,8}$= 0.119 NS ($F_{2,15}$=1.223)*							

For each interaction (row vs. column) the top line gives the result of the overall ANOVA (treatment effect). Values in bold indicate a significant difference. The remaining two lines of each interaction refers to the repeated measures ANOVA time*treatment interaction. P and M indicate significance at $P \leq 0.05$; NS indicates no significance. **P** = persistent significant preference for mycelium listed in row heading over time. P = persistent significant preference for mycelium listed in left column over time. M indicates collembola switching preference from one mycelium to the other during the course of the interaction. M = collembola switched from mycelium listed in the row heading to the mycelium listed in the left column during the interaction; **M** = collembola switched from mycelium listed in left column to mycelium listed in the row heading. Where the interaction is between the two same isolates, the mycelium listed in the top row was on the right hand side when data were collected. * The degrees of freedom for these F-values result from the use of the Huynh-Feldt correction factor (rounded to the nearest whole number).

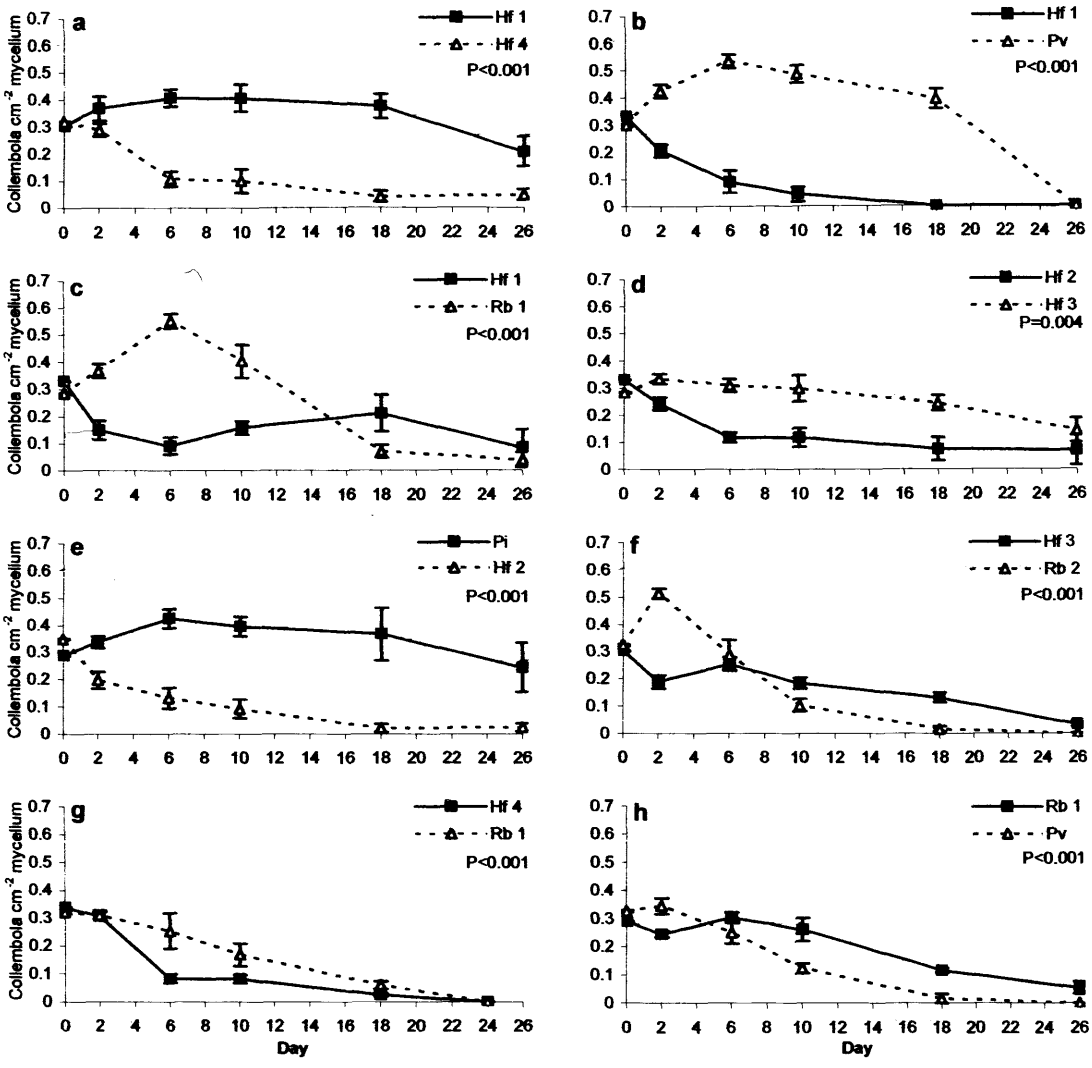


Fig 4.2 (i): Examples of collembola movement during the interactions. P-values are for repeated measures ANOVA time*treatment interaction, or where data violated assumptions, the Scheirer-Ray-Hare test time*treatment interaction. The P-value indicates whether there was a significant difference between collembola densities on the different mycelia over time. Hf 1-4 *Hypholoma fasciculare* isolates 1 to 4, Pi *Phallus impudicus*, Pv *Phanerochaete velutina*, Rb 1, 2 *Resinicium bicolor* isolates 1 and 2. (a) Hf 1 against Hf 4, (b) Hf 1 against Pv, (c) Hf 1 against Rb 1, (d) Hf 2 against Hf 3, (e) Pi against Hf 2, (f) Hf 3 against Rb 2, (g) Hf 4 against Rb 1, (h) Rb 1 against Pv. F-statistic and degrees of freedom are given in Table 4.1.

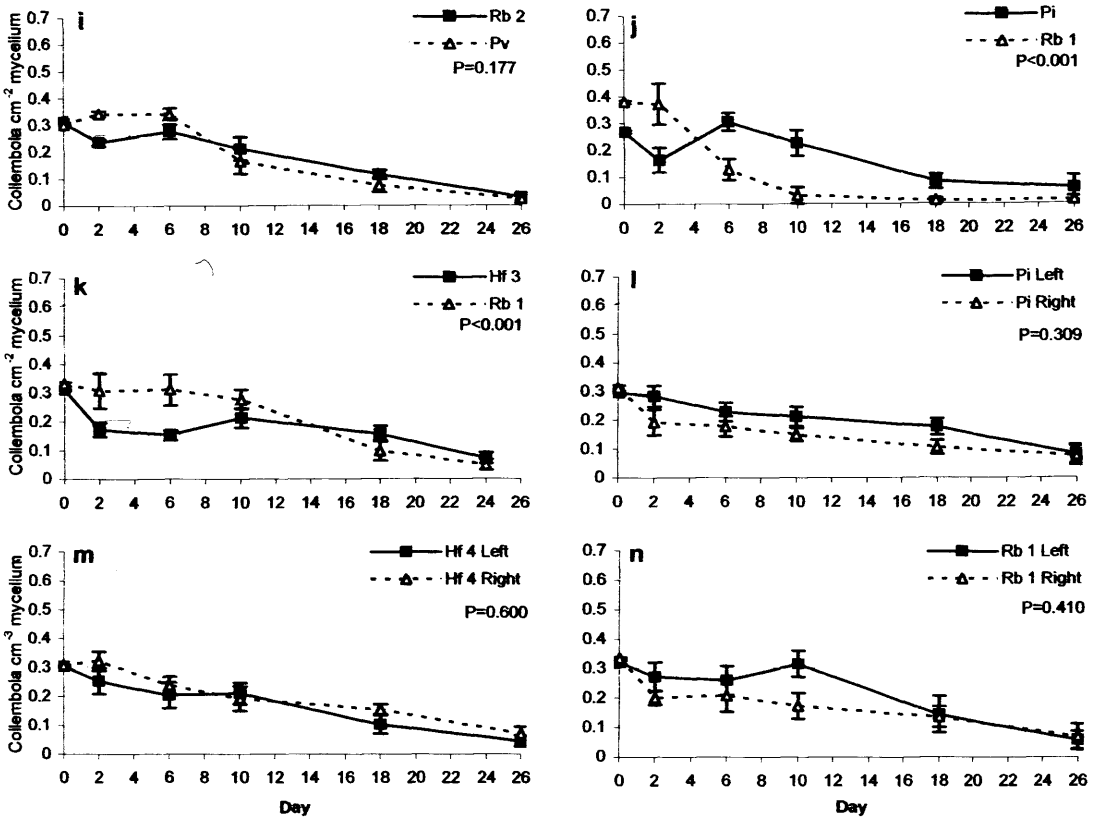


Fig 4.2 (ii): (i) Rb 2 against Pv, (j) Pi against Rb 1, (k) Hf 3 against Rb 1, (l) Pi against Pi, (m) Hf 4 against Hf 4, Rb1 against Rb 1.

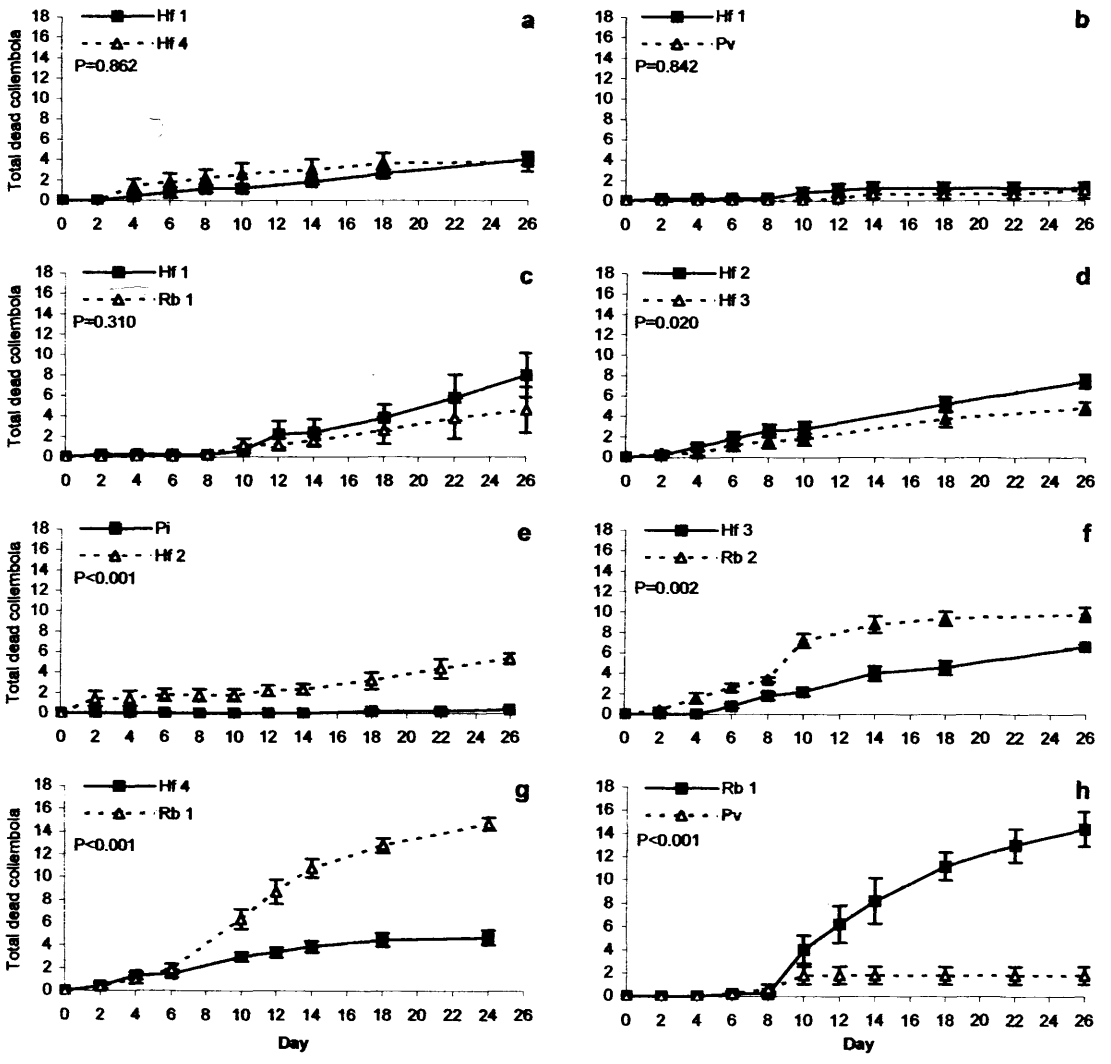


Fig 4.3 (i): Collembola death during interactions. The results are broad representation of the mortality seen on all interactions. P-values are for a one-way ANOVA on the final time point. Hf 1-4 *Hypholoma fasciculare* isolates 1 to 4, Pi *Phallus impdicus*, Pv *Phanerochaete velutina*, Rb 1 2 *Resinicium bicolor* isolates 1 and 2. (a) Hf 1 against Hf 4 ($F_{1,8}$=0.03), (b) Hf 1 against Pv ($F_{1,8}$=0.04), (c) Hf 1 against Rb 1 ($F_{1,8}$=1.17), (d) Hf 2 against Hf 3 ($F_{1,8}$=8.45), (e) Pi against Hf 2 ($F_{1,8}$= 78.13), (f) Hf 3 against Rb 2 ($F_{1,8}$=20.48), (g) Hf 4 against Rb 1 ($F_{1,8}$=144.69), (h) Rb 1 against Pv ($F_{1,8}$=56.70). Data represented in this figure correspond with the interactions listed in Fig. 2

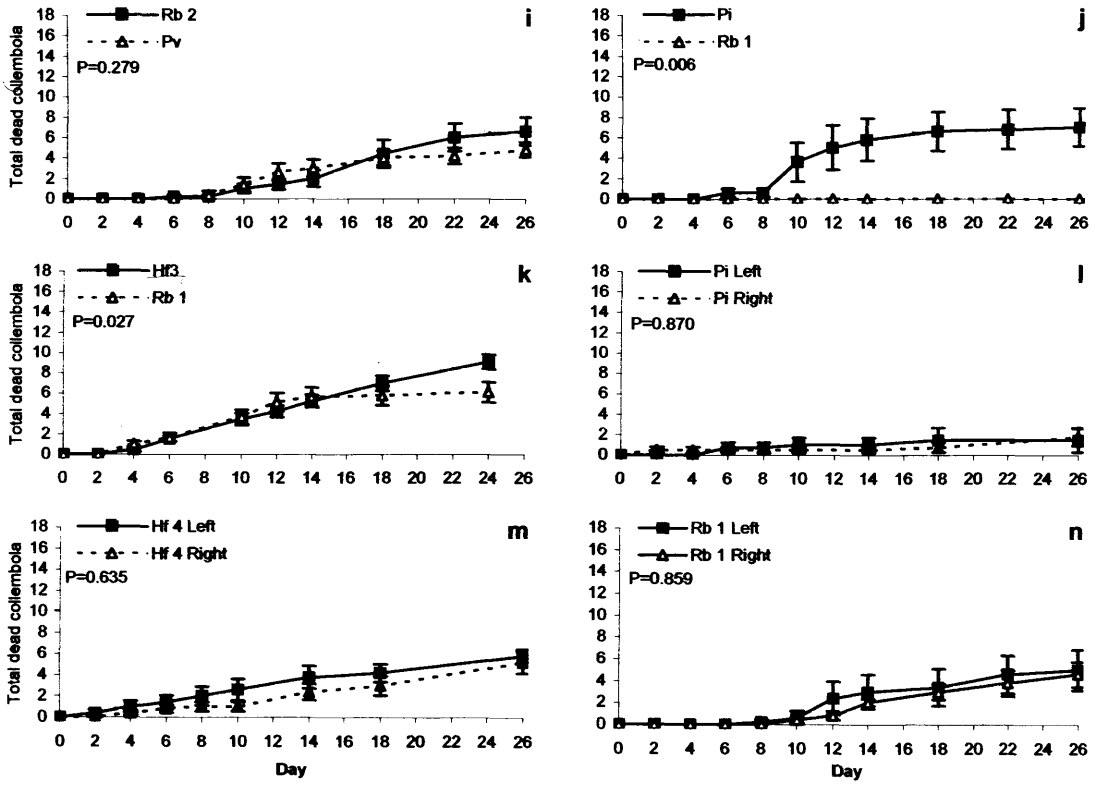


Fig 4.3 (ii): (i) Rb 2 against Pv ($F_{1,8}$=1.35), (j) Pi against Rb 1 ($F_{1,8}$= 14.0), (k) Hf 3 against Rb 1 ($F_{1,16}$= 5.90), (l) Pi against Pi ($F_{1,6}$= 0.03), (m) Hf 4 against Hf 4 ($F_{1,8}$= 0.24), (n) Rb 1 against Rb 1 ($F_{1,8}$= 0.03).

4.4 Discussion

4.4.1 Collembola response to the interaction zone

There was no evidence of collembola attraction to the interaction zone in this study although there is evidence that some invertebrates (fungus gnats *Bradysia* sp.) are attracted to, and burrow within, fungal interaction zones (Boddy *et al.*, 1983). Leakage from interacting hyphae also makes these regions likely to be nutrient rich (Wells & Boddy, 2002).

4.4.2 Collembola preferences

Collembola showed no consistent preference for one isolate during all interspecific interactions; switching from one mycelium to the other occurred in 11 interactions while a clear preference was shown in another 12 (Table 4.1). In these latter interactions, the mycelium chosen was that on which the final collembola mortality was lowest, suggesting a causal link between survivorship and resource choice. Aside from *H. fasciculare*, which was consistently the least preferred species, collembola exhibited no hierarchy of preference. A possible explanation for the avoidance of *H. fasciculare* is found in a soil microcosm study where, when growing alone, *H. fasciculare* was grazed at the mycelial margin, but as mycelia filled the microcosms, margins with relatively palatable hyphae may not have been available (Tordoff *et al.*, 2006). In the system used here there was very limited mycelial margin and possibly, therefore, restricted palatable hyphae. As a consequence of its low palatability, fecundity is likely to be low on *H. fasciculare*, although as a stress response to a suboptimal food source, egg-laying may be increased (Noel *et al.*, 2006).

Collembola preference is probably controlled by a balance between several factors. When growing alone, for example, the fine hyphae of *P. velutina* may be palatable to collembola, but the extracellular chemicals produced to attack a competing fungus (Baldrian, 2004; Xu *et al.*, 2004) may have made it unpalatable. Collembola choice is likely to be a compromise between such factors, and with different interactions the 'optimality' of a given mycelium may alter. Where the choice was limited, collembola may have chosen between two poor options neither of which would be selected in field conditions.

4.4.3 Switches in collembola preference

The pattern of switching behaviour by collembola from one mycelium to the other appeared counter-intuitive, with collembola consistently moving toward the mycelium with the highest mortality by the end of the experiment (Figs. 4.2, 4.3). The lack of complete removal of the initial mycelium indicates that the move was not a consequence of resource depletion. Initial grazing may, however, have removed fine hyphae leaving only thicker, less palatable hyphae and melanised cords (Harold *et al.*, 2005; Kampichler *et al.*, 2004). Changes in fungal gene expression, subsequent physiology (Iakovlev *et al.*, 2004) and secondary metabolite synthesis (Hynes *et al.*, 2007) through fungal colony ageing or aggressive fungal interaction may have made an initially palatable mycelium unpalatable. Equally, the initially most preferred mycelium may have produced volatile (VOC) or diffusible (DOC) organic compounds which attracted the collembola to that mycelium and/or the least preferred mycelium may have produced VOCs and DOCs that repelled collembola. Nitrogen is commonly limiting in the natural environment and collembola provide a nutrient-rich resource (Klironomos & Hart, 2001). The ability to attract nitrogen-rich collembola to their death may hold significant advantages during fungal combat, driving a selection pressure for this fungal behaviour. Collembola preference is not static; a mycelium initially preferred by collembola in one interaction was sometimes initially avoided when interacting with another species. This may be due to altered fungal physiology and chemistry with different interactions.

There was greater collembola survival in interactions in which a sustained clear preference was observed. Variable hypotheses are plausible to account for the ultimate higher mortality observed in interactions in which collembola switched preferences. Firstly, collembola may have 'misinterpreted' cues produced by the fungi and moved towards an attractive / away from an unattractive mycelium inadvertently to a poorer or even lethal resource. Secondly, fungi may have attracted collembola to their deaths but in some cases this attraction was weaker than the attraction of mycelium on which collembola were already feeding, hence switching did not occur in all interactions with a given isolate. Thirdly, depletion of edible resource in the mycelium may have forced collembola off onto the other.

Although misinterpretation of cues by collembola has been reported, this was in a mutant form of *A. funigatus* making it more palatable to collembola, suggesting that the mutation had altered the palatability but not the cues used by collembola (Scheu & Simmerling, 2004). In addition, misinterpretation of cues by collembola would be unlikely to yield such a consistent trend of movement toward 'greater mortality' across replicates. In relation to the third hypothesis, there was no visible depletion of visible resource and since the switch (in, for example, *H. fasciculare* 1 against *R. bicolor* 1; Figs 4.2 c, 4.3 c) sometimes occurred (16 d) when collembola death was already higher (from 12 d), increased mortality on that mycelium is unlikely to have resulted simply from an increased population size. This reduces the support for the third hypothesis. As increased mortality on the finally preferred mycelium is a consequence of dispersal from a diminishing resource, and the switching behaviour towards the fungus with final highest mortality was consistent, the second hypothesis therefore, seems more likely.

4.4.4 Collembola mortality

Collembola died on all interactions, but more on some than others (Fig. 4.3). Although some may be attributable to natural (age-dependent) mortality it is more likely due to the fungi as mortality on interactions was far higher than that observed in soil trays and laboratory culture (pers. obs.). In some interactions mortality was so high that, if typical, long term population survival was unlikely. This indicated that the food quality was, in some instances, low. The presence of different mycelia may allow collembola to gain essential nutrients by mixing their diet. Naturally, such interactions occur within a diverse biotic environment in which other fungi and bacteria are present. The basidiomycete defence mechanisms against invertebrate grazing include specialised killing stuctures, for example, toxic droplets from secretory mycelial appendages (Hutchison *et al.*, 1996) and defensive cystidia (Nakamori & Suzuki, 2007). It may be that the fungi in this study also have hyphae capable of trapping or killing collembola.

Genetically different isolates of the same fungal species generally resulted in similar behavioural responses. Occasionally, however, collembola showed distinct preferences for one isolate over another. In particular, interactions of *H. fasciculare* with *R. bicolor* demonstrate how different isolates can substantially alter collembola

behaviour. Many studies have used a single isolate of one fungal species to study invertebrate preference or behaviour (Hedlund *et al.*, 1991; Kampichler *et al.*, 2004; Kaneda & Kaneko, 2004); the present study implies that these should be interpreted with caution.

4.4.5 Conclusions

In conclusion, collembola showed preferences in all interactions although fungal preference did change dependent on the interacting fungal species. Collembola behaviour on different isolates of the same fungal species was generally, but not always, consistent. Collembola mortality was seen on all interactions and was generally lowest on those interactions where collembola exhibited a consistent preference for one mycelium. The study raises questions as to what cues, detectable by collembola, are produced by interacting fungi. Future studies should explore collembola sensory acuity in differentiating between fungi. This would enable determination of whether the sub-optimal choices made by collembola are a result of a lack of ability to differentiate between signals or even to detect them at all.

5.0 Collembola movement responses to fungal volatile odours

5.1 Introduction

Invertebrate population survival and growth depends on an organism's ability to locate and utilise essential resources. As resources are normally patchily distributed within a habitat, effective searching strategies are required to maximise efficiency (Godfray, 1994). Visual and chemical cues, indicating the location of possible resources, affect invertebrate behaviour (Dicke, 1994). Most studies on invertebrate responses to volatile chemicals have been conducted on above-ground organisms. For example, plant odours are exploited by insect herbivores which follow gradients of increasing volatile chemical concentration until they arrive at the host (Erickson & Feeny, 1974; Godfray, 1994). Furthermore, plant insect herbivores are often located by predators such as parasitoids through a combination of both plant and herbivore odours (Godfray, 1994).

Fungi are also known to emit volatile chemicals attractive to invertebrates. For example, parasitoids which parasitise *Drosophila* sp. found in fungal-dominated microhabitats are attracted by the habitat odours (Dicke *et al.*, 1984). In another study, the volatile chemicals emitted from cut sections of fruit bodies of the wood-decay fungi *Fomitopsis pinicola* and *Fomes fomentarius* were attractive to a range of beetles (Faldt *et al.*, 1999). Such exploitation of fungal odours can be highly specific. The bracket fruit bodies of the basidiomycete *Trametes versicolor*, for example, play host to two species of ciid beetle, *Ocetotemnus glabriculus* and *Cis boleti*. *O. glabriculus* is found in young fruit bodies, whereas *C. boleti* inhabits older brackets. These two beetle species effectively partition the fungal resource by using odour cues from the fruit bodies to determine the age of the fruit body and therefore whether it is a suitable habitat (Guevara *et al.*, 2000a).

Studies investigating the responses of below-ground invertebrates to volatile chemical cues are less abundant than for above-ground fauna. Those studies on volatiles emitted by plants below-ground have been largely restricted to two main themes: invertebrate responses to plant roots and nematode foraging behaviour. A wide range of soil root-feeders are attracted to plant root chemicals with the predominant

attractant being CO_2 (Johnson & Gregory, 2006). For example, the larvae of the root feeding clover weevil (*Sitona lepidus*) use CO_2 to orientate towards the roots of white clover (Johnson *et al.*, 2006), and a number of other generally low molecular weight root exudates are known to be attractant to root herbivores (Johnson & Gregory, 2006). As CO_2 is emitted by most soil organisms, plant roots, fungi and bacteria, as well as other invertebrates, it is a general cue although one to which invertebrates are highly sensitive (Johnson & Gregory, 2006). Secondary metabolites, on the other hand, are more specific to a given plant, or range of plant species and therefore may be exploited by highly selective feeders. For example, western corn rootworm (*Diabrotica virgifera virgifera*) larvae feed on maize (*Zea mays*) and a variety of other grasses. The volatile chemical 6-methoxy-2-benzoxazolinone is emitted from maize roots and some grasses and, along with CO_2, is used by the rootworm larvae to locate the roots (Bjostad & Hibbard, 1992). A similar example of attraction to a specific chemical has been seen in the entomopathogenic nematode *Heterorhabditis megidis*, a predator of the western corn rootworm. When attacked by the rootworm, maize plants emit a sesquiterpene (E)-β-carophyllene) attracting the nematode (Rasmann *et al.*, 2005). The recruitment of root herbivore predators mediated through plant exudates (van Tol *et al.*, 2001; Rasmann *et al.*, 2005) is highly analogous to above-ground systems (Dicke, 1994).

Aside from plant-insect interactions, there has been limited study on the attraction of soil invertebrates to other soil biota. The majority of plant root studies have eliminated much, if not all, of the soil biota (Johnson & Gregory, 2006), and yet soil organisms emit a wide variety of compounds some of which are known to trigger interspecific responses. For example, *Penicillium* and *Trichoderma* fungal species produce a range of fungistatic compounds which inhibit fungal growth (Humphris *et al.*, 2002; Kettering *et al.*, 2004). In addition, fungi also emit volatiles which attract invertebrates; termites from the genus *Reticulitermes* sp. excavate runways directly to decaying wood possibly through following a gradient of attractive chemicals (Anderson *et al.*, 1984). Collembola, a highly abundant soil invertebrate group (Hopkin, 1997), discriminate between fungal mycelial odours indicating preferences for one fungus over another (Hedlund *et al.*, 1995). In addition, circumstantial evidence of invertebrate attraction to fungal volatiles has been seen in fungus gnats (Boddy *et al.*, 1983).

Soil invertebrates must not only detect resource cues but also differentiate between cues from different resources if they are to be of functional significance. The existence of invertebrate preferences (Hedlund *et al.*, 1995; Jørgensen *et al.*, 2003; Tiunov & Scheu, 2005) indicates discrimination between resources and this may extend to exploitation of volatile or diffusible chemicals as seen with fungal fruit bodies above ground (see above).

Decomposition processes in temperate woodland are driven by wood-decay fungi (Rayner & Boddy, 1988) and aggressive interactions between them may serve to maintain their diversity. During aggressive interactions, volatile organic chemicals unique to the interaction are emitted by basidiomycete fungi (Hynes *et al.*, 2007). In woodland habitats where basidiomycete fungi and their associated interactions abound, the invertebrate mesofauna community tends to be dominated by mites and collembola (Bardgett 2005). Many collembola species are predominantly mycophagous (Hopkin, 1997) and can alter fungal morphology, foraging strategy and competitive interaction bias by their grazing activity (e.g Hedlund *et al.*, 1991; Kampichler *et al.*, 2004, Newell, 1984a, b). As is believed to occur with other mycophagous invertebrates (Boddy, 1983), collembola may exploit chemicals emitted by fungi and use them as cues to indicate the presence and nature of the potential resource. Conversely, some fungi emit chitinases (Lindahl & Finlay, 2006), which are potentially lethal to invertebrates covered in a chitinous exoskeleton. It would be of adaptive advantage to detect such chitinase synthesising fungi from a safe distance. As interactions of saprotrophic basidiomycetes lead to increased volatile compound emissions, fungal interactions form a useful system for exploring whether fungal volatile chemicals affect mycophagous soil invertebrate behaviour.

In this chapter the effects of fungal interaction volatiles alone on soil invertebrate behaviour are explored. In particular, this study aims to determine the response of *Folsomia candida* (Collembola) to volatiles emanating from interacting fungi. It is hypothesised that: (i) collembola will spend more time over attractive mycelial areas; (ii) search behaviour will increase at attractive sites; and (iii) collembola velocity will be greater at sites of low attraction.

5.2 Materials and Methods

5.2.1 Membrane frame construction

Four lengths of 25 x 2 x 2 cm kiln-dried timber, heat-sealed in strips of autoclavable biohazard bag to prevent wood volatile emissions, were attached using 40 mm panel pins to construct a square frame. A spun-bonded polypropylene membrane (Agralan Ltd, UK), chosen for low infra-red (IR) reflectance (allowing a clear contrast between collembola and membrane under experimental conditions), high volatile permeability but not inhibitory to collembola locomotion, was drawn over the wooden frame and stapled to the frame. Three membrane frames were assembled.

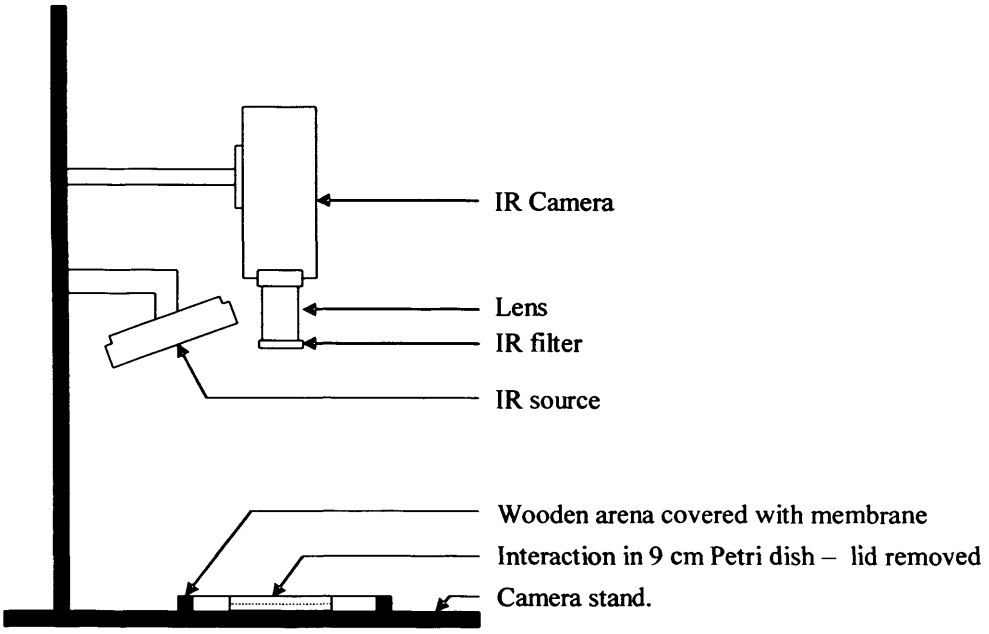
5.2.2 Fungal interactions and collembola

Fungal interactions of *Phallus impudicus* against *Resinicium bicolor* 2, *Hypholoma fasciculare* 1 against *R. bicolor* 1, and *H. fasciculare* 1 against *R. bicolor* 2 (with *P. impudicus* against *P. impudicus*, *H. fasciculare* 1 against *H. fasciculare* 1, *R. bicolor* 1 against *R. bicolor* 1, *R. bicolor* 2 against *R. bicolor* 2 and blank agar as controls), were set up and grown as described in Chapter 3. Fungal morphology during interactions was similar to that in these previous experiments. Opposing mycelia of fungal interactions had been in contact for 10 d before commencing experiments. Collembola were cultured and size selected as described in Chapter 3.

5.2.3 Experimental design

Experiments were conducted in a Sony Fitotron Constant Environment (CE) Chamber at 20°C, 95% RH and in darkness. A camera (Sanyo IR CCD, Sanyo Electric Co. Ltd, Japan), with an infra red (IR) sensitive lens (Computar H6Z0812 8-48 mm 1:1:2, CBC Co. Ltd, Japan) and filter was fixed 41 cm above the base of the camera stand on which experiments were carried out. An IR light source (Tracksys Ltd, UK) was attached below the camera (ca. 5 cm) and angled at ca. 75° from the vertical to obtain optimal, even lighting across the experimental area (see Fig. 5.1). The camera and IR equipment were connected to a PC via a port through the side of the CE chamber bunged to prevent air movement and light ingress. An agar plate, containing an ongoing interaction and with lid removed, was placed beneath the camera in the CE chamber. Different areas of the arena were defined into four 'zones' using Ethovision 3.1 software (Noldus Information Technology, Netherlands; Fig. 5.2). The zones were: outside of the Petri dish, the two separate mycelia and the interaction

zone (defined as ca. 1 cm either side of the central interaction line where morphological changes marking the interaction zone commenced). In the control self-self interactions, such as *R. bicolor* 1 against *R. bicolor* 1, one mycelium was labelled 'Rb top' and the other 'Rb bottom' based on the abbreviated fungal name (Rb, *R. bicolor*, Pi, *P. impudicus*, Hf, *H. fascicualre*) and their orientation as seen on the monitor before data recording commenced. This permitted distinction between zones during data analysis. The four zones were used to define collembola location during the tracking experiments (see below). The blank agar treatment was divided into two zones: within/outside the agar plate. To determine the speed of movement, a ruler was placed in the CE chamber next to the Petri dish and used as a scale in the Ethovision program.



Not to Scale

Fig. 5.1: Schematic representation of the side view of the experimental set up inside the constant environment (CE) chamber.

5.2.4 Experimental conduct

Before commencing a run of experiments the CE chamber was switched off to minimise air movement during the interaction. A membrane frame was carefully placed over the Petri dish so that the frame was at least 2 cm from the Petri dish edge. The chamber was closed for 10 min to allow permeation of fungal volatiles into the

membrane fabric. With minimal disturbance one collembola, which had been starved for 24 h, was added from a 1 cm diameter glass vial, and placed on the membrane frame directly above one of the two addition zones (Fig. 5.2). Under IR light, each collembola was illuminated against the membrane and for 10 min was picked up and tracked as an x, y coordinate by the Ethovision software. If the collembola left the arena for two consecutive minutes, or stopped for five consecutive minutes, the trial was terminated.

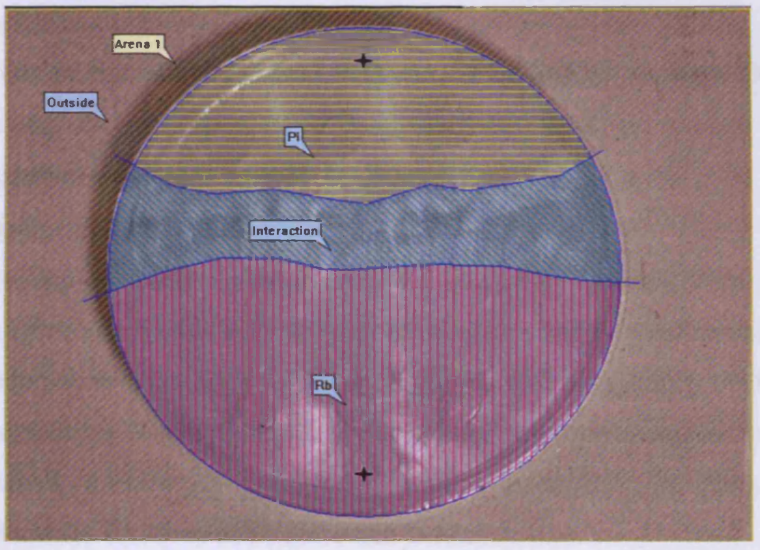


Fig. 5.2: Collembola tracking arena layout. Each coloured zone was assigned a name; Pi = *Phallus impudicus* original mycelium; Rb = *Resinicium bicolor* original mycelium; Interaction = interaction zone; Outside = zone outside of the Petri dish (diameter 9 cm). 'Arena 1' defined the entire arena area to be videoed. ✦ Point of collembola addition

Using the scale input at the start of the experiment, the software calculated speed of movement as well as changes in the direction of collembola movement (recorded as an angle change). At the end of the 10 minutes, the collembola and membrane frame were removed, a fresh membrane frame added and the CE chamber door closed for volatile permeation. Each interaction plate was run five times with collembola addition being alternated between the two points (Fig. 5.2). At the end of five runs the interaction plate was rotated through 90° to prevent bias from any extraneous sources such as materials present in the experimental setup. A naïve collembola was

used for each track and 20 tracks were recorded for each interaction plate with collembola added 10 times to each addition point in total (Fig. 5.2). To account for changes in fungal interactions over time, the plates over which collembola were tracked were interspersed; for example five tracks of *P. impudicus* against R. *bicolor* 2, followed by five tracks of R. *bicolor* 2 against R. *bicolor* 2, followed by five tracks of *P. impudicus* against *P. impudicus*. Blank agar controls were rapidly colonised by airborne organisms and were, therefore, replaced daily.

5.2.5 Data analysis

Using Ethovision, the following parameters were determined and recorded for each collembola run:

- Total time (s) spent in each of the four zones.
- Velocity (cm s^{-1}) within each zone.
- Mean angle of each turn made in a zone. A turn is described as the angle through which the collembola passed during the time from when the collembola left a straight line trajectory and turned consistently in clockwise or anticlockwise direction to the point when a straight line trajectory was resumed, or a turn on the opposite direction commenced. The mean angle for each turn is the mean of all these turn values.
- Mean angular velocity; the mean speed at which each collembola passed through each turn (as defined above) providing an indication of turn tightness.
- The sum of the total number of degrees through which a collembola passed within each zone.
- Relative time in zone; this was the time in each zone divided by the area of each zone. Zone areas were calculated from images saved in Ethovision using Image J 1.33 (National Institute of Health USA; as described in Chapter 4).
- Relative turn angle; calculated as the total absolute turn angle in each zone divided by the time spent by the collembola in each zone. This was calculated for each replicate.

5.2.6 Statistical analysis

Collembola movement as measured in each parameter (defined above) was compared within each interaction. Data for each parameter were transformed on a ladder of

powers (log_{10}, square, cube) and these, including the raw data, were analysed for kurtosis and skewness (SPSS 14). Data sets where values for kurtosis and skewness fell within ±1 were used in a univariate General Linear Model (GLM) with Tukey's honest significant difference post-hoc test (all using SPSS 14). Data violating the assumptions of the GLM were analysed using a Kruskall-Wallis test.

5.2.7 Point of addition data and qualitative track analysis

As collembola were added to two different areas on each interaction, it was possible that point of addition could bias the results. For example, collembola may have remained where they were added and results would misleadingly indicate collembola preference for the original mycelia. To determine point of addition biases, a one-way ANOVA was performed on data for time spent in zone for each mycelium and for each point of addition. For example, in the interaction between *H. fasciculare* 1 and *R. bicolor* 1, an ANOVA was performed between time spent over *H. fasciculare* mycelium and time spent over *R. bicolor* mycelium when the collembola had been added to *H. fasciculare*. This was repeated with data when collembola had been added to *R. bicolor*. The same process was repeated for relative turn angle data. This analysis was performed on *H. fasciculare* 1 against *R. bicolor* 1, *P. impudicus* against *R. bicolor* 2 and *R. bicolor* 2 against *R. bicolor* 2.

In addition to the quantitative analysis of collembola behaviour listed above, the recorded tracks were also examined quantitatively for specific behaviour patterns, such as increased turning at plate edges.

5.3 Results

5.3.1 Collembola movement behaviour

In general collembola did not display significant preferences for one mycelial region over another (Fig. 5.3). Collembola did, however, spend significantly more time (when corrected for area covered) within the boundary of the agar plate in the interaction of *R. bicolor* 2 against *P. impudicus* (Fig. 5.3 b). This did not occur in any of the other five interactions but was seen in the blank agar controls (Fig. 5.3 h). Collembola velocity, mean turn angle and angular velocity were not significantly different in any experimental or control treatment (e.g. Fig. 5.4 a, b).

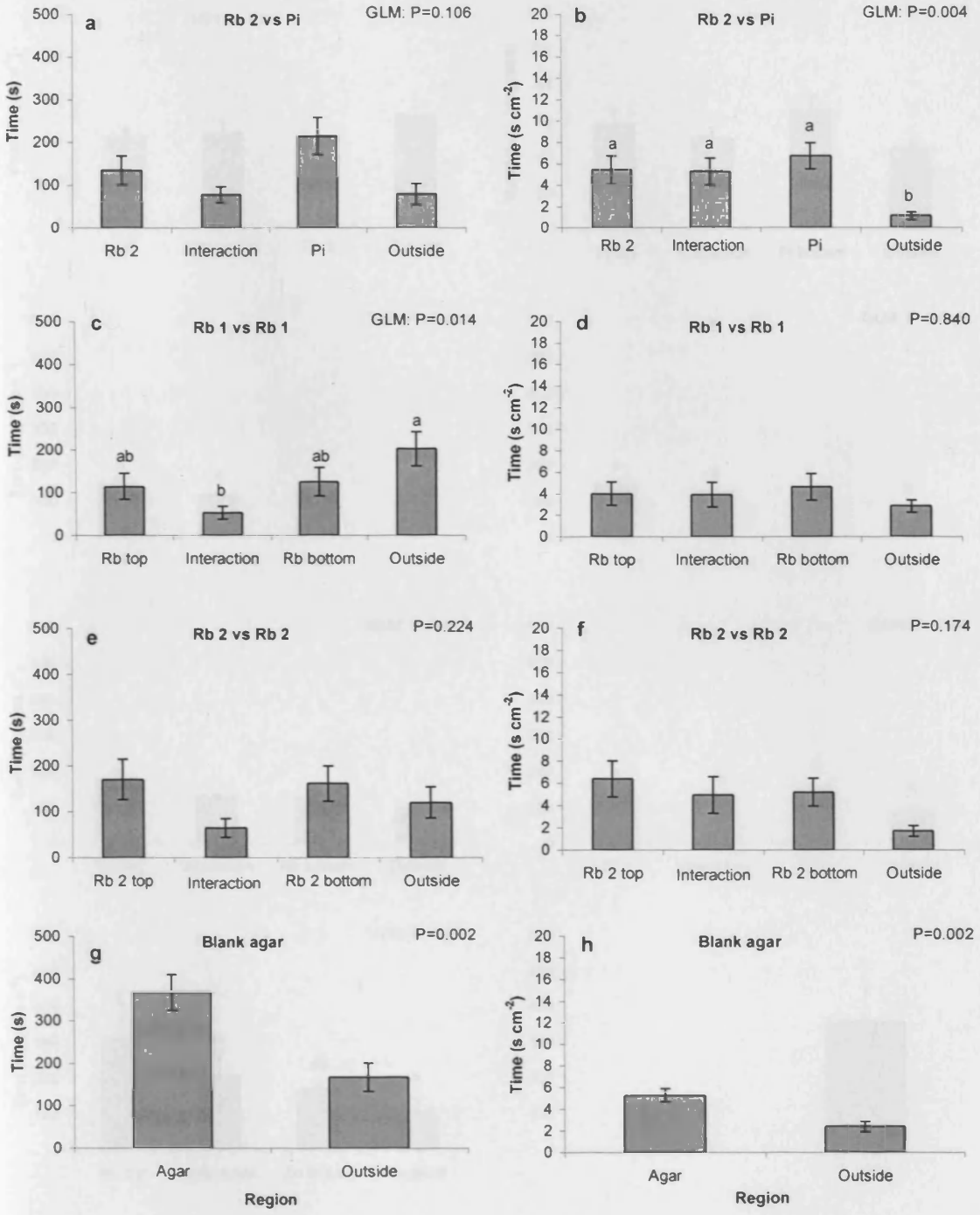


Fig. 5.3: Time (left column) and relative time (right column) spent in each zone by collembola. P-values are Kruskall-Wallis tests unless preceded by 'GLM' in which case they are one way general linear model with data transformed as necessary. Critical values: (a) $F_{3,76}$=2.113, (b) $F_{3,76}$=4.745, (c) $F_{3,80}$=3.779, (d) H_3=0.84, (e) H_3=4.37, (f) H_3=4.97, (g) H_1=9.66, (h) H_1=9.66

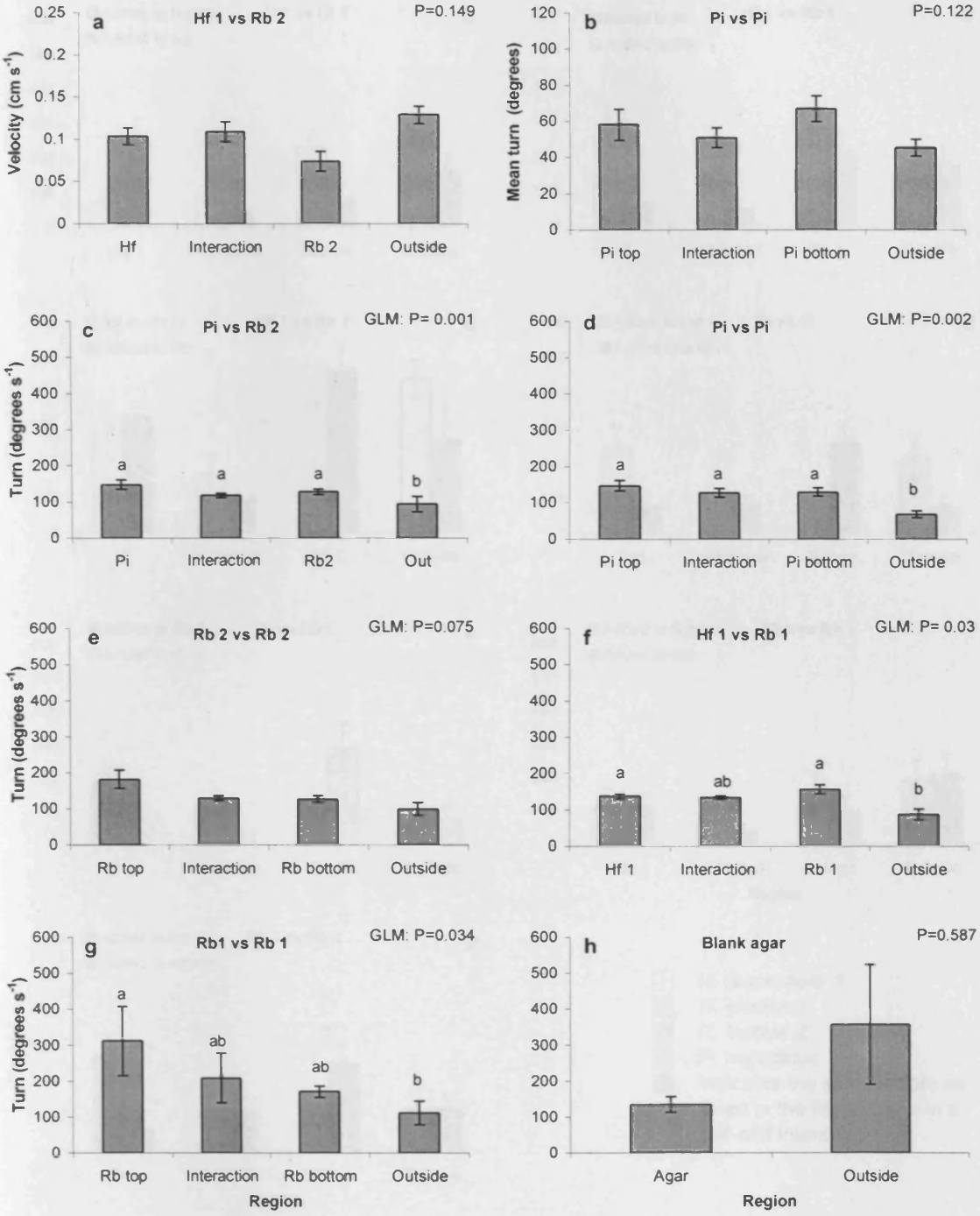


Fig. 5.4: Collembola velocity (a), mean turn (b) and total turn per second in region (c-h). P-values are Kruskall-Wallis unless preceded by GLM. Critical values: a H_3=5.34, b H_3=5.79, (c) $F_{3,56}$=6.74, (d) $F_{3,53}$=5.868, (e) $F_{3,80}$=2.387, (f) $F_{3,52}$=5.337, (g) $F_{3,48}$=3.316, (h) H_1=0.29.

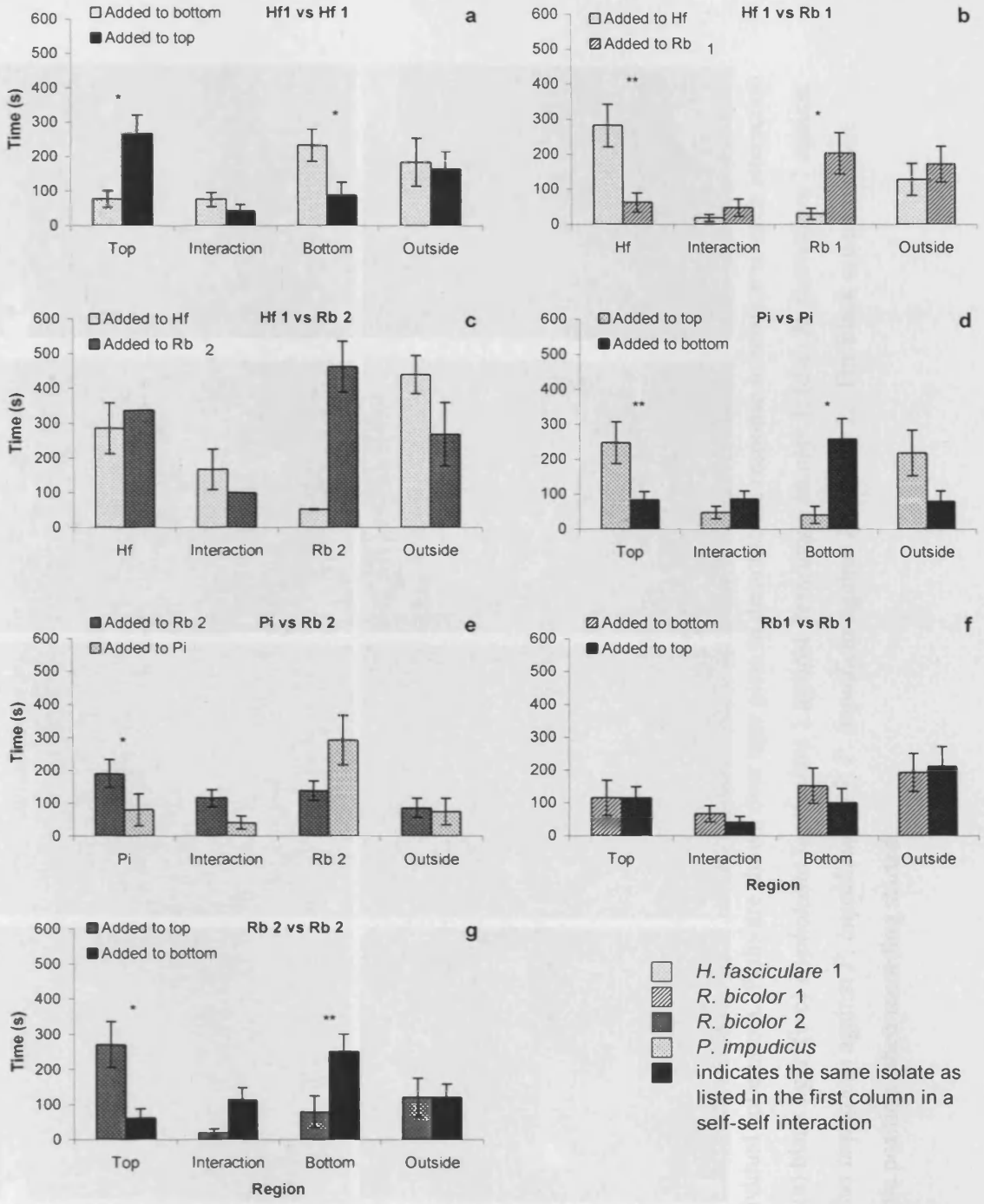


Fig. 5.5: Point of addition data for time in zone. Asterisks indicate significant difference in time spent on zone when added to the different mycelia (Kruskall-Wallis Test * $P<0.05$, ** $P<0.01$, *** $P<0.001$). Analysis was not performed on *H. fasciculare* against *R. bicolor*1 due to insufficient replicates. Critical and P-values reported as first and third column pair comparisons, respectively; (a) $H_1=5.45$, $P=0.02$, $H_1=5.22$, $P=0.022$; (b) $H_1=11.36$, $P=0.001$, $H_1=5.22$ $P=0.022$; (d) $H_1=9.87$ $P=0.002$, $H_1=6.27$ $P=0.012$; (e) $H_1=4.03$ $P=0.049$, $H_10.82$, $P=0.364$; (f) $H_1=0.33$ $P=0.566$, $H_1=1.07$ $P=0.301$; (g) H_1-$=5.561$ $P=0.002$, $H_1=8.31$ $P=0.004$.

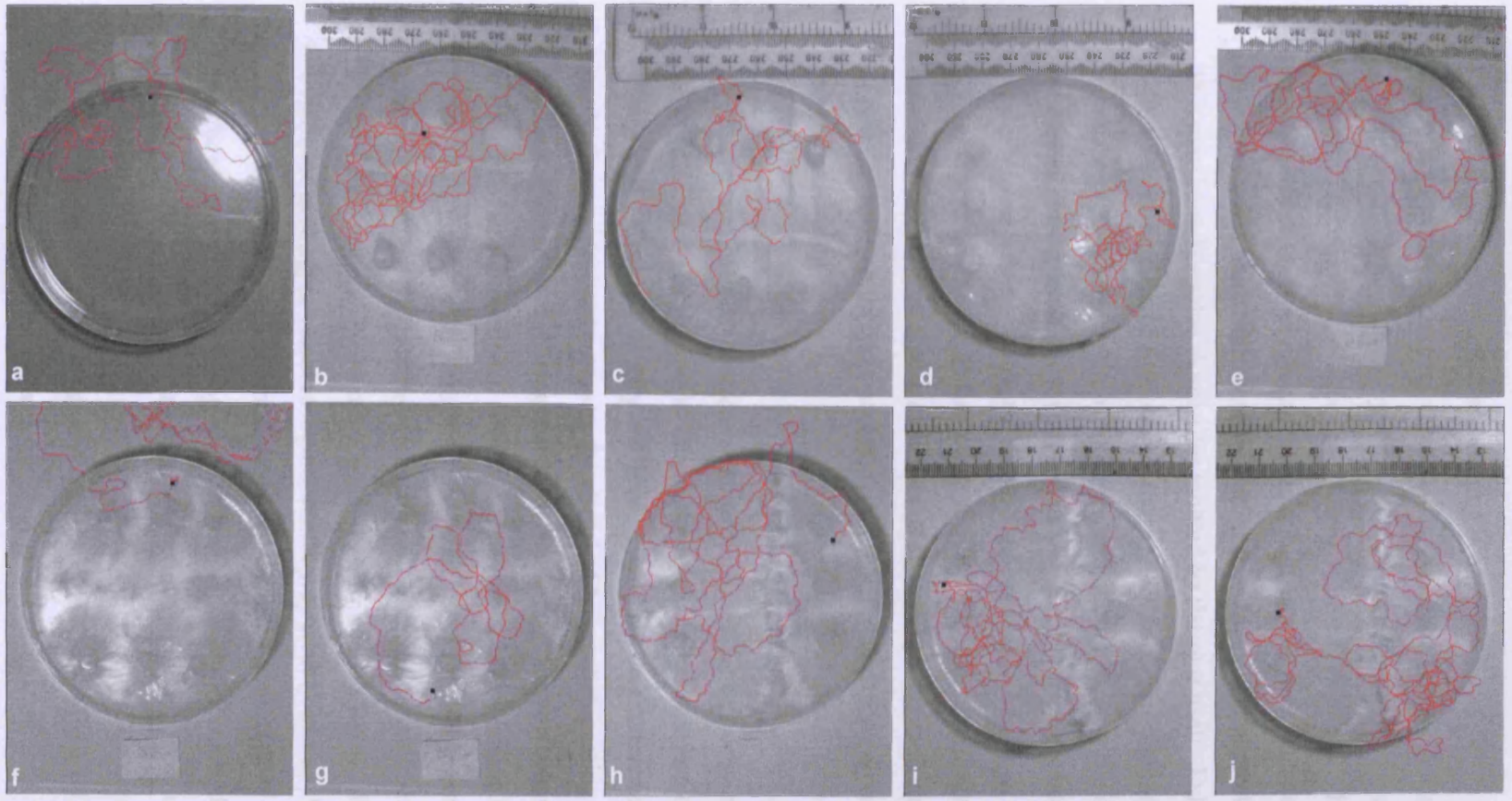


Fig 5.6: Track paths of individual collembola. Paths are shown over agar plate to demonstrate response in relation to each interaction (Petri dish 9 cm diameter). (a) blank agar; (b-c) *Hypholoma fasciculare* 1 against *Resinicium bicolor* 1; (d-e) *H. fasciculare* 1 against *H. fasciculare* 1; (f-g) *Phallus impudicus* against *P. impudicus*; (h-j) *P. impudicus* against *R. bicolor* 2. The black square on each track indicates the collembola position when recording started.

5.4 Discussion

5.4.1 Collembola movement and turning behaviour

Collembola exhibited no preference for the interaction zones in any of the fungal interactions investigated. They did, however, spend increased time over the entire Petri dish in the interaction of *P. impudicus* against *R. bicolor* 2. This suggests possible attraction to, or arrestment on, the interacting fungi. Collembola did, however, spend more time over the Petri dish in blank agar control than outside of it. This may have been due to volatiles emitted by the agar itself. Enhanced turning behaviour was exhibited by collembola on the *P. impudicus* against *R. bicolor* 2 interaction (no difference on blank agar), and qualitative analysis of collembola movement tracks indicated an edge effect, with collembola repeatedly turning at the plate edge keeping them directly above the plate (Fig. 5.5 h-j). As collembola behaviour on the *P. impudicus* against *R. bicolor* 2 interaction was significantly different from random across a number of different parameters, and was independent of point of collembola addition, behavioural differences are unlikely to be simply an experimental artefact.

The interaction between *R. bicolor* 2 and *P. impudicus* was originally selected as collembola have been shown to burrow along the interaction line of the two fungi (Chapter 3). Collembola may not have shown such clear attraction/arrestment in this particular experimental set-up as the chemical bouquet emanating may have been insufficient on its own to trigger such site-specific attraction behaviour. Such a hierarchy of stimulus-elicited response is well established among invertebrates. Parasitoids, for example, often use one set of cues to locate a resource habitat and then search for the discrete resource using other detection methods such as searching for diffusible organic chemicals (DOCs, e.g. Guillot & Vinson, 1972), active ovipositor probing or visual recognition of a prey item (Vinson, 1976). An empirical explanation for the lack of a preference for the interaction zone is that the membrane may have caused VOC mixing to occur before the stimuli reached the vicinity of the collembola, rendering different zones of the interaction indistinguishable. Further work to determine the VOCs present at the membrane surface compared to those directly above each area of the interaction would show if the VOCs become mixed or altered at the membrane surface.

5.4.2 Collembola detection of fungal volatiles

The interaction of *R. bicolor* 1 against *H. fasciculare* 1 emits a wide range of VOCs, some of which are unique to the interaction (Hynes *et al.*, 2007). The lack of any detectable collembola response was, therefore, unexpected. Collembola may require more time to exhibit a preferential behaviour; collembola response times differ between species and it can take several hours before a preference becomes obvious in some species (Kaneda & Kaneko, 2004). It is uncertain whether collembola are able to detect interaction-based VOCs and the presence of VOCs does not necessarily indicate a functional role (Gould & Lewontin, 1979). Examining collembola VOC detection ability through studying, for example, antennal response, would demonstrate which cues illicit a response and, therefore, may have functional significance. For example, the antennal responses of the two-spotted stinkbug *Perillus bioculatus* to volatiles produced by its prey the Colorado potato beetle, *Leptinotarsa decemlineata*, were successfully monitored by the use of electro-antennograms (Weissbecker *et al.*, 1999). The study revealed that among the bouquet of VOCs tested two compounds elicited a particular response, 2-phenylethanol and β-caryophyllene. Other VOCs elicited only weak antennal responses on the stinkbug and were therefore, considered to be of limited importance in the interaction studied.

5.4.3 Proposals for future work

Although this present study failed to provide unequivocal evidence of VOC exploitation by collembola during fungal interactions there remains sufficient anecdotal information to justify that a further, more refined, experiment with modified detection techniques should be executed. Such a study should:

- examine the rate of VOC transmission across the membrane to inform accurately the time for membrane saturation before collembola addition;
- determine collembola VOC detection through testing antennal response to the VOC bouquet of each interaction studied using, for example, electro-antennogram techniques; and
- test the interactions on a range of different substrates, for example, defaunated soil, to determine the role of substrate in altering collembola behaviour.

Using this information, it would be possible to determine the role of VOCs in informing the behaviour of collembola during fungal interactions and provide insight as to whether VOCs are as important in the below-ground decomposer community as they are in the plant root community.

6.0 The responses of cord forming saprotrophic fungi to collembola grazing during aggressive interspecific interactions.

6.0 Introduction

Networks of fungal cords are produced across the forest floor at the soil-litter interface (Boddy, 1999) and, as they extend across the soil, competition with other basidiomycetes for both space and resources are inevitable and frequent (Boddy, 2000). Many studies of such fungal interactions show fungi to be aggressive, often with a reproducible hierarchy of dominance between species (Donnelly & Boddy, 2001; Dowson *et al.*, 1988a; Wald *et al.*, 2004b). While abiotic conditions and duration of resource colonisation influence fungal extension rate and morphology (Donnelly & Boddy, 1997a; Harold *et al.*, 2005), various other factors including inoculum size can also alter interaction outcome (Donnelly & Boddy, 1997a; Harold *et al.*, 2005). In addition, aggressive fungal interactions may cause normally conservative fungal mycelia to become leaky, leading to nutrient loss to the soil creating a pathway for nutrient mineralisation (Wells & Boddy, 2002).

Fungal dominated ecosystems have high levels of fungal grazers such as collembola and mites (Bardgett, 2005; Hopkin, 1997). Many studies have attempted to elucidate the diets of soil invertebrates, including collembola (Moore *et al.*, 1985; Thimm & Larink, 1995). Using stable isotope techniques (Chahartaghi *et al.*, 2005), fungi have been confirmed as a major component of collembola diet. In general, however, there appears to be little dietary specialisation in soil invertebrates of all fauna groups (Maraun *et al.*, 2003). As dietary niche development is considered an explanation for above-ground invertebrate diversity (Hunter & Price, 1992), the absence of such specialisation below-ground is surprising. Laboratory preference tests indicated that collembola prefer dark pigmented fungi (Dematiacea; Mills & Sinha, 1971; Poole, 1959) while a complex field experiment revealed that the collembola *Onichiurus latus* exhibited a clear preference for basidiomycete fungi, accounting for 90% hyphae found in the gut (Newell, 1984a, b). Interestingly, *O. lata* grazing was attributed by the author to cause partitioning of two fungal species, *Marasmius androsaceus* and *Mycena galopus,* with the preferential grazing of *M. androsaceus* confining the species to the upper litter horizon which is too dry for *O. latus*. The less grazed

Mycena galopus dominated lower in the litter. This gives rise to a paradox, with soil invertebrates, including collembola, being considered to be generalists but with clear preferences often seen in empirical studies, especially those in the laboratory.

Collembola not only exhibit preferences in the fungi chosen (Bardgett *et al.*, 1993b), they also affect the fungi on which they graze. Basidiomycete fungi show reduced mycelial extension under grazing (Tordoff *et al.*, 2006) therefore potentially reducing their ability to obtain new space and resources. Other examples of fungal responses to grazing have been shown in non-basidiomycete fungi. For example, *Mortierella isabellina* changed growth pattern from 'normal' to fast growing appressed (non-aerial) and sporulating mycelium when grazed by the collembola *Onychiurus armatus* (now *Protaphorura armata*; Hedlund *et al.*, 1991). Furthermore, Bengtsson *et al.*, (1993) observed that *Verticillium bulbilosum* and *Penicillium spinulosum* increased respiratory activity in response to collembola. Collembola grazing does not, therefore, necessarily have a uniformly negative impact on mycelium. As further examples, the cord-former *Phanerochaete velutina* exhibited compensatory growth at low grazing intensity (Bretherton *et al.*, 2006) while *Hypholoma fasciculare* exhibited an occasional change in growth strategy switching from uniform growth to areas of rapid extension (Kampichler *et al.*, 2004). This latter growth change was interpreted as a fugitive response to evade grazing attack. While this change in response was caused by *Folsomia candida* grazing, which also reduced mycelial extension and cover the two other grazers used in the study, *Proisotoma minuta* and *Hypogastrura* cf. *tullbergi*, affected neither fungal extension nor cover (Kampichler *et al.*, 2004). To date, the majority of studies on collembola grazing of cord-forming decay fungi have been restricted to laboratory studies comparing the response of a single fungus to one grazer species. In the field, however, the frequent interactions between cord-forming fungi, especially in woodlands where they are very prevalent, may well alter the grazing patterns of collembola. This may feedback to affect fungal interaction outcomes and ultimately species assemblage.

This study explores the effect of collembola grazing on fungal extension, morphology and combativeness when two cord-forming fungi are interacting. Using four fungal species and two collembola grazer species, the varying nature of fungal response to grazing is explored. It is predicted that fungal preferences exhibited by collembola,

along with differences in how collembola species affect mycelial morphology and function, will influence fungal interaction outcomes. More specifically it is hypothesised that: (i) species highly susceptible to grazing, such as *Resinicium bicolor* will loose combative ability when grazed; (ii) species highly resistant to grazing, such as *Hypholoma fasciculare*, will gain a competitive advantage when grazed, and (iii) *Protaphorura armata* will have much less impact on fungi than *Folsomia candida* as it is less active and other studies employing similar systems comparing these two collembola, suggest limited impacts (Tordoff *et al.*, 2008).

6.2 Materials and Methods

6.2.1 Soil tray preparation

Soil collected from mixed deciduous woodland in Tintern, Monmouthshire (SO517069) was air-dried for 7 d, and sieved through a 4 mm and 2 mm mesh to remove organic material and stones. The soil was then frozen for 24 h to defaunate (but minimising effects on microbial community) before rewetting with 350 ml deionised water (DH_2O) kg^{-1} soil. The wet soil (200 g per tray) was evenly compacted to 4 mm depth into 24 x 24 x 2 cm non-vented lidded bioassay trays (Nunc-Gibco, Paisley, UK), which were then weighed to 0.01 g and stored in stacks of 20 double wrapped in black PVC bags to prevent desiccation (20°C ±1°C, dark). Trays were used within 7 d of being made and rewetted to original weight with DH_2O every 7 d. During the experiment any contaminating flora were removed. No non-experimental fauna were observed.

6.2.2 Wood block preparation and sterilisation

Wood blocks were cut from freshly felled beech (*Fagus sylvatica*) timber (Coed Cymru Hardwood Sawmill, Wentwood, UK) into 2 x 2 x 1 cm blocks. Blocks with discolouration and knots were discarded. Groups of 20 blocks were double-wrapped in heat-sealed biohazard bags and autoclaved for two separate 1 h sessions each being 24 h apart. Sufficient blocks were inoculated for 1.5 times the requirements of the experiment.

6.2.3 Wood block inoculation

Phanaerochaete velutina, Resinicium bicolour, Hyphloma fasciculare and *Phallus impudicus* were cultured in darkness on 2% malt extract agar (MEA, 20g L^{-1} malt,

Munton and Fison, UK, Lab M agar no. 2) and maintained in the dark at 20°C until the mycelium covered the surface of 9 cm diameter non-vented Petri dishes. Inocula excised from each culture were transferred to 14 cm diameter vented Petri dishes filled with MEA, sealed with Nescofilm® and stored at 20°C in darkness. The mycelia from a single 9 cm plate was used to inoculate two 14 cm plates. Once the mycelium had covered the entire surface of each 14 cm plate, 15 sterile beech (*Fagus sylvatica*) wood blocks (preparation described below) were added, the plates resealed, and returned to 20°C and darkness. When the mycelium had entirely covered the wood blocks the Nescofilm was removed and the plates stored in PVC bags to prevent desiccation. Wood blocks were subjected to colonisation for three months before use.

6.2.4 Experimental design

External mycelium was removed from each wood block before addition to trays. Growth rates (approximate) were determined by placing two wood blocks colonised by each species on soil trays and measuring growth under experimental conditions (20°C, dark in PVC bags) for one week. For the main experiment prepared soil trays were randomly allocated to different fungal interactions. To each tray a wood block of the slower growing of the two fungal species was added 9 cm away from a corner along a diagonal between corners, and the tray weighed. At the appropriate time (see below) the second wood block was added, 9 cm away from the opposite corner. Timing of inoculation for each of the interacting species was calculated on the basis of growth rate estimation to ensure that fungi met when the mycelia were approximately 8 cm diameter. The tray was weighed again and this was used as the target weight for subsequent rewetting.

All four fungal species were interacted in every possible combination with heterospecifics, with 20 soil trays per interaction (total 120 soil trays). Only trays with good fungal outgrowth of both species were eventually used in the experiment. To half of the trays, collembola were added (described below).

6.2.5 Collembola addition

Folsomia candida were cultured and size sorted and starved before experimental use as described in Section 3.2. For any given interaction, when 50 % or more of the

fungi had met the opposing fungus, for a minimum of 2 d, 20 collembola were added to each corner of the tray (80 per tray). *Protaphorura armata* (80 per tray) were used in two additional interactions: *R. bicolor* against *H. fasciculare* and *P. impudicus* against *H. fasciculare*. Methodology for culture and addition of *P. armata* was the same as for *F. candida.*

6.2.6 Data recording

Data were collected by digital photography using a Nikon Coolpix 5500 camera (Nikon Corporation, Japan) mounted on a stand (Kaiser RA1, Kaiser, Germany) at 47 cm with artificial illumination provided by two 1000 W spot flood lamps (Gamma 7 Al, Gamma, Chicago USA), each set 60 cm either side of the stand and 120 cm above it. Tray photography commenced on the day of collembola addition, t_0, and continued every 2 d for the first 11 d (t_{10}), then every 4 d until 23 d (t_{22}), every 10 d until 43 d (t_{42}) and finally at d 85 (t_{84}).

6.2.7 Outcomes between mycelia interacting on the soil surface

Outcome of interactions between extra resource mycelia was determined through examination of images at the final time point. Where a mycelium completely overgrew the opponent and extended to the opposite side of the microcosm, total overgrowth was recorded. Partial overgrowth was considered as any overgrowth of the opponent mycelium level with or past the opponent wood block but less than total overgrowth. Reciprocal overgrowth by both mycelia, where the mycelium of each species was seen to be overgrowing the other, was recorded as mutual overgrowth. For comparison the outcomes of interactions were attributed percentages based on the proportion of replicates exhibiting a given outcome.

6.2.8 Harvesting

All harvesting was completed within 48 h of the final photograph. Only wood blocks that had been reached by the mycelium of the opposing fungus were harvested. These blocks were cut in half and three wood chips taken from the freshly cut surface. Woodchips were then plated onto MEA (Fig. 6.1). Plates were sealed with Nescofilm and stored for 7 d (20°C, dark). Plates were then examined and the species outgrowing from the wood chips were identified on the basis of their mycelial morphology. Unless the overgrowing mycelia was isolated from the wood block the

outcome was recorded as overgrowth by the opponent but not replacement. If both fungal species were isolated the result was recorded as partial replacement. If only the opposing species was re-isolated, the outcome was recorded as total replacement. Results for each interaction were recorded as overgrowth, partial replacement or overgrowth, and expressed as a percentage of total reisolations.

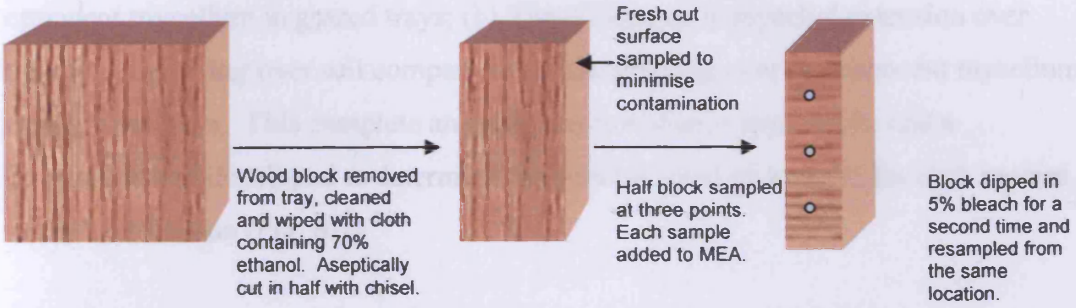


Fig. 6.1: The wood block harvesting process

6.2.9 Extension rate measurement and statistical analysis

Growth rates were measured up to the time point where at least one fungus had reached the opposing wood block in 50% or more trays, t_{reach}. Further analysis was carried out at two or three evenly spaced time intervals between the time of collembola addition (t_0) and t_{reach}.

Mycelial extent was measured as the furthest visible extent of mycelium at four equal angles through a 90° segment of the mycelium, and in the direction of the opponent (Figs. 6.2 and 6.3 a). Unless removed through grazing the mycelium in the two central measurements (30° and 60°) was invariably in contact with the opposing mycelium. Cords severed form the mycelium through grazing and not visibly connected to the wood block were not included in measurements. Both species in all replicates of each interaction were measured at t_0 and t_{reach}. Change in mycelial extent was determined from $t_{reach} - t_0$.

The comparisons made using the measurement data were: (1) the difference in mycelial extent between grazed and ungrazed trays across all four measurements at the final time point (t_{reach}); (2) the difference in mycelial extent between grazed and

ungrazed trays when growing toward (or over) the opponent mycelium (30° and 60° measurement) at the final time point (t_{reach}); (3) the difference in mycelial extent over time between grazed and ungrazed trays when growing toward (or over) the opponent mycelium; (4) the difference in mycelial extension over time between grazed and ungrazed trays when growing over soil (0°and 90°); (5) the difference in mycelial extension over time when growing over soil compared to when growing over the opponent mycelium in grazed trays; (6) The difference in mycelial extension over time when growing over soil compared to when growing over the opponent mycelium in ungrazed trays. This complete analysis was not always appropriate and a framework was developed to determine the suitable level of analysis for each species in each interaction (Fig. 6.3)

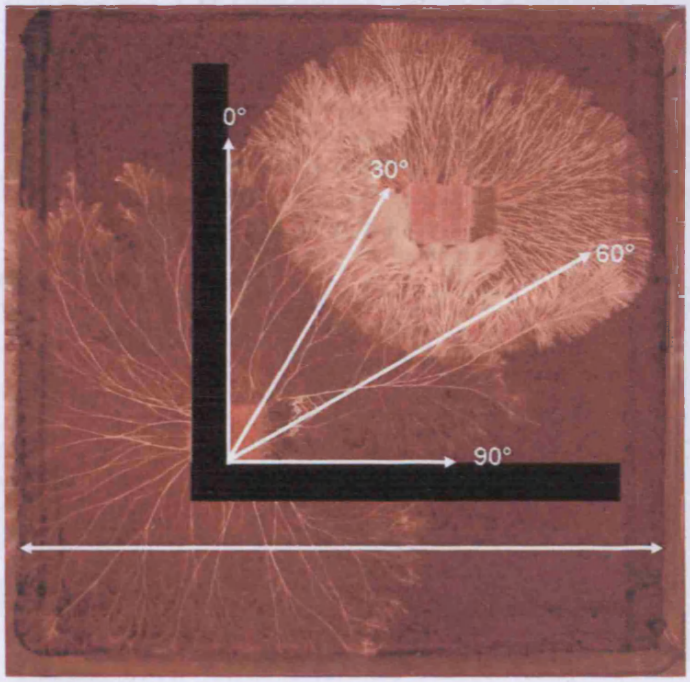


Fig. 6.2: Four measurements (indicated with white single arrows) taken for both species in every interaction at t_0 and t_{reach}. The length of the double-headed arrow (22.6 cm - internal tray width) was applied to scale all images.

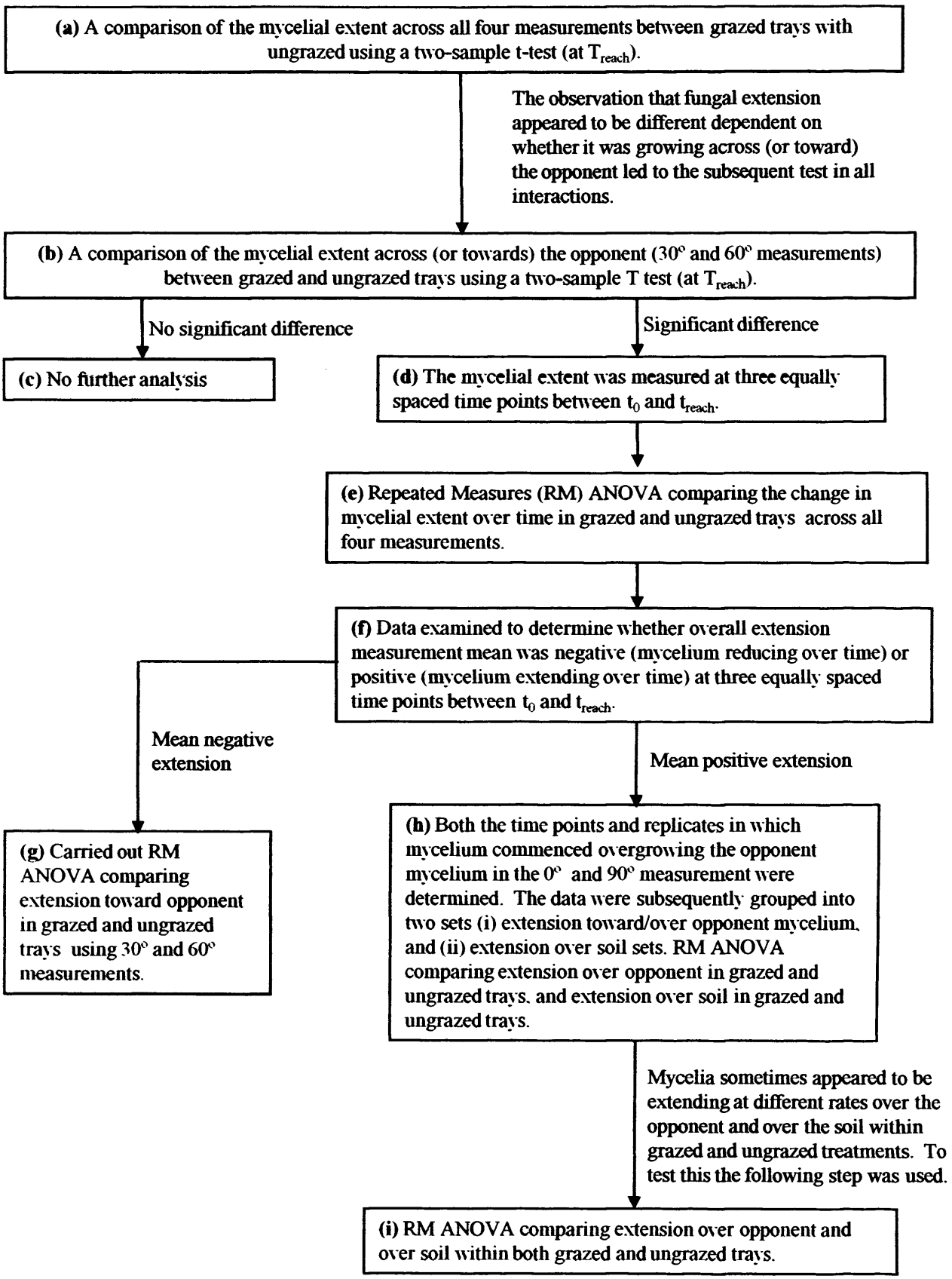


Fig 6.3: The framework applied to refine application of statistical tests in mycelial extension change analysis. Where data violated normality a Mann-Whitney U test (instead of the t-test), or a two-way ANOVA on ranked data (instead of the RM ANOVA) was used.

6.3 Results

6.3.1 Changes in interacting fungi

R. bicolor mycelia were always completely grazed away by *F. candida* (e.g. Fig. 6.4, c) whereas *P. armata* had little impact (Fig. 6.4, d). *P. velutina* was sometimes heavily grazed, but not to extinction, in some interactions, especially those with *R. bicolor* (Fig. 6.4, c). In interactions of *R. bicolor* and *P. velutina*, cords of the latter grew to both sides of the *R. bicolor* wood block forming a complete ring. This comprised a thick cord in ungrazed systems (Fig. 6.4 a, b) but the arching mycelia did not fuse to form a complete loop in grazed systems. *P. velutina* did not encircle inocula of opponents in any other combination (e.g. Fig. 6.4 f, g). *H. fasciculare* was usually only sparsely grazed, except when interacting with *P. velutina* where it was completely removed in grazed systems by 64 d (Fig. 6.4, g). In this case collembola grazed through the *P. velutina* mycelium down to the *H. fasciculare* leaving distinct holes (Fig. 6.4, i). Evidence of collembola grazing preferentially at the interaction zone when *H. fasciculare* was interacting with *P. impudicus* and *P. velutina* was seen as depleted mycelium where the two fungi met (Fig. 6.4, h, i, respectively)

6.3.2 Outcomes between mycelia interacting on the soil surface

In three (of six) interactions, grazing by *F. candida* altered the outcome between mycelia interacting on the soil surface (Table 6.1). For example, the mutual overgrowth of *P. velutina* and *R. bicolor* in ungrazed systems shifted to complete overgrowth by *P. velutina* when grazed (Table 6.1; Fig. 6.4 a, b). Grazing also conferred a competitive advantage to *P. impudicus* when interacting with *R. bicolor*. Interactions involving *H. fasciculare* were little changed when grazed except when interacting with *P. velutina*, where *F. candida* grazing shifted the balance in favour of the latter (Table 6.1). In two thirds of interactions grazing by *F. candida* had no effect on interaction outcome at the soil surface (Table 6.1). There were no outcome differences between grazed and ungrazed systems in either of the interactions grazed by *P. armata* (Table 6.1). Grazing never completely reversed any interaction outcome when compared to the ungrazed systems.

Table 6.1: Surface outcomes from interactions after 50-56 d.

Fungal species	*H.fasciculare*		*H.fasciculare*		*P. impudicus*		*P. velutina*	
	Grazed by *F.candida*	Ungrazed	Grazed by *P.armata*	Ungrazed	Grazed by *F.candida*	Ungrazed	Grazed by *F.candida*	Ungrazed
R. bicolor	**P** 100	**P** 100	**P** 100	**P** 100	**O** 100	**P** 100	**O** 100	**M** 100
P. velutina	o 100	o 70 **M** 30	-	-	**M** 100	**M** 100		
P. impudicus	o 100	o 90 **M** 10	o 100	o 100				

Outcomes were mutual overgrowth (M), partial overgrowth (P) and total overgrowth (O). For outcomes designated P and O, uppercase signifies replacement was by the fungus listed in the column whereas lowercase signifies replacement by the fungus listed in the row. Numbers refer to percentage of replicates exhibiting a given response. There were eight to 20 replicates per interaction.

6.3.3 Outcome of interactions within wood

F. candida grazing enhanced the ability of *P. velutina* to replace *H. fasciculare*; in the presence of *P. armata*, *H. fasciculare* was less able to replace *R. bicolor* but there was a high variability between both sets of ungrazed controls for this interaction (Table 6.2). In three interaction combinations, grazing did not alter the outcome. In three other interaction combinations *F. candida* grazing reduced the extent of replacement of the original incumbent in the wood block by the opponent fungus (Table 6.2). *P. velutina* mycelia reached the *H. fasciculare* wood block more rapidly when grazed than when ungrazed, although, overall, the mycelium in grazed systems appeared less extensive (Fig. 6.4 e, f, considered further below).

There was evidence of preferential grazing by *F. candida* at the interaction zone, for example with *H. fasciculare* when interacting with either *P. velutina* or *P. impudicus* (Fig. 6.4 e, h, i). In some interactions fungi produced pigments and exudates at the point of contact with the opponent (Fig. 6.4 j, k). *R. bicolor* produced a red pigment along the interaction line when interacting with *H. fasciculare* and small globules of exudate were visible where *R. bicolor* cords met those of *P. velutina* (Fig. 6.4 j, k).

Table 6.2: Outcomes of interactions after 80-84 d from woodblock reisolations.

Fungal species	*H.fasciculare*		*H.fasciculare*		*P. impudicus*		*P. velutina*	
	Grazed by *F.candida*	Ungrazed	Grazed by *P. armata*	Ungrazed	Grazed by *F.candida*	Ungrazed	Grazed by *F.candida*	Ungrazed
R. bicolor	P 20 O 80	P 40 O 60	R 29 o 71	R 42 P 29 O 29	O 100	O 100	O 100	R 20 P 20 O 60
P. velutina	r 100	r 70 R 10 P 10 O 10	-	-	M 100	M 100		
P. impudicus	o 100	o 100	o 100	o 100				

Outcomes were mutual overgrowth (M), partial replacement (P), total replacement (R) and overgrowth (O). For outcomes designated PR, R and O, uppercase signifies replacement was by the fungus listed in the column whereas lowercase signifies replacement by the fungus listed in the row. Overgrowth is where woodblock was reached but original incumbent not replaced. Numbers refer to percentage of replicates exhibiting a given response. There were four to 10 replicates per interaction treatment.

6.3.4 Radial extension

In five interaction combinations the change in mycelial radial extent was significantly different between grazed and ungrazed treatments (Table 6.3). Of these three interactions, *R. bicolor* against *H. fasciculare*, *P. impudicus* and *P. velutina*, the presence of *F. candida* resulted in a fall in mycelial extent of *R. bicolor*. This, however, was only significant over time for *R. bicolor* against *H. fasciculare* and *R. bicolor* against *P. impudicus* (Table 6.3, Fig. 6.5 a, c, e). In both interactions, growth toward the opposing mycelium was significantly reduced by grazing (Fig. 6.5, b, d).

In the other two interactions, *F. candida* grazing of *P. velutina* interacting with either *H. fasciculare* or *P. impudicus*, significant differences were seen between grazed and ungrazed treatments (Table 6.3, Fig. 6.6). In these interactions both grazed and ungrazed mycelia increase in size over time (Fig. 6.6 a-f). The radial extension of *P. velutina* (for all four extension measurements taken per replicate) was significantly less in grazed than in ungrazed treatments when interacting with *P. impudicus* but not significantly different when interacting with *H. fasciculare* (Fig. 6.6 a, b). Although *P. velutina* growth over *H. fasciculare* was slightly faster in grazed trays than in ungrazed trays, this was not significant (Fig. 6.6 c). When over soil and interacting with *H. fasciculare*, however, mycelial extension of *P. velutina* was significantly faster in ungrazed trays (Fig. 6.6 e).

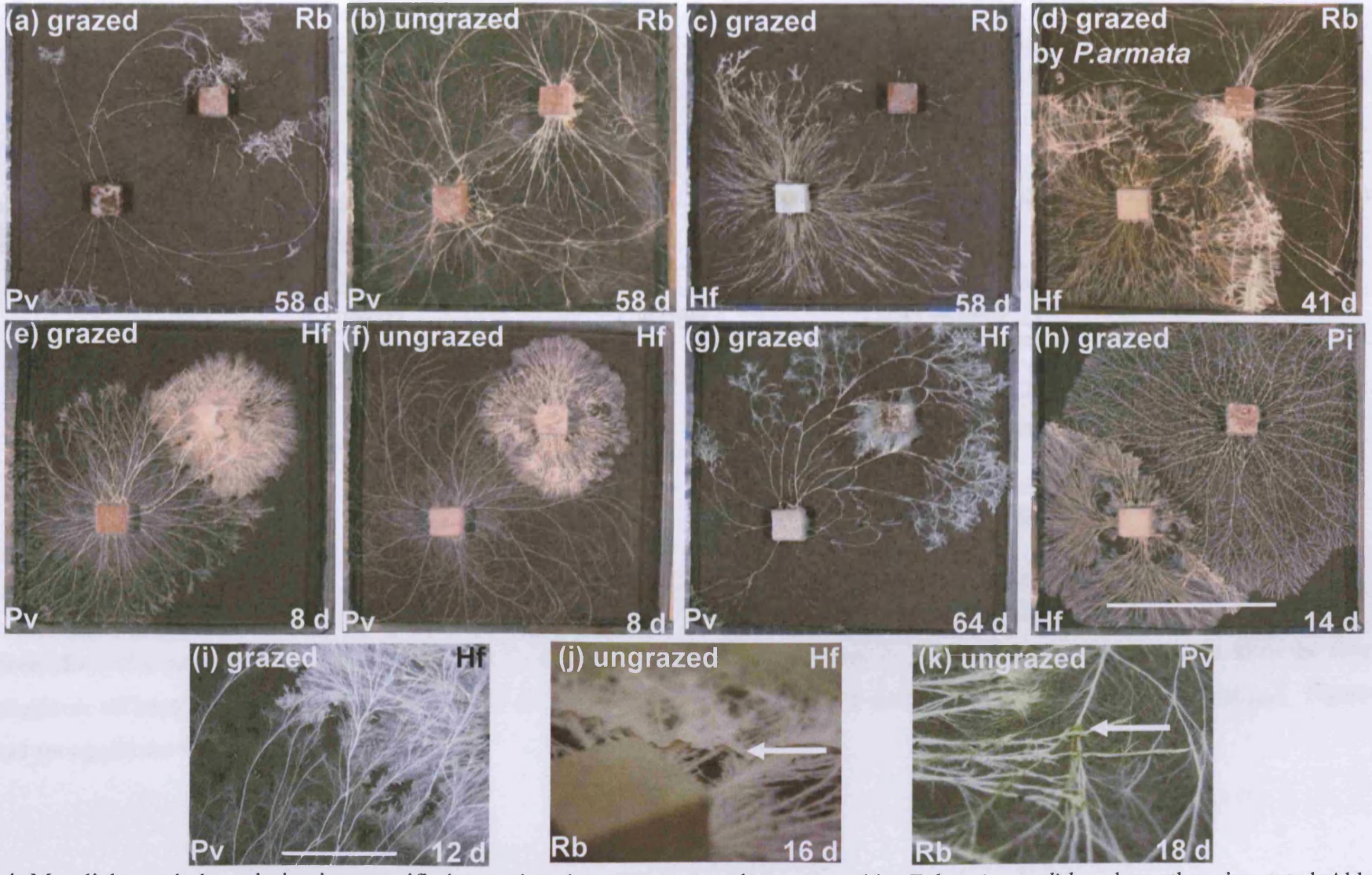


Fig 6.4: Mycelial morphology during interspecific interactions in systems grazed or ungrazed by *Folsomia candida* unless otherwise stated. Abbreviations: Hf, *H. fasciculare,* Pi, *P. impudicus,* Pv, *P. velutina,* Rb, *R. bicolor.* Scale bar (10 cm) on image h applies to images a-h. Scale bar (2 cm) on image i applies to image i alone. Images j and k show pigment and exudate production (arrowed), respectively, during interactions (scale bar inappropriate).

Table 6.3: Analysis of mycelial extent in grazed and ungrazed systems. Data are presented for each interaction combination with comparisons carried out for each species in all interactions.

	H. fasciculare grazed by *F. candida*		*H. fasciculare* grazed by *P.armata*		*P. impudicus* grazed by *F. candida*		*P. velutina* grazed by *F. candida*	
	Species in column	Species in row	Species in column	Species in row	Species in column	Species in row	Species in column	Species in row
R. bicolor	$t_{22} = 1.08$ $P = 0.291$	**$t_{22} = -6.14$** **$P < 0.001$**	$t_{26} = 0.61$ $P = 0.549$	$W = 200$ $P = 0.9085$	$t_{22} = 1.08$ $P = 0.291$	**$t_{12} = -3.61$** **$P = 0.004$**	$t_{26} = -1.32$ $P = 0.1$	**$t_{26} = -3.07$** **$P = 0.005$**
P. velutina	$W = 375$ $P = 0.351$	**$t_{40} = 2.16$** **$P = 0.037$**	-	-	$t_{36} = 0.74$ $P = 0.463$	**$t_{36} = -4.65$** **$P < 0.001$**		
P. impudicus	$W = 442.5$ $P = 0.387$	$t_{35} = 0.034$ $P = 0.734$	$W = 85$ $P = 0.398$	$t_{16} = 0.61$ $P = 0.55$				

Where data were not normal, a Mann Whitney U Test (W) was applied. The change in mycelial extent was measured from the time of collembola addition (t_0) to the point at which one at the interacting mycelia reached the woodblock of the opposing fungus (t_{reach}). Figures in bold are significant values at $P \leq 0.05$.

In the interaction between *P. velutina* and *P. impudicus* mycelial extension of *P. velutina* was significantly faster in ungrazed trays regardless of whether growing over the opposing mycelium or soil (Fig. 6.6 d, f). When mycelial extension over soil and over opponent were compared, *P. velutina* extended more rapidly over *H. fasciculare* mycelium than over soil when grazed but more rapidly over soil when ungrazed (Fig. 6.6 g, i). In contrast, with *P. impudicus* grazing had no effect with *P. velutina* mycelium extending more rapidly over soil in both grazed and ungrazed systems (Fig. 6.6 h, j).

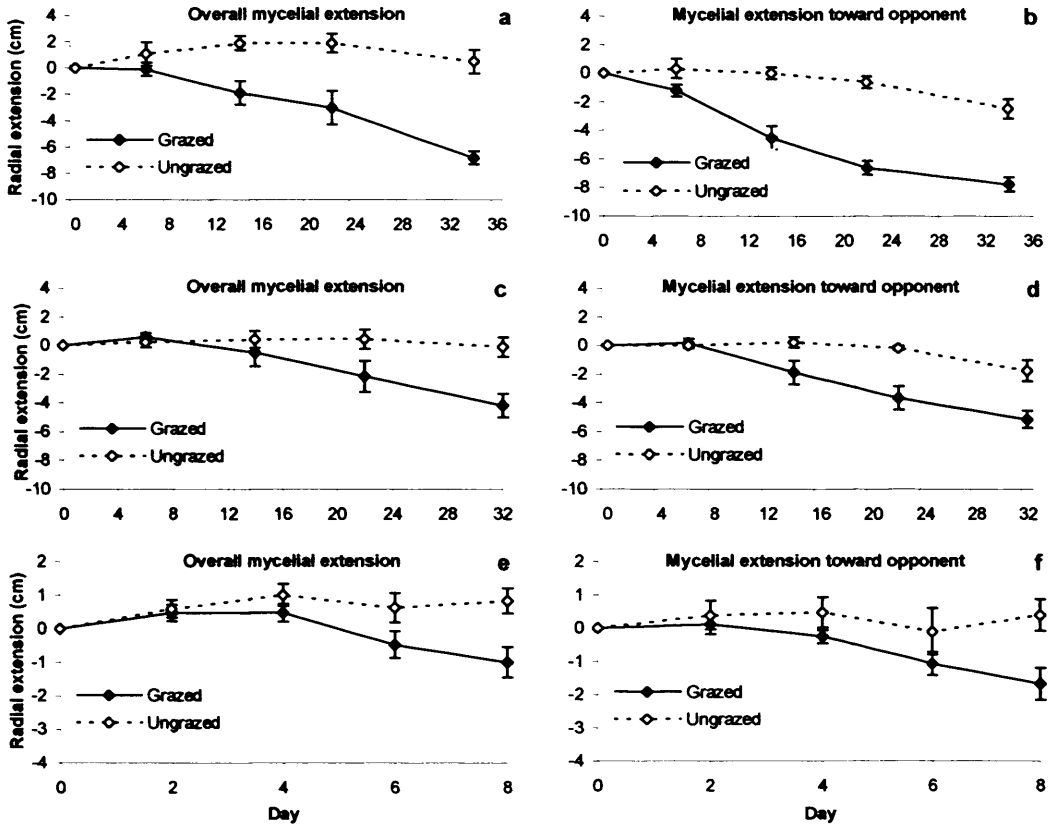


Fig. 6.5: Change in mycelial extent for *R. bicolor* based on all four (0°, 30°, 60°, 90°) extension measurements (a, c, e) and on 30° and 60° measurements (b, d, f). *R. bicolor* interacting with *H. fasciculare* (a) SRH $\chi^2 = 0.999$; $P < 0.001$, (b) Two-Way ANOVA on Ranked Data, $F_{3,88} = 15.55$, $P < 0.001$); *R. bicolor* interacting with *P. impudicus* (c) RM ANOVA $F_{2.859, 74.345} = 4.803$; $P = 0.005$, (d) RM ANOVA $F_{3.013, 36.154} = 6.461$; $P < 0.001$); *R. bicolor* interacting with *P. velutina*, (e) SRH $\chi^2 = 0.762$; $P = 0.238$, (f) Two-Way ANOVA on Ranked Data, $F_{3,104} = 2.14$, $P = 0.1$); critical values are for time*treatment interaction. Error bars are standard error of the mean.

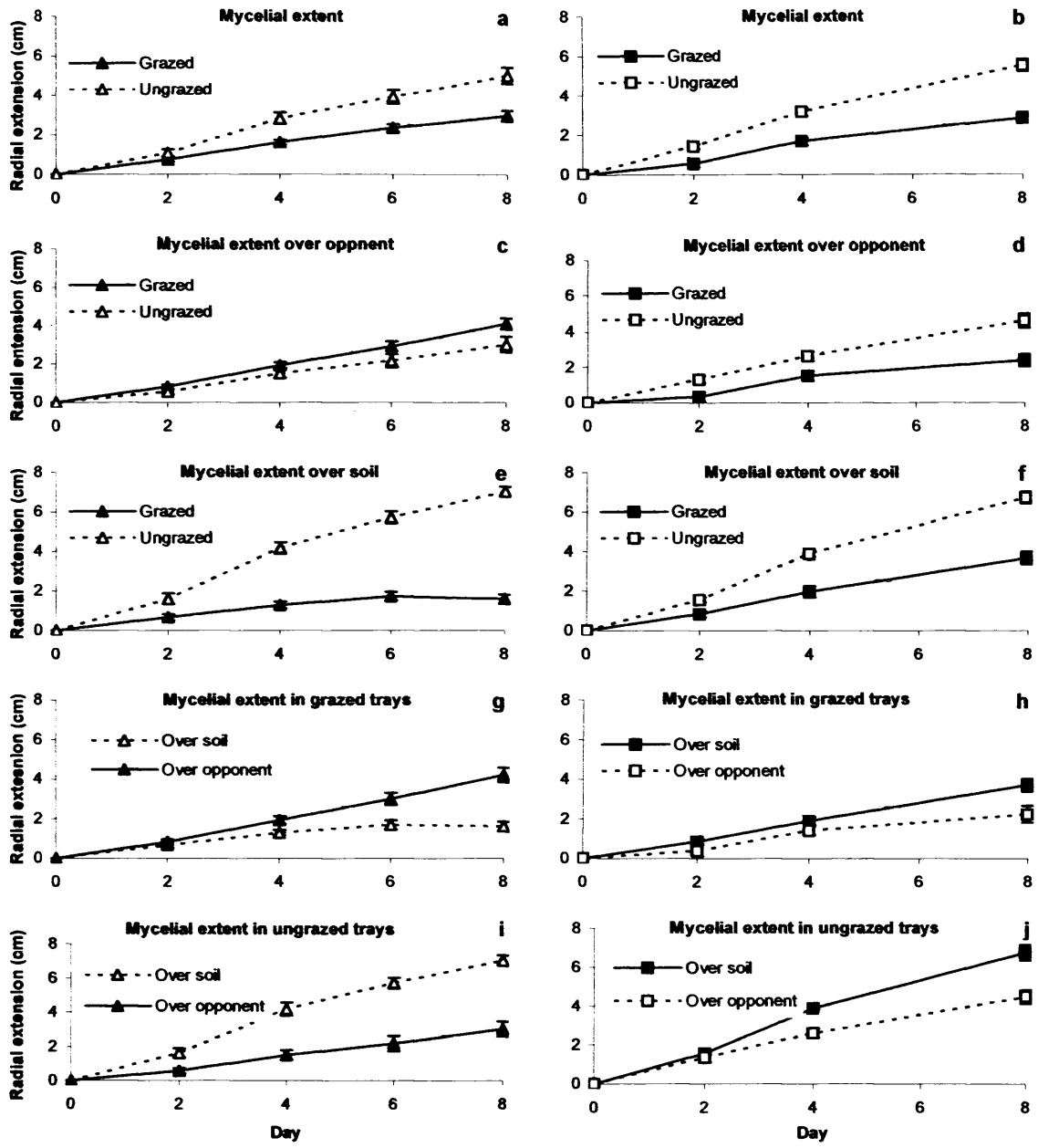


Fig 6.6: Change in mycelial extent for *P. velutina* interacting with *H. fasciculare* (a, c, e, g, i) and *P. impudicus* (b, d, f, h, j). Measurements are: mycelial extent in grazed and ungrazed systems across all four extension measurements, (a) $F_{2.443, 92.850} = 2.147$; $P = 0.112$, (b) $F_{2.051, 151.785} = 24.451$; $P < 0.001$; mycelial extent in grazed and ungrazed systems over opponent (c) $F_{2.443, 92.850} = 2.147$; $P = 0.112$, (d) $F_{2.125, 84.981} = 5.611$; $P = 0.004$; mycelial extent in grazed and ungrazed systems over soil (e) $F_{4, 140} = 212.849$; $P < 0.001$, (f) $F_{2.534, 73.490} = 25.626$; $P < 0.001$; mycelial extent over the soil and over the opposing mycelium within grazed systems, (g) RM ANOVA $F_{2.601, 46.819} = 22.264$; $P < 0.001$, (h) $F_{3, 51} = 3.651$; $P = 0.018$; mycelial extent over soil and over the opposing mycelium within ungrazed systems, (i) $F_{3.141, 56.541} = 32.797$; $P < 0.001$, (j) $F_{1.751, 26.262} = 9.835$; $P < 0.001$. Critical values are for RM ANOVA time*treatment interaction. Error bars are standard error of the mean.

6.4 Discussion

6.4.1 Changes in growth and morphology of fungal mycelia

Although many studies have considered collembola grazing on their own, there is a dearth of information on the combined effects of grazing and interspecific interactions on the fungal community. This is one of the first to investigate such a system. In brief, *F. candida* grazing played an important role in both the outcome and progression of half of the fungal interactions studied in soil microcosms; the less active *P. armata* had almost no discernable effects on fungal interactions.

Previous studies of *F. candida* grazing on individual *P. velutina* mycelia have shown a reduction in mycelial extension rate (Tordoff *et al.*, 2006, 2008) although, at low densities, collembola often stimulate fungal growth (Bengtsson *et al.*, 1993; Bretherton *et al.*, 2006; Kampichler *et al.*, 2004). When *P. velutina* was interacting with *H. fasciculare*, grazing by *F. candida* accelerated *P. velutina* extension rate when the mycelium was growing over *H. fasciculare*, compared to growing over soil. A study of the fungus *Mortierella isabellina* revealed morphological changes and accelerated mycelial growth when grazed but, unlike in this present study, the accelerated growth occurred away from the region of grazing in areas where collembola (*P. armata*) were excluded (Hedlund *et al.*, 1991). In the present study, however, the mycelium could not 'escape' grazing anywhere except within the wood blocks and there was evidence of collembola grazing at the interaction zone which was toward the region of accelerated growth (Hedlund *et al.*, 1991). This contrasts, therefore, with studies of fungi growing alone as the fungal response appears not to be an attempt to escape grazing pressure (Hedlund *et al.*, 1991; Kampichler *et al.*, 2004). Interestingly the accelerated growth of mycelia across the opponent mycelium only occurred in *P. velutina* and only when interacting with *H. fasciculare* suggesting that the interacting fungi respond differentially to one another and that grazing is a further complicating factor.

P. velutina extension appears to be regulated by a combination of opponent species identity and grazer activity. One possible mechanistic explanation for this is the unpalatability of viable *H. fasciculare* mycelium. Previous studies of *F. candida* grazing have revealed *H. fasciculare* mycelia to be unpalatable and, when grazing thereon, collembola populations perform poorly (Tordoff *et al.*, 2006). The

unpalatability, and subsequent avoidance, of *H. fasciculare* may have caused grazing to be concentrated on *P. velutina* which, already interacting with an aggressive fungal opponent, would be placed in a defensive position on two fronts. One response to this could be accelerated growth to enhance vigour and compensate for grazing loss.

The effect of grazing by *F. candida* on *R. bicolor* contrasts strongly with that of *P. velutina*. In this case grazing has a consistently negative impact on *R. bicolor* mycelial extension. The frequent severing of *R. bicolor* cords by *F. candida* in all interactions, suggests that *R. bicolor* was easily consumed. How *R. bicolor* survives in the field when it is so heavily damaged by *F. candida* in microcosm studies remains an unanswered question. *R. bicolor* may grow only short distances as exposed, extra-resource cords in the soil using litter, twigs and larger woody items as refuges for intra-resource growth. The fungus would, however, remain exposed at certain locations and be open to grazing activity severing links in the network. The growth strategy of *R. bicolor* also implies that it is a long range forager (Boddy, 1993) and this increases the likelihood of grazer contact. Cords may be less palatable in the field. *R. bicolor* is commonly found in coniferous forest (Kirby *et al.*, 1990; Woods *et al.*, 2006) where compounds found in coniferous species, such as polyphenols and terpenes (Bardgett, 2005; Rayner & Boddy, 1988), may be extracted and utilised by *R. bicolor* as it extends from a resource. Although wood decay fungi can be inhibited by these compounds those found on conifer woodlands are often capable of growing in the presence of such allelopaths (Rayner & Boddy, 1988). Beech, the wood resource used in these microcosm experiments, may not contain metabolites which could be utilised by *R. bicolor* in this way potentially weakening its protection from grazing. Although studies suggesting that fungi utilise resource-derived feeding deterrents are scarce, changes in substrate can lead to substantial alteration in fungal volatile organic compound emissions (Bruce *et al.*, 2000). This does, however, suggest that wood substrate may play a role in enabling fungi to withstand grazing. The microcosms lacked the presence of collembola predators but, in the field high population densities of *F. candida* may attract predators such as Acari, Staphylinidae and Carabidae, thus limiting mycelial damage through grazer population control (Hopkin, 1997).

6.4.2 Resilience to fungal grazing and the role of the interaction

P. impudicus and *H. fasciculare* generally exhibited a high level of resilience to grazing with few, if any, morphological differences between the mycelia of grazed and ungrazed interactions. The major exception to this was the total removal of the *H. fasciculare* mycelium when interacting with *P. velutina* and grazed by *F. candida* (discussed below). The lack of effect of *F. candida* on *H. fasciculare* extension does, however, contrast with studies where it was not interacting with another fungus where reduced extension rate (Harold *et al.*, 2005; Tordoff *et al.*, 2006) and regions of accelerated grazing have been shown (Kampichler *et al.*, 2004). Provision of food choice may have minimised the impact of collembola on *H. fasciculare* except when interacting with *P. velutina*. Complete removal of *H. fasciculare* mycelium through grazing when growing alone has not been recorded (Harold *et al.*, 2005; Kampichler *et al.*, 2004) suggesting that the interaction with *P. velutina* may have altered the palatability of *H. fasciculare*. That grazing of *H. fasciculare* was initially observed as fenestration in areas where *P. velutina* had already overgrown, suggests that interacting with *P. velutina* increased the palatability of *H. fasciculare*. As grazing took place through *P. velutina* mycelium further suggests an active searching for *H. fasciculare* mycelium by the collembola.

When loosing against *P. velutina*, *H. fasciculare* may have translocated chemicals away from the interaction zone and towards the wood block refuge. In doing so, chemicals that make the mycelium unpalatable may have also been removed from the mycelium. Collembola have been shown to prefer cords of low vitality, over healthy cords (Kaneda & Kaneko, 2004), although caution must be taken in drawing firm conclusions as a preference for actively growing hyphae has also been reported in other species (Moore *et al.*, 1985). In addition cord-forming fungi are known to move nutrients around the mycelial network and such movements can be bidirectional; such translocation may also be driven by a need to conserve nutrients (Wells *et al.*, 1998a, b).

6.4.3 The effect of grazing on combative ability

Some species are able to overgrow the mycelium of opponents but fail to replace the opponent within the woody resource. Overgrowth of another mycelia is, therefore, not necessarily indicative of combative ability for resources. For example, *P. velutina*

did not replace *P. impudicus* in either grazed or ungrazed systems, despite reaching the opponent wood block in all replicates. A similar result was seen when *P. velutina* interacted with *R. bicolor*, with *P. velutina* failing to replace *R. bicolor* in all grazed and 60 % of ungrazed systems.

Negative grazing impacts on mycelia could be expected to reduce fungal combative ability. This was not the case with *R. bicolor*. The heavy grazing of *R. bicolor* by *F. candida* led to the severing of cords and the entire network being undermined in all interactions (Fig. 6.4 a). Despite such extra-resource mycelial damage, *R. bicolor* retained possession of the wood block resource. Indeed, *R. bicolor* retained the wood block in more replicates when grazed than when ungrazed during interactions with both *H. fasciculare* and *P. velutina*. The reduced degree of replacement of *R. bicolor* in wood when grazed may, however, reflect reduced combativeness of the opponent when grazed rather than a directly positive effect of grazing on *R. bicolor*.

Grazing on one interacting species may also have an indirect effect on the combativeness of the opponent fungus. For example, the accelerated growth of *P. velutina* when grazed had a direct and negative impact on the fitness of *H. fasciculare*, limiting the ability of *H. fasciculare* to overgrow the *P. velutina* mycelium. Unlike other species, *H. fasciculare* was generally poor at defending its wood resource from *P. velutina* attack but, in 30% of ungrazed microcosms, when successfully attacking *P. velutina, H. fasciculare* was able to retain its own wood block. Whether this was due to *P. velutina* diverting resources away from its attack of *H. fasciculare* in these circumstances remains unanswered. With the exception of the interaction with *P. velutina, H. fasciculare* showed a generally high tolerance for *F. candida* grazing. This may provide a niche advantage, allowing *H. fasciculare* to proliferate in areas of high mycophagy, where more palatable species would be unable to compete. Collembola densities in soil can be very high (Hopkin, 1997) but *H. fasciculare* is ubiquitous in forest soils (Rayner & Boddy, 1988). Whether the abundance of *H. fasciculare* can be attributed, even in part, to grazing tolerance is yet to be confirmed.

6.4.4 Localised grazing

Whilst there was some evidence of grazing at the interaction zone it was not the highly concentrated grazing expected from such potentially nutrient rich areas (Wells

& Boddy, 2002). The one exception to this was grazing by *F. candida* in interactions involving *P. impudicus*. The grazing in the interaction zone, albeit intense, did not, however, alter the outcome of any interaction involving *P. impudicus*. The apparent increased density of hyphae and networking at the mycelial margin in grazed trays, does however, indicate that the mycelium was not entirely unaffected. The resilience of *P. impudicus* to *F. candida* grazing when growing alone has been established (Tordoff *et al.*, 2006) although, unlike *H. fasciculare*, *P. impudicus* always remained resilient when interacting with other mycelia. Deadlock within the wood block in all interactions indicates that, relative to the species against which it was paired, *P. impudicus*, was effective in defence but not in attack. This contrasts with a previous study (Dowson *et al.*, 1988a) where *P. impudicus* was found to be a poor competitor against a similar range of species. The species used in this present study were, however, different, the microcosms larger and the experimental period shorter.

6.4.5 Grazer identity

All the changes in morphology and extension of the fungi occurred in interactions grazed by *F. candida*. *P. armata* had limited impact on fungal growth and interaction progression. A less active species than *F. candida*, *P. armata* also has a longer generation time, at least in culture. *P. armata* is frequently the most abundant collembola species under natural conditions (Petersen & Luxton, 1982) and it is possible that, in this experiment, whilst an equivalent biomass of collembola species was used, the extent of grazer activity was lower in *P. armata* grazed interactions. In other fungal species *P. armata* grazing can affect mycelial morphology, triggering, for example, a switch in morphology and accelerated growth in *Mortierella isabellina* when growing on agar (Hedlund *et al.*, 1991).

6.4.6 Some limitations of the experimental design

Microcosm studies are always limiting in that there are restricted to arena size. In the present study the limitation was compounded by the fact that the fungi were interacted based on the radial extension of the mycelium instead of total biomass. As a consequence, the relatively sparse outgrowth of *R. bicolor* resulted in the total extra-resource *R. bicolor* mycelium at the commencement of the interaction being much lower than that of the others (Tordoff *et al.*, 2006). Quantitatively comparable mycelial damage would, therefore, be much greater as a proportion of total mycelium

in *R. bicolor*. Although comparisons between mycelia of equal hyphal coverage, and therefore differing diameter, will improve equality (Tordoff *et al.*, 2006), the resultant varying distances between wood blocks would unbalance the design. *H. fasciculare*, which grows as a diffuse mycelium with broad 'exploitative' growth search front, will encounter all in its path and employs a strategy ideal for colonising homogenously distributed resources such as leaves and small woody debris (Boddy, 1999). *R. bicolor* on the other hand exhibits 'exploratative' growth, in which a less dense narrower search front allows searching for non-homogeneously distributed resources such as fallen branches (Boddy, 1999). While these two strategies are adapted to growth and development in the field, in the microcosm the relative advantages may be lost due to the lack of additional uncolonised resources.

6.4.7 Conclusions

Mycophagous collembola are dependent on fungi to make recalcitrant nutrients accessible, and invertebrate grazing of the microbial community (including fungi) can enhance the rate of carbon mineralisation (Bardgett *et al.*, 1993a; Cole *et al.*, 2000). Collembola may also play a role in determining species combativeness during interactions with potential knock-on effects for species assemblages. According to the work presented here, the fungal response to collembola grazing during interspecific interactions appears to result from a combination grazer species and the identity of interacting fungi. This adds another variable in affecting fungal interactions as outcomes are already known to alter with changes in abiotic conditions (Boddy, 2000), inoculum size (Holmer & Stenlid, 1993, 1997) and species composition (Boddy, 2000; Holmer & Stenlid, 1993; Wald *et al.*, 2004a). Fungal grazers may be important in determining interaction outcome, acting as a further variable to maintain the diversity of fungal species on the forest floor.

7.0 Changes in the network architecture of *Phanerochaete velutina* when interacting with *Hypholoma fasciculare* in collembola grazed and ungrazed systems.

7.1 Introduction

Cord-forming saprotrophic basidiomycete fungi form extensive networks across the forest floor linking discrete resources (Boddy, 1993). These cord networks are dynamic, constantly searching for new resources and capable of translocating water and nutrients to sites of need or storage (Watkinson *et al.*, 2005). In addition to exploration, such network-forming fungi employ a sit-and-wait strategy in which resources such as branches which fall onto the existing network can be rapidly colonised in advance of other competitors (Boddy, 1999; Boddy & Jones, 2007). As well as extending into new territory, regions of the network also regress and disappear. Such patterns are particularly evident in the fairy ring fungus (*Clitocybe nebularis*) which extends out from a point as a ring with mycelium at the trailing edge of the ring regressing (Dowson *et al.*, 1989). Interestingly, this mycelial regression does not reflect a depletion of resources or an accumulation of toxins (Dowson *et al.*, 1989).

In wood-decay fungi, initial growth from a resource is often dense, filling much of the space over which it grows (Tlalka *et al.*, 2008b). Over time, however, the fine hyphae regress leaving a predominance of thicker cords. Microcosm studies of wood-decay fungi encountering new uncolonised resources show a gradual regression of hyphae between the original and the newly colonised resource following initial colonisation leaving a thick cord linking the two resources (Donnelly & Boddy, 1997b). The fungus then grows out from the second resource to continue foraging for new resources and the link between the two wood blocks can serve as a major transport connection allowing bidirectional movement of nutrients as required (Harris & Boddy, 2005, Wells *et al.*, 1998b). Similarly, during aggressive interactions, changes in mycelial organisation are also seen with reallocation of fungal biomass to the site of interaction, and regression of hyphae and cords in other regions of the mycelium (Boddy, 2000; Donnelly & Boddy, 2001; see Section 2.2.4).

Another factor affecting mycelial morphology and network architecture is invertebrate grazing. This is generally selective with fine hyphae being preferentially consumed often leaving only thick cords. Mycelial extension (Tordoff *et al.*, 2006) and colony uniformity (Hedlund *et al.*, 1991) can be dramatically altered through grazing activity. Fungal grazers also affect mycelial morphology; for example, the hyphal tips of *P. velutina* become highly fanned when grazed by *F. candida* (Tordoff *et al.*, 2006; Chapter 6). The effects on network architecture may include, for example, the removal by grazing of small links connecting two or more larger cords which may lead to a loss of efficiency in moving nutrients around the network.

Studies of cord-forming decay fungi using compressed soil microcosms with a precolonised wood block inoculum oblige the fungus to grow across the soil surface in two dimensions. Such a system reflects, to some degree, natural fungal growth as cord-forming saprotrophs tend to grow at the soil-litter interface. Microcosms are useful as changes in fungal morphology through resource encounter, interactions and grazing are easily visualised. Quantitative changes such as extension rate (Chapter 6) and an estimate of biomass, measured by calculating the number of pixels comprising a digital image of the mycelium, are also possible (Boddy, 1999). In more detailed assessments fractal dimensions of a mycelium can be estimated. Fungal mycelia exhibit a fractal structure; with smaller subsections of the mycelium mirroring larger-scale patterns albeit over limited scales. Fractal analysis of mycelial images provide information about how a mycelium permeates space and such studies have revealed subtle changes in mycelial architecture not exposed by radial extension, biomass measurements and qualitative observations (Boddy *et al.*, 1999).

Both biomass and fractal analysis require a sharp contrast between the mycelium and the substrate over which the mycelium is growing. During fungal interactions, however, contact with, or overgrowth of, opposing mycelia removes the contrast against the soil. Whilst possible (Donnelly & Boddy, 2001), separating out images manually can be inaccurate as images, necessarily magnified to distinguish between species, loose resolution. It is also a very time consuming process especially with highly intricate mycelia such as *P. impudicus*. This difficulty can, however, be overcome by adopting recent developments in network architecture analysis through creating a digitised representation of the mycelial network as a graph (Albert &

Barabási, 2002). In brief, this involves plotting a series of connected nodes at branches and interconnections (anastomoses) in the mycelium (Fricker *et al.*, 2007). By mapping the network over time changes in architecture, such as the formation of cross-linkages which increase resilience to damage, and regression of mycelia can be examined. In addition, by correlating the brightness of the mycelial cords in digital images with actual measurements of cord diameter and length, an estimate of both the transport value and cost of production of cords within the mycelium can be established (Fricker *et al.*, 2007). Such data, can provide precise quantitative analysis on the dynamics of interacting mycelia.

The preliminary study, described in this chapter, seeks to investigate the use of digital networks for the mapping and analysis of the effects of both fungal interaction and collembola grazing on mycelial organisation. As well as applying a relatively new methodology, it is hypothesised that in interaction with *H. fasciculare*, *P. velutina* will reinforce its mycelial network structure towards the interaction area while away from the interaction site, mycelial regression will be observed. Systems with collembola grazing are predicted to show a reduced the transport efficiency in *P. velutina* when compared to ungrazed systems due the damage to cords caused by collembola grazing. The biomass (or cost) of the network, is hypothesised to be greater in ungrazed systems than in grazed ones as collembola grazing removes mycelium.

7.2 Materials and Methods

7.2.1 Network digitisation

Details of the experimental set-up and data recording are given in Chapter 6 and images from that experiment were used in this study. Three replicates each of grazed and un-grazed systems were selected using randomly generated numbers from 10 available. Six images for each replicate were selected at t = 0, 4, 8, 12, 20 and 34 d after collembola addition. Each image was cropped to remove background, resized to 1773 x 1773 pixels, and saved as 8-bit greyscale .tif images. Image series were imported into a custom MatLab (The Mathworks Inc., Natick, USA) program and aligned with respect to one another. Alignment was achieved by selecting consistent landmarks on successive images, and calculating a linear spatial transformation to correct for translation, rotation and scaling. The network was extracted as a series of N nodes, each representing a branch or anastomosis, joined by a set of K links

representing the intervening cords. Node positions were stored as a list of their Cartesian (x,y) coordinates, whilst links were stored as a weighted $N \times N$ adjacency matrix, where each entry represents the diameter of the link between node i and node j (D_{ij}). As the structure of the network within the wood blocks cannot be characterised, the inoculum was represented as a single central node with multiple links leading to the cords emanating from the edge of the block (Fig7.1). At each time point new growth was added as new nodes (with associated links), and complete regression was identified by disconnecting the relevant nodes. At this stage it is not possible to discriminate a genuine cord-fusion event from cords that are growing over each other. This will cause an over-estimate of anastomoses, particularly early in development before proper junctions have had time to become established. Nevertheless, manual dissection of fully networked systems shows that more established overlying cords are almost invariably linked in *P. velutina*. Nodes connected to only two other nodes (termed k2 nodes), representing a bend in a cord were removed from the adjacency matrix during analysis and the weight of the resultant link between the junctions at either end adjusted to take into account the length and thickness of the intervening links.

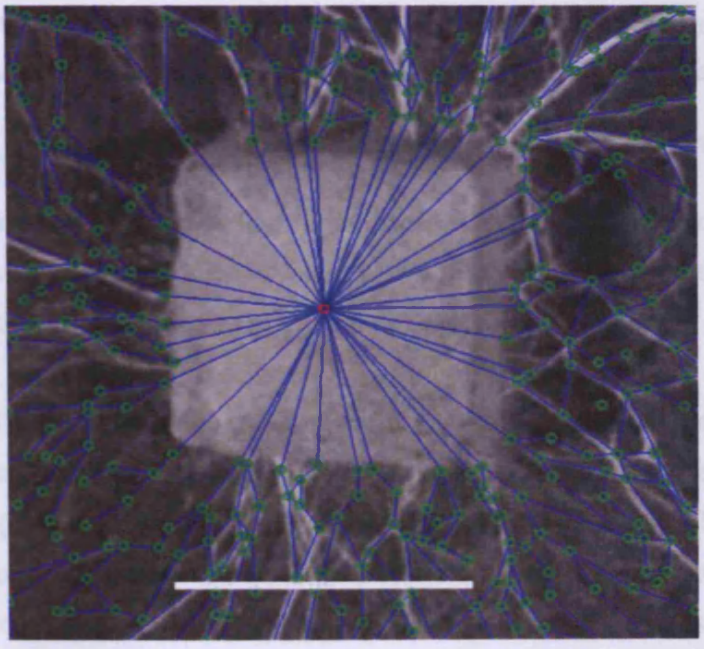
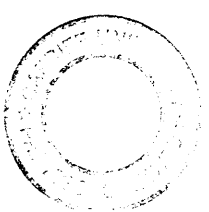


Fig. 7.1: Magnified image (scale bar 2 cm) of the network from central inoculum at 20 d. The central (red) node represents the inoculum attached to points of cord outgrowth (green).

7.2.2 Estimation of link weights

As cords differ in thickness, the links in the adjacency matrix were weighted by an estimate of the cord diameter between node i and node j (D_{ij}). The pixel resolution of the images was not sufficient to obtain direct measurements of diameter, so an average value of the local reflectance intensity was used as a proxy for cord thickness. Samples were taken 12 pixels away from each node along each cord to ensure that only intensities of the cord of interest were included. The local neighbourhood was averaged using a Gaussian smoothing filter with a radius of 5 pixels and the maximum intensity recorded. The values from both ends of each cord were averaged to give the intensity for that cord. Intensities were converted to diameter using a calibration based on the measured relationship between the reflected intensity of *P. velutina* cords with actual diameter (Bebber *et al.* 2007). The cost of each link was estimated from its length (l_{ij}) times the cross-sectional area ($a_{ij} = \pi(D_{ij}/2)^2$), whilst the predicted resistance to transport was calculated as $l_{ij}a_{ij}^{-1}$, making the simplistic assumption that a cord comprises a circular bundle of equally sized hyphae. The link weight was colour-coded across the network according to a rainbow scale, with red representing thick cords. Development or regression of links was measured as the sum of the differences in cord diameter for successive time points, $\Delta D_{ij} = D_{ij(t+1)} - D_{ij(t)}$, over the interval 8 to 34 d, normalised to the maximum range of the difference, $\sum_{t=8}^{t=34} \Delta D_{ij} \Big/ \left(D_{ij,\max} - D_{ij,\min}\right)$. This gives a value of 1 for consistently growing cords, a value of -1 for cords that shrink and 0 for cords that essentially remain constant through the time interval. Results for the link evolution were expressed as a rainbow scale, with red representing growth.

The weighted adjacency matrices, node positions and node identities were exported to R 1.9.0 (R Development Core Team, 2007) to evaluate network structure and performance (Bebber *et al.* 2007; Fricker *et al.* 2007). The area covered by the mycelium was determined from the convex hull encompassing all the outermost nodes. The total cost or biomass was estimated from the sum of the costs for each link, $\sum_{i=1}^{N} l_{ij}a_{ij}$. The predicted transport capability of the network was based on the path of least resistance between each node in the functional network, calculated using a shortest path algorithm (Gross & Yellen 2005; Carey & Long 2008), with the



assumption that low node-to-node resistance indicated better connection and more efficient nutrient distribution. The transport performance of the network was summarised as the root or local network efficiency (E_{loc}), defined as the mean of the reciprocal of shortest path lengths from the inoculum to all other nodes (Latora & Marchiori 2001, 2003). To examine the effectiveness of the way in which the fungus allocates biomass by differentially weighting cords, these measures were also calculated for networks with the same topology, but with uniform allocation of biomass to all areas of the network creating cords of equal weight (Bebber *et al.* 2007; Fricker *et al.* 2008).

7.2.3 Statistical analysis

Network development in grazed and un-grazed systems was compared using analysis of covariance. The analysis was performed for convex hull against root efficiency and convex hull against cost. For the root efficiency against convex hull data, comparisons were made between actual data and between model data assuming uniform distribution of biomass across all links. In all cases the model contained convex hull (area), treatment status (grazed or un-grazed) and an interaction between convex hull and treatment: the latter was of primary interest, testing for a difference in root efficiency-area or cost-area relationship in the presence of grazing. Because of pseudo-replication (inherent in time series on the same replicate), the linear regressions were fitted using generalised estimating equations (GEE), which adjust the regression model to allow for the potential correlations within trays, to permit valid statistical inference (Liang & Zeger, 1986). As observations were taken over time, the correlation was modelled with a first order autoregressive structure (Liang & Zeger, 1986). Models were fitted using the GEE generalised linear model (GLM) function in the geepack library in R. This form of analysis has been proposed as a suitable method for analysing datasets containing individual data points which are not statistically independent of one another, a phenomenon that is common in ecological research (Vaughan *et al.* 2007).

7.3 Results

7.3.1 Network development

P. velutina mycelia were more extensive by 34 d in ungrazed than in grazed systems, although the amount of new growth between 8 d and 34 d was greater in grazed

systems (Fig. 7.2). When grazed the mycelial growth of *P. velutina* was substantially greater over the mycelium and inoculum of *H. fasciculare* than when ungrazed (Fig. 7.2). In the grazed systems, the *P. velutina* mycelium enveloped the *H. fasciculare* inoculum with cords turning in toward the inoculum from both sides (Fig 7.2 d-f). There was a marked regression of *P. velutina* mycelium in all ungrazed systems between 8 d and 34 d and this was predominantly in areas away from the interaction. There was far less regression in the grazed systems although one replicate did show more regression than the other two and this too, was in areas away from the interaction (Fig. 7.2 e). New cross-links connecting radial cords away from the inoculum formed in grazed systems and was especially pronounced in two replicates (Fig. 7.2 d, f). Whilst there were cross-links in ungrazed systems there were fewer and many of them were cross-links that existed prior to 8 d (Fig. 7.2 a-c).

7.3.2 Changes in cord thickness

In general there were more, thick *P. velutina* cords (defined by link weight) in grazed systems than in ungrazed systems. These were particularly pronounced over the opponent *H. fasciculare* mycelium (Figs. 7.3, 7.4). As the *P. velutina* network developed in ungrazed systems (0 d – 8 d) about 12-15 major cords developed and these were connected through the network of narrower cords (Fig. 7.3 a-c). By 8 d, link weight development was polarised, with wider links developing towards the opposing mycelium (Fig. 7.3 c). Initial development in grazed systems was similar (Fig. 7.3 a, b) but polarised growth in the direction of the interactions was substantially more pronounced and was clear by 4 d (Fig. 7.3 b). The development of *P. velutina* during later stages of the interaction (12 d – 34 d) in ungrazed systems showed marked regression of mycelia from 12 d onwards (Fig. 7.3 d-f). Whilst this regression of links was most pronounced away from the interaction, it occurred across the entire mycelium including toward the opponent with both cross-links and major cords regressing by 34 d (Fig. 7.3 f). In contrast the *P. velutina* mycelium in grazed systems continued to develop over and toward the opponent mycelium with substantial regression occurring only in small cross-links away from the interaction zone (Fig. 7.4 d-f).

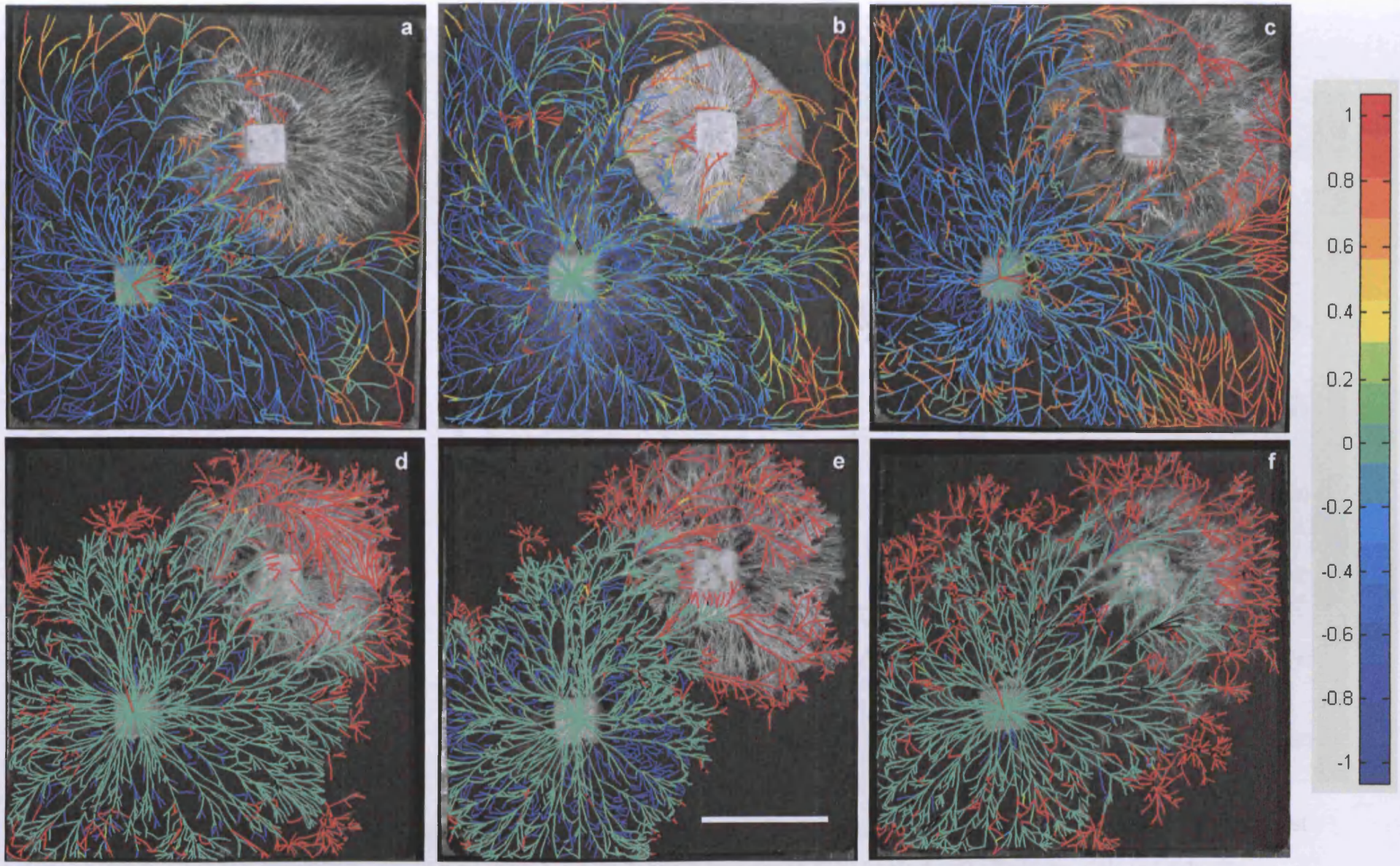


Fig. 7.2: Link evolution of *P. velutina* when interacting with *H. fasciculare* in ungrazed (a-c) and grazed (d-f) between 8 d and 34 d (White scale bar 6 cm). Rainbow scale bar values range from +1 for new growth, zero for no change to -1 for complete disappearance of a given link.

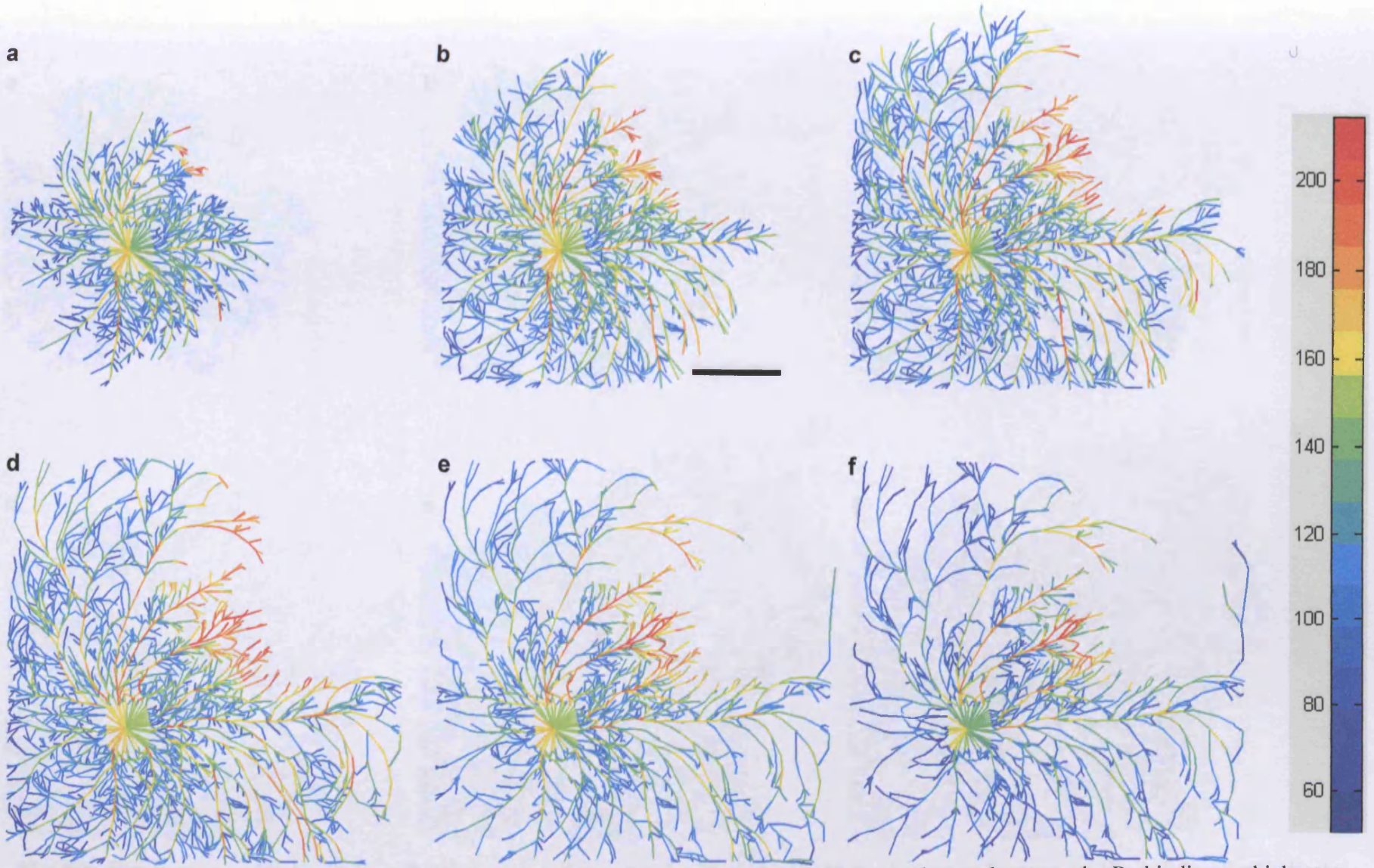


Fig. 7.3: Development of link weight in *P. velutina* mycelium when interacting with *H. fasciculare* and ungrazed. Red indicates thickest cords. Images are from (a) 0 d (b) 4 d, (c) 8 d, (d) 12 d, (e) 20 d and (f) 34 d (black scale bar is 4 cm). Rainbow scale bar indicates relative cord thickness.

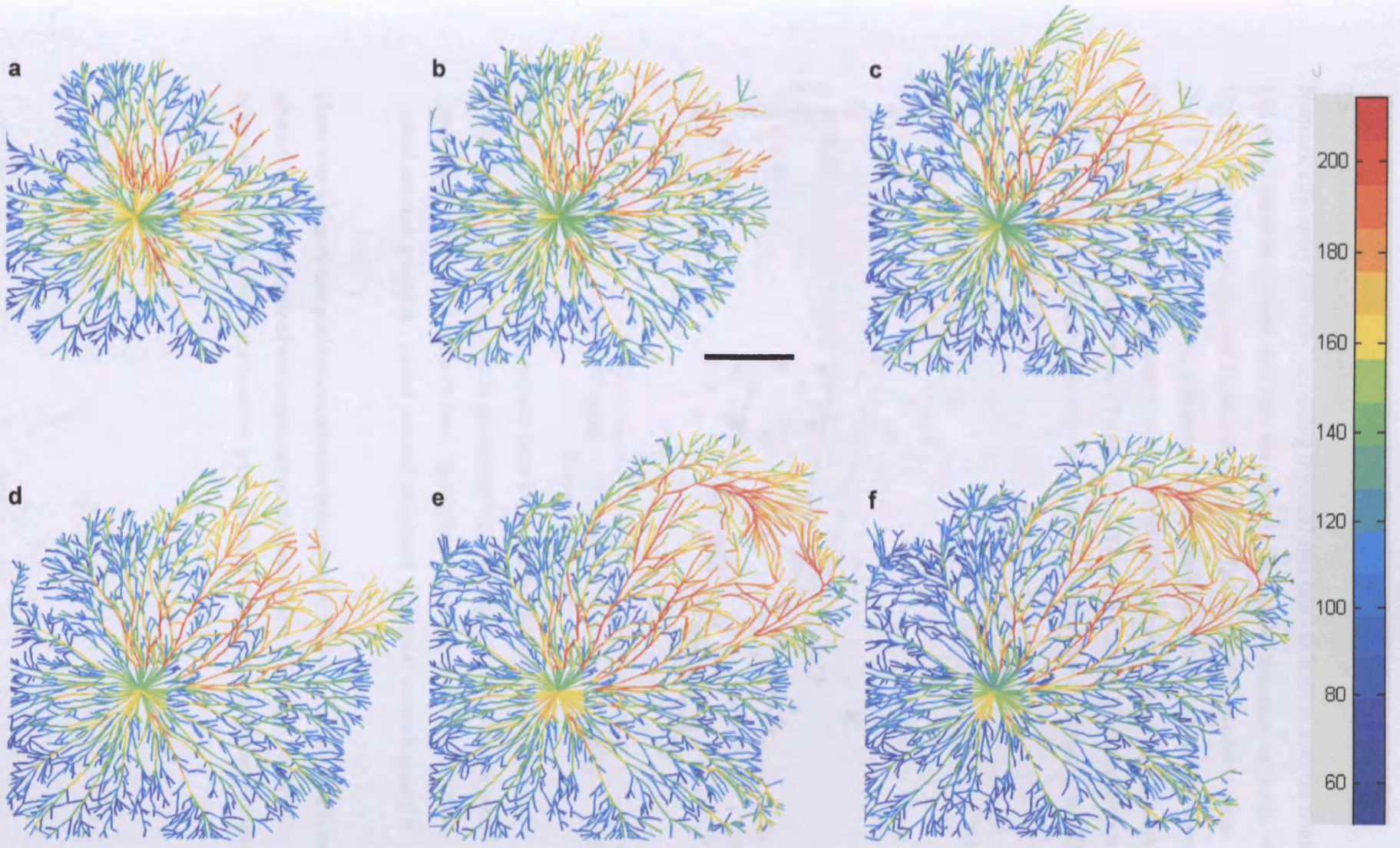


Fig. 7.4: Development of link weight in *P. velutina* mycelium when interacting with *H. fasciculare* and grazed by *F. candida*. Red indicates thickest cords. Images are from (a) 0 d (b) 4 d, (c) 8 d, (d) 12 d, (e) 20 d and (f) 34 d (black scale bar is 4 cm). Rainbow scale bar indicates relative cord thickness.

7.3.3 Network transport efficiency

Network efficiency fell with increasing mycelial area (convex hull) in both the model and actual network systems although the overall efficiency of the actual networks on a like-for-like comparison, was significantly greater than those of the model systems (Fig. 7.5, Table 7.1 c, d). The regression line for the actual grazed network indicated the greatest efficiency in relation to area covered although this was not significantly greater than the ungrazed system (Table 7.1 a). There was no significant difference between the slopes of grazed and ungrazed interactions in either the actual or model networks (Table 7.1 a, b).

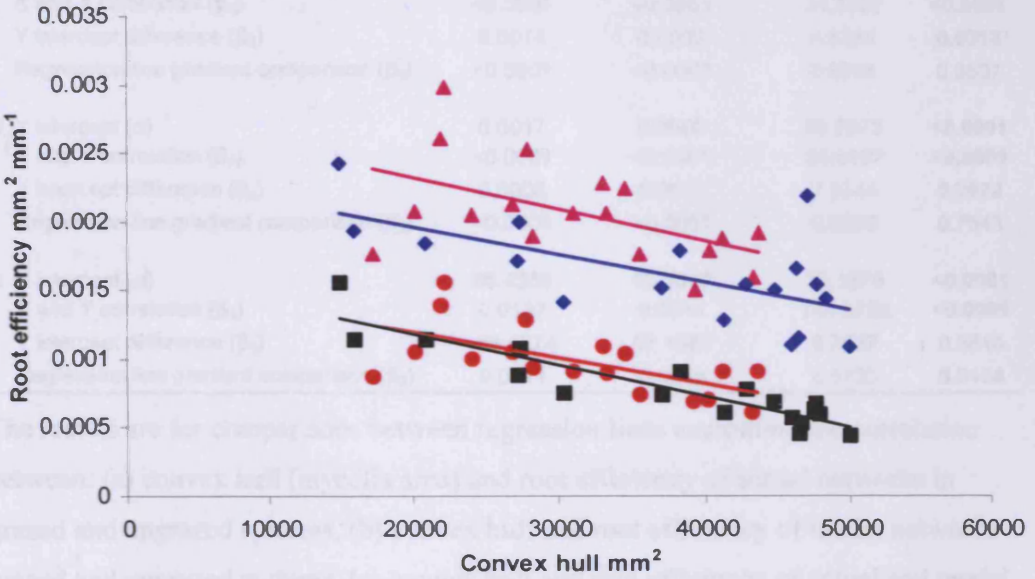


Fig 7.5: Scatter plot and regression lines for root efficiency against convex hull (mycelial area) for *P. velutina* in grazed and ungrazed systems and for both actual and uniform biomass model network data. Symbols are; actual network ungrazed ◆, actual network grazed ▲, model network ungrazed ■, model network grazed ●.

There was a significant positive correlation between mycelial area (convex hull) and network cost in both grazed and ungrazed treatments (Table 7.1; Fig.7.6). With increasing area the cost of the network grew faster in grazed than ungrazed systems (Fig 7.6).

Table 7.1: ANCOVA results for the five regression comparisons made

	Descriptor	Coefficient	Standard error	Critical value (df = 1,32)	P-value
a	Y intercept (α)	0.0025	0.0002	112.2588	**<0.0001**
	X and Y correlation (β_1)	<0.0001	<0.0001	7.8056	**0.0052**
	Y intercept difference (β_2)	0.0007	0.0006	1.3886	0.2386
	Regression line gradient comparison (β_3)	<0.0001	<0.0001	1.0843	0.2977
b	Y intercept (α)	0.0017	0.0002	73.8352	**<0.0001**
	X and Y correlation (β_1)	<0.0001	<0.0001	31.4800	**<0.0001**
	Y intercept difference (β_2)	0.0001	0.0004	0.0835	0.7727
	Regression line gradient comparison (β_3)	<0.0001	<0.0001	0.0699	0.7914
c	Y intercept (α)	0.0018	0.0004	21.6787	**<0.0001**
	X and Y correlation (β_1)	<0.0001	<0.0001	14.3782	**<0.0001**
	Y intercept difference (β_2)	0.0014	0.0007	4.6354	**0.0313**
	Regression line gradient comparison (β_3)	<0.0001	<0.0001	0.8708	0.3507
d	Y intercept (α)	0.0017	0.0002	69.2373	**<0.0001**
	X and Y correlation (β_1)	<0.0001	<0.0001	29.6107	**<0.0001**
	Y intercept difference (β_2)	0.0008	0.0003	7.2344	**0.0072**
	Regression line gradient comparison (β_3)	<0.0001	<0.0001	0.0980	0.7543
e	Y intercept (α)	86.4358	13.9927	38.1579	**<0.0001**
	X and Y correlation (β_1)	0.0137	0.0011	143.5756	**<0.0001**
	Y intercept difference (β_2)	-58.4274	67.1661	0.7567	0.3844
	Regression line gradient comparison (β_3)	0.0111	0.0043	6.5733	**0.0104**

The results are for comparisons between regression lines examining the correlation between: (a) convex hull (mycelia area) and root efficiency of actual networks in grazed and ungrazed systems, (b) convex hull and root efficiency of model networks grazed and ungrazed systems, (c) convex hull and root efficiency of actual and model networks in grazed systems, (d) convex hull and root efficiency of actual and model networks in ungrazed systems, and (e) convex hull and network cost of actual networks in grazed and ungrazed systems. For each ANCOVA: Y intercept is the estimate and standard error for the grazed (a,b,e) or actual (c,d,e) system and significance indicates a difference from zero; X and Y correlation shows whether both regression lines are correlated, Y intercept difference indicates any significant difference between the Y intercepts of the two regression lines is indicated, and the gradient comparison shows whether the two regression lines have a different gradient. Values in bold are significant at $P<0.05$. To calculate the regression lines for any comparison the formula $Y= \alpha + (\beta_1 * \text{Area}) + (\beta_2 * \text{Treatment}) + (\beta_3 * \text{Area} * \text{Treatment})$ is employed where the treatment value is 0 for ungrazed (or model in actual vs. model comparisons) and 1 for grazed or actual systems. β-values are tabulated coefficients.

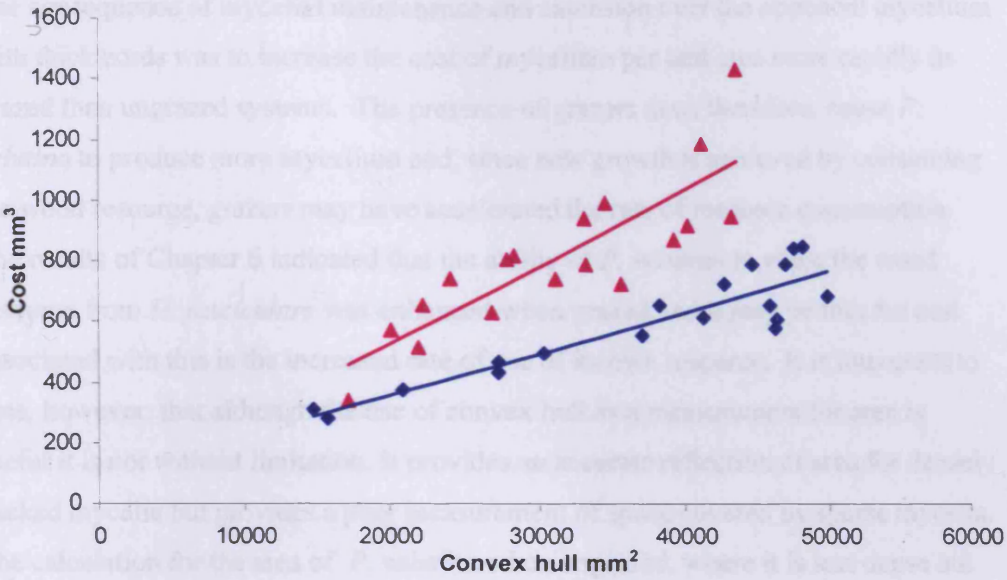


Fig 7.6: Scatter plot and regression lines for estimated cost of network construction against convex hull (mycelial area) for *P. velutina* in grazed ▲ and ungrazed ◆ systems.

7.4 Discussion

7.4.1 Network architecture and growth strategy

The change in biomass allocation over time in both grazed and ungrazed trays was clearly highlighted by the methodology employed. This was mycelial regression in both ungrazed and grazed systems away from the mycelial interaction zone. The regression in ungrazed trays was more substantial than the grazed ones suggesting that, during interactions with other fungi, more links are retained when under grazing attack. This is somewhat surprising as collembola grazing may be expected to remove hyphae and any moribund cords (Wood *et al.*, 2006). The combination of the interaction with *H. fasciculare* and grazing pressure not only reduces *P. velutina* radial extent over soil (Ch. 6) but also appears to reduce the reallocation of mycelial biomass. The formation of substantial cross-links towards the edge of the mycelium indicates the production of a more integrated network across which nutrients may be moved without the need to pass via the inoculum. Maintenance of the entire mycelium coupled with the formation of cross-links may improve the ability of the mycelium to move nutrients to the interaction zone, a site of rapid extension.

The consequence of mycelial maintenance and extension over the opponent mycelium with thick cords was to increase the cost of mycelium per unit area more rapidly in grazed than ungrazed systems. The presence of grazers may, therefore, cause *P. velutina* to produce more mycelium and, since new growth is achieved by consuming the wood resource, grazers may have accelerated the rate of resource consumption. The results of Chapter 6 indicated that the ability of *P. velutina* to wrest the wood resource from *H. fasciculare* was enhanced when grazed and it may be that the cost associated with this is the increased rate of use of its own resource. It is important to note, however, that although the use of convex hull as a measurement for area is useful it is not without limitation. It provides an accurate reflection of area for densely packed mycelia but provides a poor measurement of space covered by sparse mycelia. The calculation for the area of *P. velutina* when ungrazed, where it is less dense but more extensive than in grazed systems, may result in an over estimate of mycelial area compared to grazed systems. This has implications of the correlations performed in this study. There is, however, one advantage in using convex hull over using pixel counts as a measure of mycelial area in small microcosm systems. Litter falling onto a network only needs to be in contact with one small part of the network for it to be colonised by the fungus and, therefore, provided that litter falling onto a network is larger than the space between two cords, it is available for colonisation. The convex hull measurement gives a good estimation of the soil occupied by a mycelium and therefore the area over which that mycelium would probably be one of the first to arrive at the new resource providing it with an advantage over competitors. Convex hull may best be considered as an indicator of the ability of a mycelium to employ a sit-and wait strategy rather than as an estimate for hyphal coverage.

7.4.2 Network efficiency and biomass allocation

The decline in network transport efficiency (measured as root efficiency) with increasing area (convex hull) is to be expected as the distance over which nutrients are transported growth with increasing mycelial extent. Less predictable, however, was the lack of significant difference between the grazed and ungrazed systems. This suggests that collembola grazing does not reduce the ability of the network to transport nutrients either to or from the inoculum. This result demonstrates resilience in the *P. velutina* network as it successfully maintained efficient transport pathways despite substantial morphological alteration by grazing. Previous studies of *P.*

velutina growing alone have suggested that *F. candida* grazing activity is concentrated on the fine mycelium and that substantial cords are largely unaffected (Tordoff *et al.*, 2006). That *P. velutina* exhibits the same resilience during an interaction is particularly important due to the likely need for nutrient transport to the interaction site as it is an area of heightened metabolic activity (Baldrian, 2004).

In addition to not reducing transport efficiency, grazing did not alter the rate of efficiency change with increasing area (no significant difference in the slopes of the two regression lines). This contrasts with the effect of grazing on mycelial cost (discussed above) and further highlights the resilience of the network to grazing even as mycelial coverage became larger.

The comparison of the actual network to a model network with uniform allocation of biomass across all links demonstrated the effectiveness of *P. velutina* to 'invest' in cords in an optimal manner. This comparison has a potential use in experimental setups to test the effects of different disturbances on the network and its resilience. It would also allow comparison between species to determine whether some are better able to allocate biomass to optimise transport efficiency.

7.4.3 Methodology and future possibilities

The mapping and examination of fungal networks provides highly informative qualitative and quantitative information on mycelial development. Variables such as transport efficiency and resilience can be modelled (Fricker *et al.*, 2007) more simply than they can be tested experimentally. The methodology employed here is most effective when empirical data for real systems can be added to models to examine mycelial systems. For example, knowing that cords of *P. velutina* are formed from bundles of aligned hyphae (Bebber *et al.*, 2007) reduces the risk of an overestimation of transport rates which would occur if cord volume was calculated to be related to the quadratic of the cord radius (r^4) as opposed to the square (r^2; Fricker *et al.*, 2007). Further work is, however, required to improve modelling techniques. For example, transport efficiency modelling is limited by the current understanding of transport within cords. The current transport model assumes bidirectional transport at a fixed rate based on cord volume estimates. It is unlikely that all nutrients travel at an

equitable rate and that movement on one direction is the same as movement in the reverse.

Current quantitative analysis of the cord network is limited to the entire system but, as the results of this and other studies show, fungal development is often polarised particularly when another variable such as a resource (Bebber *et al.*, 2007), opposing fungus (Donnelly & Boddy, 1997b) or grazer (Kampichler *et al.*, 2004) is present. An useful extension of this analysis would be, therefore, to permit comparisons within regions of the network Potentially useful comparisons within the network include network connectivity and relationships between mycelial regression and extension or reinforcement. Continued development of the method along with more supporting empirical evidence with which the models used can be improved may result in an analysis tool of greater power for studying the development of fungal mycelia and their responses to a wide variety of variables.

8.0 Synthesis

8.1 Experimental studies

8.1.1 The effect of collembola on fungal morphology

Whilst both collembola grazing on fungi and combative fungal interactions have been investigated, there is little, if any, published work on the effects of collembola grazing on fungal interactions. In addition, the effects of such fungal interactions on collembola behaviour also appear not to have been reported. The experiments in this study are believed to be the first to investigate the effects of collembola grazing on fungal interactions.

Collembola had a limited impact on fungal morphology in agar microcosms (Ch. 3) but, when extending out across soil, the morphology of two of the fungal species, *R. bicolor* and *P. velutina*, was substantially altered. Removal of entire cords was observed in the former, and the production of fanned mycelia and reduced extension over soil in the latter. Substrate type is known to be an important factor in determining the combativeness of fungi (Dowson *et al.*, 1988a) but these experiments suggest that substrate identity also effects the foraging behaviour of the grazers that feed upon them. Despite the differences between the two substrates, there was one interesting similarity, the accelerated growth of *P. velutina* over *H. fasciculare* mycelium when grazed. The behaviour was consistent across replicates and only occurred in this particular interaction. Of all the responses to grazing this is the most enigmatic, with no clear explanation as to why it may occur. The analysis of the network architecture of *P. velutina* when interacting with *H. fasciculare* suggested that grazing pressure increased the cost of production of new mycelium with increasing mycelial area (Ch. 7). It would be interesting to explore this interaction in greater detail to determine what causes this change in *P. velutina* and why it only occurred when interacting with one of the three opponents against which it was pitted.

8.1.2 The effect of grazing on fungal combative ability during interactions

Eventual interaction outcomes were generally not markedly altered by invertebrate grazing in either agar or soil microcosms. This is surprising given the dramatic changes in fungal morphology caused by collembola grazing, particularly in soil systems. One possible explanation is that the collembola had very limited, if any,

access to the mycelia within the resource (agar or wood). If so, grazing could not take place, and the competition within the resource would have occurred in the absence of grazing. This is, however, not inappropriate as invertebrates cannot gain access to the wood interior until substantial decay has occurred (Rayner & Boddy, 1988). Another possibility is that the experiments were run for too short a time period for combat differences to manifest themselves under grazed or ungrazed conditions. In addition, the experimental design involved the addition of collembola once the fungi had met. Given the reduction of radial extension of the study species when growing alone (Harold *et al.*, 2005; Tordoff *et al.*, 2006), invertebrate grazing, which is active before fungi have met in the field, may have effects on the mycelium before the fungi meet resulting in a change in their combative status. It would be appropriate to repeat the experiment adding collembola at different times to determine their effect in this respect.

8.1.3 Fungal genetic isolate

Studying multiple genetic isolates of the fungi revealed genetic identity to be important factor in determining both fungal and collembola responses during aggressive fungal interactions (Chs. 3, 4). Both collembola preference and mortality were strongly influenced by genetic isolate. For example, collembola mortality was low when *P. velutina* interacted with one isolate of *R. bicolor* but high when interacting with another *R. bicolor* isolate (Ch. 4). Interspecific fungal interactions were also highly dependent on fungal isolate with a range of combative abilities being shown among the four *H. fasciculare* isolates (Ch. 3). Studies of fungal interactions and of collembola grazing on mycelia have tended to be restricted to a single genetic isolate for each study species (e.g. Harold *et al.*, 2005; Hedlund *et al.*, 1991; Kampichler *et al.*, 2004; Tordoff *et al.*, 2008), but such results may not provide a complete picture of what occurs in field conditions. Extrapolation of results must, therefore, be done with caution.

8.2 Collembola behaviour

8.2.1 Collembola grazing preference

Grazing by *F. candida* in soil microcosms was discriminating, with fungal species such as *R. bicolor* being very heavily grazed, and others such as *P. impudicus* apparently little affected. Collembola grazed more heavily on particular regions of

the mycelium. For example, despite the mycelium as a whole being substantially affected by collembola grazing, the major cords of *P. velutina* remained intact maintaining the major transport links across the network (Ch. 7). The mycelium of *H. fasciculare* was totally removed by collembola when interacting with *P. velutina*. This result was surprising as, when grown alone, *H. fasciculare* is resilient to grazing and the thick, often yellow pigmented, cords are not grazed by *F. candida* even when at high densities (Harold *et al.*, 2005). The increased palatability of the *H. fasciculare* mycelium to the collembola may be due to a weakening of the mycelium as the wood block resource, from which it was supported, was overtaken by *P. velutina*. Such a dramatic change from resilience under grazing when growing alone to complete removal through the introduction of a competitor fungus highlights the importance of fungal interactions and the need to consider their effects when investigating fungal grazing. That complete removal of the *H. fasciculare* mycelium occurred in only one of the three combinations studied evidences the importance of fungal species identity in interactions. In addition, as collembola species identity is known to affect fungal extension and morphology (Tordoff *et al.*, 2008) it would be valuable to study the effect of different collembola species on this or other interactions.

8.2.2 Collembola attraction to the interaction zone

One of the aims of this thesis was to examine whether collembola are attracted to the zone where two interacting fungi contact one another. The combination of anecdotal evidence for attraction of fungus gnats (Mycetophilidae) to fungal interactions (Boddy *et al.*, 1983), the probable leakage of nutrients at the interaction zone (Wells & Boddy, 2002) and the ability of collembola to discriminate between fungal odours (Bengtsson *et al.*, 1991; Hedlund *et al.*, 1995) provided reasons for believing that such behaviour may be seen in collembola. There was clear evidence of preferential grazing at the interaction zone in both agar and soil microcosms (Ch. 3 and 6, respectively), but the experiment specifically aimed at looking at the effects of the volatile organic compounds (VOCs) released by interacting fungi (Ch. 5) failed to provide conclusive evidence of collembola arrestment at, or attraction to, the area. The lack of conclusive results from the collembola tracking study, in contradiction to repeated anecdotal reports, suggests that either the VOCs alone were insufficient to attract collembola or that the experimental design was not sensitive enough to detect a

behavioural response. There are few, if any, studies employing video-tracking investigating collembola movement, and its feasibility suggests continuation of this area of study. The experimental design employed was informed by work on parasitoid responses to infochemicals (e.g. Waage, 1978) but this may be inappropriate for collembola. One study investigating the responses of collembola to different mycelia only detected an expression of preference between a choice of two mycelia after about 5 h (Kaneda & Kaneko, 2004). As with the use of genetically distinct fungal isolates, the use of a variety of collembola would help provide a more accurate representation of the role of these arthropods in soil processes.

8.2.3 The importance of collembola species identity

The use of *F. candida* as a model collembola is widespread. This arises from the ease with which it is cultured, its relative abundance and short generation time. Whilst using model organisms is useful it is important to examine other organisms within the same or similar guilds. The difference between *F. candida* and *P. armata* in the soil tray microcosm study of Chapter 6 was pronounced with *P. armata* having no discernable effect on fungal morphology. This work corroborates other work which suggest that *F. candida* generally has a more pronounced effect than other collembola species when grazing on cord forming fungi growing alone (Tordoff *et al.*, 2008). In reality, however, *F. candida* may not be the most appropriate species for a given study. If a species other than *F. candida* were to be substantially more abundant in a given habitat, experiments on fungi within that habitat employing *F. candida* may attribute collembola a more substantial role than is justified. In addition, not all collembola are necessarily fungal feeders (Berg *et al.*, 2004) hence selecting the appropriate species, or range of species, is necessary if ecological conclusions are to be reliably drawn from the results.

8.3 The microcosm system

8.3.1 Benefits and limitations of the microcosm approach

Agar and soil microcosms have been widely used to study both fungi and invertebrate grazers (e.g. Dowson *et al.*, 1988a; Hedlund *et al.*, 1991; Bretherton *et al.*, 2006). Their appeal lies in their simplicity offering the capability to control a wide variety of variables such as abiotic conditions, growth substrates and flora and fauna. Such

precise control allows the manipulation of one variable (e.g. inoculum size) whilst keeping other factors, to a greater or lesser degree, constant or regulated. The principal advantage of the microcosm approach is, therefore, elucidating the role of individual variables. Microcosm experiments are also valuable when investigating areas of limited previous study where so little is known about a system that a mesocosm or field-scale experiment may yield results of limited value as explanations for the results cannot be given (this can also occur in microcosms as discussed above).

In reality, however, variables are often correlated and microcosm experiments generally fail to account for this. As seen in the present studies, substrate, fungal isolate identity and collembola species are all important factors in determining fungal interaction outcome. In these cases temperature, water potential and other fauna were controlled! To devise a microcosm experiment in which all variables are considered and varied relative to one another is logistically difficult, if not impractical. Whilst a useful tool, microcosms can simply form a part of research in a given area of ecology and can not be relied upon as a method for investigating all aspects of a system.

8.3.2 Future work – developing a mesocosm

There is now a growing body of research into collembola-fungal interactions and a logical next step would be to develop larger, less uniform and more representative systems (mesocosms) in which a range of fungal species and invertebrate grazers are employed. The soil microcosms employed in Chapters 6 and 7 are useful for examining changes in mycelial morphology but three dimensional systems with multiple fungal species and resources of varying size and status (colonised and uncolonised) may be more realistic. In the compressed soil microcosm used in this study, neither fungi nor collembola can avoid contact. In a three dimensional mesocosm, however, with a stratified soil structure and refugia, as occurs naturally, a range of behaviours employed by both fungi and invertebrates more representative of natural conditions and not evidenced in the microcosms may be observed. In such a system, fungal morphological change is not easily examined and will involve disturbance of the system. The continued exploration and development of imaging techniques such as the use of radiotracers and the techniques investigated in Chapter 7 may provide solutions to non-destructive study of mycelial change (Tlalka *et al.*, 2008a, b).

Collembola populations can increase dramatically in soil microcosms, particularly when *R. bicolor* is present (Tordoff *et al.*, 2008; T. Harward unpublished data). As with many soil arthropods, collembola aggregate (Hopkin, 1997) and the combination of high populations and aggregative behaviour may attract predators. A possible future path for research would be to introduce predators of fungal grazers to determine whether they are attracted to fungal grazer populations. If this were the case it may reveal a mechanism for controlling collembola populations that, when unchecked, can destroy an entire mycelium (Tordoff *et al.*, 2008). Of particular interest would be to determine whether fungi recruit predators of grazers following attack as has been seen in maize roots (*Zea mays*) when attacked by larvae of the western corn rootworm (*Diabrotica virgifera virgifera*; Rasmann *et al.*, 2005). Whilst soil fungi are known to emit volatile chemicals (e.g. Hynes *et al.*, 2007) it remains unknown whether these serve a functional role regarding invertebrates and their predators.

8.4 Conclusions

Fungal interactions can be substantially altered through collembola grazing activity although the substrate on which the mycelia grow plays an important role in determining the strength of that effect.

1. Fungal genetic isolate is an important variable in determining the outcome of fungal interactions and also affects both collembola preference and mortality.

2. Collembola grazing can dramatically alter the morphology of interacting fungi when growing across soil but appears to have limited effects in agar microcosms.

3. Collembola do appear to show increased grazing at the fungal interaction zone although it has not yet been possible to demonstrate attraction to fungal interaction volatiles.

4. Fungal combativeness can be affected by collembola grazing. Collembola species, and the identity of the interacting fungi and the substrate upon which

they are interacting, however, all play a role in determining the magnitude of that effect.

5. Collembola grazing is not random. It is directed at finer mycelium and is heavier on some species than on others.

6. The fungus with which another fungus is interacting can determine the level to which fungal grazing has an effect.

7. The network architecture of *P. velutina* is substantially altered by *F. candida* grazing when interacting with *H. fasciculare* but transport efficiency remains high.

Overall, these studies, have shown that collembola have a substantial impact on fungal interaction progression and outcome. In return, fungal interactions can also have an effect on collembola preference and survival. As the major agents of wood decay, saprotrophic fungi are central to woodland decomposer processes with different species operating different growth strategies and decomposing wood at varying rates (Rayner & Boddy, 1988). The possible impacts on fungi by collembola through preferential grazing altering fungal combativeness and morphology may have substantial implications for fungal species assemblage. The diversity of fungi present is likely, therefore, to have impacts on the rate of wood decay (Rayner & Boddy, 1988; Tordoff *et al.*, 2008), and the cycling and translocation of nutrients within woodland (Boddy & Watkinson, 1995). The possible role of collembola in maintaining fungal diversity on the woodland floor with potential implications for other organisms found in the soil food-web and even above-ground processes merits greater exploration.

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Appendix I Source and authority for study organisms.

Species and Authority	Isolate	Reference in thesis	Source
Fungi			
Hypholoma fasciculare (Huds.: Fr) Kummer	DD2	Hf 1	Cardiff University Culture Collection
	WV2	Hf 2	Cardiff University Culture Collection Isolated from fruit body August 2003 Trelleck Common (UK National Grid Reference SO517619)
	DD3	Hf 3	Cardiff University Culture Collection
	JHC002065	Hf 4	Cardiff University Culture Collection
Phallus impudicus (L.) Pers.	JHY4	Pi	Cardiff University Culture Collection Isolated from cord at fruit body base August 2003 Trelleck Common (UK National Grid Reference SO517619)
Phanerochaete velutina (DC.: Pers.) Parmasto	KC1685	Pv	Cardiff University Culture Collection
Resinicium bicolor (Abertini & Schwein.:Fr.) Parmasto	Rb1	Rb 1	University of Aberdeen UK
	RbM6A	Rb 2	Cardiff University Culture Collection
Collembola			
Folsomia candida Willem	-	-	CEH Lancaster, UK
Protaphorura armata Tullberg	-	-	National Environment Research Institute, Silkeborg, Denmark

Appendix II Publications associated with the thesis

Rotheray, T. D., Boddy, L., Jones., T. H. (2008) Collembola foraging responses to interacting fungi. *Ecological Entomology* (in press).

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