The role of fungal secondary metabolites in Collembola – fungi interactions

TECHNISCHE UNIVERSITÄT DARMSTADT

Vom Fachbereich Biologie der Technischen Universität Darmstadt

zur Erlangung des akademischen Grades

eines Doctor rerum naturalium

genehmigte Dissertation von

Dipl.-Biol. Swantje Staaden

aus Hamburg

Berichterstatter:	Prof. Dr. Stefan Scheu
Mitberichterstatter:	PD. Dr. Ulrich Brose
Tag der Einreichung:	17.12.2009
Tag der mündlichen Prüfung:	14.06.2010

Darmstadt 2010

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Nimm dir Zeit, den Himmel zu betrachten.

Suche Gestalten in den Wolken,

Höre das Wehen des Windes

und berühre das kalte Wasser.

Gehe mit leisen behutsamen Schritten.

Wir sind Eindringlinge

die von einem unendlichen Universum

nur für eine kurze Zeit geduldet werden.

(Indianische Weisheit)

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SUMMARY

Soil organisms, in particular fungi and decomposer insects are primary drivers of organic matter recycling and energy fluxes (Swift et al. 1979; Cadish and Giller 1997; Bardgett et al. 2005). Fungi play a crucial role in the cycling of carbon, nitrogen and phosphorus in terrestrial ecosystems functioning while having to deal in the same time with relentless attacks from fungivores. Only few studies, however, investigated the structuring forces of the population dynamics of fungi and the abundant decomposer fungivores, such as Collembola, with whom they continuously interact. This thesis investigated the interactions between fungi and Collembola focussing particularly on the effects of fungal secondary metabolites from different perspectives. Fungal secondary metabolites are believed to be one of the main vectors driving this interaction. Aiming to get specific insights into the nature of the mechanisms driving this interaction I focused on testing three overarching hypothesis:

H1. Fungal secondary compounds mediate the Collembola – fungi interaction

H2. Collembola have evolved means to detect fungal toxicity

H3. Genetic evidence (transcript regulation) can be used to understand the molecular nature of the Collembola – fungi interaction

The above three overarching hypothesis have been addressed in three experimental studies, each with several pointed hypothesis.

H1. The first experimental study consisted of a feeding choice experiment offering single and mixed fungal diets using labelled fungal species (C_3 and C_4; ^{13}C and ^{15}N) of different toxicity. Collembola fractionation and carbon/ nitrogen incorporation of fungal species were assessed via stable isotope analysis. Four knock out mutants of *Aspergillus nidulans* with the sterigmatocystin production blocked at different steps along the biosynthetic pathway were combined in mixed diets with either the high quality fungus *Cladosporium cladosporioides* or the low quality fungus *A. nidulans* (wildtype). This study aimed at understanding the impact of fungal secondary metabolites and more specifically sterigmatocystin (ST) on Collembola performance in single and mixed diets and stabile isotope fractionation. It was hypothesised that (i) presence of sterigmatocystin (ST) impairs Collembola performance with increasing fungal toxicity of the *A. nidulans* strains, (ii) mixed diets will be beneficial to Collemboal fitness due to toxin dilution and (iii) the fractionation of ^{13}C and ^{15}N it is more pronounced in more toxic diets. We found that ST generally but not uniformly diminished springtail

fitness partially supporting the idea that secondary compounds act as shield against fungivory. However, the use of knockout mutants *A. nidulans* of the ST pathway (S3-S6) led to rather idiosyncratic responses. Although Collembola fitness was not uniformly increased in mixed diets (suggesting a species specific response) the results still support the toxin dilution hypothesis since no correlation between fungal N content and ingestion could be found. Strong and specific responses of the two Collembola species to mixed diets, knock out mutants and toxins suggest the evolution of species specific strategies to cope with the constraints associated with living in different soil layers. The hypothesis suggesting a link between stable isotope fractionation and fungal toxins has been partially supported with the results suggesting that fungal toxin content may be more important than the nutrient content in controlling stable isotope fractionation of ^{13}C and ^{15}N.

H2. The second study focused on the olfactory ability of Collembola to perceive fungal toxicity via olfactory/volatile cues**.** By means of an olfactometer approach this experiment hypothesized that (i) Collembola are able to olfactorily perceive and distinguish fungal species/strains differing in secondary metabolism, (ii) that Collembola are able to sense and respond to fungal grazing by avoiding to forage on grazed fungi and that (iii) grazing by Collembola triggers in secondary metabolite gene expression in one Basidiomycete and one Ascomycete fungal species using a custom made cDNA microarrays (Chapter 3). All investigated Collembola species recognized fungal olfactory cues and directed their movement to fungal patches and moreover towards fungal strains with suppressed secondary metabolites, in particular towards the mutant ΔlaeA with the main part of secondary metabolites silenced. The volatile cues of conspecifically grazed fungi provoked a movement from two of the three Collembola species (*H. nitidus* and *S. furcifera*) towards ungrazed fungi. However, the response of *S. furcifera* was restricted to fungi extensively exposed to grazing (5 days) suggesting that the response varies between Collembola species. Surprisingly, the investigated fungal gene spectrum did not significantly respond to grazing by Collembola. The results support the first and second hypothesis indicating that Collembola are able to olfactorily differentiate fungi of different toxicity, orientate their movement towards more palatable fungi and avoid movement towards fungi previously exposed to grazing. The lack of changes in fungal gene regulation by grazing suggests that refined methods need to be adopted to investigate the genetic response of fungi to grazing.

H3. The third study investigated the impact of fungal secondary metabolites on transcript regulation of stress related expressed sequence tags (ESTs) of *Folsomia candida*, the Collembola species used as model species in ecotoxicology. *Aspergillus nidulans* wildtype (WT; Ascomycota) able to produce secondary metabolites including sterigmatocystin (ST) and a knockout mutant with reduced secondary metabolism (*A. nidulans* ΔLaeA) were combined with the high quality fungus *C. cladosporioides* as mixed diets or offered as single diets. I hypothesized that (i) *A. nidulans* WT triggers more genes associated with stress responses compared to the *A. nidulans* ΔlaeA strain with suppressed secondary metabolism, (ii) *C. cladosporioides* causes significantly different transcript regulation than the *A. nidulans* strains ΔlaeA and WT, and (iii) mixed diets will cause significantly different transcript expression levels than single diets. All three hypotheses are generally supported despite the fact that many functions of the affected ESTs are unknown. The results bring molecular evidence for the existence of a link between fungal secondary metabolites and responses in springtails supporting the hypothesis that fungal secondary metabolites act as a shield against fungivory.

Overall, the work conducted in this thesis suggests that fungal secondary metabolites act as a structuring force in Collembola-fungi interactions and population dynamics. Using multiple approaches (food choice, olfactory and genetical) the results brings new insights supporting the hypothesis that fungal secondary metabolites act as a shield against fungivory.

ZUSAMMENFASSUNG

Bodenorganismen, insbesondere Pilze und Mikroarthropoden bilden treibende Kräfte für die Wiederaufbereitung organischen Materials und beeinflussen hierdurch die Energieflüsse terrestrischer Ökosysteme. Pilze unterliegen einer Vielfalt antagonistischer Einflüsse, z.B. werden sie von Pilzfressern konsumiert. Potenzielle Abwehrmechanismen gegen Fraßfeinde, wie die im Boden häufig vorkommenden Collembolen, sind dabei wenig untersucht. In der vorliegenden Arbeit wurde der Einfluss von Sekundärmetaboliten von Pilzen auf Collembolen untersucht. In Analogie zu den detailliert untersuchten Wechselwirkungen zwischen Pflanzen und Herbivoren wurde angenommen, dass pilzliche Sekundärmetabolite eine wichtige Rolle für die Abwehr von Fraßfeinden spielen. Es wurden drei übergreifende Hypothesen untersucht:

H1. Sekundärmetabolite spielen eine wichtige Rolle in der Kommunikation zwischen Collembolen und Pilzen.

H2. Collembola detektieren olfaktorische Signale der Pilze, pilzliche Toxizität und modifizieren ihr Verhalten.

H3. Pilzliche Sekundärmetabolite in der Nahrung von Collembolen veränderen deren Genexpression, was in Transkriptanalysen detektiert werden kann.

Zur Untersuchung dieser Hypothesen wurden drei experimentelle Studien durchgeführt.

H1. Das erste Experiment untersuchte den Einfluss pilzlicher Sekundärmetabolite auf Reproduktionsparameter von Collembolen. Verfüttert wurden Einzel- und Mischdiäten bestehend aus Pilzarten/-stämmen verschiedener Toxizität, die mit stabilen Isotopen markiert waren (^{13}C und ^{15}N). Vier toxindefiziente knock out Mutanten von *Aspergillus nidulans,* eine regulatorische mit ausgeschalteter Sterigmatocystinproduktion und drei funktionale biosynthetische Vorstufen, wurden in einem Fütterungsexperiment als Einzel- und Mischdiäten eingesetzt. Der von Collembolen aufgenommene Kohlenstoff einzelner Pilze in Mischdiäten wurde durch den Einbau von stabilen Isotopen verfolgt. Die Untersuchung diente einem tieferen Verständnis der Bedeutung pilzlicher Sekundärmetabolite, insbesondere Sterigmatocystin, für die Fitness von Collembolen. Zudem wurde die Fraktionierung von stabilen Isotopen in Abhängigkeit pilzlicher Sekundärmetabolite in der Nahrung analysiert.

Es wurde angenommen, dass (i) die Präsenz von Sterigmatocystin und seiner Vorstufen in Mutanten von *A. nidulans* die Fitness von Collembolen beeinträchtigt, (ii) sich Mischkost durch Verdünnung toxischer Subtanzen vorteilhaft auf die Fitness von Collembolen auswirken, und dass (iii) die Fraktionierung von ^{13}C und ^{15}N im Gewebe von Collembolen mit steigender Toxizität der Pilzstämme zunimmt.

Tatsächlich veränderte Sterigmatocystin die Fitness der Collembolen, wobei die toxische Wirkung allerdings nicht parallel zu knockout Mutanten mit vermindertem Sekundärstoffwechsel abnahm. Die Hypothese, dass Mischdiäten zu einer Verdünnung von Toxinen führen, wurde generell bestätigt, wobei die untersuchten Collembolenarten jedoch unterschiedlich reagierten. Insgesamt weisen die Ergebnisse auf artspezifische Anpassungen von Collembolen an pilzliche Toxine hin.

Die Hypothese, dass die Fraktionierung stabiler Isotope (^{13}C und ^{15}N) von pilzlichen Toxinen abhängt, wurde teilweise bestätigt. Die Ergebnisse deuten daraufhin, dass pilzliche Toxine für die Fraktionierung stabiler Isotope von größerer Bedeutung sind als der Gehalt von Nährstoffen.

H2. Das zweite Experiment untersuchte die Fähigkeit von Collembolen, Toxizität von Pilzen durch olfaktorische Signale wahrzunehmen. In separaten Ansätzen wurden die folgenden Hypothesen geprüft: (i) Collembolen erkennen Pilzarten/–stämme mit variierendem Sekundärmetabolitgehalt olfaktorisch, (ii) Collembolen differenzieren zwischen Pilzen, die von Artgenossen befressen wurden, und nicht zuvor attackierten Pilzen, und (iii) Fraß von Pilzen durch Collembolen verändert die Transkription pilzlicher Sekundärmetabolite in Ascomycota (*A. nidulans*) und Basidiomycota (*Laccaria bicolor*).

Collembolen nahmen olfaktorische Signale von Pilzen wahr, was sich in veränderter Bewegungsrichtung äußerte. Sie bevorzugten dabei Pilzstämme mit vermindertem Gehalt von Sekundärmetaboliten, insbesondere *A. nidulans* ΔLaeA mit stark reduziertem Sekundärmetabolitanteil. Olfaktorische Signale von Pilzen, die von Artgenossen befressen worden waren, veränderten das Wahlverhalten bei zwei der drei getesteten Collembolaarten (*Heteromurus nitidus* und *Supraphorura furcifera*), wobei die Tiere den unbefressenen Pilz bevorzugten. Jedoch trat die Bevorzugung bei *S. furcifera* nur bei zuvor intensiv befressenen Pilzen auf, was wiederum für eine artspezifische Reaktion bei Collembolen spricht. Erstaunlicherweise wurde durch Fraß von Collembolen keine signifikante Änderung der Genexpression in *A. nidulans* und *L. bicolor* festgestellt, was allerdings auf methodische Limitierung zurückzuführen sein könnte. Insgesamt stützen die

Ergebnisse die erste und zweite Hypothese, was darauf hindeutet, dass das olfaktorische System von Collembolen in der Lage ist, Pilze entsprechend ihrer Toxizität zu differenzieren. Collembolen meiden zudem bereits befressene Pilze, was auf induzierte Abwehrsubstanzen in den Pilzen hindeutet.

H3. Das dritte Experiment untersuchte den Einfluss pilzlicher Sekundärmetabolite auf die Transkriptregulation eines Spektrums von Gensequenzen (ESTs) von *Folsomia candida*, einem Modellorganismus in der Ökotoxikologie. Der Wildtyp von *A. nidulans* (WT) mit vollständig exprimiertem Sekundärmetabolismus, einschließlich Sterigmatocystin (ST), die knock out Mutante *A. nidulans* ΔLaeA mit reduziertem Sekundärmetabolitanteil und der Referenzorganismus *Cladosporium cladosporioides* wurden in einem Fütterungsexperiment als Einzel- oder als Mischkost angeboten. Es wurde angenommen, dass (i) *A. nidulans* WT mit hohem Toxinanteil die Transkription von stressassoziierten Genen in *F. candida* stärker beeinflusst als *A. nidulans* ΔLaeA mit stark reduzierter Toxinbildung, dass (ii) *C. cladosporioides* die Transkription von Genen in *F. candida* weniger beeinflusst als die *A. nidulans* Stämme ΔLaeA and WT und, dass (iii) Mischdiäten verglichen mit Einzeldiäten andere Expressionsmuster verursachen.

Alle drei Hypothesen wurden generell unterstützt trotz der meist unbekannten Funktion der regulierten ESTs. Die Ergebnisse geben Hinweise auf die molekulare Wirkungsweise von pilzlichen Sekundärmetaboliten in Collembolen. Die Ergebnisse deuten damit auf evolutive Anpassungsmechanismen von Collembolen auf pilzliche Toxine hin.

Insgesamt bestätigen die Experimente die Hypothese, dass pilzliche Sekundärmetabolite als strukturierende Kraft in Collembolen-Pilz Wechselbeziehungen fungieren. Collembolen können den pilzlichen Sekundärstoffwechsel beeinflussen, wohingegen Pilze über konstitutive und induzierte Abwehrsubstanzen den Metabolismus und Lebenszyklus von Collembolen verändern. Die Verwendung von unterschiedlichen Methoden (stabile Isotopenanalyse, Verhaltenstests zur olfaktorischen Wahrnehmung und molekulare Analyse der Genexpression) erlaubte neue Einblicke in die Wechselbeziehung zwischen Pilzen und Pilzfressern. Die Ergebnisse unterstützen die Hypothese, dass pilzliche Sekundärmetabolite zur Abwehr von Fraßfeinden evolviert sind. Dies deutet auf kooevolutionäre Prozesse zwischen Pilzen und Collembolen als Pilzfresser hin.

1.1 DECOMPOSITION - AN ESSENTIAL PROCESS FOR ECOSYSTEM FUNCTIONING

Decomposition is the fundamental ecosystem process by which complex organic materials break down into simpler forms of organic and inorganic matter (Hayes 1979; Moore et al. 2004). It is an essential recycling process without which dead organic matter would accumulate irreversibly (Swift et al. 1979; Cadish and Giller 1997; Schlesinger 1997) and the development and growth of organisms would not be possible. It is also a vital part of the terrestrial biogeochemical cycles of carbon, nitrogen and phosphorus (Wardle 2002) and hence for plant growth and community structure (Hättenschwiler et al. 2005). Consequently, it determines plant growth and community structure (Wardle 2002; Bardgett 2005). The availability of macronutrients, such as nitrogen and phosphorus as well as sulphur, strongly depends on decomposition processes as these elements form an integral part of organic matter (Perry 1995).

During the decomposition process dead organic molecules are physically broken down and biochemically transformed into simpler molecules (Juma 1998) in a cascading process with two distinct phases. Solid organic matter is transformed into a liquid phase followed by vapour production. However, this process may also be described with four phases: the autolysis, the bloating (putrefaction), the decay (putrefaction and carnivores) and the drying (diagenesis) of matter (Statheropoulos et al. 2007; Eberhardt and Elliot 2008). The main three factors driving these transformations are: (I) the physicochemical environment, (II) litter quality and (III) the composition of the decomposer community (Berg et al. 1993; Brussaard 1994; Couteaux et al. 1995; Cadish and Giller 1997).

The fresh residues which enter the decomposition system contain plant litter, deceased microorganisms and animals (Swift et al. 1979; Berg and McClaugherty 2003; Bot and Benitez 2005). Plant derived residues predominantly consist of complex carbon compounds derived from plant cell walls (Mulder 2006), however different compounds decompose at different rates. Generally, sugars, starch and proteins are rapidly decomposed, cellulose, aliphatic and aromatic compounds slowly and lignin very slowly (Bot and Benitez 2005; Begon et al. 2009). Lignin (class of phenylpropanoids) comprises between 5-45% of plant dry mass and ~ 30% of all organic carbon in the biosphere (Goodman 2004) can only be degraded by specialised bacteria and fungi (particularly Basidiomycetes; Trojanowski et al. 1984).

Decomposition of structural compounds affects the stoichiometry of biota with implications for the long term decomposition patterns (Polis et al. 1997; Ruess and Ferris 2004). In the short term, the detritus quality strongly impacts the trophic structure and dynamics of the ecosystem (Moore et al. 2004). Hairston and Hairston (1993) suggested that

both detritus quality and quantity alters community characteristics e.g., promote species diversity and support larger predator biomass and longer food chains than in presence by living autotrophs only (Schindler 1990; Williamson et al. 1999).

Decomposer fauna play a crucial role in accelerating decomposition processes either indirectly by modifying the biomass, composition and activity of soil microbial communities or directly by consuming detritus and releasing inorganic nutrients. Microarthropods are able to modify the structure of microbial communities; mites and Collembola can affect the fungal to bacterial ratio and affect fungal competition through selective grazing (Parkinson et al. 1979). Transformation of organic matter from detritus, via biota, into inorganic compounds have a global impact by affecting carbon storage, nutrient translocation and climate change (Cebrian and Duarte 1995; Bardgett 2005), and Collembola can play a crucial role via their impacts on the primary and most common decomposers of litter in many ecosystems which are the saprophytic fungi.

1.2 FUNGAL ECOLOGY AND DECOMPOSITION

1.2.1 Systematics and physiology

Fungi (Eumycota or Mycota) are unique organisms which belong to their own kingdom, completely separated from plants, animals and bacteria (Deacon 2006). Fungi are eukaryotic modular organisms with haploid nuclei as special feature and little morphological differentiation (Carlile 1994). They also exhibit a definite cell wall throughout their somatic phase and are mainly heterotrophic (Alexopoulos et al. 1996; Kendrick 2000). Hawksworth (1991, 2001, 2003) estimated a total of approx. 1,5 million species, of which ~ 4,000–120,000 have been named, with a current description rate of approx. 1,100 new fungal species per year. The oldest fossil finding of a fungus, resembling the modern Glomerales, originated in the Ordovician period between 460 and 455 million years ago. Very likely aquatic fungi were present long before that in the Proterozoic eon, presumably approx. 1,430 billion years ago (Deacon 2006; Lucking et al. 2009).

Over 75% of the identified fungi belong to the monophyletic Ascomycota which is the most important and diverse phylum, at least 300 Mio. years old (Deacon 2006). Its prominent feature is the ascus, a cell with two compatible haploid nuclei of different mating types, which fuse to diploid nuclei and end in ascospores after meiosis resulting in haploid sexual spores or in asexual mitospores after mitosis as in *Aspergillus fumigatus* (Adams et al. 1998). The second important phylum, the monophyletic Basidiomycota, comprises around 30,000 known species with the characteristic basidium in which meiosis takes place. Based on molecular phylogenetic analyses fungal systematics have been revised recently resulting in an increase from five to seven phyla (Schüßler et al. 2001; Cracraft and Donoghue 2004; Hibbett et al. 2007).

Fungal hyphae contain several nuclei within each hyphal compartment contrasting other eukaryotic organisms (Kendrick 2001). They grow apically through tip extension of filaments which is stimulated by a vesicle supply center, the "Spitzenkörper" (Wessels 1994; Riquelme et al. 1998). A plastic deformable tip continuously extends and the wall behind rigidifies (Bartnicki-Garcıa and Lippman 1969; Gooday 1971). During growth hyphae are continuously fused by forming anastomoses, yielding a network of interconnected hyphae, the mycelium, representing the fungal individual (Glass et al. 2000). Fusions may enhance the protoplasmatic flow which affects hyphal pattern formation (Buller 1933; Rayner et al. 1994; Davidson et al. 1996). Total hyphae in the mycelium with a multitude of hyphal tips are defined as one hyphal growth unit. Some dimorphic fungi switch between a yeast and hyphal phase (Dix and Webster 1995; Alexopoulos et al. 1996; Kendrick 2001; Deacon 2006).

Fungi are heterotroph (chemoorganotroph) organisms absorbing simple soluble nutrients through the cell wall (Carroll and Wicklow 1992; Griffin 1994). Hyphal tips excret a wide range of enzymes degrading complex polymers including starch, cellulose, chitin, keratin and lignin (Trojanowski et al. 1984; Wood et al. 1989; Lindahl et al. 2005). In the vicinity of fungi the concentration of (partially) decomposed organic matter may be high and therefore attract decomposer animals (external rumen hypothesis; Swift et al. 1979; Maraun et al. 2003). Fungi are major agents driving nutrient cycling, organic matter decomposition and disease suppression (Boddy 2001; Mulder 2006) and thus indispensable for soil functioning (Domsch and Gams 1969; Anderson and Domsch 1975; Swift et al. 1979).

One of the most characteristic features of fungi is the formation of radially expanding, circular colonies (Rayner et al. 1994). This growth habit is one of the reasons of their ecological impact and ubiquity (Riquelme et al. 1998). For instance, more than half of the carbon mineralized originates from the metabolic activity of fungi in forest soils (Anderson and Domsch 1975).

1.2.2 Importance of fungal symbiosis

Fungi are engaged in a broad range of close symbiotic associations with other organisms forming e.g., lichens and mycorrhiza (Allen 1991). Mycorrhiza is defined as fungus and plant root association (Smith and Read 2008). At least 80% of all vascular plants (angiosperms, gymnosperms, many pteridophytes and some bryophytes) form symbiotic associations with fungi (Read and Perez-Moreno 2003; Smith and Read 2008). The fungus – plant symbiosis serves multiple functions (Christensen 1989; Bonfante and Anca 2009). Fungi provide limiting mineral nutrients, such as nitrogen and phosphorus, in exchange for carbon as energy source from the plant (Read and Moreno 2003; Hobbie and Colpaert 2003; Smith and Read 2008). The contribution of the fungal partners such as ericoid (ERM), ecto-(ECM) and arbuscular (AM) mycorrhizas to plant nutrition might be strikingly different since they occur under distinctive soil conditions (Read and Moreno 2002). Allen (1991) proclaimed ectomycorrhiza to form major components ensuring the functioning of ecosystems. Fungal hyphae extend into a network of individual hyphae or form conglomerated mycelial cords (Mummey and Rillig 2008). These complex interconnected mycelial networks are enmeshing soil organic matter (Rayner 1996) and are difficult to describe using linear growth models for filamentous fungi (Davidson et al. 1996; Davidson 1998). The widespread fungal network may link plant individuals within one habitat (Simard et al. 1997). Hence, tree seedlings may profit from the transport of nutrients from nitrogen fixing to non-fixing plant species (Smith and Read 1997).

Conservative estimations indicate trees to invest 15-30% of their annual net production into fueling fungal mutualists (Leake et al. 2004; Leake et al. 2008). Plants, on the other hand, benefit from enhanced nutrient acquisition via mycorrhizal hyphae thereby increasing their competitive strength and resistance against environmental stress. Soil phosphate rapidly forms insoluble complexes and therefore is particularly difficult to acquire by plants (Rodriguez and Fraga 1999). Notably, a variety of mycorrhizal fungi with different characteristics and ecological roles evolved independently (Fogel 1980; Harley and Smith 1983; Hobbie 2006).

1.2.2.1 Ectotrophic mycorrhiza

More than 5,500 ectotrophic mycorrhizal species, primarily of the Basidiomycota, are known (Malloch et al. 1980; Agerer 2006). They constitute a very advanced group of true fungi which coevolved with terrestrial plants and exploit highly complex organic substrates (Gange 2000). Ectomycorrhizae like *Laccaria laccata* primarily occur on woody plants, such as coniferous and broad leaved trees (Agerer 2006). Characteristically, plant roots covered by fungal tissue are short and thick without root hairs (Deacon 2006). Underneath the fungal sheath the fungus invades between the root cortical cells forming a "hartig net" (Nylund 1980). Fungal hyphae extend into a network of individual hyphae or form conglomerated mycelial cords (Mummey and Rillig 2008). In forests, fungal mycelia derived from single spores may form carpets of several square meters and survive for hundreds of years (Korhonen 1978; Kile 1983; Smith et al. 1992). The most outstanding giant fungal individuum of the tree killing *Armillaria ostoyae* species grows in the Malheur National Forest, Oregon USA (Rishbeth 1991); it covers around 10 ha and has an estimated age of up to approx 8,500 years (Ferguson et al. 2003).

1.2.3 Fungi as drivers of decomposition processes

Fungi play an important role in soil as they are the main decomposers of organic material, such as dead wood or leaf litter with the help of a wide range of extracellular enzymes (Sims et al. 2007; Boddy et al. 2008). Gadd (2007) postulated that fungi play a fundamental role in biogeochemical transformations at local and global scales with special emphasis in terrestrial aerobic habitats. Since in terrestrial ecosystems, the above- and belowground plant-litter input constitutes the main resource of energy and matter for life in soil lignocellulose degraders play a vital part for sustaining global carbon cycle and nutrient availability (Hättenschwiler et al. 2005). Fungi affect the sink - source balance of carbon residues (Read

et al. 2004). For instance, in grasslands the biomass of fungal hyphae can reach approx. 250 kg dry mass ha^{-1} within the upper 5 cm of soil, exceeding by far the biomass of plants, animals or bacteria (Kjoller and Struwe 1982; Christensen 1989; Bardgett 2005). Perez-Moreno and Read (2001) postulated mycorrhiza to be substantial parts of direct nutrient mobilization overcoming the long lasting reductionistic approach of strictly separated functional soil fungi groups. Some ericoid and ectomycorrhizal fungi are highly efficient scavengers for N and P through the degradation of both, structural and organic polymers. Hence, they restrict nutritional supplements for decomposer communities and accordingly, to a certain extent plant benefit from this extra source of nutrient acquisition (Hawkins et al. 2000). These symbioses therefore control ecosystem functioning, nutrient cycling, species composition and productivity (Read et al. 2004). Nevertheless, the relative contributions of symbionts and saprotrophs to carbon storage and cycling particularly in the context of global climate change and impacts of anthropogenic pollutant N deposition remain unknown.

Fungi on decomposing litter materials follow a successional pattern. Epiphytic fungi are followed by weak parasites, pioneer "sugar" saprotrophic fungi, polymer degraders, secondary opportunistic fungi and degraders of recalcitrant compounds (Harper and Webster 1964; Chang and Hudson 1967; Hudson 1968; Carroll and Wicklow 1992). The more senescent a leave, the more microbes and dimorphic fungi are present. In the beginning of senescence weak parasites and pathogens colonize leaf tissue exploiting easily available carbon compounds (Lu et al. 2004). Mitosporic fungi with melanized hyphae, such as *Alternaria alternata* and the leaf surface fungus *Cladosporium cladosporioides*, as well as pioneer saprotrophs, such as *Mucor* spp., exploit sugars and soluble carbon compounds ('sugar fungi'; Garrett 1951; Hudson 1968). With a short exploitation phase and low competitive ability they cannot degrade complex structures like cellulose. Further, they cannot cope with antibiotics and other fungal growth inhibitors. The polymer degrading fungi defend their resources by producing secondary metabolites and by sequestering nitrogen, which often limits fungal growth. They occur on various substrates, environments and phases of litter decay and include e.g., species of the genera *Chaetomium*, *Trichoderma*, *Fusarium* and *Mortierella* (Deacon 2006). Thereafter, certain fungi degrade or modify recalcitrant polymers such as lignocellulose (Lindahl and Olsson 2004). Secondary opportunistic invaders grow intimately linked with polymer degrading fungi utilizing the breakdown products and tolerating other fungal metabolites. They colonize dead hyphal remains, faecal pellets or exoskeletons of soil arthropods. Only humus material is left forming soil organic matter that enhances soil structure and water retention.

1.2.4 The importance of fungal secondary metabolites

Since decades secondary metabolites from different taxonomic groups (e.g., plants, bacteria and fungi) provide a variety of molecules of pharmaceutical, medical and economic value (Fox and Howlett 2008). Continuously, new metabolites of high industrial potential are discovered, with those produced by fungi being of paramount importance. Fungi produce a plethora of potentially toxic substances, such as antibiotics (mainly bacteria specific) or phytotoxins (plant specific; Graniti 1972), pigments or growth factors. Although they are defined as not essential for the survival or development of an organism (Aharonowitz and Demain 1980; Demain and Fang 2000) there is evidence that they play a key role in defence (as antibiotics and toxins) and signalling (as agents for inter and intra-specific communication), thereby affecting the organism's survivability by increasing its competitive strength (Keller et al. 2005; Bhatnagar and Cary 2006; Deacon 2006). Secondary metabolism is linked to particular environmental conditions or developmental stages (Vining 1992). Key substances of the primary metabolism form the basis for the synthesis of secondary metabolites over a number of precursors and pathways (Kueck et al. 2009). Interspecific gene transfer may be one of the important drivers in their evolution (Vining 1992). Approximately 50,000 different secondary metabolites are discovered so far, but this is only a small fraction of the total existing in plants, bacteria, protists and fungi (Demain and Fang 2000).

Fungi produce a diversity of secondary metabolites via unique and unusual pathways (Calvo et al. 2002; Keller et al. 2005). Genes involved in primary metabolism are generally scattered throughout the fungal genome, but genes of secondary metabolism are arranged in clusters similar to the bacterial secondary-metabolite operons (Keller et al. 2005). Several thousands of secondary metabolites are known (Turner and Aldridge 1983) sharing the following characteristics:

1. They are usually synthesized at the end of the exponential growth phase.
2. They derive from common metabolic intermediates but are encoded through specific genes in specific enzymatic pathways.
3. They are not essential for the survival of an organism.
4. The composition is genus, species or even strain specific.

Depending on their origin in the primary metabolism there are four main groups: polyketides with fatty acid derivates, non-ribosomal peptides, isoprenoids and alkaloids (Keller et al. 2005; Kueck et al. 2009; Fig. 1). The most relevant pathway is the polyketide pathway, which

has Acetyl-CoA as a precursor with a number of important endproducts, such as griseofulvin or aflatoxins (Payne and Brown 1998; Bennett and Klich 2003).

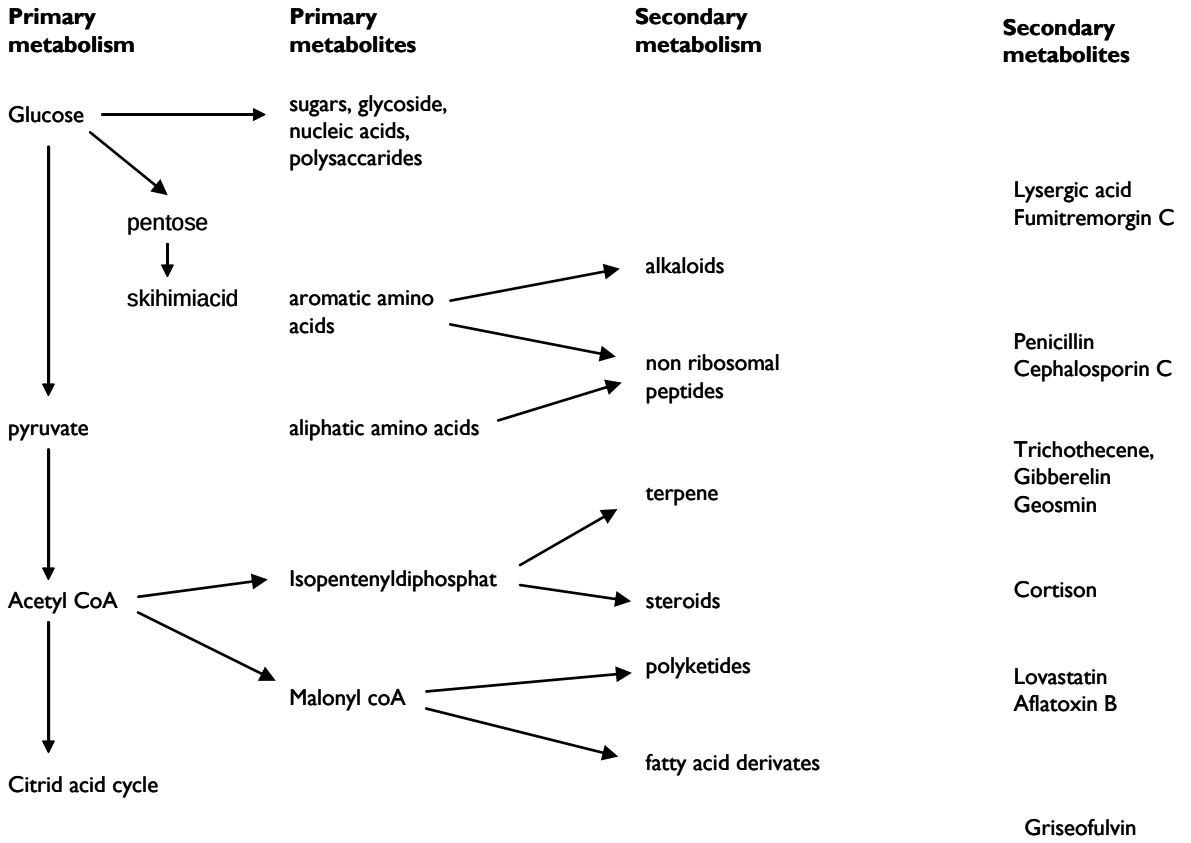

Figure 1 Linkage between fungal primary and secondary metabolism (modified after Kueck et al. 2009).

Some secondary metabolites have significant economic and pharmaceutical value (Calvo et al. 2002; Keller et al. 2005). In 1928 the β-lactam antibiotics like Penicillin derived from the saprotrophic fungus *Penicillium* spec. were discovered by Fleming (Kettering et al. 2004). Antibiotics are by definition restricted to particular active compounds which affect specific cellular targets (Vining 1985). About 1,500 antibiotic substances, such as bacteria suppressing penicillins, cephalosporins and fungi suppressing griseofulvins, are presently known (Keller et al. 2005; Pelaez 2005). Deacon (2006) estimated the number of mycotoxins to exceed 300. Secondary metabolites are most common in Ascomycota and mitosporic fungi, including species of the genera *Penicillium*, *Aspergillus, Fusarium* and *Trichoderma* (Demain and Fang 2000). Basidiomycota also produce antibiotics, whereas Chytridiomycota, Zygomycota and Oomycota produce only a limited number of antibiotics, which likely is related to their short life cycle and the limited necessity for defending resources (Deacon 2006).

1.2.4.1 Ecological significance of secondary metabolites

Immobile organisms, such as plants and fungi, evolved defence strategies against enemies and competitors (Spiteller 2008). Plant defence mechanisms have been studied in detail (Steglich 1989; Bennett and Wallsgrove 1994; Kessler and Baldwin 2001; Sudha and Ravishankar 2002), whereas fungal defence has been widely neglected and research so far focussed on economically valuable bioactive compounds (Demain and Fang 2000).

Higher fungi protect their mycelia and the fruiting bodies against competitors and enemies (Anke 1995; Stadler and Sterner 1998). For their distribution mycelia need space and nutrients whereas the fruiting bodies are exposed to fungivores, such as arthropods and vertebrates or mycoparasites (Spiteller 2008). The bodies are equipped with bitter, pungent or toxic compounds to repel consumers (Palmerino et al. 1980; Böllmann et al. 2009). Fungal defence may be constitutive or induced. Constitutive chemical defence mechanisms are characterized by the continuous presence of defence compounds whereas the production of induced defence compounds is linked to threads e.g., are only produced after wounding (Spiteller 2008). The latter mechanism is characterized by the transient enzymatic conversion of inactive precursors to active agents. Induced chemical defence is characterized by either de novo synthesis or an increase in the production of a constitutive defence compound (Zenk and Juenger 2007). Since the process includes several metabolic steps the induced production of defence compounds takes longer than e.g., the wound activated defence process where enzymes immediately convert precursors into the products (Spiteller 2008). In plants the presence of wounding hormones, such as jasmonic acid, activates the genetic response (McConn 1997; Halim et al. 2006). This results in the production of chemical defence substances like phytoalexines (Grisebach and Ebel 1978) and volatiles, such as terpenes, which might act as an attractant for predators of the plant herbivores (Arimura et al. 2005).

The defence response in fungi is generally little studied (Spiteller 2008). Wounded fungi produce a number of volatiles including 3-octanon or 1-octen-3-ol, but it is unknown whether this leads to an induced chemical response as in plants (Pasanen et al. 1997; Kishimoto et al. 2007). Since strobilurin production strongly increase in response to other fungal competitors chemical defence responses likely occur (Spiteller 2008). Similar to the wide variety of plant metabolic responses to injury, higher fungi evolved also a diversity of defence mechanisms but their ecological role is still little known (Calvo et al. 2002; Yu 2005). Only few studies have investigated the relationship between fungi and fungal feeding microarthropods in an evolutionary context e.g., the impact of fungal secondary metabolites

on fitness parameters or selected genes of Collembola. Rohlfs et al. (2007) showed Collembola to preferentially feed on a mutant of *Aspergillus nidulans* lacking a global secondary metabolite regulator. The results suggest that sterigmatocystin may act as a shield against fungivory. Moreover, fungal toxic metabolites or crystals on hyphal surface repel Collembola, which indicates once more the existence of mechanical defence traits in fungi (Böllmann et al. 2009).

However, communication traits between fungi and fungal consumers have been largely overlooked (Fig. 2). This is surprising as interactions between fungi and fungal feeders are likely to play an important role in essential ecosystem processes like decomposition and nutrient cycling (Klironomos and Kendrick 1995).

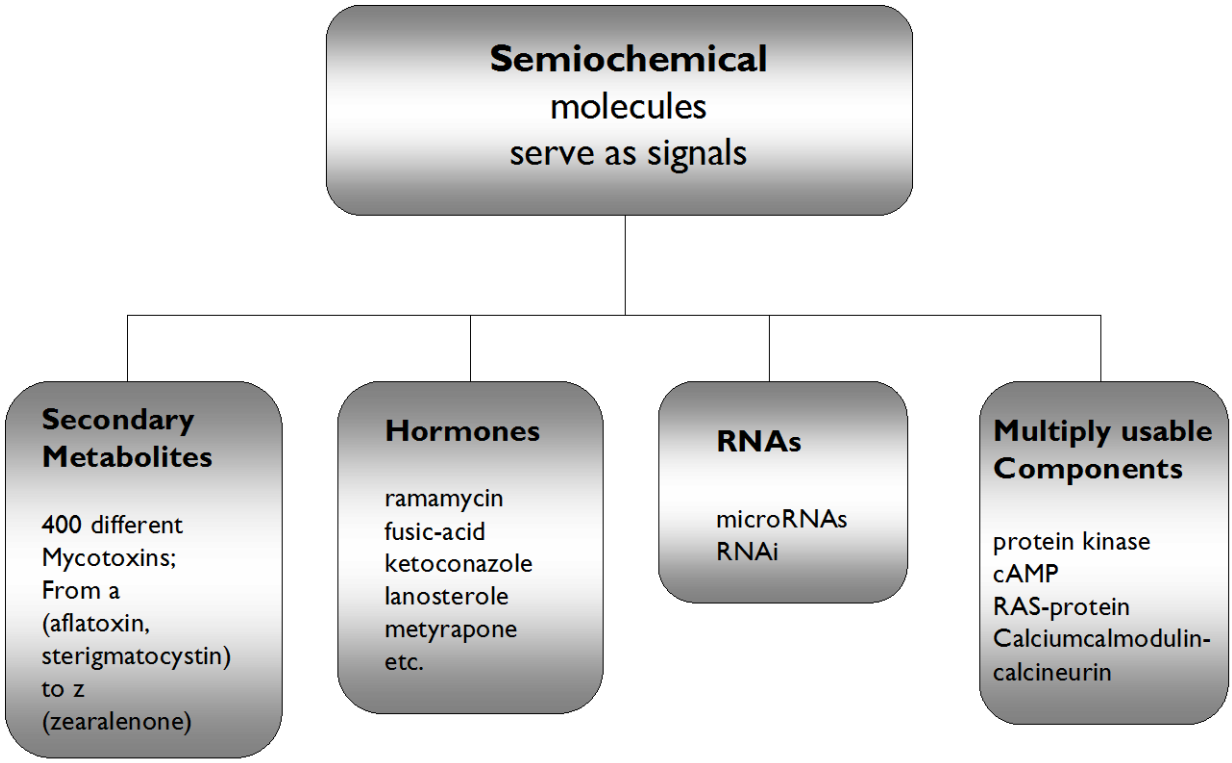

Figure 2 Examples of potential mediators in fungal communication channels (modified after Witzany 2009).

1.3 COLLEMBOLA ECOLOGY

Collembola (springtails) are tiny wingless animals of the mesofauna which form the largest of the three lineages of endognathous insects (Hopkin 1997). The name Collembola, derived from the Greek "*cole*" meaning glue and "*embol*" meaning a wedge, refers to a peg-shaped structure, the collophore, on the underside of the first abdominal segment (Hopkin 1997). A ventral furca at the fourth abdominal segment is another characteristic feature which helps Collembola to disperse over large distances and to prevent predation. Collembola are the most omnipresent and widely distributed insects, abundant on every continent and occurring even under extreme conditions including Antarctica and the Australian desert (Rusek 1998). Collembola are exceedingly abundant in soil and litter. With about 50,000 ind./m^2 they occur densely in open habitats like meadows, pastures and arable fields, but reach maximum density in boreal forests with around 10^5 to 10^6 ind./m^2 (Petersen and Luxton 1982). Densities are particularly high in forests with a duff layer (Stierhoff 2003).

Collembola comprise around 7,900 species worldwide (Bellinger et al. 2009; Doblas-Miranda et al. 2008) with approx. 2,000 species in central Europe, but diversity presumably is much higher as e.g., tropical forests have been little studied. New species are continuously described. Collembola are among the oldest terrestrial animals; the first known fossils were from the Devonian (approx. 390 Mio. years ago; Hopkin 1997). This long-term success is certainly an indicator for successful adaptions.

There are three main orders of Collembola. The Arthropleona (about 5,500 species) characterized by an elongated body and including active surface-dwelling species and less mobile ones living deeper in soil. One prominent example of the Arthropleona family Isotomidae is the well known model organism in ecotoxicology, *Folsomia candida*, the "standard test soil organism" for the assessment of new chemicals (Fountain and Hopkin 2005). The Symphypleona with about 1,000 species have a round habitus and are often attractively coloured surface-living species. The Neelipleona are soil-living tiny springtails with an average length of 0.5 mm and with no more than 25 species known worldwide. They have a rounded body shape and bear a superficial resemblance to Symphypleona.

A distinctive feature of Collembola compared to other insects is the lifelong growth combined with constant moulting. If Collembola consume toxic food they accumulate heavy metal ions in intracellular granulae from undisolvable salts. This is considered to be the major detoxifying pathway since the midgut epithel is moulted together with the cuticula and regenerated thereafter (Humbert 1979; Joosse and Verhoef 1983).

1.3.1 Collembola as decomposers

Soil organisms play a crucial role in decomposing organic matter and nutrient mineralization (Bradford et al. 2002; Wardle 1999). These processes are mainly carried out by soil microorganisms, but their activity, spatial distribution and community composition is altered by decomposer invertebrates, such as Collembola and earthworms (Scheu and Setälä 2002; Partsch et al. 2006). Presence and density of Collembola depend on environmental factors, such as humidity, temperature and organic matter (Hopkin 1997). However, Collembola densities are also affected by the presence of other decomposer species e.g., the presence of Lumbricidae may detrimentally affect Collembola (Schaefer and Schauermann 1990; Eisenhauer et al. 2007).

Occasionally it has been shown that Collembola can act as root herbivores, grazing on the fine roots but also as predators of nematodes (Filser 2002; Endlweber et al. 2009). However, it is still debated to what extent Collembola feed on other food sources than fungi such as nematodes, pollen, algae, bacteria and eggs of other Collembola (Cassagnau 1972; Lee and Widden 1996; Jørgensen et al. 2003). Generally, Collembola are regarded as selective generalists (Hopkin 1997) feeding predominantly on fungi (Verhoef et al. 1988; Visser and Whittaker 1987; Chen et al. 1995; Jørgensen et al. 2005). Collembola structure soil microbiota incl. fungal population dynamics (Parkinson et al. 1979; Klironomos et al. 1992).

Selective grazing on fungal hyphae alters decomposition rates and soil respiration rates (Moore et al. 1987; Bakonyi et al. 2002) depending on grazer density and frequency dependent (Bengtsson and Rundgren 1983). Their impact on fungal biomass and community composition affects nitrogen mineralisation, leaching of dissolved organic carbon and plant growth (Gange 2000; Scheu et al. 2005). Collembola promote mycelial growth and therefore enhance fungal dispersal or feeding might eliminate certain fungal species from the community (Hanlon 1981; Newell 1984a, b; Klironomos and Ursic 1998). Another way by which Collembola can impact on decomposition processes is by acting as dispersal agents; around 100 fungal species may adhere to the body surface of *Onychiurus subtenuis* (Visser et al. 1987). It has been suggested that selective grazing plays an important role in controlling basidiomycete dispersal (Thimm and Larink 1995).

Many of the aforementioned impacts of Collembola on decomposition are density-dependent and due to the indirect" or "catalytic" contribution of Collembola to decomposition their overall impact to decomposition processes is difficult to quantify. Through their impact on nutrient turnover and availability, Collembola can stimulate plant

growth and alter the structure of plant communities, which in turn may affect the decomposition processes via their litter inputs (Scheu and Setälä 2002; Wardle 2002; Scheu et al. 2005). Davidson (1993) argued that due to their ubiquity, high abundances and significant impacts on ecosystem functioning Collembola serve as keystone decomposers.

1.3.2 Functional groups of Collembola

Soil biota are commonly grouped into functional types according to their feeding habits. Differences in grazing modes of Collembola may result from morphological features of their mouthparts such as styliform parts or molar plates. Collembola species were classified in seven feeding guilds according to their digestive enzyme composition assuming an existing link between mouthpart morphology, specific carbohydrase activity and food choice (Berg et al. 2004). This suggests distinct effects of functional features in soil decomposers on nutrient fluxes, dynamics and structure of soil microbial community (Bardgett and Chan 1999). But these biocommunication mechanisms are still not understood. However, it has also been argued that soil animal species are functionally redundant and that Collembola diversity therefore may not significantly affect ecosystem functions, such as N mineralization and plant growth (Cragg and Bardgett 2001; Liiri et al. 2002).

1.4 COLLEMBOLA – FUNGI INTERACTIONS AND DECOMPOSITION

In general, biotic interaction types are differentiated as mutualism, antibiosis and contramensalism - the latter including parasitism, predation, phytophagy and competition (Hodge and Arthur 1996). Three categories may be distinguished in the interaction between Collembola and fungi:

1. *Commensalism* and *mutualism*, i.e. the ability of species to coexist with one or both partners benefiting from the other, respectively. Besides *obligate mutualists* which include the microflora permanently associated with Collembola, e.g. in microhabitats like the gut (Czarnetzki and Tebbe 2004b), *facultative mutualists* play an important role since Collembola may utilize cellulases produced by fungi and ingested with the incorporated detritus (Begon et al. 2009). The latter reflects the *external rumen* concept stating that detritivores benefit from incorporating decomposing matter which contains enzymes produced by microorganisms that liberate easily digestible compounds.
2. *Interference competition* or *combat*, i.e. the ability to exclude or substitute another species via, e.g. direct interaction by physical forces or the production of allelopathic substances such as antibiotics.
3. *Exploitation competition*, i.e. the ability of one species to inhibit another indirectly via more efficient or faster resource use. This might apply to Collembola and fungi competing for nematode prey but there is no experimental proof that this really occurs in the field (Duddington et al. 1973; Hauser 1985; Lee and Widden 1996).

Collembola - fungi interactions may have strong implications for the performance of both groups of organisms and these are described in more detail below.

1.4.1 IMPACT OF COLLEMBOLA ON FUNGI

Although Collembola are known to feed on a multitude of substrates, it is generally accepted that fungi form their main diet (Jørgensen et al. 2005). Therefore, the main interaction between Collembola and fungi, i.e. fungivory, is similar to the plant herbivore-consumer relationship. The converse (fungi incapacitating and using Collembola as a source of nutrients) exists although as an exception (Barron 2003). The main impacts of Collembola on fungi with consequences for decomposition processes are discussed below.

1.4.1.1 Fungal community composition

Collembola prefer to feed on certain fungal species, in particular on dark pigmented fungi (Maraun et al. 2003) and selective grazing may impact the fungal community composition (Tordoff et al. 2008). Interestingly, soil microarthropods such as Collembola preferably graze in contact zones of fungal colonies and this may play an important role in determining fungal community composition (Gormsen et al. 2004). Nutrients accumulating in contact zones between fungal colonies are the main explanation put forward as stated in Chapter I.2 (Rayner et al. 1994; Simard and Durall 2004; Fricker et al. 2007).

1.4.1.2 Fungi-to-bacteria ratio

Via preferential grazing Collembola affect the fungi-to-bacteria ratio. Bengtsson (1992) showed that compensatory fungal growth is common after grazing and this can shift the fungi-to-bacteria ratio. Collembola are also known to act as vectors for both bacterial and fungal species. Furthermore, Borkott and Insam (1990) presented evidence for a mutualistic symbiosis of Collembola (*Folsomia candida*) with chitin-degrading bacteria, not only intra-intestinal but also involving an extra-intestinal phase, resulting in enhanced chitin degradation to the benefit of both species. Thimm et al. (1998) described the gut of *Folsomia candida* as an unstable but specific habitat for bacteria. Therefore, microarthropods may significantly alter the structure of soil microbial communities.

1.4.1.3 Fungal biomass, mycelial physiology and chemistry

Surprisingly, only few studies investigated induced fungal reactions to microarthropod grazing, such as changes in extracellular enzyme composition, morphology of mycelia and fungal growth rate or biomass production (Hedlund et al. 1991; Tordoff et al. 2008; Rotheray 2009). After grazing fungi temporarily respond by compensatory growth. Hyphae become thinner which may increase nutrient uptake (Bengtsson et al. 1993). Further, grazing induces the development of fast-growing hyphal morphotypes and promote the production of extracellular enzymes, such as proteases and amylases, followed by nutrient release in the close neighbourhood of the hyphae (Hedlund et al. 1991). Fungal grazers are attracted by volatile compounds of fungi (Bengtsson et al. 1988, 1991), but from an evolutionary point of view fungi should avoid being located via volatile compounds and their metabolism and growth should be impaired rather than enhanced by grazing (Calvo et al. 2002; Yu and Keller 2005). Some defence mechanisms of Basidiomycota caused by Collembola grazing have been unravelled (Rotheray 2009). For instance, *Pleurotus* species excrete toxins through aerial

stalks (Barron and Thorn 1987; Hibbett and Thorn 1994), *Hohenbuehelia* species develop adhesive structures on their hyphae or conidia (Thorn and Barron 1984) and *Hyphoderma* species emit adhesive molecules from stephanocysts (Tzean and Liou 1993). Further, the length of mycelia may increase as a result of grazing (Hedlund et al. 1991).

1.4.1.4 Dispersal of fungi

Collembola are important dispersers of mycelial fragments and fungal propagules (Wiggins and Curl 1979; Lussenhop and Wicklow 1984; Visser et al. 1987). Fungal spores adhere to the cuticle of Collembola or are transported in the gut and excreted in other habitats. The fitness gained by attractive fungi from spore dispersal may well compensate for losses due to grazing, especially if the fungi are patchily distributed. Preferential grazing on the contact zones of fungal colonies may play an important role in controlling fungal dispersal (Boddy et al. 1983; Gormsen et al. 2004). Some of the potentially released nutrients after fungal tissue injury are exclusively found in these interaction zones in dissolved phase (Bardgett 2005). Furthermore, Collembola promote dispersal of arbuscular mycorrhizae but this varies between fungal species (Klironomos and Moutoglis 1999). Recently, Collembola feeding on fungal fruiting bodies have been shown to seriously damage fungal spores, showing that Collembola can have a negative impact on fungal fitness (Nakamori and Suzuki 2009).

1.4.2 IMPACT OF FUNGI ON COLLEMBOLA

Presumably, fungal community is a major factor in structuring Collembola abundance and diversity, however, this has not been thoroughly investigated.

1.4.2.1 Fungal enzymes

Soil fungi produce an array of complex and diverse enzymes with higher potential than that of microarthropods (Maraun et al. 2003). They are capable of degrading cellulose which is limited to only few soil animals and moreover lignin. The occurrence of exoenzymes may attract decomposer animals feeding on the digested material in hyphal vicinity (external rumen hypothesis; Swift et al. 1979) which can be seen as resource quality improvement and therefore a structuring force in Collembola community composition. Another enzymatic trait with consequences for Collembola is chitinolysis. Chitinolytic fungi such as *Trichoderma, Penicillium, Paecilomyces* and *Mortierella* may digest Collembola (Maraun et al. 2003).

1.4.2.2 Predatory fungi

Exceptions to the rule that Collembola feed on fungi include a couple of predatory fungi feeding on soil microarthropods (Duddington 1973; Hauser 1985). Fungi are well known for preying on nematodes employing special hyphal traps (Migunova and Byzov 2005) with the degree of functioning as predators (rather than as saprotrophs) depending on the availability of the respective food. Mycorrhizal fungi such as *Laccaria bicolor* are able to kill Collembola and exploit their nitrogen pools (Klironomos and Hart 2001). They paralyse Collembola and thereafter infect and digest Collembola tissue. Similarly, saprotrophic fungi may paralyse insects through the excretion of the toxin ostreatin (Klironomos and Hart 2001). This reversed trophic interaction between ectomycorrhizal fungi and soil arthropods indicates that animal nitrogen and phosphorus is traded for carbon from the host tree by mycorrhizal fungi.

1.4.2.3 Fungal morphology and physiology

Fungi attacked by fungivores may alter their morphology and physiology. The length of fungal mycelium is one structuring force for Collembola dispersal rates, with dispersal rate decreasing with the mycelial length (Bengtsson et al. 1994). The authors even suggested that fungal odour attract Collembola from large distances and enhance their movement rates more than a three-fold increase (Bengtsson et al. 1994). Collembola foraging on fungi may even trigger fungal stress response pathways resulting in the production of fungal secondary compounds. In response Collembola may avoid grazing on previously grazed mycelia. Some Basidiomycota bear deterrent or repulsive substances on or in their cell walls, such as calcium oxalate (Horner et al. 1995; Connolly et al. 1999) or melanin (Rayner and Boddy 1988; Jacobson 2000; Butler et al. 2005) which may repel Collembola grazing. Responding to grazing or interspecific mycelial interactions, fungi emit a plethora of volatile organic compounds and dissolved organic compounds (Faldt et al. 1999; Rosecke et al. 2000; Xu et al. 2004) whose quality and quantity may change depending on the kind and intensity of external cues (Stadler and Sterner 1998; Woodward and Boddy 2008). Ascomycota synthesize toxic secondary compounds, such as aflatoxin and sterigmatocystin (Calvo et al. 2002). There is evidence that these fungal secondary metabolites play an important role in Collembola food selection and fitness (Rohlfs et al. 2007; Böllmann et al. 2009) and may have evolved as a shield against fungivory (Kempken and Rohlfs 2009).

1.5 AIM AND OVERARCHING HYPOTHESES

This thesis aims at improving our knowledge on the interactions between Collembola and fungi. Three studies have been conducted aiming at improving our understanding of specific issues of these interactions. Three overarching hypotheses have been tested:

H1. Fungal secondary compounds mediate the Collembola – fungi interaction.

H2. Collembola have evolved means to detect fungal toxicity

H3. Genetic evidence (transcript regulation) can be used to understand the molecular nature of the Collembola – fungi interaction.

I. The first study aimed at understanding the impact of the fungal secondary compounds, more specifically the impact of the fungal toxin sterigmatocystin (ST), on the performance of springtails in a feeding preference experiment. It was hypothesised that the presence of ST in *Aspergillus nidulans* (Ascomycota) impairs Collembola performance with their fitness decreasing when gene silencing takes place at a later stage of the ST biosynthetic pathway. Further, it was hypothesized that the intake of mixed diets will be beneficial due to toxin dilution. Also, we expected that ^{13}C and ^{15}N fractionation will be increased in Collembola feeding on more toxic diets (Chapter 2).

II. The second study focused on the ability of Collembola to perceive fungal toxicity via olfactory/volatile cues. By means of an olfactometer approach this experiment hypothesized that Collembola are capable of olfactorily sensing fungi with varying toxicity and direct their movement towards more palatable (less toxic) fungal strains/species. Furthermore, the experiment investigated if Collembola avoid fungi which had been grazed by conspecifics before. Further, we investigated changes in secondary metabolite gene expression due to grazing by Collembola in one Basidiomycete and one Ascomycete fungal species using a custom made cDNA microarrays (Chapter 3).

III. The third study investigated the impact of fungal secondary metabolites on transcript regulation of *Folsomia candida*, the Collembola species used as model species in ecotoxicology. We expected that expression levels of selected transcripts will be significantly affected by secondary metabolites of *A. nidulans*, in particular compared to high quality food

like *Cladosporium cladosporioide*s. Furthermore, the experiment investigated if transcript regulation in *F. candida* is affected by ingesting mixed (as compared to single) diets (Chapter 4).

CHAPTER 2

FUNGAL TOXINS (STERIGMATOCYSTIN) AFFECT THE FITNESS AND STABLE ISOTOPE FRACTIONATION OF COLLEMBOLA

2.1 Abstract

We investigated the effect of the fungal toxin sterigmatocystin on the fitness and stable isotope fractionation of two Collembola species (*Folsomia candida* and *Heteromurus nitidus*) feeding on mixed *vs.* single diets. Four knock out mutants of *Aspergillus nidulans* with the sterigmatocystin production blocked at different steps along the biosynthetic pathway were combined in mixed diets with either the high quality fungus *Cladosporium cladosporioides* or the low quality fungus *A. nidulans* (wildtype). Using fungi labeled with stable isotopes (^{13}C and ^{15}N) we evaluated the incorporation of carbon and nitrogen from individual fungi. We hypothesised that (i) Collembola fitness decreases with the putative toxicity of the fungi (ii) Collembola benefit from ingestion of mixed diets due to toxin dilution and (iii) fractionation of ^{13}C and ^{15}N is more pronounced in more toxic diets. Mixed diets did not uniformly improve fitness. Toxin dilution, however, played an important role in Collembola fitness. The fractionation of ^{13}C and ^{15}N varied with sterigmatocystin mutant strains, and Collembola species often differed from the expected enrichment per trophic level. The results show that fungal toxin production may affect stable isotope fractionation, presumably by altering consumer excretion rates necessary for detoxification.

2.2 Introduction

Collembola are abundant soil and litter dwelling microarthropods and are known to affect decomposition processes by interacting with saprotrophic fungi. They are commonly considered to be generalist feeders (Hopkin 1997), however, the factors that control Collembola food choice are little understood. As Collembola are secondary decomposers, feeding predominantly on fungi (Moore et al. 1987; Chen et al. 1995; Jorgensen et al. 2005), one would expect a certain degree of specialisation resulting from co-evolutionary processes similar to those between plants and herbivores. Yet, Collembola appear to be uniformly polyphagous and it has been documented that mixed diets increase their fitness (Scheu and Folger 2004) as in polyphagous herbivores (Bernays et al. 1994).

Dilution of toxins and a more balanced nutrient supply are the two commonly hypothesised explanations of why generalists benefit from mixed diets (Pulliam 1975; Rapport 1980).

It has been assumed that fungal identity is of little importance for Collembola nutrition, however, food choice experiments demonstrated preferences for dark pigmented (melanised) saprotrophic fungi ('Dematiacea') irrespective of Collembola species (Aitchinson 1984; Maraun et al. 2003). This is surprising since there is evidence that the melanin production pathway is linked to the aflatoxin pathway (Brown and Salvo 1994) responsible for the production of some of the most toxic fungal toxins, sterigmatocystin and aflatoxin (Keller et al. 2005). Furthermore, melanin is indigestible and therefore should reduce food quality (Kuo and Alexander 1967; Butler et al. 2005). Even though there is evidence that Collembola benefit from melanin and sterigmatocystin deficient strains of *Aspergillus* (Ascomycota) (Scheu and Simmerling 2004; Rohlfs et al. 2007) the role of fungal secondary metabolites (toxins and pigments) for the fitness of fungal feeding microarthropods is poorly understood. Further, it is unknown to what extent their responses vary between species and functional groups.

Another little understood aspect is the often observed deviation from the expected trophic level fractionation in fungivorous invertebrates (Chahartaghi et al. 2005; Haubert et al. 2005). Belowground foodwebs are notoriously difficult to assess. Recently, shifts in $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios have been used to uncover the trophic structure of decomposer systems (Schmidt et al. 2004; Tiunov 2007). However, there is a wide range of factors that affect stable isotope fractionation questioning the applicability of the method for belowground foodwebs (Post 2002; Vanderklift and Ponsard 2003). Although it is known that excretion processes drive stable isotope fractionation (via preferential processing of light isotopes during excretion) and that toxins affect excretion, so far no study investigated links between toxins in the diet and stable isotope fractionation.

We used *Aspergillus nidulans,* a genetically well characterised fungus which is widespread in soil, to investigate the effect of the toxic fungal metabolite sterigmatocystin on Collembola fitness and isotope fractionation. Four sterigmatocystin deficient strains of *A. nidulans* (knock out mutants with increasing putative toxicity) were used. The influence of these knock out mutants on Collembola performance was assessed in single and mixed diets with *A. nidulans* wildtype (WT) and *Cladosporium cladosporioides* as reference fungi of low and high food quality, respectively (Scheu and Simmerling 2004).

Using stable isotope labeling, the incorporation of carbon into Collembola tissue from individual fungi offered in mixtures was evaluated together with stable isotope fractionation.

The following hypotheses were investigated: (1) The presence of sterigmatocystin impairs Collembola performance with their fitness decreasing with increasing toxicity.
(2) Collembola benefit from ingestion of mixed diets due to toxin dilution. (3) Fractionation of ^{13}C and ^{15}N is more pronounced in more toxic diets as toxic compounds alter excretion rates and stable isotope fractionation is affected by excretion.

2.3 Material and Methods

2.3.1 Fungal strains

Aspergillus nidulans is a widespread filamentous fungal species of the Ascomycota and an important model organism for gene expression profiling since its genome is fully sequenced. We used the wildtype strain RSCS2 of *A. nidulans* (WT) (C-to-N ratio 15.3 ± 1.1) as reference fungus of low quality which naturally synthesises sterigmatocystin. The sterigmatocystin deficient strains of *A. nidulans* used in this study, labelled S3, S4, S5 and S6, are well characterised isogenic strains of *A. nidulans* that are blocked at different steps along the sterigmatocystin biosynthetic pathway, i.e., *aflR*, *stcJ*, *stcE*, *stcU* (Wilkinson and Ramaswamy 2004; Fig. 1). The C-to-N ratio of the S3, S4, S5 and S6 strains did not differ from the wildtype ($F_{1,16}$ = 1.09, P = 0.310),

Cladosporium cladosporioides (C-to-N ratio 18.9 ± 1.0) is a common soil fungus which does not contain sterigmatocystin. It was taken from laboratory cultures and used as reference fungus of high nutritional quality (Scheu and Simmerling 2004). The lack of sterigmatocystin and previous results of Jørgensen et al. (2003) and Maraun et al. (2003) show that this fungus is highly preferred as diet by Collembola and therefore suited as reference fungus of high food quality.

All fungi were grown on Czapek-dox agar (Warcup 1950; Caddick et al. 1986) and kept at 28°C in permanent darkness. Chloroform extraction (Bok and Keller 2004) followed by evaporation and chromatographic analysis of the extracts was used to check the presence of sterigmatocystin in the fungal strains. As expected, sterigmatocystin was only present in *A. nidulans* WT.

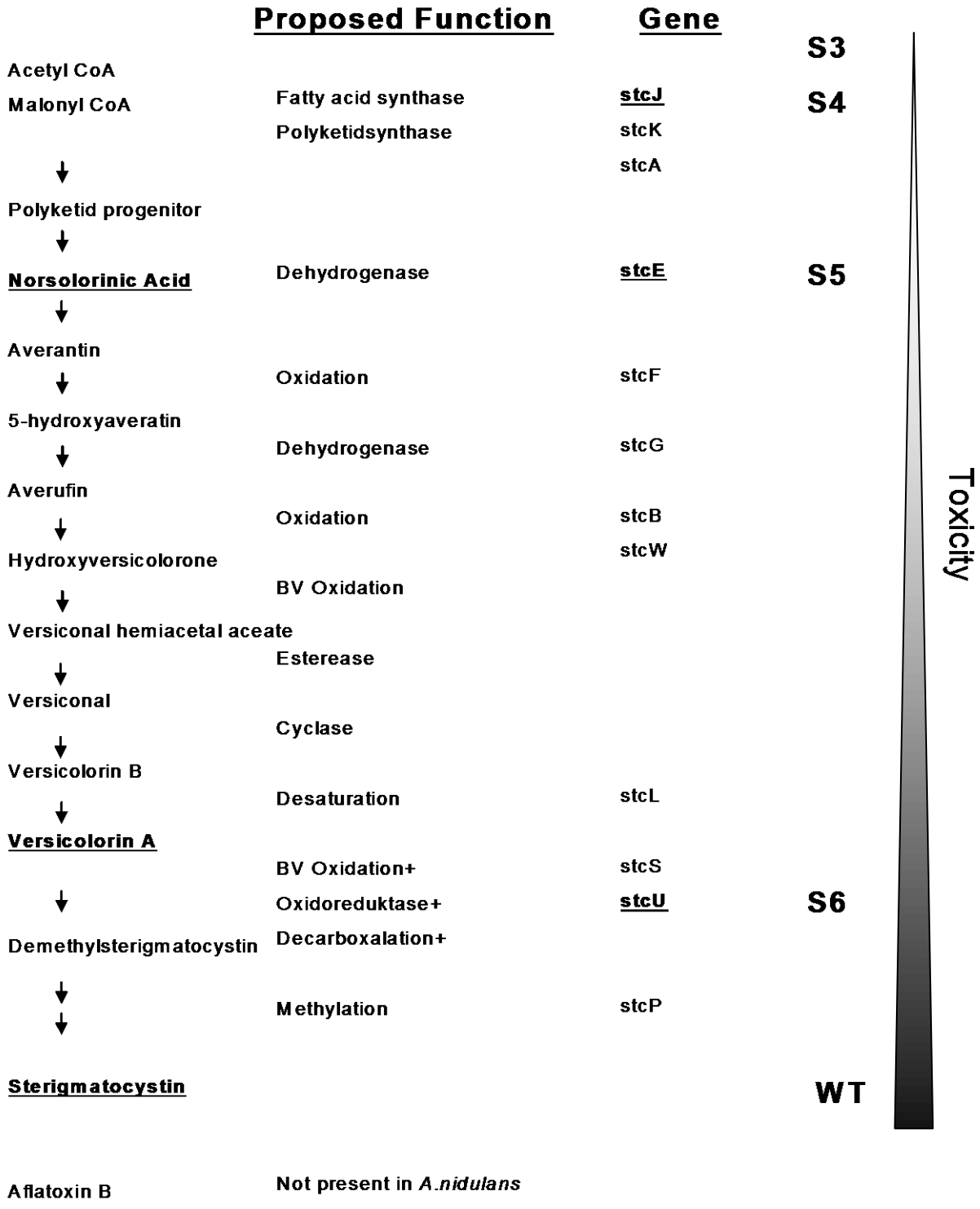

Figure 1 Gene function of the sterigmatocystin biosynthetic pathway in *Aspergillus nidulans* wildtype (WT). The four isogenic strains of *A. nidulans* used were blocked at different steps along the pathway: S3, (ΔalfR – not shown, regulatory gene coding for several gene clusters), S4 (ΔStcJ), S5 (ΔStcE) and S6 (ΔStcU) modified after Wilkinson and Ramaswamy (2004).

2.3.2 Collembola species

We used two species of Collembola which are easy to cultivate in the laboratory (*Folsomia candida* and *Heteromurus nitidus*). *F. candida* is a common parthenogenetic euedaphic cosmopolitan species. It has been used as a standard test organism for pesticide research for more than 40 years (Fountain and Hopkin 2005). *H. nitidus* is an epedaphic species which is widespread in forest and grassland soils in Europe. Juveniles of *F. candida* (body length of 0.8-1.0 mm) and subadults of *H. nitidus* (body length ~1 mm) were used. *H. nitidus* is a facultative parthenogenetic species and no males were found in a subsample of 100 animals. The Collembola cultures have been raised on baker's yeast (^{13}C and ^{15}N signatures of −24.19‰ ± 0.1 and 1.57‰ ± 0.24, respectively; C-to-N ratio 5.83 ± 0.05). This resulted in stable isotope signatures of −23.26‰ ± 0.06 and 5.5‰ ± 0.17 as well as −23.71‰ ± 0.15 and 3.94‰ ± 0.08 for ^{13}C and ^{15}N in *F. candida* and *H. nitidus*, respectively.

2.3.3 Experimental design

The experiments were established in perspex vessels (diameter 7 cm, height 5 cm) with a base layer of a mixture of plaster of Paris and activated charcoal (5 : 2) of ca. 1 cm thickness. Fungal cuts (10 mm diameter) were taken from young and actively growing hyphae of 7 d old cultures avoiding contamination with agar. The fungal cuts were renewed at regular intervals. During the experiment the boxes were incubated at 17±0.5°C in the dark for five weeks and kept humid. Treatment effects were assessed by counting the eggs daily. Every day eggs, exuvia and fecal pellets were removed to prevent animals to feed on them.

Single diet experiment

Ten juveniles of *F. candida* and 15 subadults of *H. nitidus* were exposed separately to six single fungal diets (*C. cladosporioides, A. nidulans* WT and its sterigmatocystin deficient strains S3, S4, S5 and S6). Four disks were placed per vessel ensuring food supply in excess. Five replicates were set up per treatment. The experiment lasted for 36 days. At the end of the experiment Collembola were sampled and from each replicate stable isotope ratios of adults were analyzed.

Mixed diet experiment

The experiment was set up in a similar way to the single diet experiment. The fungal species/strains used in the single diet experiment were offered in two species combinations. Either *C. cladosporioides* as fungus of high food quality or *A. nidulans* WT as fungus of low food quality were combined with the four sterigmatocystin deficient strains of *A. nidulans* (Table 1). From each diet two discs were offered, ensuring that it was available in excess. After 36 days Collembola were sampled and stable isotope signatures of adult specimens were determined.

To analyze the contribution of each diet to Collembola nutrition, fungi differing in ^{13}C signals were used. For differential labelling of fungi either sucrose from sugar cane (C_4 plant; Merck, Darmstadt, Germany; $\delta^{13}C$ −10.92‰) or from sugar beet (C_3 plant; Sigma, St. Louis, USA; $\delta^{13}C$ −26.03‰) was used for preparation of Czapek-Dox Agar medium. $\delta^{13}C$ signatures of the C_3 and C_4 agar medium were −23.55‰ and −13.31‰, respectively; respective $\delta^{15}N$ signatures were 3.01‰ and 1.21‰. Incorporation of carbon from each of the diets into *F. candida* and *H. nitidus* was calculated using a two-source mixing model (Gearing, 1991). Isotopic signatures of Collembola of the two respective single diet treatments ($^{13}C_{K1}$ and $^{13}C_{K2}$) were used to calculate the relative contribution of the first diet to the body carbon in each Collembola species (K1) according to the following formula:

$K1(\%) = [(^{13}C_{mix} - ^{13}C_{K2})/(^{13}C_{K1} - ^{13}C_{K2})] \times 100$. The contribution of the second diet (K2) is given by the difference of the first from 100. Based on the contribution of each diet to the body carbon of the two Collembola species, fractionation of nitrogen was calculated. The calculation assumed that nitrogen was assimilated from the two dietary species in the same proportion as carbon. This assumption appears reasonable considering the low specificity of Collembola digestion (Hopkin 1997).

Table 1 Design of fungal species combination fed in the mixed diet treatment. Diet quality, represented through *Cladosporium cladosporioides* as high quality reference and *Aspergillus nidulans* wildtype (WT), as low quality reference and putative toxicity represented through sterigmatocystin deficient strains of *A. nidulans* (S3, S4, S5 and S6) were used. Fungi differed in $\delta^{13}C$ signatures to reveal ^{13}C incorporation of both components in each combination. For agar preparation sucrose from sugar beet (C_3 plant, $\delta^{13}C$ −26.03‰) for the factor food quality and corn (C_4 plant, $\delta^{13}C$ −10.92‰). This resulted in 8 combinations offered as mixed diets.

Reference fungus	ST deficient mutant of *A. nidulans*
A. nidulans (C_3) *with*	*A. nidulans* S3 (C_4)
	A. nidulans S4 (C_4)
	A. nidulans S5 (C_4)
	A. nidulans S6 (C_4)
C. cladosporioides (C_3) with	*A. nidulans* S3 (C_4)
	A. nidulans S4 (C_4)
	A. nidulans S5 (C_4)
	A. nidulans S6 (C_4)

2.3.4 Stable isotope analysis

Samples of sucrose, agar, fungi and Collembola were dried (3 days, 60°C), weighed into tin capsules and stored in a desiccator until analysis. Fungi and Collembola were frozen at −80°C prior to drying. Collembola were kept without food for 6 h prior to freezing to clear their gut. Whole animals were used for measurement of stable isotope signatures and all individuals per replicate were bulked to one sample. Isotope ratios were determined using a coupled system of an elemental analyzer (NA 1500, Carlo Erba, Milan) and a mass spectrometer (MAT 251, Finnigan, Bremen). Accuracy of the measurement is 0.1‰ and 0.2‰ for ^{15}N or ^{13}C, respectively. Stable isotope abundance is expressed using the δ notation with $X\ (‰) = (R_{sample} - R_{standard})/R_{standard} \times 1000$, where X represents ^{15}N or ^{13}C, and R_{sample} and $R_{standard}$ represent the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratios of the sample and standard, respectively. The fractionation of ^{13}C in the mixed diets could not be calculated due to the two different sources (C_3 and C_4).

2.3.5 Statistical analysis

Due to very large differences in reproductive and moulting rates between Collembola species, the impact of diets was analysed individually for each species. In the single diet experiment single factor ANOVA was used for analysing the effect of diet (six levels: *A. nidulans* WT, *A. nidulans* S3, *A. nidulans* S4, *A. nidulans* S5, *A. nidulans* S6 and *C. cladosporioides*). Two factor ANOVAs were used to test for the effect of the reference fungi [two levels: *A. nidulans* WT (low food quality) and *C. cladosporioides* (high food quality)] and the *A. nidulans* STdeficient strains (four levels: S3, S4, S5, S6) and their interaction on reproduction and moulting. An additional ANOVA with pooled data from single and mixed diet experiments was used to test whether the reproductive and moulting rates differ between the single and mixed experiments.

The ^{13}C and ^{15}N signatures in fungi were compared with those of the agar medium on which they grew by single factor ANOVAs. For the single diet experiment the effect of diet on fractionation (for both ^{13}C and ^{15}N) was analysed by two factor ANOVAs with Collembola species (two levels) and diet (four levels) as factors. In the mixed diet experiment stable isotope fractionation and incorporation into collembolan tissue was analysed by three factor ANOVAs with Collembola (two levels), reference fungi (two levels) and ST deficient strains (four levels) as factor. Additional ANOVAs were also performed on pooled data from single and mixed diet experiments to analyse the effect of single and mixed diet on ^{15}N signatures and incorporation. Tukey's HSD posthoc test was used to compare means of treatments with more than two levels. Regression analysis was used to test if the fractionation of ^{13}C and ^{15}N increased with the putative fungal toxicity. Data were analysed using Statistica 7.1 software package (StatSoft, Inc., Hamburg), inspected for homogeneity of variance and log-transformed if required.

2.4 Results

2.4.1 Fitness parameter

2.4.1.1 Reproduction in single diets

The number of eggs laid per Collembola individual differed by more than an order of magnitude between the two springtail species (81.34 *vs.* 3.86 eggs ind.$^{-1}$ 5 weeks^{-1} for *F. candida* and *H. nitidus*, respectively; $F_{1,48} = 634.8$, $p < 0.001$). In both Collembola species reproduction strongly varied with fungal diet ($F_{5,24} = 15.94$, $p < 0.001$ and $F_{5,24} = 5.43$, $p = 0.002$ for *F. candida* and *H. nitidus*, respectively).

The reproduction of *F. candida* feeding on *C. cladosporioides* significantly exceed that when feeding on *A. nidulans*, S5, S6 and WT (Tukey's HSD test, $p < 0.001$; Fig. 2a). Reproduction of *H. nitidus* did not differ significantly between the *C. cladosporioides, A. nidulans* WT and S6 treatments (Tukey's HSD test, $p > 0.560$), however, reproduction of *H. nitidus* in these treatments was higher than in the S3, S4 and S5 treatments (Fig. 2b).

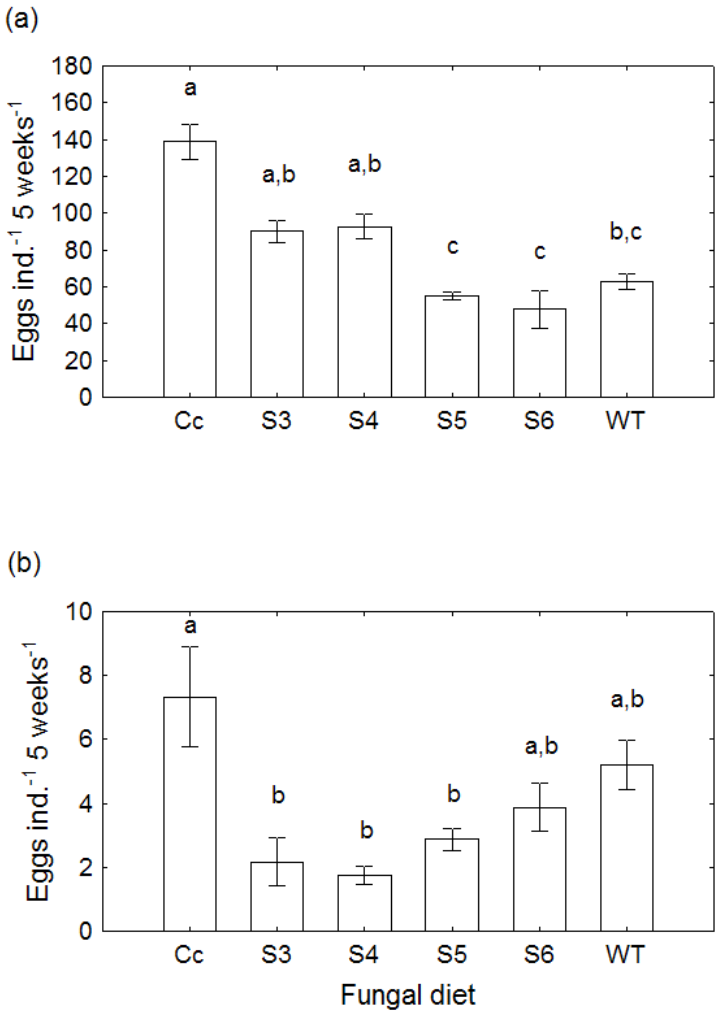

Figure 2 Reproduction (eggs ind.$^{-1}$ 5 weeks^{-1}) of *Folsomia candida* (a) and *Heteromurus nitidus* (b) as affected by feeding on single diets (CC: *Cladosporium cladosporioides*, *A. nidulans* S3, S4, S5, S6 and WT). For abbreviations see Fig. 1. Means of 5 ± 1 SE replicates of 10 individuals each. Different letters above the bars indicate significant differences (Tukey's HSD test, $p < 0.05$).

2.4.1.2 Reproduction in mixed diets

Reproduction of both Collembola species was significantly higher in diets containing *C. cladosporioides* as compared to those containing *A. nidulans* WT [111 *vs.* 71 eggs ind.$^{-1}$ 5 weeks^{-1} for *F. candida* ($F_{1,32}$ = 64.95, p < 0.001) and 22 *vs.* 8 eggs ind.$^{-1}$ 5 weeks^{-1} for *H. nitidus* ($F_{1,32}$ = 44.49, p < 0.001); Fig. 3a,b]. Further, the reproduction of both Collembola species depended on the interaction between the reference fungus (*C. cladosporioides* and *A. nidulans* WT) and ST deficient strains ($F_{3,32}$ = 7.11, p < 0.001 for *F. candida* and $F_{3,32}$ = 8.35, p < 0.001 for *H. nitidus*). Reproduction of *F. candida* varied with the added ST deficient strains; mixing *A. nidulans* WT with S6 reduced the reproduction by ~ 40% and 56% compared to the S4 and S5 mixtures respectively (Fig. 3a). In *H. nitidus* reproduction in the mixture of *C. cladosporioides* with *A. nidulans* S4 significantly exceed that of the mixture containing *C. cladosporioides* and *A. nidulans* (Fig. 3b).

Generally, the oviposition in mixed diets was significantly higher than in single diets ($F_{1,138}$ = 3.24, p < 0.01). Reproduction in mixed diets was significantly higher in *H. nitidus* (+361.4%; $F_{1,67}$ = 56.10, p < 0.001), but only marginally higher in *F. candida* ($F_{1,67}$ = 3.24, p = 0.076) where reproduction rate was at a maximum in the single diet with *C. cladosporioides* (+59.6%) compared to all other diet types and combinations.

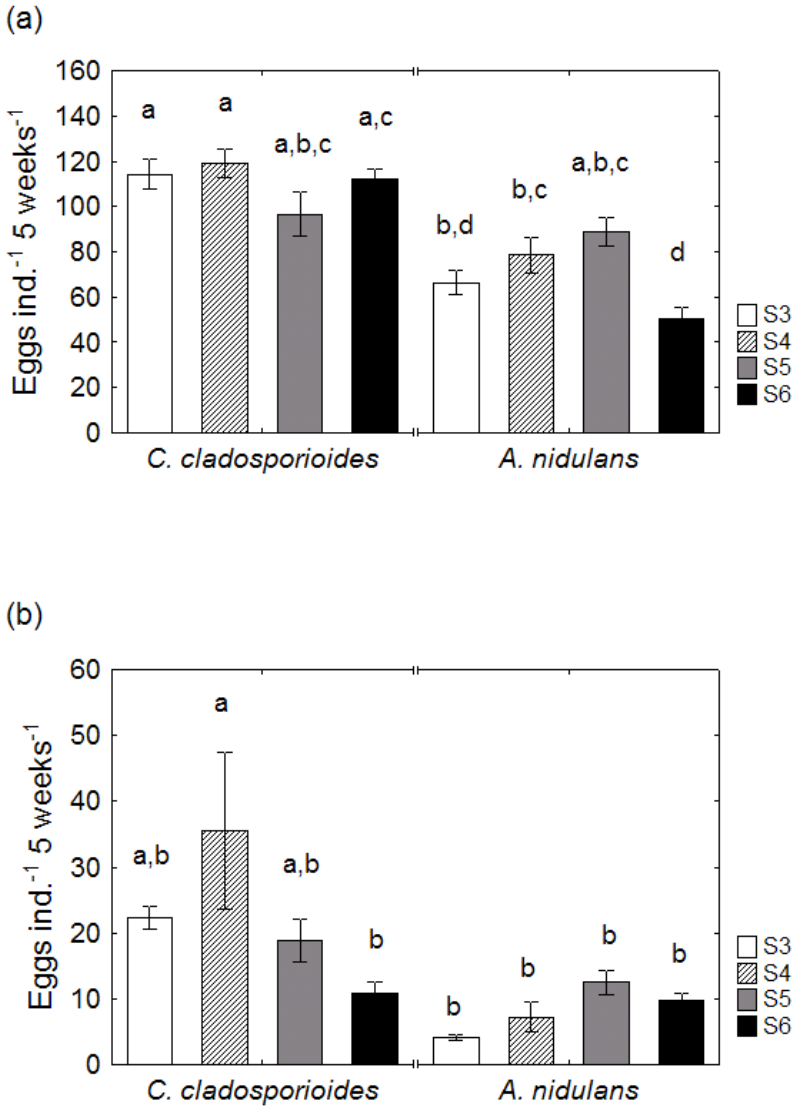

Figure 3 Reproduction of *Folsomia candida* (a) and *Heteromurus nitidus* (b) as affected by feeding on mixed diets (CC: *Cladosporium cladosporioides* and *A. nidulans* WT each combined with S3, S4, S5 and S6). For abbreviations see Fig. 1. Means of 5 replicates ± 1 SE of 10/ 15 individuals each. Different letters above the bars indicate significant differences (Tukey's HSD test, $p < 0.05$).

2.4.1.3 Moulting in single diets

The moulting rate of *H. nitidus* (3.8 moults ind.$^{-1}$ 5 weeks^{-1}) significantly exceeded that of *F. candida* (2.6 moults ind.$^{-1}$ 5 weeks^{-1}; $F_{1,48} = 49.9$, $p < 0.001$). Moulting rates varied with fungal diet ($F_{5,48} = 15.53$, $p < 0.001$) and this was similar in both Collembola species ($F_{5,48} = 0.90$, $p = 0.48$ for the interaction between fungal diet and Collembola). Moulting rates did not differ significantly between *C. cladosporioides* and *A. nidulans* WT (Tukey's HSD test, $p = 0.99$ and

p = 0.77 for *F. candida* and *H. nitidus*, respectively), but in the S5 and S6 treatments they were significantly lower than in *C. cladosporioides* and *A. nidulans* WT treatments (Tukey's HSD test, $p < 0.001$; Fig. 4).

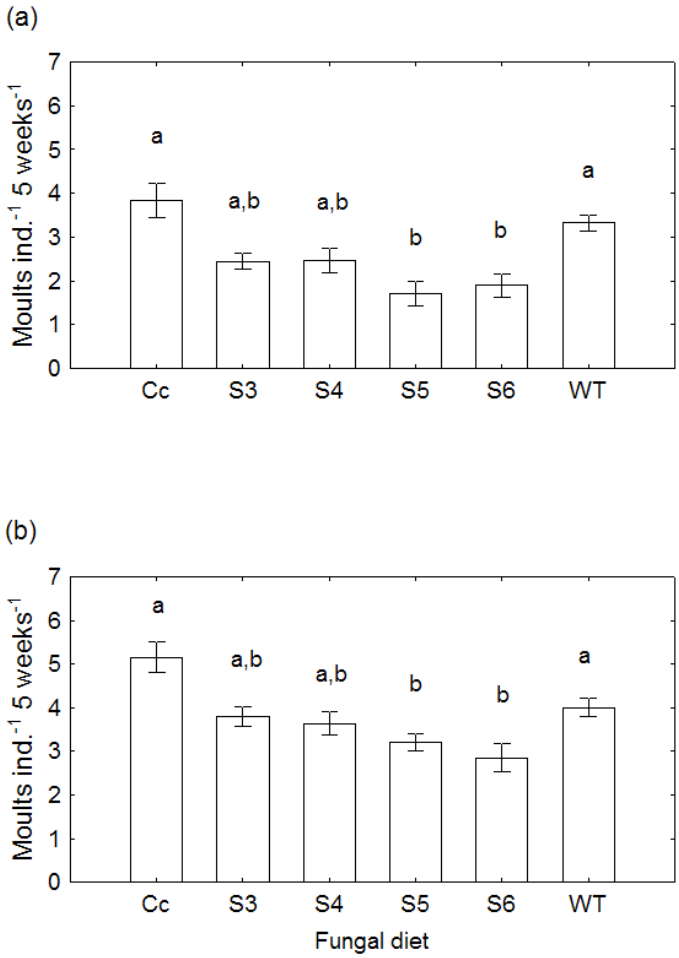

Figure 4 Moulting of *Folsomia candida* (a) and *Heteromurus nitidus* (b) as affected by feeding on mixed diets (CC: *Cladosporium cladosporioides*, *A. nidulans* S3, S4, S5, S6 and WT). For abbreviations see Fig. 1. Means of 5 replicates ± 1 SE of 10/15 individuals each. Different letters above the bars indicate significant differences (Tukey's HSD test, $p < 0.05$).

2.4.1.4 Moulting in mixed diets

Moulting rates of *F. candida* in the mixed diet treatment were similar to those in the single diet treatment (2.6 *vs.* 2.8 moults ind.$^{-1}$ 5weeks^{-1}; $F_{1,67} = 1.68$, $p = 0.19$; Fig. 5a). However, moulting rates in *H. nitidus* in the mixed diet treatment significantly exceeded those in the single diet treatments (4.4 *vs.* 3.8 moults ind.$^{-1}$ 5weeks^{-1}; $F_{1,67} = 11.69$, $p < 0.005$; Fig. 5b). In both Collembola species moulting rates varied with the fungal species/strains combined with

A. nidulans WT or *C. cladosporioides* ($F_{1,38}$ = 52.67, p < 0.001 and $F_{1,38}$ = 8.83, p < 0.001 for *F. candida* and *H. nitidus*, respectively). When the sterigmatocystin deficient strains were combined with *C. cladosporioides* moulting rates of *F. candida* and *H. nitidus* were on average 3.4 and 4.7 moults ind.$^{-1}$ 5 weeks^{-1}, respectively, whereas respective rates when combined with *A. nidulans* WT were 2.2 and 4.1 moults ind.$^{-1}$ 5 weeks^{-1}.

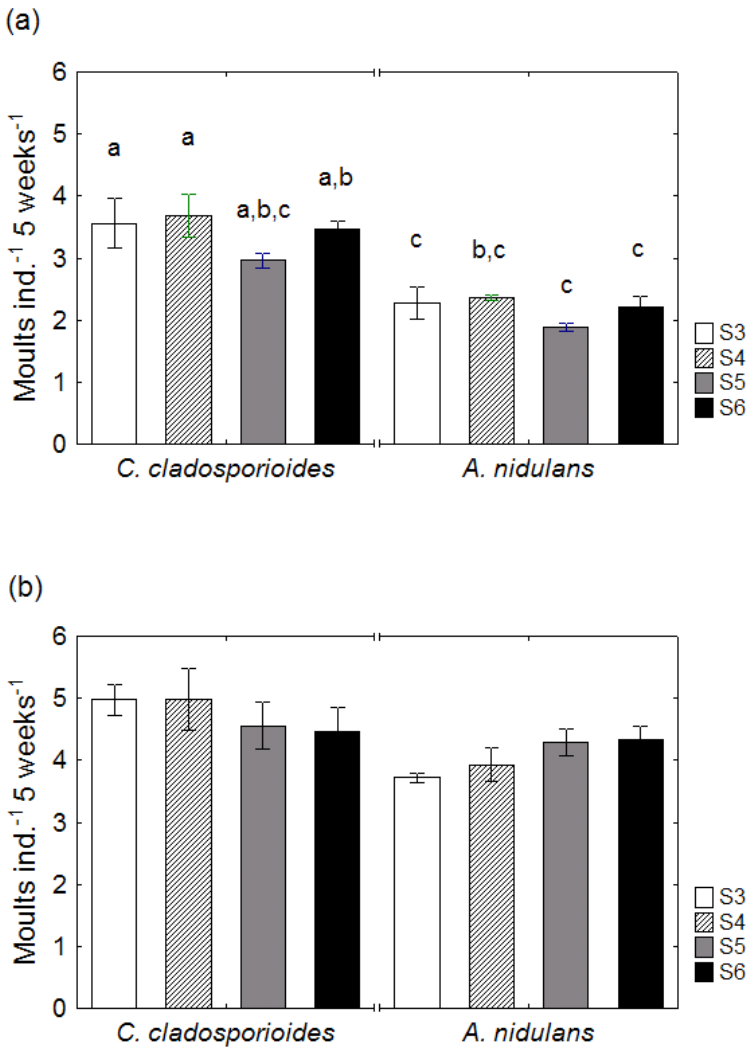

Figure 5 Moulting of *Folsomia candida* (a) and *Heteromurus nitidus* (b) per individual feeding on mixed diets (CC: *Cladosporium cladosporioides* and *A. nidulans* WT each combined with S3, S4, S5 and S6). For abbreviations see Fig. 1. Means of 5 replicates of 10/15 individuals each. Different letters above the bars indicate significant differences (Tukey's HSD test, p < 0.05); lack of letters in Fig 5b denotes no significant differences between the treatments. Means of 5 replicates ± 1 SE.

2.4.2 Stable isotope analysis

2.4.2.1 Fractionation in single diets

Stable isotope signatures of the fungi differed significantly from the agar medium on which they were grown for $\delta^{13}C$ but not for $\delta^{15}N$ (Table 2). $\delta^{13}C$ signatures of fungi grown on C_3 agar (-25.2‰) were significantly depleted compared to the agar (-23.6‰; $F_{1,7}$ = 16.16, p = 0.005); the same was true for fungi grown on C_4 agar (-14.0 *vs.* -13.3‰; $F_{1,13}$ = 5.21, p = 0.04; Fig. 6).

Table 2 Stable isotope signatures of *Cladosporium cladosporioides, Aspergillus nidulans* wildtype (WT) and sterigmatocystin deficient strains of *A. nidulans* (S3, S4, S5 and S6) and $\Delta^{13}C$ represents the difference in $\delta^{13}C$ signatures between fungal strains and their growth medium. For agar preparation sucrose from sugar beet (C_3 plant, $\delta^{13}C$ −26.03‰) and corn (C_4 plant, $\delta^{13}C$ −10.92‰) was used. Means (± SD) of five replicates.

		$\delta^{13}C$ (‰)			$\delta^{15}N$ (‰)		
Diet	Sucrose	Mean	SD	$\Delta^{13}C$ agar	Mean	SD	$\Delta^{15}N$ agar
C. cladosporioides	C_3	-25.74	0.21	-2.19	-5.81	1.28	-8.82
S3	C_4	-13.48	0.05	-0.18	1.18	0.28	-0.03
S4	C_4	-13.91	0.42	-0.60	1.28	0.03	0.07
S5	C_4	-13.82	0.15	-0.51	1.06	0.04	-0.15
S6	C_4	-14.57	0.22	-1.27	1.25	0.20	0.04
A. nidulans	C_3	-24.65	0.49	-1.11	3.59	0.46	0.58

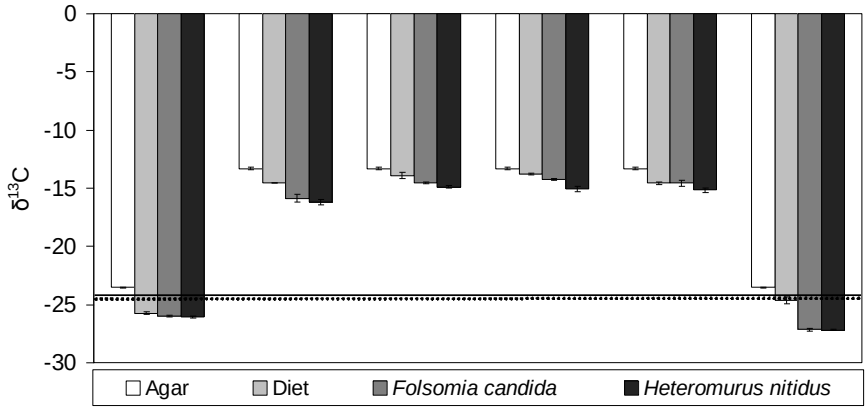

Figure 6 Signatures of ^{13}C of the six diets studied in the single diet experiment. Collembola diets consisted of *Cladosporium cladosporioides* (C_3), toxin deficient strains of *A. nidulans* S3, S4, S5 and S6 (C_4) and *Aspergillus nidulans* wildtype (C_3). Fungi were grown on agar medium (see Table 2). The dashed line represents the signature of *Folsomia candida* and the uncut line that of *Heteromurus nitidus* at the start of the experiment. For abbreviations see Fig. 1. Means of 5 replicates ± 1 SE.

When feeding on single fungal diets fractionation of ^{13}C ($\Delta^{13}C$ of -1.12 *vs.* -1.43‰; $F_{1,44}$ = 8.28, p = 0.006) and ^{15}N ($\Delta^{15}N$ of 5.27 *vs.* 2.67‰; $F_{1,44}$ = 98.5, p < 0.001) in *H. nitidus* significantly exceeded that in *F. candida*. Fractionation of ^{13}C strongly depended on the ingested diet ($F_{5,21}$ = 27.28, p < 0.001 and $F_{5,23}$ = 33.93, p < 0.001 for *F. candida* and *H. nitidus*, respectively; Table 3), with the highest fractionation in the *A. nidulans* S3 and WT diets (Fig 7a). The fractionation of ^{13}C did not increase with the putative toxicity of the diets (R^2 = 0.07, p = 0.17 for *F. candida* and R^2 = 0.10, p = 0.09 for *H. nitidus*).

Fractionation of ^{15}N in Collembola significantly varied with fungal diet ($F_{5,44}$ = 6.07, p < 0.001), but the variation differed between Collembola species ($F_{1,44}$ = 6.0, p < 0.001; Fig. 7b). Fractionation of ^{15}N in both Collembola species was more pronounced when feeding on *C. cladosporioides* than when feeding on *A. nidulans* WT (Tukey's HSD test, p < 0.001). Surprisingly, the fractionation of ^{15}N decreased with increasing putative toxicity of the fungal strains in *F. candida* (R^2 = 0.36, p < 0.001; Fig. 7b) and *H. nitidus* (R^2 = 0.19, p = 0.020).

Table 3 Fractionation of ^{13}C and ^{15}N in *Folsomia candida* and *Heteromurus nitidus* feeding on single and mixed fungal diets for 48 days (*Cladosporium cladosporioides* (CC), *Aspergillus nidulans* wildtype (WT) and sterigmatocystin deficient strains (S3, S4, S5 and S6)). Fractionation of ^{15}N in mixed diets was calculated assuming that nitrogen was used in the same proportion as carbon from the respective resources (see Materials and Methods). Means (± SD) of five replicates.

	$\delta^{13}C$				$\delta^{15}N$					$\delta^{15}N$			
Collembola	*F. candida*		*H. nitidus*		*F. candida*		*H. nitidus*			*F. candida*		*H. nitidus*	
Single	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mixed	Mean	SD	Mean	SD
CC	-0.25	0.19	-0.31	0.17	5.42	1.39	5.08	0.62	CC + S3	1.99	0.39	8.77	0.51
S3	-2.40	0.74	-2.71	0.53	2.06	0.61	6.00	0.20	CC + S4	2.26	0.55	8.16	0.42
S4	-0.64	0.23	-0.99	0.24	2.39	0.71	5.85	0.39	CC + S5	2.50	1.03	8.32	0.29
S5	-0.43	0.23	-1.25	0.51	2.73	1.78	6.26	0.82	CC + S6	2.20	0.38	8.09	0.83
S6	-0.18	0.62	-0.82	0.42	3.24	1.28	5.74	0.98	WT + S3	2.53	0.66	2.38	0.83
WT	-2.50	0.28	-2.52	0.14	0.60	0.68	2.73	0.55	WT + S4	2.33	1.27	2.47	0.47
na	na	na	na	na	na	na	na	na	WT + S5	2.03	0.91	3.90	1.19
na	na	na	na	na	na	na	na	na	WT + S6	2.19	1.74	3.66	0.31

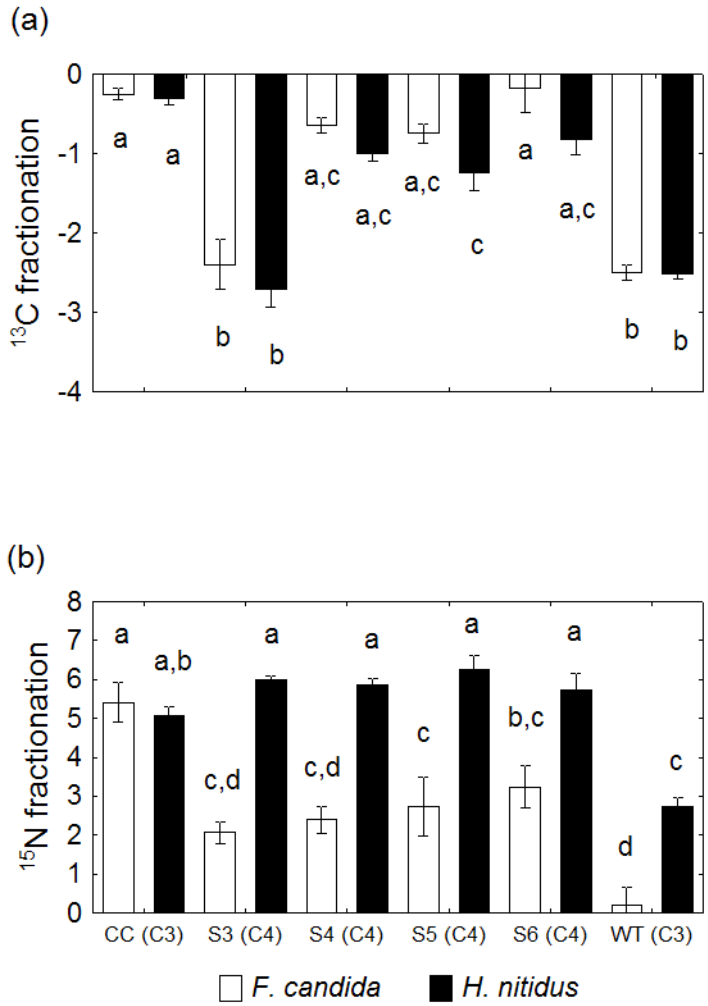

Figure 7 Fractionation of ^{13}C (a) and ^{15}N (b) in *Folsomia candida* and *Heteromurus nitidus* as affected by different single diets: CC (*Cladosporium cladosporioides*) and the four isogenic strains of *Aspergillus nidulans* blocked at different steps along the Sterigmatocystin biosynthetic pathway, i.e. *A. nidulans* S3, S4, S5, S6 and WT. Means of 5 replicates ± 1 SE. For abbreviations see Fig. 1.

2.4.2.2 Fractionation in mixed diets

As in the single diet experiment, fractionation of ^{15}N in *H. nitidus* exceeded that in *F. candida* (Δ^{15}N of 5.72 *vs.* 2.25‰; $F_{1,61}$= 378.1, p < 0.001). Also, in general fractionation in the diets including the high quality reference fungus *C. cladosporioides* exceeded those including the low quality reference fungus *A. nidulans* WT (Δ^{15}N of 5.28 *vs.* 2.68‰, $F_{1,61}$= 215.3, p < 0.001). However, this was more pronounced in *H. nitidus* than *in F. candida* as showed by the interaction between Collembola species and reference fungi. ($F_{1,61}$= 199.69, p < 0.001; Table 3). Furthermore, fractionation of ^{15}N significantly depended on the interaction between Collembola species, reference fungi and ST deficient strains ($F_{3,61}$= 4.18, p = 0.009) with the fractionation being at a maximum in *H. nitidus* feeding on mixtures containing

C. cladosporioides ($\Delta^{15}N$ of 8.3‰). Fractionation of ^{15}N was significantly higher in single diets ($\Delta^{15}N$ of 3.0‰) compared to mixed diets ($\Delta^{15}N$ of 2.2‰) in *F. candida* ($F_{1,63} = 5.19$, $p = 0.03$) but not in *H. nitidus* ($F_{1,64} = 0.22$, $p = 0.64$; Table 3).

2.4.2.3 Carbon incorporation

Both *F. candida* and *H. nitidus* incorporated carbon from both reference fungal species offered, however, the amount incorporated from *C. cladosporioides* generally exceeded that incorporated from *A. nidulans* WT ($F_{1,61} = 157.16$, $p < 0.001$; Fig. 8a). The amount of C incorporated from the ST deficient strains varied between the Collembola species and the reference fungi; *H. nitidus* generally incorporated less C from the ST deficient strains than *F. candida* ($F_{1,61} = 4.22$, $p = 0.041$, Fig 7a). In the mixtures containing *A. nidulans* WT but not in those containing *C. cladosporioides*, the amount of C incorporated decreased with increasing putative toxicity from S3 to S6 ($F_{1,18} = 11.18$, $p = 0.005$, $R^2 = 0.38$, $p = 0.004$; Fig. 8b).

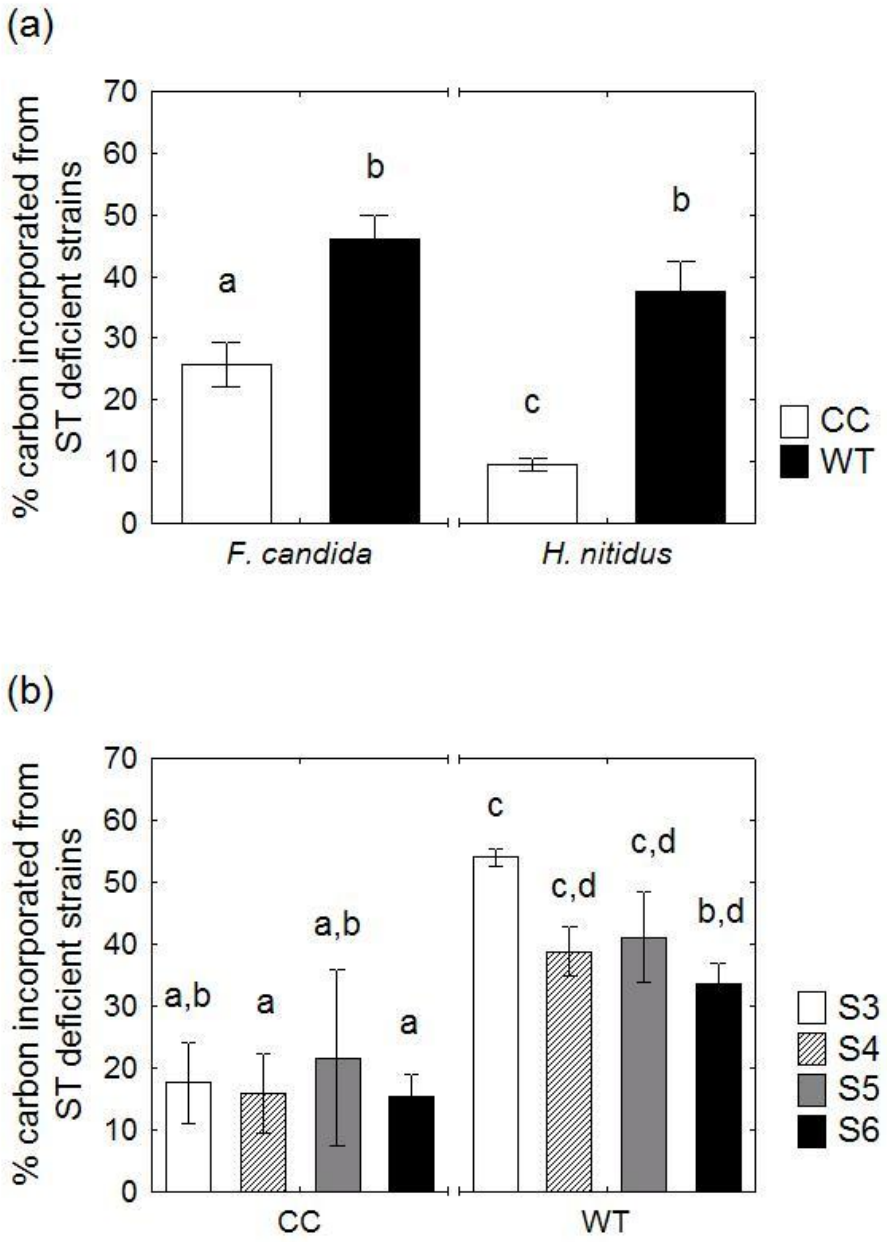

Figure 8 Percentage carbon incorporated (calculated from $\delta^{13}C$ signatures) as affected by (a) Collembola species (*Folsomia candida* and *Heteromurus nitidus*) and the presence of the two reference dietary species of high and low quality (*Aspergillus nidulans* WT and *Cladosporium cladosporioides*) and (b) sterigmatocystin (ST) deficient mutants of *A. nidulans* (S3-S6) and the reference dietary species. Means of 5 replicates ± 1 SE. For abbreviations see Fig. 1.

2.5 Discussion

2.5.1 Diets, sterigmatocystin and Collembola fitness

Benefits of feeding on mixed diets are commonly ascribed to the dilution of toxins or a more balanced uptake of nutrients and this may also apply for Collembola (Scheu and Folger 2004; Scheu and Simmerling 2004). In the present study mixed diet only increased the fitness (measured as reproductive rates) in one of the two species of Collembola (*H. nitidus*). Both Collembola species preferentially ingested *C. cladosporioides* in mixed diets, but only *H. nitidus* increased egg production when *A. nidulans* was added suggesting that the effect of mixing diets on Collembola performance is species specific. Since both Collembola species preferred the fungus with the highest C-to-N ratio (*C. cladosporioides*) and without sterigmatocystin, this suggests that toxins played a more important role in Collembola nutrition than fungal protein content. Using *A. nidulans* strains defect in ΔLaeA, a global regulator for secondary metabolites (Bok and Keller 2004), Rohlfs et al. (2007) observed higher reproduction of *F. candida* feeding on the strain lacking secondary metabolites including sterigmatocystin. Furthermore, Shaw (1985) showed that Collembola prefer to feed on hyphae containing low concentrations of toxins. Thus, fungal secondary metabolites may act as shield against fungivory and consequently, we expected a decrease in reproduction with increasing putative fungal toxicity. As expected, reproduction of *F. candida* feeding on the putatively low toxicity mutants of *A. nidulans* (S3 and S4) exceeded that when feeding on putatively high toxicity mutants (S5 and S6) and the wildtype (WT). In contrast, reproduction of *H. nitidus* increased with increasing putative toxicity of *A. nidulans* mutants with no differences between the single diets containing *A. nidulans* WT and *C. cladosporioides*. However, the differential response of *H. nitidus* to the fungal treatments should be interpreted with caution as the very different reproductive output of the two species studied suggest that they differ in their resource allocation to growth (moulting) and reproduction. Nevertheless, the higher reproduction of *H. nitidus* feeding on *A. nidulans* WT compared with the sterigmatocystin deficient strains suggest that this species is well adapted to detoxify sterigmatocystin. Potentially, this is related to the habitat the two species live in. Compared to *F. candida* which preferentially colonizes the upper mineral soil (hemiedaphic species), *H. nitidus* predominantly lives in the litter layer (epedaphic species) a habitat where melanized and toxic fungi are most abundant (van der Wal et al. 2009).

Moulting rates are generally considered to reflect animal growth. Fungal diets did not significantly affect moulting in mixed *vs.* single diets, however, the two Collembola species differed significantly in their moulting rates. More frequent moulting in *H. nitidus* than in

F. candida might be linked to its ability to selectively feed on the high quality reference fungus and this is supported by the results on C incorporation. This is in line with previous studies indicating that in epedaphic species metabolic rates and foraging on high quality food resources exceed that in hemiedaphic species (Petersen 1980; Hopkin 1997).

2.5.2 Stable isotope fractionation

Previous diet switch experiments (from C_3 to C_4 materials) showed that springtail biomass turnover rates are fast and that it is unlikely that any highly abundant compounds in Collembola would have a half-life longer than six days (Ostrom et al. 1997; Chamberlain et al. 2004). Therefore, Collembola isotopic fractionation can be assessed after switching from C_3 to C_4 materials given sufficient time for tissue element turnover. As the current study lasted 5 weeks we assume that the observed differences in isotopic signatures are due to fractionation and do not reflect the previous diet.

One of the limitations in interpreting natural variations of ^{15}N and ^{13}C signatures in microbivorous soil invertebrates is that isotope fractionation may vary with dietary species, e.g., fungi, bacteria and algae (Haubert et al. 2005). Indeed, in the present study the isotope signatures of the fungal species/strains studied often were significantly depleted relative to the growing substrate (agar) for both ^{13}C and ^{15}N. The fractionation of ^{13}C and ^{15}N in Collembola varied with diet and often differed from the expected values of 0-0.4‰ and 2.5-3.4‰ enrichment per trophic level for ^{13}C and ^{15}N, respectively (Post 2002; Vanderklift and Ponsard 2003). In single diets the fractionation of ^{13}C in both Collembola species was generally negative and the observed depletion in ^{13}C varied with fungal species and mutants of *A. nidulans*. In particular when feeding on *A. nidulans* S3 and WT, Collembola were more depleted in ^{13}C compared to other diets. Furthermore, fractionation of ^{15}N in Collembola also depended on the species of fungi ingested and consistently exceeded the expected trophic level shift of decomposers of 2.54‰ (Vanderklift and Ponsard 2003) for *H. nitidus* except in the *A. nidulans* WT diet.

In contrast to our third hypothesis, fractionation of ^{13}C did not increase with putative toxicity in single diets, mainly because the fractionation of ^{13}C in both Collembola species was significantly higher when feeding on *A. nidulans* S3 and WT. Brown et al. (1996) showed that knocking-out the regulatory genes in the S3 mutant may impact not only sterigmatocystin precursors but also other gene clusters. This might explain the unusual fractionation in the *A. nidulans* S3 strain. Also in contrast to the third hypothesis, the fractionation of ^{15}N did not increase with the toxicity in both Collembola species. Similarly,

^{15}N fractionation in both Collembola species was consistently lower when feeding on *A. nidulans* WT which contains sterigmatocystin than when feeding on the other fungal strains/species. It has been reported that food of high quality (particularly high N content) results in an enrichment in ^{15}N due to increased protein turnover resulting in higher fractionation (Rothe and Gleixner 2000; Haubert et al. 2005); however, N content and fractionation was not correlated in this study. Although toxicity of the mutants and stable isotope fractionation was not correlated closely, the results suggest that the observed difference in fractionation at least in part are related to fungal toxins, presumably, by altering excretion rates necessary for detoxification.

Synergistic effects in ^{15}N fractionation occurred in mixed diets; often fractionation in *H. nitidus* feeding on mixed diets exceeded that when feeding on both single diets and varied in the range 8.09-8.77‰. However, considering that Collembola in the field are likely to feed on a variety of food substrates, an average fractionation in the range of 2.5-3.4‰ as proposed earlier (Post 2002; Vanderklift and Ponsard 2003) might still be a reasonable estimate. Yet, fractionation of ^{15}N in Collembola as mainly fungal feeders may exceed that of decomposers feeding typically on detritus since food quality of fungi exceeds that of decomposing litter materials.

In conclusion, our first hypothesis that Collembola fitness decreases with increasing toxicity was only supported for *F. candida*. The second hypothesis that Collembola benefit from mixed diets was also only partly supported for *H. nitidus*; increased reproduction of this species in mixed diet treatments presumably was due to more pronounced food selection as compared to *F. candida*. In contrast, reproduction in *F. candida* was at a maximum in the single diet with *C. cladosporioides* and generally the two Collembola species responded differently to sterigmatocystin presence. Strong and specific responses of the two Collembola species to mixed diets, knock out mutants and toxins suggest the evolution of specific strategies to cope with the constraints associated with living in different soil layers. The third hypothesis assuming that fractionation of ^{13}C and ^{15}N will be more pronounced in more toxic diets was not supported, however, the results suggest an intricate link between fungal toxins (i.e. sterigmatocystin) and stable isotope fractionation.

CHAPTER 3

OLFACTORY CUES ASSOCIATED WITH GRAZING INTENSITY AND SECONDARY METABOLITES PATHWAY MODULATE COLLEMBOLA FORAGING BEHAVIOUR

3.1 Abstract

Fungal secondary compounds play an important role for springtail food choice and fitness. Little is known, however, on the role of olfactory cues for Collembola foraging behaviour and whether Collembola can olfactorily perceive volatiles associated with fungal secondary metabolite pathways. We investigated the ability of three species of Collembola (*Folsomia candida*, *Heteromurus nitidus* and *Supraphorura furcifera*) to use olfactory cues for discriminating between fungi of different phylogenetic affiliation (*Aspergillus nidulans*, *Cladosporium cladosporioides*, Ascomycota; *Laccaria bicolor*, Basidiomycota) and toxicity using fungal strains of *A. nidulans* with reduced secondary metabolite production. Furthermore, we studied if olfactory cues from hyphae injured by grazing affect the foraging behaviour of Collembola. We hypothesized that (i) Collembola are able to olfactorily perceive and respond to fungal species/strains with different secondary metabolite pathways, that (ii) Collembola are able to sense fungal mycelia injured by grazing and that (iii) grazing by Collembola changes the expression of genes in fungi related to the production of secondary metabolites. Each of the Collembola species studied preferentially oriented their foraging towards fungal strains of *A. nidulans* with suppressed secondary metabolites, and in particular towards the mutant where the global regulator for secondary metabolites (ΔlaeA) has been silenced. Two of the three Collembola species (*H. nitidus* and *S. furcifera*) sensed olfactory cues of previously grazed fungi and moved towards ungrazed fungi, however, the response of *S. furcifera* was restricted to fungi extensively exposed to grazing (5 days) suggesting that the response varies between Collembola species. Surprisingly, the investigated fungal gene spectrum did not significantly respond to grazing by Collembola.

Overall, the results support the first and second hypothesis indicating that Collembola (1) are able to olfactorily differentiate fungi of different toxicity and move towards more palatable fungi, and (2) avoid movement towards fungi previously exposed to grazing. The lack of changes in fungal gene regulation by grazing suggests that refined methods need to be adopted to investigate the genetic response of fungi to grazing.

3.2 Introduction

Collembola are among the most widespread, abundant and diverse decomposer invertebrates (Hopkin 1997). They are known to feed on a wide variety of food resources, however, they show strong preferences for certain types of food resulting in the occupation of distinct niches in the field (Parkinson et al. 1979; Chahartaghi et al. 2005). Despite the wide range of food types, including nematodes, fecal pellets of other animals, fine rootlets and detritus, fungi are presumably the major food source for most species of Collembola (Jørgensen et al. 2003). By grazing on fungi and detritus they are able to affect essential ecosystem processes, such as soil nitrogen and carbon turnover (Rusek 1998; Filser 2002). Alongside plants, fungi are known for producing a wide range of secondary metabolites (fungal toxins) and there is evidence that these compounds play an important role in Collembola food selection and fitness (Demain and Fang 2000; Scheu and Simmerling 2004; Rohlfs et al. 2007). Analogue to the plant-herbivore system in which plants evolved specific signalling pathways regulating plant defence responses to insect herbivores (Walling 2000; Kessler and Baldwin 2001; Schoonhoven et al. 2005), one would expect signalling pathways in fungi regulating their response to fungal grazers. Indeed, similar pathways as those regulating the response of plants to herbivores exist in fungi (Spiteller 2008) but it is unknown if they serve similar functions. Further, similar to plants, fungi may have evolved ways of signalling their toxicity. Surprisingly, however, in contrast to the plant-herbivore system (Belovsky and Schmitz 1994; Kessler and Baldwin 2001; Dearing et al. 2005) little is known on the role of fungal volatiles for food selection by fungal feeders.

Fungal choice depends on a number of factors including the concentration and composition of nutrients, secondary metabolites and volatiles, and the morphology and physical strength of mycelia and conidia (Tordoff et al. 2008). It has been shown that olfactory cues are important for foraging; Bengtsson et al. (1991) proved that Collembola perceive volatile compounds to a concentration of one nanogram while other studies showed that Collembola aggregate in zones of high microbial activity, and are able to sense and direct their movement towards CO_2 sources (Moursi 1962; Hassal et al. 1986).

Although toxin dilution plays an essential role in selecting fungal food, it is unknown whether Collembola are able to olfactorily perceive the presence of fungal toxins. Furthermore, it has been shown that fungi respond to mechanical injuries by increasing fungal respiration (Bengtsson and Rundgren 1983) and altering the production of secondary compounds including volatile emissions similar to the induced response in plants (Stadler and Sterner 1998). Based on this, one would expect Collembola to avoid grazing on fungi

previously attacked by fungal grazers, but it is not known if Collembola are able to olfactorily perceive these cues and adjust their foraging behaviour accordingly.

We investigated the ability of the three Collembola species to use olfactory cues for discriminating between fungi of different phylogenetic affiliation (*Aspergillus nidulans*, Ascomycota and *Laccaria bicolor*, Basidiomycota) and fungi differing in secondary metabolites, i.e. knockout mutants of *A. nidulans* with suppressed secondary metabolism and fungi exposed to different grazing intensities. Furthermore, we investigated grazing induced changes in fungal gene expression in *A. nidulans* and *L. bicolor*.

We hypothesized that Collembola (i) are able to olfactorily perceive and distinguish fungal species/strains differing in secondary metabolism, (ii) are able to sense and respond to fungal grazing by avoiding to forage on grazed fungi and that (iii) grazing by Collembola changes the expression of genes in fungi related to the production of secondary metabolites in fungi.

3.3 Material and Methods

3.3.1 Collembola

Three Collembola species of different functional groups were investigated. *Folsomia candida* (Willem, 1902) is a common parthenogenetic euedaphic widely distributed Collembola species which can be cultured easily in the laboratory. It is used as model organism in soil biology (Cragg and Bardgett 2001) and for pesticide research since more than 40 years (Hopkin 1997; Fountain and Hopkin 2005). *Supraphorura furcifera* (Börner, 1901) is a northern hemisphere hemiedaphic species. *Heteromurus nitidus* (Templeton, 1835) is a widespread epedaphic Collembola species of forest and arable soils. Adults of the three Collembola species were taken from laboratory cultures where they had been raised in polypropylene boxes consisting of a mixture of activated charcoal and plaster of Paris. The stock cultures were kept humid at 17°C in darkness and reared with soy flour. Prior to all experiments Collembola were starved for 24 hr to avoid faecal contaminations.

3.3.2 Fungi

Aspergillus nidulans is a widespread saprotrophic filamentous fungal species of the Ascomycota. The wildtype (WT) strain was taken from laboratory cultures and hereafter is labelled *A. nidulans* WT. For more than 50 years it has served as a model organism in cell biology and genetics and its genome is fully sequenced (Galagan et al. 2005).

We conducted three experiments on the olfactory response of the Collembola and one on the transcriptional response of certain fungal genes. For the first olfactometer experiment we used *A. nidulans* WT strain RDIT2.3 and a mutant strain with the secondary metabolism silenced (ΔlaeA mutant strain RJW46.4; labelled as *A. nidulans* ΔlaeA below). ΔlaeA is a global regulator for secondary metabolites and silencing of ΔlaeA in *A. nidulans* has been shown to significantly reduce the amount of secondary metabolites (Bok and Keller 2004). All *A. nidulans* strains were inoculated from a spore culture (0.6 M KCl solution) on autoclaved sterile Miracloth membrane covered by agar. Since there is evidence that medium quality affects fungal palatability (Leonard 1984), secondary metabolite (Dennis and Webster 1971) and volatile compound production (Wheatley et al. 1997) we opted for a high quality agar (Czapek Dox) which contains nutrient supplements and a defined pH value (Caddick et al. 1986). The spore solution (50 µl containing about 1.2 x 10^5 spores) was inoculated in the middle of a Petri dish (5.5 cm Ø) and kept in darkness at 37°C. For the second and third olfactometer and for the gene expression experiment we used the strain RSCS2 obtained by crossing RSCS1 and FGSC26 (Wilkinson and Ramaswamy 2004; acquired from Fungal Genetics Stock Center, Missouri, USA). The sterigmatocystin (ST) deficient strains of *A. nidulans* used in this study, labelled S3, S4, S5 and S6, are well characterized isogenic strains of *A. nidulans* that are blocked at different steps along the ST biosynthetic pathway, i.e., *aflR*, *stcJ*, *stcE*, *stcU* (Wilkinson and Ramaswamy 2004).

Cladosporium cladosporioides, a common soil fungus, was used as reference fungus of high nutritional quality (Scheu and Simmerling 2004). Fungi were grown on Czapek-Dox agar (pH = 7.3 +/- 0.2 at 25°C) and kept at 28°C in permanent darkness. Fungal cuts (1 cm Ø) were taken from young and actively growing hyphae of seven day old cultures avoiding contaminations with agar; fungal mats were separated from the agar to use fungi without agar.

Laccaria bicolor strain S238N (Orton) is a widespread symbiontic ectomycorrhizal basidiomycete with a completely sequenced genome (Martin et al. 2008). A fungal cut (1 cm Ø) was inoculated in each Petri dish on cellophane covered Pachlewska Agar P5 at 25° for 15 d (Paschlewski and Pachlewska 1974; di Battista et al. 1996). Each Petri dish contained 20 ml of modified Pachlewska medium (0.5 g tartrate, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 1 g glucose, 1 ml 1/10 diluted Kanieltra microelement solution and 20 g agar l^{-1} at pH 5.5).

3.3.3 Experimental designs

3.3.3.1 Olfactometer experiments

The experiments were performed in four chamber olfactometers made of PVC pipes modified after Steidle and Schöller (1997). They consisted of a cylinder (4 cm height, 9 cm inner Ø) divided by vertical plates into four chambers. On the top of the cylinder a walking arena (1 cm height 10 cm Ø) was placed consisting of cellulose filter paper (Rotilabo, no 70027071, Karlsruhe, Germany) with a rim of acrylic glass (0.9 cm height) and covered with a perspex plate. The fungal cuts were placed in two opposing chambers with the remaining two chambers (with background odour) serving as control. The olfactometers were rinsed with ethanol, detergent without additives and then with aqua bidest, and used only once a day to avoid carry over effects. Batches of 25 individuals were placed on the cellulose filter and the number of Collembola individuals foraging above each chamber was counted every 30 min for 3 h. The cellulose filter paper constituting the walking arena for Collembola was kept moist during the experiments by adding distilled water. The following combinations were tested for each of the three Collembola species as three experimental runs:

Experiment 1: *A. nidulans* RDIT2.3 (WT) was offered together with the mutant strain of *A. nidulans* ΔlaeA where the whole secondary metabolism is putatively silenced (ΔlaeA RJW46.4).

Experiment 2: The high quality reference fungus *C. cladosporioides* and the wild-type strain *A. nidulans* RSCS2 (WT) were offered in combination with each of the four knock out mutants of the ST pathway (S3, S4, S5, S6).

Experiment 3: Ungrazed and grazed cuts of *A. nidulans* and *L. bicolor* S238N exposed to grazing of the same intensity for different periods of time (3 h, 20 h and 5 d) were offered separately to each of the three Collembola species. Conspecific individuals were used to establish the grazing treatments and in order to prevent that food choice by Collembola was affected by the presence of other fungal grazers (or their odour) all individuals have been removed from the grazed patches 2 h before adding the fungal cuts to the arenas.

3.3.3.2 Gene expression experiments

We tested fungal gene expression responses to Collembola grazing time for *L. bicolor* S 238N (no grazing, before grazing, 3h and 20h grazing) and *A. nidulans* WT RSCS2 (no grazing, grazing for 5 days) in two experimental runs with slightly different setups (details below):

Laccaria bicolor S238N experiment

The in vitro bioassay was set up in Petri dishes (9 cm inner diameter) with four replicates per treatment. The gaze cylinders (0.4 mesh, 1 cm Ø, 1 cm height, perspex cover with 1 mm hole in the centre) were sterilized in 70% ethanol and dried under UV light for 1 h. A mycelial plug of 1 cm diameter was inoculated in the middle of the Petri dish. In each Petri dish we arranged four gaze cylinders around the mycelial plug in order to achieve the before grazing treatment. Five Collembola (*Supraphorura furcifera*) were transferred into each gaze cylinder, each Petri dish therefore contained 20 Collembola. After exposure the mycelium was removed from the cellophane with a scalpel, pooled per treatment for RNA extraction and briefly immersed in liquid nitrogen (-170°C). Then, samples were stored at -80°C until analysis. Trizol Reagent was used to extract fungal RNA according to the instruction for small RNA quantities of the manufacturer (Invitrogen, Stockholm, Sweden). PCR was used to test for DNA contaminations. RNAse-free 1% agarose electrophoresis was performed to check the RNA quality. RNA was converted into cDNA using RT-PCR (SMART PCR cDNA Synthesis Kit, Clontech, Palo Alto, USA). The array consisted of 4992 *L. bicolor* S238N clones (Deveau et al. 2007). The mycelium cDNA library consists of 768 expressed sequence tags and the fruiting body cDNA libraries contained 4224 clones from which 965 are sequenced. A total of 4992 *L. bicolor* PCR amplified cDNA inserts were spotted from 384 microtiter plates onto nylon membranes with the BioGrid arrayer (BioRobotics, Cambridge, UK) as recommended by the manufacturer (Eurogentec, Saraing, Belgium; Peter et al. 2003; Deveau et al. 2007). Each unique transcript was spotted twice on the membrane. Arrays were then wrapped in plastic foils and exposed to a phosphorimaging screen (Eastman Kodak Company, Rochester, USA) for varying periods (12 h to 3 d) and the target intensities were visualized by scanning at a resolution of 50 μm per pixel in a Personal Molecular Imager FX (BioRad Laboratories, Hercules, USA) that generated a 16-bit TIFF image. Each spot of the 16-bit TIFF image was detected and quantified after the 'volume quantification' method with the X –DOT-READER program (version 2.0; Cose, Paris, France). cDNA array hybridization was performed as described by Peter et al. (2003), Duplessis et al. (2005) and Deveau et al. (2007).

Aspergillus nidulans experiment

The in vitro bioassay was set up in Petri dishes (5.5 cm inner diameter) with fifteen replicates per treatment. Each Petri dish contained 70 Collembola (*Folsomia candida*). Seven to ten different 25-mers (oligomers consisting of 25 nucleobases) corresponding to the 3-prime half of each presented gene were spotted in hexaplicates on an *Aspergillus* secondary metabolism

array (ASMA). Custom spotted (BF-Biolabs, Denzlingen, Germany) CodeLink glass slides (GE Healthcare, Munich, Germany) were used (Schroeckh et al. 2009). Samples were pooled resulting in a minimum amount of 200 mg fungal mycelium. The Master Pure Yeast RNA Purification Kit in combination with Baseline-ZERO DNase (Epicentre® Biotechnologies, Madison, USA) was used to isolate total fungal RNA. Quantification and quality control were performed with the 2100 bioanalyzer (Agilent, Waldbronn, Germany) using the RNA 6000 LabChip Kit. QiaQuick PCR Purification columns were used for DNA purification after the manufacturer manual (Qiagen, Hilden, Germany). SuperScript™ Indirect cDNA Labeling System (Invitrogen, Carlsbad, USA) generated fluorescently labeled cDNA. Differing from the manual of the manufacturer the cDNA was labelled with Dyomics DY547 NHS/ DY647 NHS Chip Pack (Dyomics, Jena, Germany). Hybridization was performed in the HS400 Pro Hybridization Station (Tecan, Crailsheim, Germany) in buffer containing 4xSSC, 0.2% (w/v) SDS at 45°C for 16 h. Dye purification took place with the Reaction Clean Up II Concise Guide to cDNA Microarray Analysis II (Hedge et al. 2000). After scanning with the Axon GenePix 4200AL dual wavelength scanner (Molecular Devices, Union City, USA) the slides were analyzed with the GenePix Pro 6.1 software. A transcript was represented as a triplicate of probesets. Each probeset contains 10 probes (different 25-mer oligonucleotides) per gene.

3.3.3.3 Volatile experiment

Experimental setup

Glass petridishes (5.5 cm diameter, 1 cm height) were washed with 70% ethanol, rinsed with aqua bidest. and dried under UV-light for 1 h to remove contaminants. Volatile production of the fungal wildtype strain RSCS2 *Aspergillus nidulans* and one Collembola species *Supraphorura furcifera* were investigated in a preliminary experiment. Prior to the experiments Collembola were starved for 24 h to avoid faecal contaminations. Three treatments with three replicates each were set up and equipped with one Polydimethylsiloxan (PMDS; 5 mm length) tube each. 75 Collembola individuals were stored in each Collembola treatment replicate. Treatments were Czapek Dox Agar, Agar + Fungus (*A. nidulans* WT) and Agar + Fungus (*A. nidulans* WT) + Collembola *S. furcifera*. The experiment included five day old fungal cultures and exposure time was 48 hrs. At the end of the experiment all tubes were removed with a featherweight forceps and immediately sealed in glass vials.

Collection and analysis of volatile components

5 mm pieces of PMDS tubes (1.5 mm ID x 2.3 mm OD, Reichelt Chemietechnik, Heidelberg) were conditioned in a flow of helium gas for 15 min and 250°C. After exposure and before the measurement each piece of tubes was halved and one half transferred in a closed glass tube fitting in the Thermodesorptions-Injektor. The automatic change of glass tubes was performed with an adapted CONCEPT autocollector (PAS Technologies, Magdala, Germany). The utilized GC-MS-system consists of a HP 6890 Gaschromatograph (Agilent Technologies, Santa Clara CA, USA) equipped with a Optic 3 Injector as thermodesorption intake, a coldtrap (both ATAS Benelux B.V., Zoetermeer, Netherlands) and a time-of-flight-mass spectrometer (GCT, Micromass, Manchester, UK).

The substances were separated in a ZB-5MSi capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness, Phenomenex, Torrance, CA, USA). As the carrying gas Helium displayed a linear flow rate of 1 ml x min^{-1}. After insertion of the glass tubes the injector was heated with a heating rate of 30°C x s^{-1} from 45°C to 220 °C while keeping the subsequent cold trap at -150°C. After a transfer time of 180 s the cold trap was heated to 220 °C with a heating rate of 30°C x s^{-1}.

Initially the oven temperature was kept to 45 °C for 4 min, increased to 280°C with a heating rate of 10°C min^{-1} and kept for 2.5 min. Measurements were performed splitless and with a split ratio of 30:1. Retention indices were determined after Kovats (1958) through concurrent respective separated measurement of an alcane standard C_8-C_{20} (Aldrich, Taufkirchen, Germany). Comparisons of mass spectra were carried out with a software and libraries of Wiley, NIST und Massfinder 3.5 (Dr. D. Hochmuth, Hamburg, Germany) and manually with Adams (2007). Retention indices were compared with Massfinder 3.5.

3.3.4 Statistical analysis

Data were summarised as counts resulting from averaging the number of Collembola foraging on the different fungi every half an hour for 3 h and then transformed into percentages. Arcsin transformed percentages were used to test for differences between the treatments using a factorial analysis of variance approach ANOVA as integrated into the General Linear Models module in Statistica 9 (StatSoft, Inc.). This was used to analyse the effects of Collembola species, fungal strains (A. nidulans WT vs. ΔlaeA in Experiment 1; C. cladosporioides vs. WT, S3-S6 vs. WT and differences between S3-S6 in Experiment 2; A. nidulans and Laccaria bicolor in Experiment 3), grazing (grazed vs. ungrazed in Experiment 3)

and grazing intensity (3h, 20h and 5 days in Experiment 3). Untransformed percentages were used to generate graphs in Statistica 9.

***Gene expression data in Laccaria bicolor* S238N**

Gene expression assessment was performed with t-test and a Bayesian statistical framework implemented in the Cyber-T web interface (http://www.igb.uci.edu/servers/cybert/) (Long et al. 2001; Baldi and Hatfield 2002). Based on the statistical analysis, a gene was considered significantly up- or down-regulated if it met all three criteria: (I) the trend (up- or down-regulation) was consistent in all replicates; (II) grazed *vs*. control fold change > 2.5; (III) t-test *p*-value < 0.001. For the final analysis, fold changes of genes significantly differentially expressed were averaged.

Gene expression in Aspergillus nidulans

The signal intensity of a probeset was calculated with the robust estimation procedures Tukey's biweight (Mosteller and Tukey 1977). Data were processed using quantile normalization. Background correction was performed using the "minimum" method. To obtain the genes with changed expression a linear model fit was calculated for each gene using the 'LIMMA' (Linear Models for Microarray Data, Open Source; Gentleman et al. 2004; Smyth 2005). Reproducible differences between samples were investigated as well.

3.4 Results

3.4.1 Olfactometer experiment

In each of the three experiments Collembola were attracted by fungal volatiles with significantly higher numbers of individuals selecting the area with fungi (83%) as compared to the background without fungi ($F_{1,24}$ = 3413.4, p < 0.001; $F_{1,234}$ = 926.7; p < 0.001; $F_{1,174}$ = 304.59, p < 0.001 for Experiment 1, 2 and 3, respectively; Fig. 1abc). Whilst no significant difference could be detected in selectivity of areas with fungi between the three Collembola species in Experiment 1 and 3, in Experiment 2 a higher percentage of *H. nitidus* and *S. furcifera* individuals foraged above the fungal areas ($F_{2,234}$ = 61.13, p < 0.001; Fig 1b).

In Experiment 1 each of the three Collembola species were generally more attracted by the *A. nidulans* strain that lacked secondary metabolites (*A. nidulans* ΔlaeA) than by *A. nidulans* WT ($F_{1,24}$ = 332.7, p < 0.001; Fig. 2a). However, as indicated by the significant interaction between Collembola species and *A. nidulans* strain ($F_{1,24}$ = 15, p < 0.001) attraction to the *A. nidulans* ΔlaeA strain lacking secondary metabolites varied among Collembola species (Fig. 2a); in *S.*

furcifera 32.5% of the individuals foraged over the WT strain whereas in *H. nitidus* it were only 16.0%.

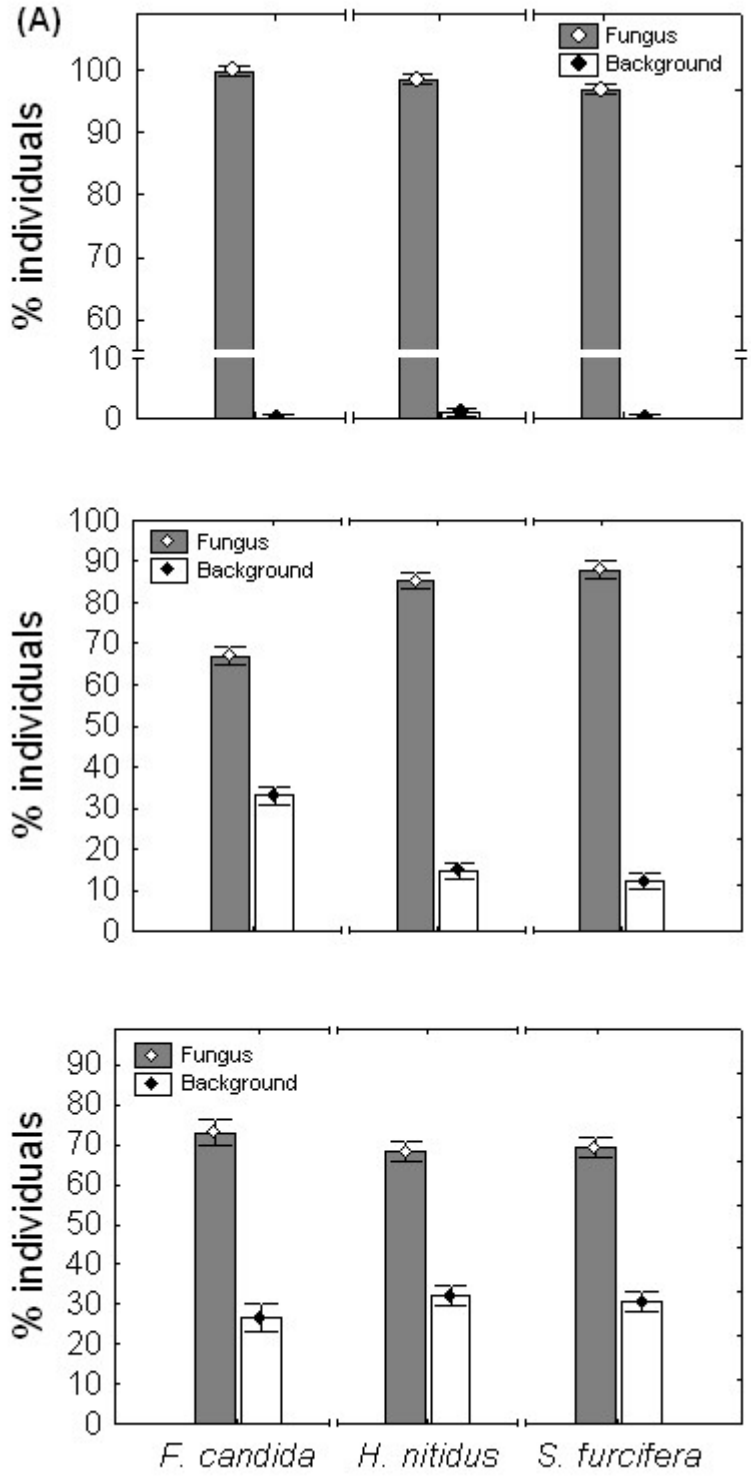

Figure 1 Percentages of total number of the three collembola species (*Folsomia candida*, *Heteromurus nitidus* and *Supraphorura furcifera*) which moved into the fungal odour area as compared to the background (no fungus) area in Experiment 1 (A), 2 (B) and 3(C).

In Experiment 2 Collembola were generally attracted by the high quality reference fungus *C. cladosporioides* as compared to the well defended *A. nidulans* WT ($F_{1,114}$ = 77.7, p < 0.001; Fig. 2b). However, preferences varied with Collembola species ($F_{2,114}$ = , p < 0.001; Fig. 2b). Both *H. nitidus* and *S. furcifera* preferred *C. cladosporioides* (64.0% and 69.9% of the individuals selected this species, respectively), whereas preferences in *F. candida* remained unclear with the species randomly selecting one of the two fungal species (Fig. 2b). Similarly, when confronted with *A. nidulans* WT and one of each of the four knockout mutants in which the sterigmatocystin synthesis pathway is blocked, *F. candida* foraged randomly, whereas *H. nitidus* and *S. furcifera* were more attracted by the odour of the sterigmatocystin deficient mutant ($F_{2,114}$ = 8.0, p = 0.001; Fig. 2c). In particular, *H. nitidus* and *S. furcifera* were more attracted to the odours of the *A. nidulans* S3 sterigmatocystin deficient mutant ($F_{6,108}$ = 2.38, p = 0.033; Fig 3a).

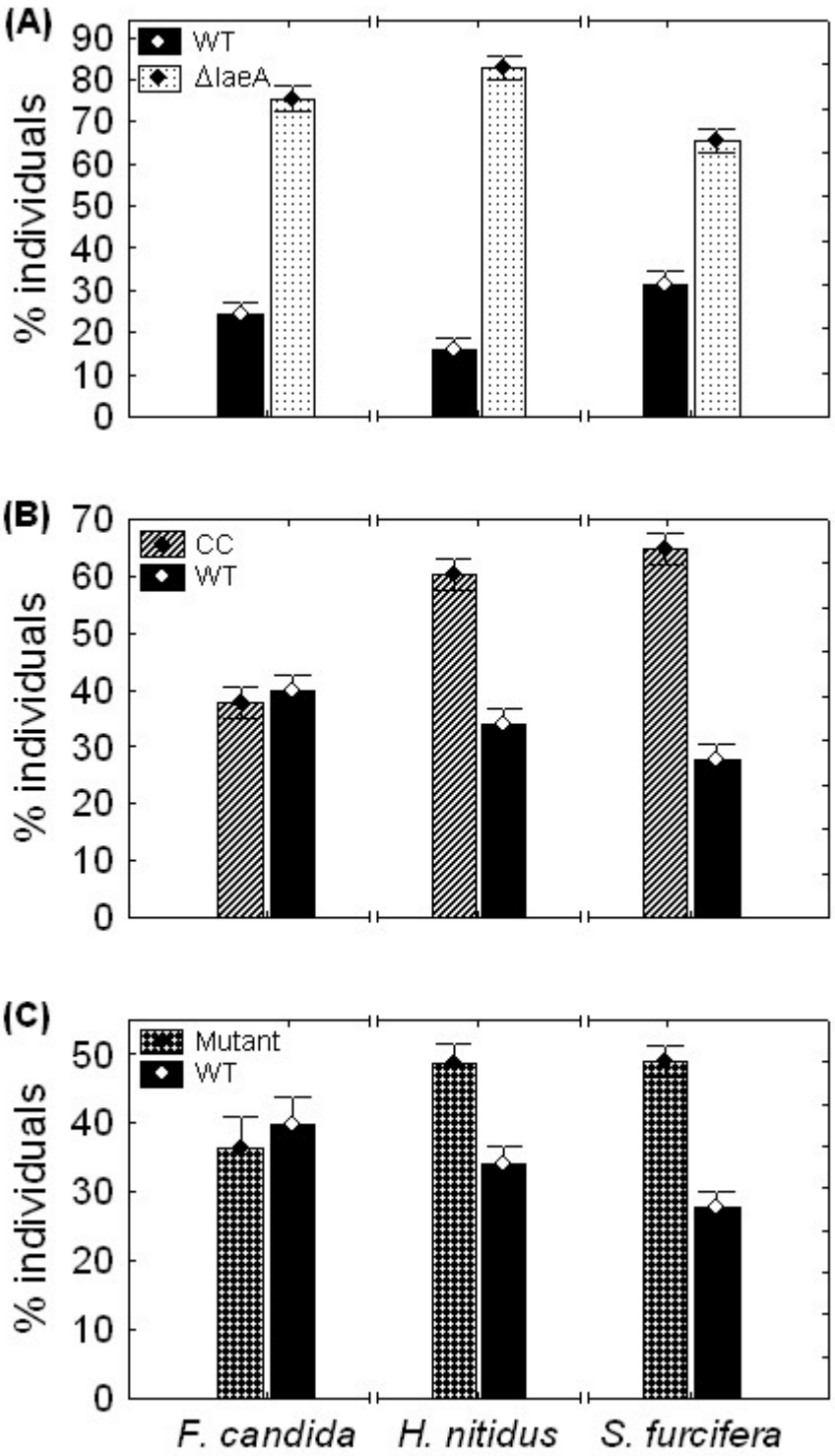

Figure 2 (A) Percentages of total number of the three collembola species (*Folsomia candida*, *Heteromurus nitidus* and *Supraphorura furcifera*) which moved into the *Aspergillus nidulans* wildtype (WT) area as compared to the area above the mutant strain of *A. nidulans* (ΔlaeA) lacking secondary metabolites in Experiment 1. (B) Collembola individuals (percentages of total) which moved into the *Cladosporium cladosporioides* area (CC) as compared to the

Aspergillus nidulans wildtype area (WT) in Experiment 2. (C) Collembola individuals (percentages of total) which moved into the *Aspergillus nidulans* wildtype area (WT) as compared to the area of the four mutant strains of *A. nidulans* (Mutant) deficient in the production of sterigmatocystin (S3-S6; see Methods) in Experiment 2.

In Experiment 3 the volatiles of ungrazed fungi were generally preferred over grazed fungi ($F_{1,168} = 38.62$, $p < 0.001$), however, preferences varied among Collembola species ($F_{2,168} = 10.58$, $p < 0.001$) with *H. nitidus* and *F. candida* preferring to forage over the area with ungrazed fungi (Fig. 3b). Increasing grazing intensity reduced marginally the percentage of foraging individuals ($F_{2,81} = 2.52$, $p = 0.086$) from 32.1% after 3h to 27.9% after 20h and 23.4% after 5 days. Further, the significant interaction between grazing time and Collembola species ($F_{4,81} = 2.82$, $p < 0.030$) indicates that Collembola's foraging behaviour was affected by the intensity of grazing, however, no relationship is evident between Collembola species, grazing intensity and preference (Fig. 3c). The effect of grazing intensity varied with fungal species with significantly less individuals being attracted by the *L. bicolor* after 20h of grazing exposure ($F_{2,84} = 3.13$, $p < 0.048$; Fig 3d).

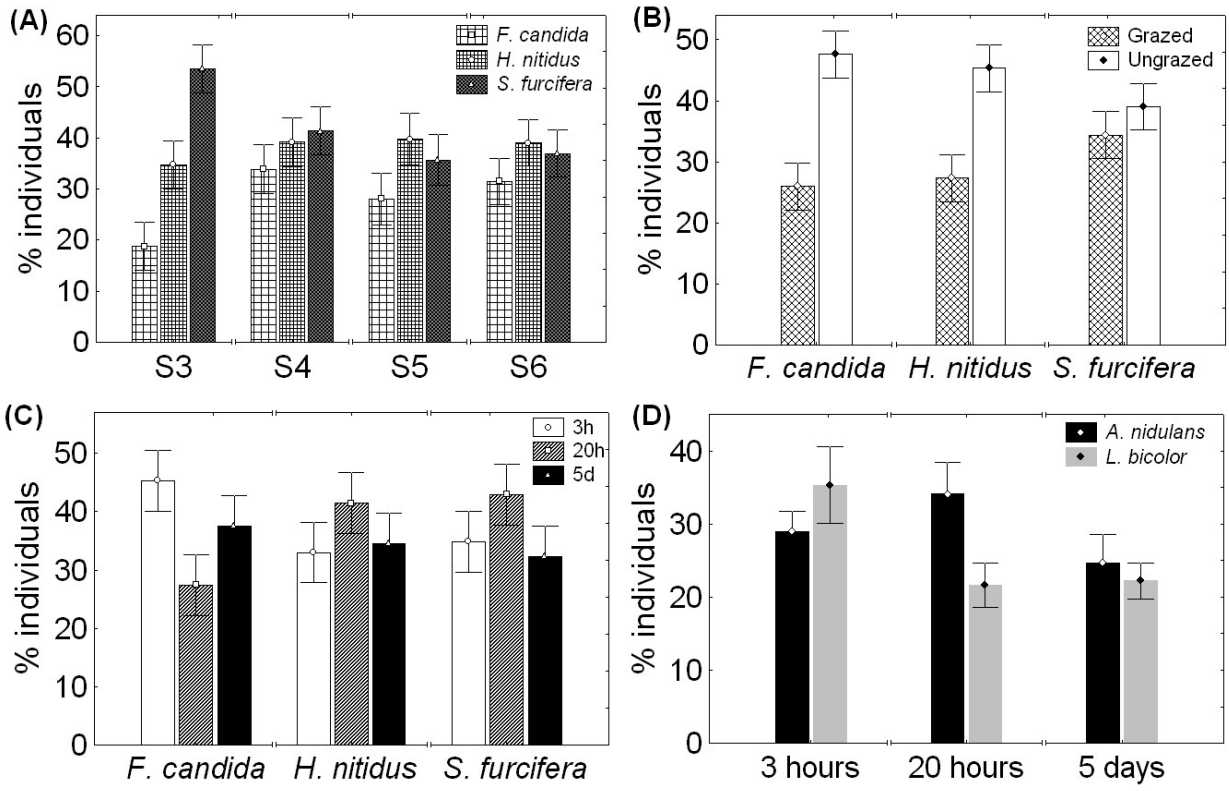

Figure 3

(A) Percentages of total number of the three collembola species (*Folsomia candida*, *Heteromurus nitidus* and *Supraphorura furcifera*) which foraged over the four mutant strains of *Aspergillus nidulans* deficient in the production of sterigmatocystin (S3, S4, S5 and S6 see Methods) in Experiment 2. (B) Collembola individuals (percentages of total) moving into the area of ungrazed *Aspergillus nidulans* and *Laccaria bicolor* S238N as compared to the area of respective grazed fungi (see Methods) in Experiment 3. (C) Collembola individuals (percentages of total) belonging to the three species ((*Folsomia candida*, *Heteromurus nitidus* and *Supraphorura furcifera*) which moved into the area of *Aspergillus nidulans* and *Laccaria bicolor* S238N exposed to grazing for different time periods (3 hours, 20 hours, 5 days) as compared to the respective ungrazed fungi in Experiment 3. (D) Percentages of Collembola individuals chosing to forage over the areas with fungi (*Aspergillus nidulans* and *Laccaria bicolor* S238N) exposed to three different grazing intensities (3 hours, 20 hours and 5 days) in Experiment 3.

3.4.2 Gene regulation of Laccaria bicolor S238N and *Aspergillus nidulans*

Based on the criteria presented in the material and methods section (Duplessis et al. 2005) grazing did not significantly changed the expression patterns of the investigated transcripts in both fungi *L. bicolor* and *A. nidulans*.

3.4.3 Volatile experiment

In two of three replicates *A. nidulans* revealed nine volatile compounds compared to emitted Czapek Dox agar volatiles (Table 1; Figure 4). Several of these substances are already confirmed in *Aspergillus* spp. by Fischer et al. (1999) and Schnürer et al. (1999).

Table 1 Volatiles produced by *Aspergillus nidulans* grown on Czapek Dox Agar classified after element groups. The confirmation type is listed under comments. „Reference" means the particular chemical agent is measured under equal conditions. It showed exactly the same retention time in GC and an identical mass spectrum as the compound of the experiment. „MS-Library + Retention Index" means the chemical agents have a high consistency with mass spectra taken from literature and a similar retention index (using a standardized retention time). Bold substances are confirmed.

No	Compound	Comment
	Ketone	
1	3-octanon	reference
	Ester	
2	**(Z)-Hex-3-enyl acetate**	reference
3	(N)-Hex-3-enyl acetate	MS-Library + Retention Index
	Terpene	
	Monoterpenes	
4	Limonene	reference
5	(Z)-Ocimenone	MS-Library + Retention Index, oxygenated monoterpene
	Sesquiterpenes	
6	(E)-b-Farnesene	MS-Library + Retention Index
7	(Z)-g-Bisabolene	reference
8	(E)-a-Bisabolene	reference
9	(RI 1571, M 204, BP 93)	unknown

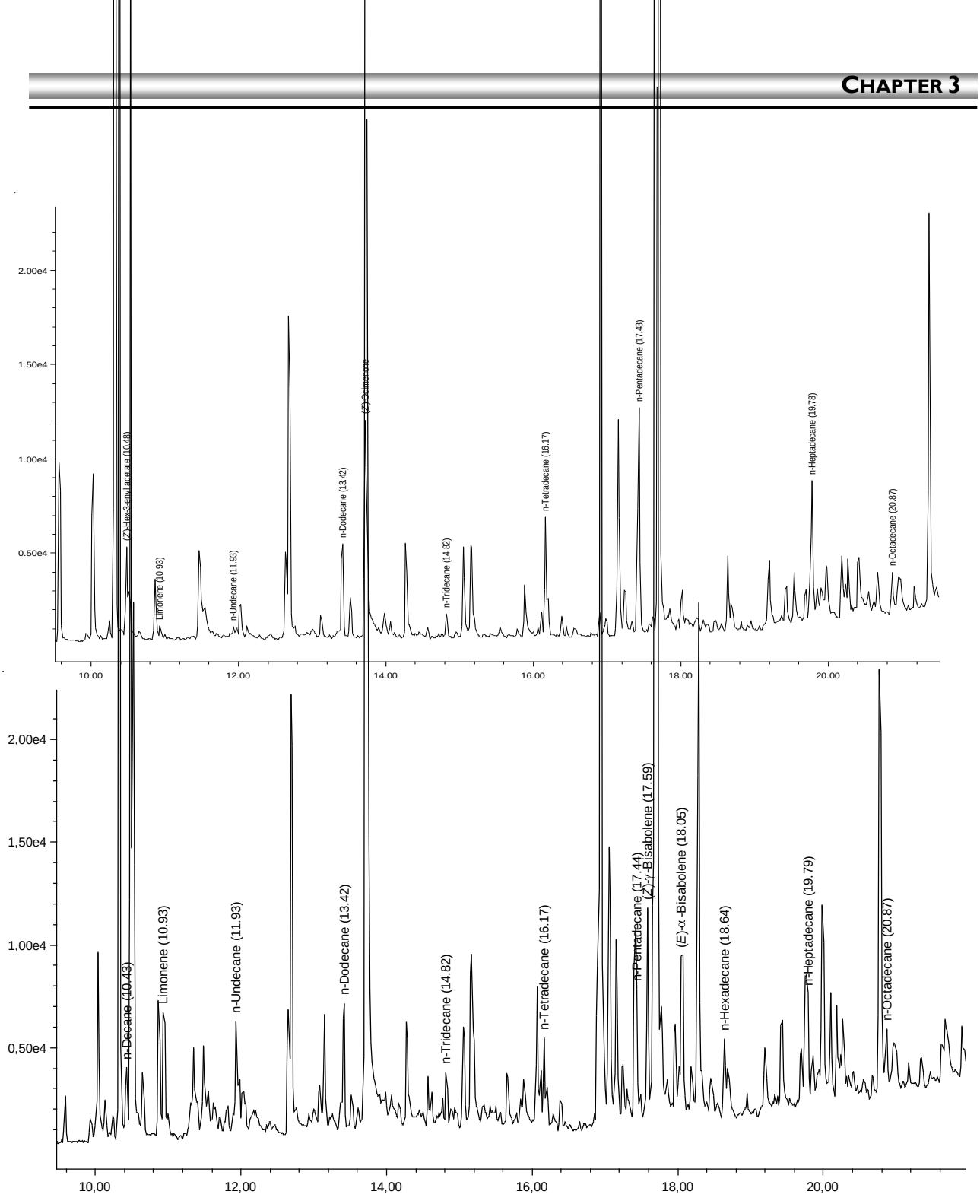

Figure 4 GC chromatogram section of volatiles emitted by Czapek Dox Agar (first) and Czapek Dox Agar + *Aspergillus nidulans* (second). Alkan standards from n-Decan bis n-Octadecan added for retention indices determination. Non marked substances either not determinable or subsidiary signals of additional compounds occurring in the second treatments as listed in Table 1.

Two of three replicates of grazed treatments of *A. nidulans* emitted a substance with the mass spectrum M 272, BP 257 which may be classified as a diterpen similar to Isopimara-8,15-dien respective Rimuene (Bartram 2009, unpublished). This occurred only when grazed by *S. furcifera*.

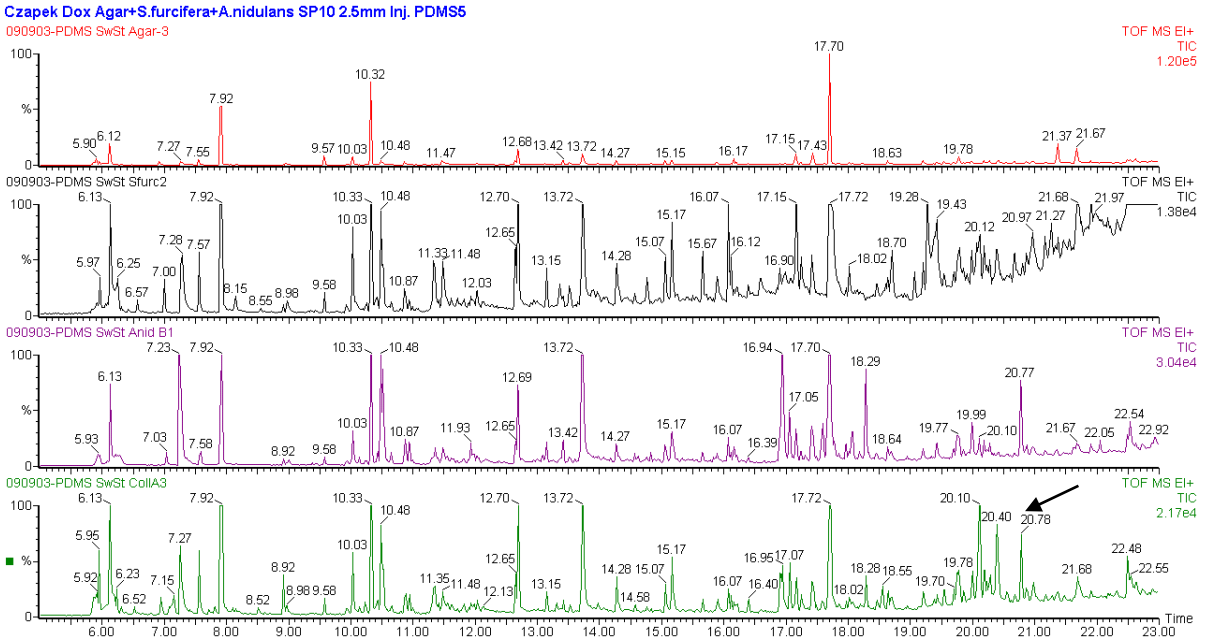

Figure 5 GC chromatogram section of Czapek Dox Agar + *S. furcifera* (first), Czapek Dox Agar + *A. nidulans* (second) and Czapek Dox Agar + *A. nidulans* + *S. furcifera* (third). Arrow shows the retention time of a substance occurring in two of three replicates (mass spectrum M 272, BP 257).

3.5 Discussion

Collembola are able to recognise high quality food and to avoid or adjust the intake of toxic fungi in order to increase their fitness (Hopkin 1997). Although there is evidence that for most species odour perception is playing an important role in food selection (Verhoef et al. 1977) and foraging (Hedlund et al. 1995) the ability of Collembola to perceive volatiles related to fungal secondary metabolites has not been investigated. Fungi are known to emit volatile compounds and the composition of fungal odours varies with species (Fischer et al. 1999), the growth substrate and age of the colony (Karahadian et al. 1985; Bengtsson et al. 1988; Klironomos and Kendrick 1996) and Collembola species may be attracted or repelled by volatile compounds or the ratio between volatile compounds.

Results of this study suggest that each of the three Collembola species was able to sense the presence of fungi and to direct their movement towards them. Further, and more importantly, Collembola were able to differentiate fungal strains with putatively knocked out secondary metabolism (*A. nidulans* ΔlaeA) from the wildtype strain. Similarly, when confronted with a fungal strain able to produce toxic defence compounds (*A. nidulans* WT) as compared to *A. nidulans* sterigmatocystin deficient mutants or the high quality reference fungus *C. cladosporioides,* two (*H. nitidus* and *S. furcifera*) out of the three Collembola species investigated directed their movement away from the wildtype. Furthermore, when comparing the foraging behaviour of the Collembola when exposed to odours of the sterigmatocystin deficient mutants (S3-S6), two of the three species (*H. nitidus* and *S. furcifera*) preferred the S3 mutant where the sterigmatocystin production is interrupted very early in the biosynthetic pathway. Overall, these results are in line with the findings of Bengtsson et al. (1988; 1991) showing that Collembola select fungi due to their volatile odour and confirms the results of Rohlfs et al. (2007) for more than one Collembola species and using a more appropriate agar medium (see Materials and Methods). Although we did not assess the changes in volatile composition associated with the different mutant strains in this study, the results show that Collembola are able to olfactorily perceive changes in fungal secondary compounds and adjust their foraging behaviour in order to avoid toxic fungi.

As shown in Table 1 various fungi specific volatiles including terpenes, esters and a ketone are produced by *A. nidulans* RSCS2 WT. Several of them are already verified for *Aspergillus* species (Pasanen et al. 1997; Fischer et al. 1999; Fischer et al. 2000). Their composition strongly depends on the fungal growth medium (Börjesson et al. 1990; Sunesson et al. 1995). Grazed *A. nidulans* produced an unconfirmed substance presumably a terpene, but only species specifically for *S. furcifera.* This substance displays a mass spectrum (M 272, BP 257) similar to Isopimara-8,15-dien respective Rimuene (Bartram, Staaden, Scheu unpublished). Terpenes are also induced herbivore specific defence substances of the tobacco plant *Nicotiana attenuata* (Halitschke et al. 2000). This suggests the presence of induced defence mechanisms in fungi. Interestingly, the olfactormeter experiments showed *S. furcifera* moving away from grazed fungi after five days grazing exposure and *A. nidulans* emitted a certain volatile only being exposed to *S. furcifera* Collembola orientate away from grazed fungi to ungrazed which one would expect being the case for epedaphic species as the litter layer has a higher metabolic activity. This is a small hint for a species specifically developed perception system between fungal feeders and fungi itself.

Interestingly, *F. candida* and *H. nitidus* turned their movement away from fungi which had been exposed to grazing before and overall, there was a trend of decreasing foraging above the fungi exposed to a higher intensity of grazing. In plant-herbivore systems it has been shown that herbivore induced plant volatiles may help defending plants by attracting predators (Kessler and Baldwin 2001). It is unknown, however, if volatile driven indirect defences also operate in the fungi–fungivore system. Further, experiments analysing the odour of fungi exposed to grazing are necessary to evaluate if changes in the composition or concentration of chemicals are responsible for the perception of grazed *vs*. non-grazed fungal mycelium by Collembola.

In conclusion, the results of this study showed that Collembola are able to sense olfactory cues of fungi of different species and, more importantly, of strains with modified secondary metabolism as we had hypothesized. This allowed the Collembola to orientate their movement away from fungi of high toxicity. Using olfactory signals Collembola also avoided movement towards fungi previously exposed to grazing which supports our second hypothesis that Collembola are able to sense and respond to grazing on fungi by other Collembola individuals.

CHAPTER 4

IMPACT OF FUNGAL SECONDARY METABOLITES ON TRANSCRIPT REGULATION OF *FOLSOMIA CANDIDA*

4.1 Abstract

Genetic and molecular evidence to support the hypothesis that fungal secondary metabolites play a significant role in protecting the fungi against fungivory is scarce. We investigated the impact of fungal secondary metabolites on transcript regulation of stress related expressed sequence tags (ESTs) of the Collembola *Folsomia candida* feeding on mixed *vs*. single diets. *Aspergillus nidulans* wildtype (WT; Ascomycota) able to produce secondary metabolites including sterigmatocystin (ST) and a knockout mutant with reduced secondary metabolism (*A. nidulans* ΔLaeA) were combined with the high quality fungus *Cladosporium cladosporioides* as mixed diets or offered as single diets. We hypothesized that (i) *A. nidulans* WT triggers more genes associated with stress responses compared to the *A. nidulans* ΔlaeA strain with suppressed secondary metabolism, (ii) *C. cladosporioides* causes significantly different transcript regulation than the *A. nidulans* strains ΔlaeA and WT, and (iii) mixed diets will cause significantly different transcript expression levels than single diets. All three hypotheses are generally supported despite the fact that many functions of the affected ESTs are unknown. The results bring molecular evidence for the existence of a link between fungal secondary metabolites and responses in springtails supporting the hypothesis that fungal secondary metabolites act as a shield against fungivory.

4.2 Introduction

Filamentous fungi synthesize a wide array of secondary metabolites such as pigments and toxic or repellent substances (Yu and Keller 2005; Karlovsky 2008). Although they are not essential for the survival and development of an individual (Fox and Howlett 2008), they serve multiple functions with subtle effects on the fitness of the organism (Leonard 1977; Klittich and Bronson 1986; Coccia et al. 2001). Further, secondary metabolites are of paramount importance as pharmaceutical substances (Yu and Keller 2005; Deacon 2006). Fungal secondary compounds received particular interest as they constitute some of the most important antibiotics (Yim et al. 2007). However, their role in increasing the fitness of the fungus itself remains little understood (Calvo et al. 2002; Schiestl et al. 2006). Many saprotrophic fungi, like the abundant *Aspergillus* species, live in soil and there is evidence that

their secondary metabolites may provide protection against other soil organisms, e.g. by increasing their competitive strength (Calvo et al. 2002; Sherratt et al. 2005). Toxic or repellent compounds in soil are prominent drives of evolutionary processes among soil organisms (Roelofs et al. 2008).

Analogue to the plant-herbivore system (Bennett and Wallsgrove 1994; Halitschke et al. 2000; Halim et al. 2006) in which plants evolved specific signalling pathways regulating plant defence responses (e.g. the jasmonate pathway; McConn 1997; Li et al. 2002), one of the most important biosynthetic pathways for fungal secondary compounds is the polyketid pathway (Langfelder et al. 2001) leading to the production of some of the most toxic secondary compounds known, such as aflatoxin and sterigmatocystin (Wright et al. 2000; Dezotti and Zucci 2001; Deacon 2006). There is evidence that these fungal secondary metabolites play an important role in Collembola food selection and fitness (Rohlfs et al. 2007; Böllmann et al. 2009). Collembola are highly abundant soil and litter dwelling microarthropods (Hopkin 1997) which feed extensively on fungi (Thimm and Larink 1995; Jørgensen et al. 2003, 2005). If fungal secondary metabolites act as a shield against fungivory as suggested by Rohlfs et al. (2007), then their ingestion should trigger stress response pathways in Collembola.

Transcriptomics is a promising tool to disentangle coevolutionary processes at the mRNA level but has rarely been applied to soil organisms (Roelofs et al. 2008). Through gene expression microarray and gene ontology analysis Roelofs et al. (2008) identified general and specific genomic responses of soil organisms to multiple abiotic factors.

It is unknown how fungal secondary metabolites such as toxic or repellent metabolites affect the gene expression in Collembola. In this study we investigated the impact of fungal secondary metabolites such as sterigmatocystin (ST) on transcriptional regulation of selected expressed sequence tags (ESTs) of *F. candida*. *F. candida* is a commonly used model organism in terrestrial ecotoxicology (Fountain and Hopkin 2005) and is on the way of becoming a suitable model organism for investigating gene expression responses as currently about 6000 ESTs are known from its genome (http://www.collembase.org; Timmermans et al. 2007).

We focussed on the comparison between the effects of foraging on a wildtype strain of *Aspergillus nidulans* (Ascomycota) able to produce secondary metabolites including ST and a knockout mutant of *A. nidulans* where the global secondary metabolite transcription regulator LaeA, is silenced (Bok and Keller 2004; Perrin et al. 2007). It has been shown that LaeA regulates the expression of a number of secondary metabolites including mycotoxins such as ST in *Aspergillus* species (Bok and Keller 2004; Bok et al. 2006). Furthermore, it has

been documented that mixed diets increase the fitness of Collembola (Bernays et al. 1994; Scheu and Folger 2004). Dilution of toxins or a more balanced nutrient supply are the two commonly hypothesised explanations of why generalist feeders benefit from mixed diets (Pulliam 1975; Rapport 1980). Thus we investigated the impact of mixed *vs.* single diets on the transcriptional expression of selected ESTs.

We hypothesized that (i) genes associated with stress responses will be triggered when foraging on the *A. nidulans* WT (with an intact secondary metabolism) but not when foraging on the *A. nidulans* ΔlaeA strain with suppressed secondary metabolism, (ii) the saprotrophic fungus *Cladosporium cladosporioides* which is of high food quality causes significantly different transcript regulation than the ΔlaeA and the WT *A. nidulans* strains, and (iii) mixed diets will cause significantly different transcript expression levels than single diets.

4.3 Material and Methods

4.3.1 Fungi

Aspergillus nidulans is a widespread saprotrophic filamentous fungal species of the Ascomycota. We used the wildtype *A. nidulans* (WT) strain RDIT2.3, labelled as WT below and a mutant strain with the whole secondary metabolism putatively silenced (ΔlaeA mutant strain RJW46.4; Bok and Keller 2004) taken from laboratory cultures provided by Nancy Keller, Wisconsin, USA, labelled laeA below. For more than 50 years it has served as a model organism in cell biology and genetics and its genome is fully sequenced (Galagan et al. 2005).

We conducted a microarray experiment on the transcript regulation of selected expressed sequence tags (ESTs) of *Folsomia candida*. All fungal strains were inoculated from fungal cuts on Czapek Dox Agar and kept at 28°C in permanent darkness. The C-to-N ratio of *A. nidulans* WT and the knockout strain ΔlaeA was 13.5 and 8.1, respectively. Concurrent with previous studies, the common soil fungus *Cladosporium cladosporioides* (C-to-N ratio 18.9), hereafter labelled CC, was used as reference fungus being preferred by Collembola (Scheu and Simmerling 2004).

4.3.2 Collembola

One parthenogenetic euedaphic Collembola species, *F. candida* (Willem, 1902) was cultured for the exposure experiments ('Berlin strain'; Free University Amsterdam). Before exposure Collembola cultures were age synchronized following standardized methods (ISO 1999).

F. candida is used as model organism in soil biology (Cragg and Bardgett 2001) and for pesticide research since more than 40 years (Hopkin 1997; Fountain and Hopkin 2005).

4.3.3 Experimental design

The experiments were established in perspex vessels (diameter 7 cm, height 5 cm) with a base layer of a mixture of plaster of Paris and activated charcoal (5 : 2) of ca. 1 cm thickness. Fungal cuts (25 mm diameter) were taken from young and actively growing hyphae of 5 day old cultures avoiding contamination with agar and subsequently renewed in daily intervals offering food in excess. During the experiment the boxes were incubated at 17 ± 0.5°C in the dark for 5 days and kept humid. Eggs and exuvia were removed daily to prevent egg predation.

4.3.4 RNA extraction and labelling

After five days of exposure to the five diets mentioned above four replicates of thirty animals each and snap frozen in liquid nitrogen. Total RNA was extracted with the SV Total RNA Extraction Kit (Promega, Madison, USA) and checked for purity and integrity on the Agilent 2100 Bioanalyzer. The labelling reaction was done with 500 ng of total RNA (Quick Amp Labeling Kit, Agilent, Santa Clara, USA). Two replicates were labelled with Cy-3 and two were labelled with Cy-5, and for quality control purposes the reactions were spiked with Spike A and B mix, respectively (Agilent RNA Spike-In Kit). Subsequently, 300 ng of a Cy-3 and a Cy-5 cRNA sample were hybridized to every array, according to the interwoven loop hybridization scheme in Fig. 1, with the Gene Expression Hybridization Kit (Agilent). Bias introduced by dye specific labelling efficiencies was circumvented by performing dye swaps between the biological replicates.

After washing with the Gene Expression WashBuffer Kit (Agilent), the arrays were scanned with an Agilent DNA microarray scanner. The intensities of the fluorescence were measured with the Feature Extraction software (Agilent, v 9.5). A custom Agilent 8x15k oligo array has been designed by Nota et al. (2009), which contains 5069 gene-specific probes printed in triplicate (GEO platform GPL7150). The sequences of these probes originated from contigs generated in an EST sequencing project (Timmermans et al. 2007) and their description and annotation was updated by a Blast2GO and Annot8er (Gotz et al. 2008) launched on September 30th 2009.

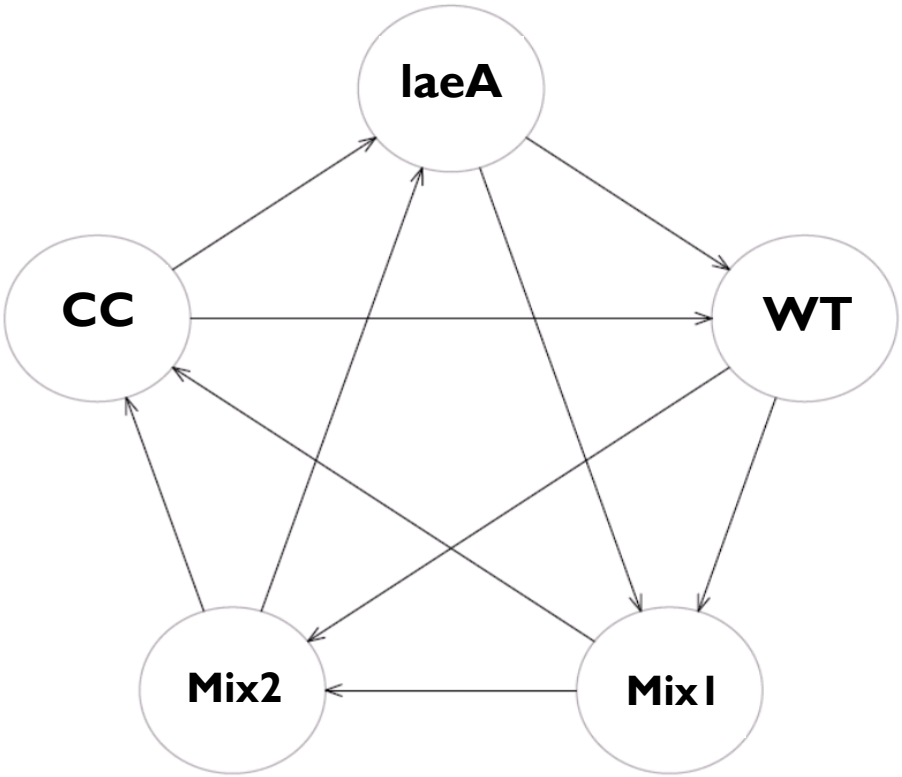

Figure 1 Hybridization scheme. The treatment codes reflect *Folsomia candida* fed *ad libitum* with CC (*Cladosporium cladosporioides)*, laeA (*Aspergillus nidulans* ΔLaeA), WT (*Aspergillus nidulans* wildtype). Mix1 represents the mixed diet of CC and WT, Mix2 the mixed diet of CC and ΔLaeA. The arrows reflect hybridizations of which the sample at the arrowhead is labelled with Cy-3.

4.3.5 Statistical analysis

The raw microarray files were analysed in the R software package version 2.9.2 with the help of the *limma* library version 2.18.3. (Smyth 2005). As a quality control, first the intensities of the fluorescence of both the foreground and background channels were compared. The normexp background correction (Ritchie et al. 2007) was used in order to correct for background fluorescence, but an arbitrary minimal fluorescence (offset = 30) was kept. Normalizations of the background corrected data were conducted within *limma* (Smyth and Speed 2003) and those within arrays consisted of global lowess normalization. Subsequently MA plots were created to check the relationships between the $\log_2$ ratios and the average intensities of the spots. After lowess normalization the remaining dye bias was removed and $\log_2$ ratios were symmetrically positioned around 1. The average intensities between the arrays were scaled to each other by making use of the Aquantile normalization method between the arrays. The statistical analyses were conducted on the $\log_2$ ratios

among the five treatments and consisted of using the linear models on sets of triplicated probes on the array (Smyth et al. 2005) with empirical Bayes methods and taking a dye effect into account. Probes exhibiting a dye effect were removed from the dataset. Data were corrected for multiple testing by using the Benjamini-Hochberg correction. First the *C. cladosporioides* exposure was used as a reference and the applied contrast were the two separate *A. nidulans* strains and the mixed diets. Additionally, the *A. nidulans* ΔLaeA mutant was tested against the wildtype as a reference. For every contrast between treatments an average $\log_2$ fold change was calculated. The $\log_2$ fold change values of genes with a differential expression in at least one of the treatments data were visualized in a heatmap, which was created by using the hierarchical clustering method (Euclidian distance, complete linkage) in the integrOmics package (Le Cao et al. 2009; Fig. 2).

Based on the gene lists of up and down regulated genes a gene set enrichment analysis was conducted in the R package topGO 1.12.0 (Alexa et al. 2006). The weighted algorithm was used for assessment of the significance of the gene ontology (GO) term nodes, because in previous simulation runs this algorithm proved to give the lowest false positive and false negative results. GO terms with only one significant or annotated gene were omitted from the output. Several new terms such as molecular function (MF), biological process (BP) and cellular components (CC; abbreviation only used in Table 3) of the regulated ESTs are described via this analysis.

4.4 Results

The probes of ten genes did show a dye bias and were removed from the analysis. In Table 1 the results of all the treatments versus the *C. cladosporioides* diet are summarized. The order of the genes is the same as the heatmap in Figure 2 and the shaded rows reflect the three main gene clusters in the heatmap. The mixed diets had a lower number of differentially expressed genes (respectively zero and 11 genes) relative to the single diets of the *A. nidulans* WT (31 genes) and the ΔLaeA mutant (20 genes). The transcriptional patterns of the two mixed diets clustered together with the *A. nidulans* ΔLaeA mutant diet.

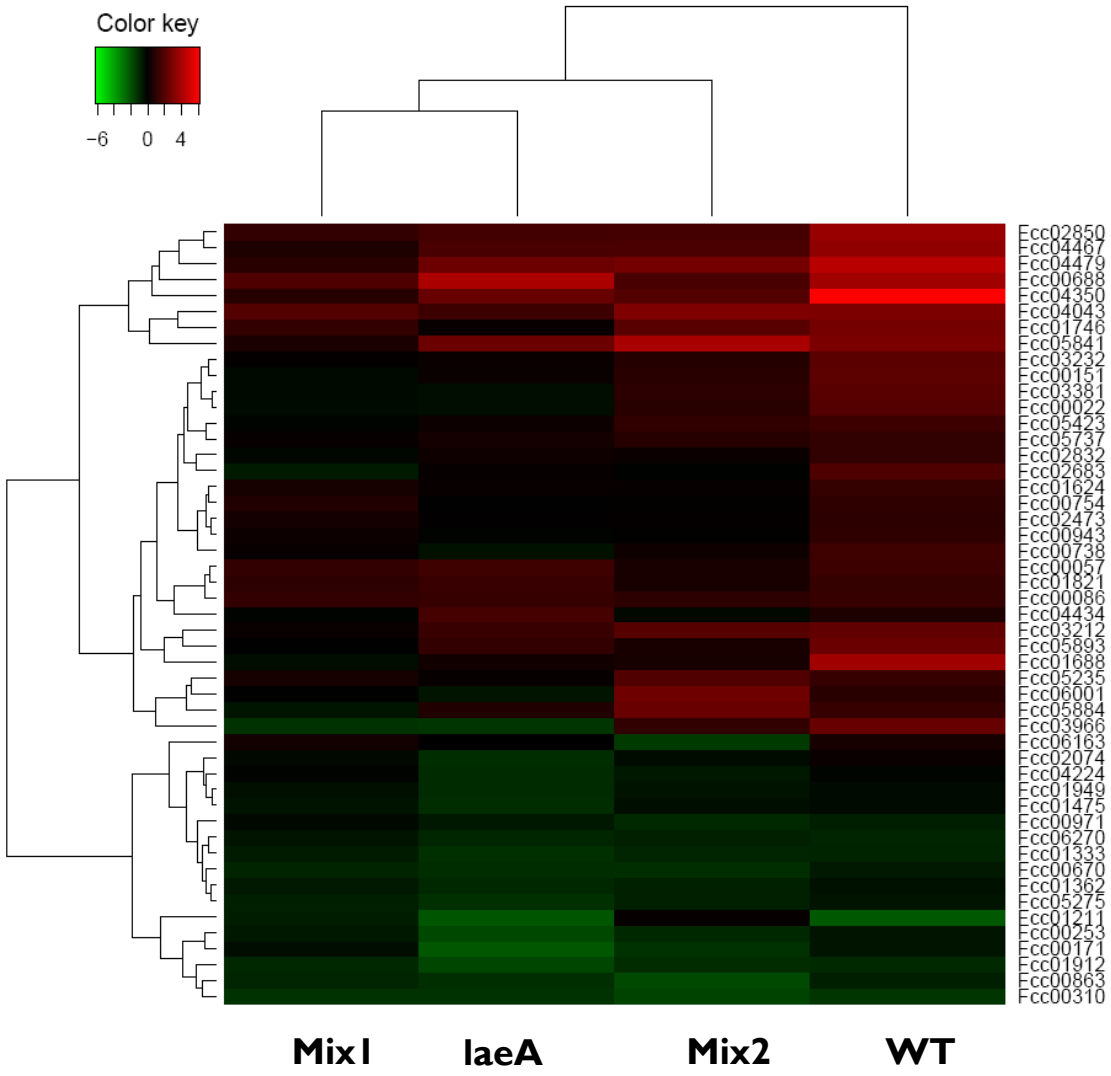

Figure 2 Heatmap with hierarchical clustering (Euclidian distance, complete linkage) using log_2 fold changes (treatment/reference) for transcripts differentially expressed for all diet combinations compared to the reference fungus *Cladosporium cladosporioides*. Log_2 fold changes are indicated as colors. Red indicates up regulation, green down regulation and black no difference. Transcripts are named by their gene cluster in Collembase followed by their predicted function. For treatment codes see Figure 1.

Table 1 Calculated log_2 fold changes of the *limma* method on the different treatments relative to the reference diet of *Cladosporium cladosporioides* (CC). laeA (*Aspergillus nidulans* ΔlaeA), WT (*Aspergillus nidulans* wildtype). Mix1 represents the mixed diet of CC and WT, Mix2 the mixed diet of CC and laeA. *p*-values *** < 0.001, ** < 0.01, * < 0.05.

GeneName	Description	Mix1	laeA	Mix2	WT
Fcc02850	No hits	1.22	1.62	1.66	**3.72** **
Fcc04467	No hits	0.7	1.78	1.8	**3.53** **
Fcc04479	No hits	0.99	2.64	2.84	**4.49** **
Fcc00688	No hits	1.91	**4.15** **	1.79	**3.92** **
Fcc04350	Cuticle protein 66cb	0.93	2.51	2.04	**6.22** ***
Fcc04043	SMAP-1	2.03	1.46	**3.11** *	**3.02** *
Fcc01746	No hits	1.25	0.21	2.14	**2.83** *
Fcc05841	No hits	0.64	**2.6** *	**4.11** ***	**2.99** **
Fcc03232	No hits	0.09	0.23	0.91	**2.21** **
Fcc00151	No hits	-0.22	0.22	1	**2.28** **
Fcc03381	No hits	-0.23	-0.31	1.11	**2.15** **
Fcc00022	chorion peroxidase	-0.25	-0.33	0.96	**2.04** *
Fcc05423	No hits	-0.09	0.32	1.16	**1.49** *
Fcc05737	No hits	0.14	0.52	0.92	**1.19** *
Fcc02832	lectin 2a	-0.15	0.34	0.25	**1.21** *
Fcc02683	No hits	-0.64	0.15	-0.06	**1.91** **
Fcc01624	angiotensin converting enzyme	0.55	0.2	0.15	**1.29** **
Fcc00754	No hits	0.77	-0.01	0.06	**1.12** **
Fcc02473	No hits	0.52	0.05	0.1	**1.09** **
Fcc00943	No hits	0.32	-0.07	0.07	**1.15** **
Fcc00738	No hits	0.2	-0.42	0.36	**1.54** **
Fcc00057	isopenicillin n synthetase	1.25	**1.53** **	0.6	**1.48** **
Fcc01821	isopenicillin n synthase	1.06	**1.39** **	0.61	**1.27** **
Fcc00086	laminin a	1.21	**1.31** *	1.08	**1.32** *
Fcc04434	sonic hedgehog	-0.08	**1.68** **	-0.2	0.7
Fcc03212	No hits	0.21	1.37	**2.1** *	**2.35** **
Fcc05893	No hits	-0.02	1.24	0.57	**2.58** ***
Fcc01688	No hits	-0.32	0.42	0.56	**3.92** *
Fcc05235	Ca2+ activated chlorine channel	0.53	0.17	**1.98** *	1.32

GeneName	Description	Mix1	laeA	Mix2	WT
Fcc06001	niemann-pick type c-2	-0.02	-0.48	**2.69** *	0.99
Fcc05884	No hits	-0.5	0.79	**2.53** *	1.32
Fcc03966	No hits	-1.2	-1.28	1.19	**2.51** *
Fcc06163	No hits	0.46	0.03	**-1.42** *	0.55
Fcc02074	No hits	-0.19	**-1.09** *	-0.27	0.24
Fcc04224	No hits	-0.08	**-1.03** *	-0.58	-0.12
Fcc01949	Hypothetical protein CBG14366 [Caenorhabditis briggsae AF16]	-0.37	**-1.05** *	-0.46	-0.24
Fcc01475	No hits	-0.44	**-1.11** *	-0.35	-0.25
Fcc00971	adam metallopeptidase domain 33	-0.2	-0.56	**-1** *	-0.73
Fcc06270	No hits	-0.48	**-0.92** **	**-0.78** *	**-0.91** **
Fcc01333	AGAP010917-PA [Anopheles gambiae str. PEST]	-0.65	**-1.16** *	-0.9	-0.84
Fcc00670	glycoside hydrolase family 16	-0.87	**-1.1** *	-1.11	-0.61
Fcc01362	bacterial leucyl aminopeptidase	-0.62	**-0.98** *	-0.82	-0.42
Fcc05275	aquaporin 10	-0.74	**-1.16** *	-0.78	-0.49
Fcc01211	No hits	-0.7	**-2.09** *	0.17	**-2.17** **
Fcc00253	No hits	-0.59	**-1.74** *	-0.97	-0.49
Fcc00171	No hits	-0.31	**-2.12** ***	-1.19	-0.48
Fcc01912	No hits	-0.99	**-1.68** *	-1.08	-1.01
Fcc00863	titin (connectin)	-0.9	-1.12	**-1.78** *	-0.72
Fcc00310	No hits	-1.19	**-1.2** *	**-1.65** **	**-1.28** **

The genes with significantly different transcription profiles towards some of the dietary treatments of the two upper clusters all show up regulation, while the transcription of the ones from the lower cluster was repressed. Twenty-nine genes were up regulated in the springtails fed on the *A. nidulans* WT diet relative to the *A. nidulans* ΔlaeA mutant. One gene was down regulated (Table 2).

Table 2 Calculated log_2 fold changes of the *limma* method of the *Aspergillus nidulans* WT diet relative to the *A. nidulans* ΔLaeA diet. *p*-values *** < 0.001, ** < 0.01, * < 0.05. Gene name and blasts according to Collembase.

GeneName	Description	logFC
Fcc03966	No hits	3.79***
Fcc00738	No hits	1.96**
Fcc00171	No hits	1.64**
Fcc00943	No hits	1.22**
Fcc04350	Cuticle protein 66Cb	3.71**
Fcc03381	No hits	2.46**
Fcc02473	No hits	1.05**
Fcc02074	No hits	1.33**
Fcc03721	No hits	1.05**
Fcc00504	No hits	3.28*
Fcc02932	No hits	1.45*
Fcc03232	No hits	1.97*
Fcc05876	NiemannPick disease, type C2 precursor	1.92*
Fcc00022	chorion peroxidase	2.36*
Fcc00754	No hits	1.14*
Fcc00557	No hits	1.74*
Fcc01311	GE13745 [*Drosophila yakuba*]	1.17*
Fcc00151	No hits	2.06*
Fcc06058	hypothetical protein [*Lepeophtheirus salmonis*]	-1.3*
Fcc01769	No hits	2.34*
Fcc00137	cyclopropane fatty acyl phospholipid synthase	0.96*
Fcc02683	No hits	1.76*
Fcc05229	No hits	2.62*
Fcc00255	serin protease	1.45*
Fcc01746	No hits	2.62*
Fcc05688	No hits	1.51*
Fcc00598	No hits	2.67*
Fcc04617	follistatin	1.48*
Fcc01585	No hits	1.94*
Fcc02565	No hits	1.14*

The gene ontology enrichment analysis clustered genes into user defined known cellular components and the putatively resulting biological functions. The table shows significantly regulated transcripts related to them as cellular compounds and their biological function. Statistically significant patterns were shown for the upregulated gene lists of *A. nidulans* WT and *A. nidulans* ΔLaeA compared to the reference fungus *C. cladosporioides*. Table 3 represents overrepresented gene ontology enrichment analysis (GO) of biological and

cellular components. Both fungal diets caused an effect considering the cell surface and in the extracellular matrix. Shared biological processes were antibiotic synthesis and regulation of cell migration. *A. nidulans* ΔLaeA mutant exposure caused transcriptionally regulated developmental growth effects at the axon level whereas *A. nidulans* WT caused a slight overrepresentation of genes involved in the extracellular matrix organization and redox reactions. One cannot apply general GO terms for springtails as it also contains terms like skeletal functions and there is no GO especially for springtails.

Table 3 Gene ontology enrichment analysis (GO) via the weight algorithm in the top GO package of up regulated genes in the *A. nidulans* ΔLaeA mutant and WT diets. Only the biological process (BP) and cellular component (CC) results are given.

Biological Process						**Cellular Component**					
GO.ID	Term	Annotated	Significant	Expected	p-value	GO.ID	Term	Annotated	Significant	Expected	p-value
laeA up						**laeA up**					
GO:0017000	antibiotic biosynthetic process	14	2	0.04	0.001	GO:0009986	cell surface	44	2	0.06	0.001
GO:0030334	regulation of cell migration	14	2	0.04	0.001	GO:0005615	extracellular space	56	2	0.07	0.001
GO:0007411	axon guidance	16	2	0.04	0.001						
GO:0045944	positive regulation of transcription from RNA polymerase II promoter.	23	2	0.06	0.001						
GO:0048704	embryonic skeletal system morphogenesis	3	2	0.01	0.004						
GO:0048589	developmental growth	15	2	0.04	0.026						
GO.ID	Term	Annotated	Significant	Expected	p-value	GO.ID	Term	Annotated	Significant	Expected	p-value
WT up						**WT up**					
GO:0017000	antibiotic biosynthetic process	14	2	0.05	0.001	GO:0009986	cell surface	44	3	0.09	2.30E-005
GO:0030334	regulation of cell migration	14	2	0.05	0.001	GO:0005615	extracellular space	56	2	0.11	0.004
GO:0030198	extracellular matrix organization	16	2	0.05	0.001						
GO:0055114	oxidation reduction	196	3	0.66	0.019						

4.5 Discussion

From the total of 5069 Collembola transcripts of expressed sequence tags (ESTs), 51 ESTs were significantly affected by the *A. nidulans* WT or ΔLaeA strains compared to the highly preferred fungus *C. cladosporioides* (CC) (Figure 2, Table 1 and 3.) Relative to CC, more transcripts of ESTs were differentially regulated in *A. nidulans* WT than *A. nidulans* ΔLaeA (31 *vs*. 20). Thirty genes were significantly different regulated in *A. nidulans* WT compared to *A. nidulans* ΔLaeA, which supports the first hypothesis. The gene ontology enrichment analysis (GO) indicated that genes associated with developmental growth and reproduction are not up-regulated in the *A. nidulans* WT treatment but upregulated in *A. nidulans* ΔLaeA treatment.

In mice and rats Sterigmatocystin (ST) acts as a potent toxin. If exposed to ST rats exhibited biotransformation processes and the production of reactive oxygen species, such as hydrogen peroxide, causing lipid peroxidation (Sivakumar et al. 2001) and mouse lungs exhibited extensive levels of inflammation-associated transcriptional patterns (Miller et al. 2010). Moreover, ST can be biotransformed to a potent carcinogenic derivat causing DNA-adducts (Bunger et al. 2004; Huang et al. 2004).

The cuticle protein 66cb was the most upregulated gene in the *A. nidulans* WT treatment. Previously, other cuticle proteins have been shown to be upregulated in response to cold stress in an Antarctic springtail (Purac et al. 2008) and to dietary cadmium (Roelofs et al. 2009). Also, the significantly upregulated hypothetical chorion peroxidase in the Collembola exposed to *A. nidulans* WT suggests a stress response towards the presence of elevated hydrogen peroxide levels. Originally, this gene product causes a post-translational mechanism of chorion proteins, through the cross linking of tyrosine residues with hydrogen peroxide by chorion peroxidase as a substrate in developing eggs of arthropods (Li and Li 2006; Konstandi et al. 2006; Roelofs et al. 2008).

In the *A. nidulans* WT and Mix2 (CC x laeA) treatment the ADP-ribosylation factor GTPase-activating protein SMAP-1 was upregulated. It regulates the clathrin-coated pit dependent endocytosis of the transferring receptor and E-cadherin (Tanabe et al. 2006). Cell lines overexpressing SMAP-1 accumulate E-cadherin at the extra-cellular membrane and exhibited decreased cell migration (Kon et al. 2008). Overexpression of SMAP-1 in *F. candida* could point to a similar cellular mechanism like the tumor-suppressing in other species.

The zinc-metallopeptidase, angiotensin converting enzyme is a key player in the biotransformation of neuropeptides and gut hormones in *Spodoptera littoralis* (Lemeire et al. 2008) and the interaction of *Drosophila* sp. with the endosymbiont *Wolbachia* sp. (Xi et al.

2008). In sheep, ROS modulation by an angiotensin converting enzyme has been observed in the nucleus (Gwathmey et al. 2010). Since *Wolbachia* sp. is a common maternally inherited cytoplasmatic bacterium located in the reproductive tissue of *F. candida* (Czarnetzki and Tebbe 2004a) an upregulation may lead via an altered infection ratio to expression of abnormal reproductive phenotypes which lastly result in decreased egg hatchability and female ratio similar to the process occurring in spider mites (Gotoh et al. 2003).

A. nidulans WT and *A. nidulans* ΔLaeA caused a two fold upregulation of isopenicillin-N-synthase genes have been identified as internally expressed gene products in the midgut of *F. candida* (Nota et al. 2008) not derived from gut inhabiting microorganisms. Nota et al. (2008; 2009) proved these genes responding to various toxicants to represent a backup innate immune system.

Laminin A was upregulated in both the *A. nidulans* WT and *A. nidulans* ΔLaeA treatments. It belongs to laminins, structural proteins of the basal lamina involved in morphogenesis (Miner and Yurchenco 2004). In *Drosophila* sp. laminin A affects the anterior-posterior axis determination of the oocyte (Deng and Ruohola-Baker 2000) and in grasshoppers laminins are involved in the outgrowth of axons (Bonner and O'Connor 2001).

Comparing the *A. nidulans* WT with *A. nidulans* ΔLaeA treatment, alongside the cuticle protein 66cb, the chorion peroxidase, a neuronal morphogenetic regulator follistatin (Pentek et al. 2009) but also a Niemann-Pick disease type C2 precursor is upregulated. The latter is a membrane-based protein involved in (chole)sterol trafficking (Storch and Xu 2009).

The transcriptional patterns generated by *A. nidulans* WT (and for some genes also the *A. nidulans* ΔLaeA offered as single or mixed diet) exposure and the resulting gene ontology analysis (Table 3) point towards putative modes of action related to oxidative stress, tumorogenesis, developmental disruption and represents homeostatic mechanism. Since the fungal diets contain a wide variety of secondary metabolites such as melanin one cannot account transcriptional modifications only to ST. Melanins, such as the derivate occurring in the reference diet *C. cladosporioides* are known to inhibit lipid peroxidation, which could contribute to the observed contrasts in transcriptional patterns (Jacobson 2000; Byshneva and Senchuk 2001). Newer studies suggested melanin features an increased growth compared to non-melanized cells after exposure to ionizing radiation (Dadachova et al. 2007). Furthermore, melanin is known to function as an environmental stress protection (Henson et al. 1999).

Although generally fewer ESTs associated to stress responses have been differentially regulated than in recent studies of exposure of *F. candida* to common pollutants (Nota et al.

2009), the results suggest a link between transcript regulation and fungal secondary metabolite content.

The impact of mixed diets on the transcript regulation strongly depended on the fungal species in the mixtures and can be interpreted with additional knowledge from previous food choice experiments. Mix1 (CC x WT) showed no differences to *C. cladosporioides*. This can be explained by predominant ingestion of CC despite the offered mixed diet. Indeed, previous food choice studies showed that *F. candida* preferentially ingests CC over *A. nidulans* WT if offered in combination (Scheu and Simmerling 2004; Chapter 2). Mix2 (CC x laeA) caused slightly more (11) significantly regulated transcripts than Mix1 (CC x WT), although fewer than in the *A. nidulans* ΔLaeA treatment (20). Again this can be explained considering the results of previous studies on food preference in mixed diet experiments showing that *A. nidulans* ΔLaeA is much more palatable than *A. nidulans* WT for Collembola and that Collembola ingest more *A. nidulans* ΔLaeA than *A. nidulans* WT when offered together with *C. cladosporioides* (Scheu and Simmerling 2004; Chapter 2).

It needs to be stressed here that this was a pilot experiment and the microarray platform used needs to be validated via quantitative RT-PCR (qPCR). In this experiment one could conduct RT-qPCRs for the most significant regulated genes in the single diet *A. nidulans* WT compared to the reference fungus *C. cladosporioides* in order to confirm the microarray log fold changes. Six potential candidate genes are Fcc00057 (isopenicillin n synthetase), Fcc01821 (isopenicillin n synthase), Fcc00086 (laminin A domain), Fcc06001 (niemann-pick type c-2), Fcc04350 (Cuticle protein 66cb) and Fcc01211 (No hits).

Crucially, more information on the investigated sequences for a more diverse annotation of the *F. candida* transcriptome is required. Moreover, there is limited knowledge on the chemical and molecular functions of the compounds generated by the regulated ESTs and the biological processes in which they are involved. Further, in some cases the fold changes were too low to speculate about possible gene functions even if these changes do not always reflect biological impact.

Ideally, one would repeat these experiments as soon as more sequencing data are available. One should conduct a more complex setup including the exposure of the most prominent toxins as pure extracts such as ST occurring in *A. nidulans* strains grown under defined conditions at a certain EC_{50} or EC_{10} concentration (half maximal effective concentration) as a spike-in in yeast or soil. This should result in a time and intensity peak of transcriptional patterns revealing the tolerance capacity of the investigated organism. This approach would lead to a more balanced gene set enrichment analysis since with few

differentially transcribed genes, biases may occur in the output of overrepresented GO terms questioning its biological relevance. Further, a quantitative confirmation of the ST content within the fungus *A. nidulans* WT and a mass spectrum analysis of all occurring chemical compounds related to toxins should be done. Also, one may repeat this experiment including the exposure to a wildtype strain of *A. nidulans* with a mutant where only ST as the most prominent toxin is silenced. However, with transcriptomic experiments one can never investigate, e.g. posttranscriptional factors and there are several methodological and handling errors in all different approaches.

In conclusion, despite methodological limitations, the results suggest that all three hypotheses are generally supported. Therefore, the study for the first time brings molecular evidence that fungal secondary compounds trigger stress responses in springtails.

CHAPTER 5

GENERAL DISCUSSION

The fundamental role of the decomposer subsystem is still underestimated despite its importance for ecosystem functioning and services. Nutrient cycling and organic matter turnover are key drivers for primary production and global carbon stocks. Soil organisms, in particular fungi and bacteria, have a major impact on the recycling processes and energy fluxes (Swift et al. 1979; Cadish and Giller 1997; Bardgett et al. 2005). Turnover rates vary with successional stage which in turn, determines the importance of bacterial and fungal based energy channels (Wardle et al. 2004). The bacteria-to-fungi ratio is modulated by soil fauna and litter quality with direct consequences for mineralization rates (Hanlon and Anderson 1979; Scheu et al. 1999). Above- and belowground plant-litter constitutes the main resource of energy and matter for an extraordinarily diverse community of soil organisms driven by highly complex interactions (Hättenschwiler et al. 2005). Coevolution of plant litter traits such as recalcitrant secondary (e.g. polyphenolics) and structural compounds (e.g. lignin) with individually adapted mycorrhizal associations constitutes one of the defining features in ecosystems (Read et al. 2004). The mechanisms of coexistence between plants and their predators (herbivores) have been intensively investigated (Bennett and Wallsgrove 1994; Wink 2003; Schoonhoven et al. 2006), however, the counterpart mechanisms between fungi and their predators (fungal grazers) have been little studied. There is evidence that in both cases the defence against multiple enemies, such as pathogens and herbivores, is regulated via signalling pathways (Mayer 2004).

Defence mechanisms play an important role and are intensely investigated between plants and herbivores (Biere et al. 2004) and a plethora of secondary metabolites with defence roles against herbivores, pests and pathogenic fungi (Bennett and Wallsgrove 1994) have been identified in plants. From an evolutionary perspective they are adaptive characters subjected to natural selection (Wink 2003) and one would expect similar mechanisms to operate between fungi and their predators. Indeed, a multitude of secondary metabolites are present in fungi and despite their low molecular weight (~1,500 kDa) they exhibit very diverse and complex structures with their function often being unknown but likely contributing to fungal fitness (Vining 1992; Wink 2003; Deacon 2006). From an evolutionary perspective one would expect Collembola to also have evolved means to cope with fungal toxic compounds. Unfortunately, only few studies investigated the signalling pathways and defence mechanisms that mediate interactions between fungi and Collembola. This thesis is

an attempt to improve our understanding about fungi-Collembola interactions and the three overarching hypothesis formulated in the Introduction are discussed below.

H1. Fungal secondary compounds mediate the Collembola – fungi interaction.

Fungal feeding is a constant pressure for fungal populations (Scheu et al. 2005). Highly abundant decomposers, like Collembola with well differentiated ecomorphological life forms and feeding guilds (Rusek 1998) play a vital role in modulating fungal community composition. Since secondary metabolites presumptively reduce the nutritional value of fungi they might function as repellent or deterrent against consumers (Stadler and Sterner 1998; Demain and Fang 2000; Karlovsky 2008) or as stated by Rohlfs et al. (2007), "shield against fungivory". Böllmann et al. (2009) postulated that the evolution of repellent metabolites and crystalline structures on the hyphal surface is much more important than the palatability and represents a prevailing and dominating fungal defence trait against fungal feeders.

In this study (presented in detail in Chapter 2) three hypothesis have been tested:

- **H1.1** Presence of sterigmatocystin (ST) impairs Collembola performance and their fitness is expected to decrease with increasing toxicity.

This hypothesis is only partially supported. The results generally support the claim of Rohlfs et al. (2007) about secondary compounds acting as shield against fungivory, however, the use of knockout mutants *A. nidulans* of the ST pathway (S3-S6) led to rather idiosyncratic responses. This is presumably related to our limited knowledge of the interconnectedness of the ST pathway, with some intermediate regulatory genes affecting the synthesis of other unknown pathway compounds such as repellent substances which might have influenced Collembola fitness. Furthermore, we measured only quality and not the quantity of fungal toxins like sterigmatocystin.

- **H1.2** Collembola benefit from ingestion of mixed diets due to toxin dilution.

Although Collembola fitness was not uniformly increased through mixed diets (suggesting a species specific response) the results still support the toxin dilution hypothesis over the more balanced nutrient supply hypothesis since no correlation between fungal N content and ingestion could be found. The toxin dilution hypothesis assumes that the ingestion of deleterious compounds will not exceed the organism's capability of coping with toxins. There are two potential mechanisms invoked to support this hypothesis. First, the "eat more

mechanism" as a result to habituation may cause improved fitness due to a greater food intake (Bernays et al. 1994). Second, the possibility of "less-than-additive-poisoning" effects suggests that the ingestion of multiple secondary metabolites can cancel each other out or ameliorate their negative effects due to anergistic (antagonistic) or biochemically contradictory effects (e.g., tannin as antidote of human alkaloid intoxication and behavioural antagonistic (Freeland et al. 1985).

The most likely explanation, however, is a combination of both aforementioned hypotheses in species specifically varying degrees with several traits determining the foraging behaviour. For example, aposematic species (e.g. antipredator adaption such as warning coloration) may employ toxin dilution mechanisms due to toxin sequestering while the ingestion of food by cryptic species may be driven by nutritional balance (Bernays et al. 1994). More investigations are required to define the relative importance of these hypotheses.

- **H1.3** Fractionation of ^{13}C and ^{15}N it is more pronounced in more toxic diets.

This hypothesis suggesting a link between stable isotope analysis and fungal toxins such as ST through excretion rates for detoxification has been partially supported. The results suggest that fungal toxin content may be more important than the nutrient content in controlling stable isotope fractionation of ^{13}C and ^{15}N. The results are in line with previous experiments showing that care is necessary in using fractionation for determining trophic levels belowground due to deviance from the expected enrichment per trophic level for both ^{13}C and ^{15}N. Some unexpected values of fractionation were found at the interface agar-fungus and varied between the fungi raised on C_3 and C_4 agar. Rossman et al. (1991) have shown that ^{13}C is not randomly distributed within the glucose molecule and also that the distribution in glucose molecules produced by a C_3 plant (beet) and a C_4 plant (maize) are different. Henn and Chapela (2000) showed that fractionation in basidiomycetes occurs during sugar uptake and is sensitive to the nonrandom distribution of stable isotopes in the source molecule (e.g. glucose or sucrose). This suggests that chemical species derived from C_3 or C_4 sucrose are routed through specific biochemical pathways at different kinetic rates, resulting in the observed total cellular isotopic discrimination. Overall, the balance between respiratory physiology and fermentative physiology modulates the degree of fungal fractionation, however, the question remains as to whether fractionation patterns observed in the field result from intrinsic fungal processing or are due to substrate effects. The same is true for Collembola fractionation, however, as mainly fungal feeders their fractionation may

exceed that of decomposers feeding typically on detritus since food quality of fungi exceeds that of decomposing litter materials.

H2. Collembola have evolved means to detect fungal toxicity

Insects evolved highly resolved patterns of semiochemical information processing via several quantitative and qualitative olfactory receptors accounting for precise recognition of volatile cues (Visser and de Jong 1988). Collembola aggregate at patches of high microbial activity and are able to follow gradients in CO_2 concentration to locate these microsites (Moursi 1962; Hassal et al. 1986). The perception system of Collembola captures substances with a resolution of one nanogram (Bengtsson et al. 1991). Furthermore, fungal respiration rate (Bengtsson and Rundgren 1983) and secondary metabolite content (Stadler and Sterner 1998) is modified depending on intensity and frequency of injuries raising questions about perception mechanisms of fungivores and accordingly foraging behaviour modulation. It has been suggested that volatile cues are used by Collembola for locating and discriminating fungal species (Bengtsson et al. 1988; Bengtsson et al. 1991; Hedlund et al. 1995).

In this study (presented in detail in Chapter 3) two hypothesis have been tested:

- **H2.1** Collembola are able to olfactorily perceive and distinguish fungal species/strains differing in secondary metabolism.

All three investigated Collembola species perceived fungal odour cues and orientated their movement towards fungi. Fungi are known to emit volatiles (Fischer et al. 1999; Schnürer et al. 1999) and this was also the case for our fungal species as preliminary experiments showed that volatile spectrum of fungi differs from that of agar. Furthermore, two out of three Collembola species could distinguish between fungal species and strains of the same species with modified secondary metabolism. They were repelled from the fungus with an intact secondary metabolism and attracted by fungi with suppressed secondary metabolism, supporting our hypothesis. This suggests that Collembola have developed a resolved perception mechanism in order to selectively forage on nutritious food and avoid toxic or repellent fungi. Hence, they presumably perceive differences in secondary metabolite content and can avoid toxic fungi. This supports the assumption of a coevolutionary link between fungi and fungivores.

- **H2.2** Collembola are able to sense and respond to fungal grazing by avoiding to forage on grazed fungi.

This hypothesis has been generally supported with the results showing that Collembola (two of three species investigated, *H. nitidus* and *S. furcifera*) oriented their foraging towards ungrazed fungi. Fungi increase their respiration (Bengtsson and Rundgren 1983) and alter the production of secondary compounds, e.g. volatile emissions (Stadler and Sterner 1998), in response to mechanical injuries. Also, cord forming fungi are known to induce morphophysiological shifts after grazing (Tordoff et al. 2008; Rotheray et al. 2009). Since *S. furcifera* is only repelled by intensive grazing (5 day grazing), this suggests the existence of an intraspecific communication channel signalling the physiological condition of the fungal food. This is supported by the volatile analysis of the grazed fungus *A. nidulans* showing one extra compound compared to ungrazed fungi, however, only when grazed by *S. furcifera*. This substance, presumably rimuene (S. Bartram, pers. comm.), belongs to the terpene family which are known defence substances in plants (Halitschke et al. 2000). Terpenes, especially sesquiterpenes, are also produced in fungal mycelial interactions (Hynes et al. 2007) but also in single fungal individuals. This strongly urges for more investigations with varying time and density exposures of fungi to Collembola, but this preliminary experiment suggests the presence of induced defence mechanisms in fungi.

- **H2.3** Grazing by Collembola changes the expression of genes in fungi related to the production of secondary metabolites in fungi.

Furthermore, we investigated grazing induced changes in fungal gene expression in *A. nidulans* and *L. bicolor*. Surprisingly, the investigated fungal gene spectrum did not significantly respond to grazing by Collembola. The lack of changes in fungal gene regulation by grazing suggests that refined methods need to be adopted to investigate the genetic response of fungi to grazing.

H3 Genetic evidence (transcript regulation) can be used to understand the molecular nature of the Collembola – fungi interactions

It is unknown how fungal secondary metabolites such as toxic or repellent metabolites affect the gene expression in Collembola. In this study we investigated the impact of fungal secondary metabolites such as sterigmatocystin (ST) on selected expressed sequence tags (ESTs) of *F. candida*.

In this study (presented in detail in Chapter 4) three hypothesis have been tested:

- **H3.1** *A. nidulans* WT triggers the expression of more genes in *F. candida* associated with stress responses compared to the *A. nidulans* ΔlaeA strain with suppressed secondary metabolism.

A total of 30 genes were significantly differently regulated in *A. nidulans* WT compared to *A. nidulans* ΔlaeA which supports the hypothesis. Unfortunately, our level of knowledge on the biological functions of these genes is very limited. Nevertheless, from the few known functions the data suggest that processes related to growth and development are hampered in the *A. nidulans* WT strain, which is in line with our hypothesis.

- **H3.2** *C. cladosporioides* causes significantly different transcript regulation in *F. candida* than the *A. nidulans* strains ΔlaeA and WT.

As expected, more differentially regulated transcripts (relative to *C. cladosporioides*) where observed in *F. candida* fed *A. nidulans* WT (31) than when fed with *A. nidulans* ΔlaeA (20). Presumably, this is related to the presence of ST in *A. nidulans* WT. Again, according to the gene ontology analysis, genes presumably linked with developmental growth and reproduction was not up regulated in the WT treatment. In particular, two genes involved in cuticular protein functions and which have been previously identified to be linked with stress responses in an antarctic springtail (Konstandi et al. 2006; Roelofs et al. 2008) have been up-regulated in *F. candida*.

- **H3.3** Mixed diets cause significantly different transcript expression levels than single diets.

This hypothesis is partly supported since the impact of mixed diets on transcript regulation depended on fungal species combination. The results would have been difficult to interpret without additional information from previous food choice experiments. Previous food choice studies showed that *F. candida* preferentially ingests *C. cladosporioides* over *A. nidulans* WT if offered in combination (Scheu and Simmerling 2004). Mix2 (*C. cladosporioides* x *A. nidulans* ΔlaeA) caused slightly more (11) significantly regulated transcripts than Mix1 *C. cladosporioides* x *A. nidulans* WT), although fewer than in the *A. nidulans* ΔlaeA treatment (20). Again this can be explained considering the results of previous studies on food preference in mixed diet experiments showing that laeA is much more palatable than *A. nidulans* WT for

Collembola and that Collembola ingest more *A. nidulans* ΔlaeA than *A. nidulans* WT when offered together with *C. cladosporioides* (Scheu and Simmerling 2004).

Transcriptomics itself is a promising but nevertheless limited method; only transcripts of a specific status can be recorded. Artefacts such as cross hybridising can be caused by special sequence and spatial structures of the probes. Posttranslational modifications such as molecule folding or other modifications such as the addition of carbohydrate groups cannot be taken into account. The amount of a specific mRNA in a cell at a certain time point is not only influenced by the level of transcriptional activity but also by the stability of the mRNA (Dale and von Schantz 2002). A gene transcribed at low levels but resulting in a stable product may cause higher amounts of mRNA than a more active gene with an unstable mRNA. Further, the amount of mRNA present does not necessarily correlate with the amount of protein produced.

Although gene ontology analysis is not the perfect tool for investigating biological stress responses in invertebrates as many functions of the affected ESTs are unknown, the results still suggest a link between transcript regulation and fungal secondary metabolite content. Therefore, the results suggest the existence of a link between fungal secondary metabolites and stress responses in springtails.

PROSPECT

The results of the experiments in this thesis call for more studies disentangling the interactions between dominating decomposer organisms. These results represent a first glance of the multiple paths of fungi-Collembola interactions and give hints towards possible coevolutionary processes. Further experiments on the impact of food on Collembola fitness should include investigations of at least two generations of Collembola (best until F_2 generation) fed with the same fungal species and including a large number of reproductive and growth parameters. The first generation should already be synchronized. Future experiments on stable isotope fractionation should include data on the quantity and quality of the secondary metabolites present in the fungal species used. Since the composition of fungal secondary metabolites may function syn- or antagonistic, care is necessary ensuring well defined growth conditions and optimal growth medium. Further, more Collembola species per functional group should be included to allow more general conclusions.

Experiments addressing Collembola olfactory responses could be improved by combining an olfactory behavioural setup with a GC-MS fungal volatile analysis and a following electroanntenogram (EAG) analysis, ideally for several functional types of Collembola. An

optimal olfactory design would generate airflow through an Y-olfactometer, test in advance the role of the aggregation pheromones and adjust the experimental design accordingly. Behavioural experiments should be investigated in darkness ideally with the help of infrared cameras. Fungal volatile analysis will reveal several fungal odours which should be ordered and tested as pure chemical substances. Ultimately, Collembola antennae need to be exposed to individual fungal volatile. Another improvement would be the investigation of undisturbed fungi, i.e. without using fungal cuts which injure the young growing hyphae.

The gene expression investigation of grazed fungi such as the exemplary Basidiomycota *Laccaria bicolor* and the Ascomycota *Aspergillus nidulans* needs to be repeated with an improved design especially because a couple of months after finishing this experiment the whole genome *Laccaria bicolor* has been sequenced. The setup needs improvement as for instance gauze cylinders cause artefacts. They are contaminated after 24 hrs of contact with Collembola which function as vectors for e.g. bacteria and fungi. One way would be to produce a Collembola extract, determine the exact chemical quantitative composition and expose fungi to this extract. Collembola must have the same age (which is not trivial to achieve) and must be reared under the same physiological conditions. Ideally, whole genome arrays for both *A. nidulans* and *L. bicolor* should be used. Another promising transcriptome profiling tool will be a deep sequencing technology, the RNAseq which will generate far more precise measuring of transcripts and isoforms than traditional methods (Wang et al. 2008).

Further, one should only extract RNA from the interacting fungal tissue; this may be achieved by simulation of Collembola grazing by mechanically injuring fungal hyphae. A critical point in such experiments is the intensity and time of grazing. Another question interesting to address is if grazed fungi emit volatiles that are recognized by conspecific fungi and alter their gene expression. Also, it would be interesting to investigate whether Collembola predators, such as mites or nematodes, react to emitted volatiles of grazed fungi. For analysing Collembola gene expression responses RT qPCR is recommended for refining parameters. The analysis of gene expression changes in *F candida* as the standard arthropod in ecotoxicology should be redone as soon as more functions are known from the ESTs and one can consider the whole genome.

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ACKNOWLEDGEMENTS

I am grateful to Prof. Dr. Stefan Scheu for the support during this PhD.

Thanks to Prof. Dr. Ulrich Brose, Prof. Dr. Heribert Warzecha and Prof. Dr. Christoph Schüth for accepting to take part as examiners for my PhD dissertation.

Financial support by the German Science Foundation within the framework of the graduate college 340 and subsequently by the Fazit- Foundation is gratefully acknowledged.

I am thankful to all my cooperation partners for providing work possibilities and help:

- Hans Knoell Institute, Molecular and Applied Microbiology, Functional Genomics, Dr. Volker Schroeckh, Jena, Germany. Dank Dir und auch Christina für die intensive Einführung in die Thematik und den warmen Empfang in Eurem Labor.
- MPI for Chemical Ecology, Department of Bioorganic Chemistry, Dr. Axel Mithoefer and Dr. Stefan Bartram, Jena, Germany.
- Vrije University Amsterdam, Department of Animal Ecology, Dr. Dick Roelofs and Dr. Thierry Janssens, Amsterdam, Netherlands.
- INRA de Nancy, UMR 1136 Interactions Arbres Microorganismes, Dr. Annegret Kohler and Dr. Aurelie Deveau, Nancy, France.

Ganz besonderer Dank an Dr. Alexandru Milcu für alles. Danke, dass Du immer an mich geglaubt und mir dadurch die Kraft gegeben hast, diese Arbeit zuende zu bringen. Danke für deine Geduld, Diskussionsbereitschaft und alleinige, sehr gute Betreuung.

Monika Medina danke ich für ihre unkomplizierte, außergewöhnlich schnelle und korrekte Bearbeitung sämtlicher Abrechnungen und die Vermittlung einer RBT.

Ich bedanke mich bei der AG Thiel für den Zugang zu ihrem Nanodrop, ohne den ich meine RNA Messungen nicht hätte durchführen können, insbesondere danke ich Sylvia für die Unterstützung durch Petrischalen, wenn unser Bestand mal wieder leer war. Der AG Layer sei ebenso gedankt für die Bereitstellung von Sterilbänken, wenn ich keinen Platz fand im Keller bzw. zwingend kontaminationsfrei arbeiten musste. Danke an Arnulf Kletzin, der mir problemlos spontan ein Mikroskop zur Verfügung stellte. Katja Rosenberg danke ich für die Hilfe und die Versuche, das beste draus zu machen bei sämtlichen Laborangelegenheiten.

Vielen Dank auch an die Werkstatt für all die geduldige Unterstützung bei doch sehr umständlichen Basteleien, speziell Udo Pelger für seine Extrahilfe, der Gerüchten zufolge bereits Filme wie den Illusionist unter dem Pseudonym Edward Norton gedreht haben soll. Danke auch an Helmut für die spontanen Fahrten für meine eigentlich ständig defekte externe Festplatte.

Karl Schuller sei gedankt für die Einführung in die rumänische „Poesie“ und für die freundlichen Worte zwischendurch.

Dank an die Fitneßschnecke Dora für die Hilfe und Gespräche und Marius für die Hilfe bei Renovierung und so. Bernhard sei gedankt für seine spezielle, aber immer ehrliche Art, was die Arbeitsatmosphäre immer aufgelockert hat. Georgia danke ich für ihre freundliche, offene und auch ehrliche Art, die sehr gut getan hat.

Uta und Agathe sei gedankt für die tolle Arbeitsatmosphäre, in der man sich nur wohl fühlen kann. Liebe Agathe, herzlichen Dank für Deine aufbauenden warmherzigen Worte.

Meiner Mutter danke ich für ihr stets offenes Ohr trotz der Umstände und meinem Vater, dass er nie den Glauben an mich verloren hat.

Sämtlichen lieben Menschen sei gedankt für die oft wochen- bis monatelange Betreuung und Bespaßung meiner charaktervollen Hündin Kutzu, die den Klischees über Rumänen alle Ehre macht.
Und auch Lea für ihre Freundschaft.

PUBLICATIONS

Staaden S, Milcu A, Rohlfs M, Scheu S 2009 Fungal toxins (sterigmatocystin) affect the fitness and stable isotope fractionation of Collembola. Accepted in Soil Biology and Biochemistry.
Chapter 2

Staaden S, Milcu A, Rohlfs M, Scheu S 2009 Olfactory cues associated with grazing intensity and secondary metabolite pathway modulate Collembola foraging behaviour. Under review in Soil Biology and Biochemistry.
Chapter 3

Staaden S, Janssen T, Ylstra B, Scheu S, Roelofs D Impact of fungal secondary metabolites on transcript regulation of *Folsomia candida*. Under review in Pedobiologia.
Chapter 4

POSTER

Staaden S, Scheu S. Growth and fitness of fungal feeding Collembola influenced by melanin and toxin deficient mutants of *Aspergillus nidulans*.
Poster at the BES Oxford 2006 and GfOE Bremen 2006.

Die vorliegende Arbeit wurde unter der Leitung von Prof. Dr. Stefan Scheu am Institut für Zoologie der Technischen Universität Darmstadt durchgeführt und von der Deutschen Forschungsgemeinschaft (DFG) im Rahmen des Graduiertenkollegs GRK340: Communication in Biological Systems: „*From the Molecule to the Organism in its Environment*“ und von der FAZIT Stiftung gefördert.

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Hilfsmitteln angefertigt habe. Ich habe noch keinen Promotionsversuch unternommen.

Darmstadt, den 17.12.2009

Swantje Staaden

„The answer to life, the universe and everything is …. 42”.

The Hitchhiker's Guide to the Galaxy

Douglas Adams