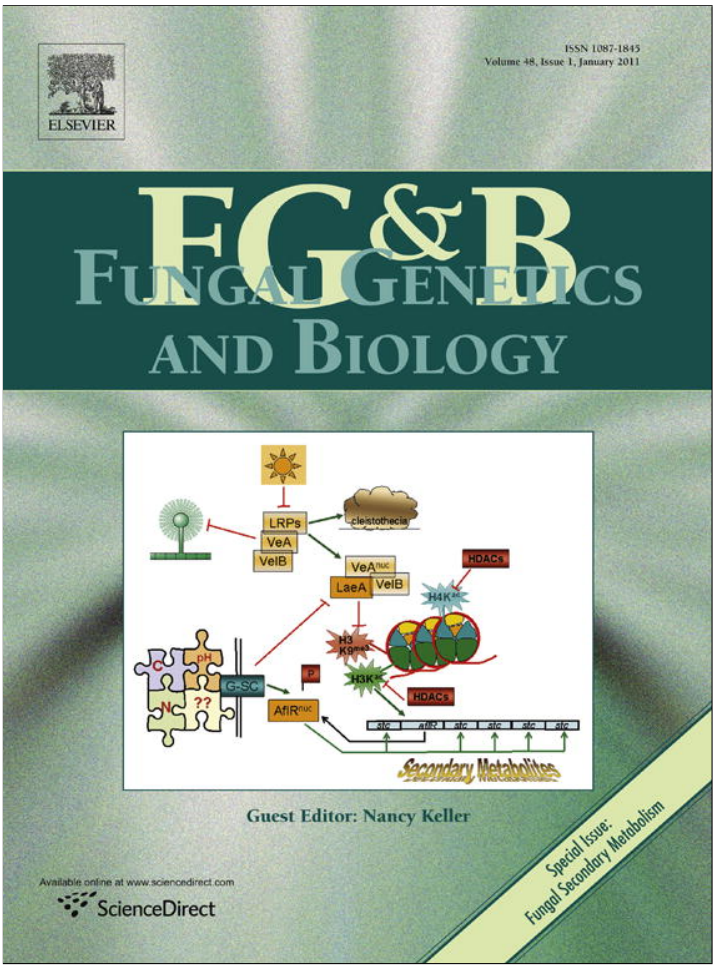

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Review

Fungal secondary metabolites as modulators of interactions with insects and other arthropods

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ABSTRACT

Fungi share a diverse co-evolutionary history with animals, especially arthropods. In this review, we focus on the role of secondary metabolism in driving antagonistic arthropod–fungus interactions, i.e., where fungi serve as a food source to fungal grazers, compete with saprophagous insects, and attack insects as hosts for growth and reproduction. Although a wealth of studies on animal–fungus interactions point to a crucial role of secondary metabolites in deterring animal feeding and resisting immune defense strategies, causal evidence often remains to be provided. Moreover, it still remains an unresolved puzzle as to what extent the tight regulatory control of secondary metabolite formation in some model fungi represents an evolved chemical defense system favored by selective pressure through animal antagonists. Given these gaps in knowledge, we highlight some co-evolutionary aspects of secondary metabolism, such as induced response, volatile signaling, and experimental evolution, which may help in deciphering the ecological importance and evolutionary history of secondary metabolite production in fungi.

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1. Introduction

The biosynthesis of a still underexplored diversity of secondary metabolites is an outstanding hallmark of fungal organisms. Given the increasing knowledge about the molecular genetic mechanisms underlying the tight regulation of secondary metabolite formation, it has been suggested repeatedly that this metabolic machinery is favored by natural selection because the products formed supply the host with a chemical arsenal that increases its fitness under challenging ecological conditions. Antagonistic interactions with other organisms co-occurring in the fungal habitat have an especially strong impact on fungal growth and reproduction, i.e., on evolutionary fitness. In decomposer systems, saprotrophic fungi may be engaged in competition with con- and heterospecifics and prokaryotic microorganisms (Vining, 1990), as well as saprophagous insects exploiting the same resource patches (Lam et al., 2009; Lussenhop and Wicklow, 1985; Trienens et al., 2010). Because fungi have the ability to accumulate high amounts of nitrogen and phosphorous, they are an important food source in soil ecosystems and are, thus, under heavy attack by fungivorous animals, especially arthropods, such as collembola, mites and insects (Boddy and Jones, 2008; Ruess and Lussenhop, 2005). Moreover, as endosymbionts of plants, fungi may suffer substantial fitness losses when their host plants are under attack by herbivorous insects (Lane et al., 2000). Since many fungal secondary metabolites are potentially toxic to antagonistic arthropods, the massive biosynthetic machinery driving the formation of mycotoxins may be maintained to provide direct chemical shields against animal antagonists.

Interestingly, some fungi have taken another evolutionary route of resource exploitation and have been selected for using living arthropods as a hub for growth and reproduction (Roy et al., 2006; Vega et al., 2009). While some pathogenic fungi have become highly specialized natural enemies of arthropods, others are able to exploit both dead and living resources associated with plants and soils (St. Leger, 2008). Secondary metabolites with immunosuppressive or otherwise toxic functions may help fungi invading their animal hosts by overcoming cellular and humoral defense systems (reviewed in Gillespie et al. (2000)). Here, the ability to form secondary metabolites may be maintained by the need to employ chemicals to withstand, or attack and overcome, the hosts' immune system. This strategy enables the fungus to consume an otherwise well-protected resource, rather than producing metabolites to serve solely as chemical shields to fend off animal antagonists as in decomposer communities.

In this review, we evaluate the extent to which the existing literature supports the hypothesis that fungi harbor the ability to produce secondary metabolites because they function as defensive "chemical weapons" in interactions with arthropods. In analogy with plant–herbivore interactions, we attempt to distinguish between secondary metabolites as "resistance" or "defensive" traits (Rausher, 2001). This may be one important step towards an evolutionary understanding of fungal secondary metabolites.

2. Regulation of secondary metabolite formation

Nonribosomal peptides, alkaloids, terpenes, and polyketides are the main classes of fungal secondary metabolites whose expression and secretion appear to be controlled by various genetic and cellular regulatory mechanisms (Chanda et al., 2009; Hoffmeister and Keller, 2007). We regard, in line with numerous other authors, this sophisticated regulatory network of secondary metabolite expression as a flexible device to meet the various ecological challenges encountered during the lifetime of a fungus, though this review is not intended to provide a detailed description of these regulatory mechanisms. Rather, we direct the reader to several thorough review articles for in-depth coverage of these molecular genetic and genomic topics (Calvo, 2008; Fox and Howlett, 2008; Georgianna and Payne, 2009; Hoffmeister and Keller, 2007; Keller et al., 2005; Shwab and Keller, 2008; Yu and Keller, 2005). Some regulatory mechanisms and pathways, however, will be considered when we discuss case studies, which provide strong causal evidence of a significant role of secondary metabolites in interactions with arthropods.

3. Definitions of "resistance" and "defense"

In both purely saprotrophic and facultative or obligatory entomopathogenic fungi, secondary metabolites may mediate resistance to animal traits that reduce fungal fitness, viz. secondary metabolites limit the burden of and have a negative effect on the animal antagonists (cf. Råberg et al., 2009). From this definition of resistance, the following hypotheses about fungal secondary metabolites can be derived. The negative influence on the antagonists implies a reduction in animal evolutionary fitness, which can be measured in terms of (1) avoiding a toxic but otherwise alimentary fungal diet, i.e., reduced food intake, and/or (2) impaired growth, reproduction and survival if the fungus is consumed. In addition to passive external barriers impeding the penetration of the host cuticle, the most hazardous arthropod defense against parasitic fungi is an immune system capable of forming melanin, antimicrobial peptides, eicosanoids, protease inhibitors and detoxification proteins, in combination with attack by circulating hemocytes (Vilcinskas and Götz, 1999; Bulet and Stöcklin, 2005; De Gregario et al., 2002; Stanley, 2006; Takeda and Akira, 2005). Because some secondary metabolites from entomopathogenic fungi have been shown to have immunosuppressive effects (Pedras et al., 2002; Vey et al., 2002; Fiolka, 2008; Pal et al., 2007; Vilcinskas and Götz, 1999), interactions with the host immune system likely constitute an important interface where secondary metabolites serve as a means to resist or suppress animal defenses (Gillespie et al., 2000). Therefore, measurements of their negative effects on arthropods should be evaluated in greater detail at the "fungal secondary metabolite-host immune system" interface. The apparent induction of apoptosis in insect host hemocytes by the destruxin metabolites of *Metarhizium anisopliae* (Vilcinskas et al., 1997b; Gillespie et al., 2000) and the cyclosporin A-mediated arrest of host detoxification mechanisms by *Beauveria bassiana* (Podsiadlowski et al., 1998) are two examples of how fungal secondary metabolites may act to incapacitate the immune systems of arthropod hosts (Gillespie et al., 2000). Since fungal secondary metabolites also have the demonstrated or potential ability to cause changes in host development, behavior, reproduction, and the onset of host morbidity (Andersen et al., 2009; Roy et al., 2006; Lefèvre et al., 2009; Krasnoff et al., 2007; Pal et al., 2007; Xu et al., 2008, 2009), understanding their effects on host regulatory networks and signal transduction pathways is of interest. In entomopathogens, general studies of these pathways have begun only recently and show great promise for increasing our understanding of secondary metabolite-mediated effects on global regulatory networks (Fang et al., 2009; Zhang et al., 2009, 2010).

If secondary metabolite biosynthesis functions, in part, as a resistance trait for fungi, thereby reducing the negative effects of animal attacks or defense reactions, fungal species or genotypes that are "armed" to protect themselves from their natural enemies (e.g., by inducing insect avoidance behavior or mortality) should be favored. This fungal evolutionary response, in turn, can be expected to exert selection on insect populations leading to animal counter-adaptation. Some of these counter-adaptations may result in a co-evolutionary arms race between insects and fungi (Thompson, 2005). Some of the literature reviewed below indeed suggests

a causal relationship between fungal secondary metabolite production and resistance to animal antagonists. Knowledge about such relationships cannot, however, automatically be used to imply that fungal secondary metabolism is an *evolved defense* system whose underlying *resistance mechanisms* are maintained by the impact of natural enemies. Reductions in fungivore damage correlated with secondary metabolite production also can be explained by incidental, pleiotropic fungal traits selected for other reasons (Jermy, 1984; Sherratt et al., 2006). For instance, recent investigations indicate an additional, if not exclusive, role of secondary metabolites in controlling central physiological processes, as well as communication, in microbial communities (Dietrich et al., 2008; Linares et al., 2006; Mlot, 2009). A more specific example is the role of gliotoxin as a virulence factor in mediating systemic mycosis by *Aspergillus fumigatus* in vertebrates (Brakhage and Langfelder, 2002; Latgé, 2001; Bok et al., 2006; Spikes et al., 2008; Kwon-Chung and Sugui, 2009) and, possibly, in insects (Reeves et al., 2004). Even though gliotoxin appears to play a significant role in frequently fatal aspergillosis (Askew, 2008), vertebrate bodies certainly do not provide a selective environment for the very simple reason that fungal reproductive success may be close to zero in this environment. Thus, other yet unknown ecological forces must have favored the ability of *A. fumigatus* to synthesize gliotoxin, e.g., there may have been selection in natural environments where the fungus is saprotrophic, but opportunistic, and potentially interacting with insects.

4. Secondary metabolite formation as a resistance trait in fungi

Investigations considering secondary metabolites as putative resistance mechanisms against animal antagonists come from various disciplines, including molecular genetics, biochemistry and ecology. While genetic/genomic approaches often have worked well in combination with biochemical methods, and vice versa, to address questions of the roles of secondary metabolites in biological systems (e.g., Bayram et al., 2008; Bok et al., 2009; Chiang et al., 2008; Fischbach et al., 2008; Jenke-Kodama and Dittmann, 2009; Palmer and Keller, 2010; Perrin et al., 2007; Reyes-Dominguez et al., 2010), integration of these perspectives into ecological research programs remains to be done. Although earlier reviews have nevertheless interpreted the principle function of secondary metabolites to be resistance characters or even as evolved defenses against their natural enemies (Demain and Fang, 2000; Vining, 1990), the evidence for such functions of fungal metabolites is largely circumstantial and often lacks experimental scrutiny.

4.1. Non-pathogenic interactions

4.1.1. Interactions with fungal feeders

As a source of nutrients to a plethora of animals in the decomposer network, saprotrophic fungi are subject to predation by fungal feeders (Ruess and Lussenhop, 2005). There is plenty of work demonstrating that fungivorous arthropods, such as collembolans and mites, display distinct preferences when offered different fungal diets (Jørgensen et al., 2003; Klironomos et al., 1999; Klironomos and Kendrick, 1996; Maraun et al., 2003; Sabatini and Innocenti, 2000; Sadaka-Laulan et al., 1998; Scheu and Simmerling, 2004), and they also appear to be selective feeders under field conditions (Jørgensen et al., 2005). These consistent patterns in preference often positively correlate with arthropod life-history traits, such as survival, growth and reproduction (Jørgensen et al., 2008; Klironomos et al., 1999; Scheu and Simmerling, 2004). Even though some studies tend to argue in favor of a defensive function of fungal toxins (Böllmann et al., 2010), such choice experiments cannot distinguish between the proposed effects of secondary metabolites and any other species-specific fungal traits that may be relevant to decision-making in fungivores. Moreover, only very few food choice studies quantified in parallel the effect of fungivore grazing on fungal growth. Recording in detail the damage an arthropod causes to a fungus, however, provides basic information on fungal fitness loss, which is a prerequisite for detecting (chemical) resistance traits in fungi and for differentiation of resistance and tolerance of fungivore attack, e.g., the ability to buffer the negative effects by compensatory growth (Bretherton et al., 2006) or to form more resilient mycelial networks (Boddy et al., 2010). This differentiation is a critical point in investigating the proposed resistance function of secondary metabolites since there may be tradeoffs between these two mechanisms that allow mainly modular organisms to cope with predator attacks (Fineblum and Rausher, 1995).

The increasingly intensified use of molecular biology techniques for disentangling the pathways and regulatory mechanisms involved in secondary metabolite formation (see review recommendations above) opens new opportunities for ecologists to study the proximate causes driving outcomes of antagonistic interactions of fungi with fungal grazer and/or insect competitors rather than speculating on the basis of correlative data. Until now, very few studies have made use of this possibility. Scheu and Simmerling (2004) hypothesized that melanin in fungi may reduce the quality of a fungal diet since it cannot be digested by fungivores and may additionally reduce the digestibility of other essential fungal compounds. To test this, they used a melanin-deficient transgenic strain of *A. fumigatus* (Δ*pksP*) in feeding and food choice experiments with two fungivorous collembolans. According to their prediction, the melanin-deficient strain better supported collembolan life-history traits, such as growth and reproduction, than did the melanin-producing wild type strain (Scheu and Simmerling, 2004). Interestingly, in food choice experiments, the fungal grazers did not discriminate between the wild type and the transgenic strain and, hence, did not adjust their behavior to optimize food source and reproduction.

From the fungal perspective, the melanin-deficient strain was not more susceptible to predation. Thus, the wild type did not profit immediately from being able to produce melanin. Melanin pigments are wide-spread in fungi and have been shown to protect against ultraviolet radiation and reactive oxygen species (Langfelder et al., 2003; Nosanchuk and Casadevall, 2006; Dadachova et al., 2007; Taborda et al., 2008). The negative effects on springtail evolutionary fitness may be just a by-product of another evolutionary force acting upon the ability to synthesize melanins. Moreover, a criticism of the study by Scheu and Simmerling is that they used circular disks cut from fungal cultures as the source of the diet. Physical injury of fungi can elicit various chemical responses related to wound-activated chemical defenses (Spiteller, 2008; Stadler and Sterner, 1998). Therefore, severe damage of fungal tissue may superimpose a chemical effect above and beyond that of melanin biosynthesis alone, which could influence the chemical milieu springtails use to sense the presence of detrimental melanins.

The ability of the springtail *Folsomia candida* to discriminate between wild type and chemically-deficient strains of *A. nidulans* has been demonstrated using a Δ*laeA* mutant strain (Rohlfs et al., 2007). LaeA, a putative methyltransferase, is known to regulate secondary metabolic gene clusters in *Aspergillus* spp. (Bok and Keller, 2004; Bok et al., 2005; Perrin et al., 2007), including sterigmatocystin (an insecticidal mycotoxin), penicillin, and terrequinone A pathways in *A. nidulans* and the gliotoxin pathway in *A. fumigatus* (Bok et al., 2005). *F. candida* significantly preferred colonies of the Δ*laeA* strain over the wild type strain. This preference for the chemically-deficient mutant appears to be adaptive since enforced feeding on the Δ*laeA* strain resulted in higher reproductive outputs in comparison to those resulting from feeding on the wild type

strain. Moreover, the Δ*laeA* strain suffered from higher biomass loss due to springtail grazing than did the wild type strain (Rohlfs et al., 2007). A similar beneficial effect for *F. candida* was found when the animals were offered various transgenic *A. nidulans* strains lacking the expression of sterigmatocystin biosynthetic genes (Wilkinson et al., 2004), as well as the pathway-specific regulator AflR (Staaden et al., 2010). However, another soil springtail, *Heteromurus nitidus*, did not respond differentially to wild type and sterigmatocystin-deficient *A. nidulans* strains, suggesting that different species of Collembolan fungal grazers respond or adapt uniquely to the presence of mycotoxins in their diet (Staaden et al., 2010), perhaps due to species-specific differences in detoxification or sensory abilities.

4.1.2. Interactions with insect competitors

In addition to a possible role of some secondary metabolites in fending off competing microbes, secretion of insecticidal mycotoxins, such as aflatoxin B_1 (Chinnici and Bettinger, 1984; Rohlfs and Obmann, 2009), by a well-coordinated vesicle transport machinery (Chanda et al., 2009) may create an adverse environment in decaying fruits, dung, carrion and the like (Hodge and Mitchell, 1997; Rohlfs et al., 2005; Trienens et al., 2010) that harms insects exploiting the same resource patch. Similar to the phenomenon of allelopathy among plants, the export of toxic secondary metabolites might drive competition between insects and fungi. Yet, insects appear to reduce the impact of competing molds by communal attack and impairment of fungal colonies (Rohlfs, 2005; Rohlfs et al., 2005), possibly assisted by mutualistic microbes (Rohlfs and Kürschner, 2010) and/or by the ability of some insects to efficiently detoxify mycotoxins, e.g., by means of an induction of cytochrome P450 monooxygenases (Niu et al., 2008).

Trienens et al. (2010) investigated the effects of loss of *laeA* on the outcome of interactions of each of *A. nidulans*, *A. fumigatus*, or *A. flavus* with competing insect larvae. The impact of wild type strains on the survival of *Drosophila* larvae very much depended on the time that had elapsed between the colonization of dead organic matter by fungi and the settlement of the insect larvae. Generally, *A. flavus* was the most deadly competitor, followed by *A. fumigatus* and *A. nidulans* (Trienens et al., 2010). Survival of *Drosophila* larvae in the presence of the Δ*laeA A. nidulans* mutant was almost as high as under mold-free conditions, which suggests that LaeA controls the expression of almost all insecticidal properties of *A. nidulans* in this system. Interestingly, even though loss of LaeA in *A. fumigatus* and *A. flavus* appeared to mitigate the negative effects of the fungi on *Drosophila* larvae, these strains nonetheless caused high mortality among the insects; Δ*laeA A. flavus*-borne insect mortality was higher than that caused by Δ*laeA A. fumigatus*. It has been shown that LaeA does not control all genomic regions where secondary metabolite gene clusters are located (Kosalková et al., 2009; Perrin et al., 2007), which may be one reason for the species-specific effects of the various Δ*laeA Aspergillus* mutants on the survival of competing *Drosophila* larvae. The generally higher diversity of secondary metabolites in *A. flavus* and *A. fumigatus*, possibly reflecting greater diversity in secondary metabolite clusters, may lead to a higher proportion of metabolic pathways that are not controlled by LaeA and, hence, still continue to produce insecticidal compounds.

Similar to the results by Rohlfs et al. (2007) (see above), the Δ*laeA A. nidulans* and *A. flavus* strains suffered more from competition with *Drosophila* larvae than did the wild type strain. Yet there were no differences in the effects of the insects on the Δ*laeA A. fumigatus* and wild type strains (Trienens et al., 2010). Thus, although gene expression regulation by LaeA appears to be related to the expression of insecticidal properties, this study suggests strong species-specific effects of LaeA on those fungal traits that mediate resistance to competing saprophagous insects. In this regard, several anti-insectan metabolites predicted to prevent fungivory in *Aspergillus* spp. occur in the overwintering structures of the fungus, i.e., sclerotia (Whyte et al., 1996; Wicklow et al., 1996). A recent paper by Georgianna et al. (2010) reports the presence of six unknown secondary metabolite gene clusters in *A. flavus* that appear to be expressed only in the dark during conditions favorable for sclerotia formation. Their lack of expression in the *A. flavus* Δ*laeA* strain, which does not produce sclerotia (Kale et al., 2008), suggests their production could be sclerotia-specific. Whether they also play roles in anti-insectan feeding remains to be determined, i.e., sclerotia that contain mycotoxins may have a survival advantage compared to those from atoxigenic isolates (Bhatnagar et al., 2003).

4.1.3. Endophytic fungi and herbivores

Fungi that are symbiotic endophytes may be directly or indirectly affected by herbivorous insects feeding on fungal host plants. In these systems, fungal secondary metabolite production has been suggested to play a key role in maintaining plant–fungus symbioses because endophytic fungi have the ability to alter the feeding behavior of invertebrates and reduce invertebrate growth rates, hence, protecting plants from herbivory (Schardl, 1996; Lane et al., 2000; Rodriguez et al., 2009). Toxic metabolites produced by endophytic fungi (e.g., *Epichloë* and *Neotyphodium* spp.) found in agronomically-relevant fescue grasses greatly reduce the populations of associated herbivorous insects, increasing plant fitness but reducing insect fitness (Clay and Schardl, 2002; Rudgers and Clay, 2008). Tanaka et al. (2005) found plants infected with a transgenic *Epichloë festucae* strain that lacked production of the insect-deterring compound peramine were as attractive to the Argentine stem weevil, *Listronotus bonariensis*, as fungus-free plants (plant specimens harboring the wild type strain usually deter this herbivorous beetle). In a non-grass system, researchers reported the anti-feeding activity of the toxin rugulosin, which is produced by the fungal endophyte *Phialocephala scopiformis* that inhabits the white spruce needles eaten by the forest pest spruce budworm (*Choristoneura fumiferana*) (Miller et al., 2002, 2008; Sumarah et al., 2008; Sumarah and Miller, 2009). More recently, anti-insect extracts were isolated and several insect-toxic metabolites were characterized from endophytic fungi of red spruce needles (Sumarah et al., 2010). Further studies may reveal how endophytic fungi could be used in protection of forests against insect pests.

Interestingly, recent studies of endophyte-containing native grasses have produced results that appear counter to the defensive mutualism hypothesis supported by the agronomic grass systems. Jani et al. (2010) reported that endophyte-produced alkaloids are associated with increased herbivore abundances and species richness, suggesting that the high alkaloid levels measured in native grasses may negatively impact natural enemies of herbivores rather than protect host grasses from arthropod herbivores. Faeth and Shochat (2010) also measured higher herbivore abundances on another endophyte-infected native grass. Others observed that some insect pests are attracted by and survive longer on plants infected with *Fusarium verticillioides* (Schulthess et al., 2002), one of the most common mycotoxin-producing pathogens of maize. Further studies are warranted to determine: (1) whether such differences between alkaloid-impacted target populations on native and agronomic grasses are consistently identified and correlated with high in planta alkaloid levels and (2) who are the target populations impacted by the production of endophyte alkaloids in particular host systems – arthropod herbivores, herbivore enemies, or both.

4.2. Host–parasite interactions

Entomopathogenic fungi have been used and developed worldwide as biocontrol agents for invertebrate pest control and as alter-

natives to chemical pesticides (de Faria and Wraight, 2007; Hajek et al., 2001, 2007; Li et al., 2010; Shah and Pell, 2003). They are prolific producers of bioactive secondary metabolites (Isaka et al., 2003, 2005; Molnar et al., 2010), which are predicted to play key roles as virulence factors for fungi infecting arthropods. Only a few studies to date have unequivocally demonstrated the significance of entomopathogen secondary metabolites in the disease process.

Two of the most commonly used and best studied entomopathogenic fungi worldwide are *M. anisopliae* and *B. bassiana*, each of which produces a set of diverse secondary metabolites exhibiting a wide range of biological activities in vitro against mammalian, insect, microbial, or plant cells (summarized in Moon et al., 2008; Xu et al., 2008; Lemmens-Gruber et al., 2009; Molnar et al., 2010). Recent application of targeted gene disruption methodologies in both fungi has provided opportunities for gene function analyses in attempts to identify whether specific secondary metabolite pathways play significant and measurable roles in fungal mediated-insect disease and death. In *M. anisopliae* var. *anisopliae* strain ARSEF 2575 (recently renamed *Metarhizium robertsii* [Bischoff et al., 2009]), disruptions of key genes for the biosynthesis of the novel serinocyclins (Krasnoff et al., 2007) and the mutagenic NG-391 compound (Krasnoff et al., 2006) demonstrated that neither are required for virulence of the fungus against beet armyworm (*Spodoptera exigua*) (Moon et al., 2008; Donzelli et al., 2010); the serinocyclins, also, do not contribute to virulence of the fungus against Colorado potato beetle (Moon et al., 2008). Although functional roles for these metabolites have not been identified, Krasnoff et al., (2007) demonstrated that the highly polar serinocyclin A compound has the biological effect of altering mosquito larvae swimming ability in in vitro assays. The degrees to which other secondary metabolites, such as the destruxin compounds produced by several members of the *M. anisopliae* complex (Bischoff et al., 2009), necessarily contribute to *Metarhizium*-mediated invertebrate disease remain yet to be determined genetically.

In *B. bassiana*, Xu et al. (2008, 2009) used targeted gene disruption approaches to demonstrate that both bassianolide and beauvericin function as highly significant insecticidal virulence factors against multiple insect hosts. In contrast, tenellin does not contribute to the virulence of *B. bassiana* against the lepidopteran insect host *Galleria mellonella* (Eley et al., 2007). It is notable that tenellin and NG-391 from *M. robertsii* are structurally similar compounds, are biosynthesized by the same class of genes (i.e., a hybrid PKS/NRPS) (Donzelli et al., 2010), and neither plays a measurable role in killing lepidopteran insects. Whether they play similar, as yet undefined, biological roles in their respective host–fungal pathosystems is unknown. It is possible that these and other fungal metabolites having no measurable effect on insect death contribute to the disease process in more subtle ways not measured during the course of an insect bioassay, which is based primarily on speed of kill at a particular pathogen dose, e.g., by perturbing invertebrate physiological or immunological processes that have the potential to exert subtle effects on downstream host responses to pathogen challenge. More frequent use of molecular genetic and biochemical assays with higher sensitivities for measuring insect responses to secondary metabolites will increase our understanding of where and how these compounds act in biological systems (see destruxin discussion below), especially since the majority of metabolites produced by entomopathogenic fungi in vivo probably do not act as "brute force" cytotoxins that are a direct cause of arthropod death.

In addition to their roles as cytotoxins, fungal secondary metabolites also play significant roles in other processes contributing to successful host–fungus interactions, including fungal development, e.g., sporulation (Brodhagen and Keller, 2006), melanization of various fungal structures contributing to infection, oxidative stress responses, and environmental resistance (Henson et al., 1999; Ebbole, 2007; Liu and Nizet, 2009), signal transduction (Böhnert et al., 2004; Collemare et al., 2008; Tsitsigiannis and Keller, 2007), and nutrition, e.g., as siderophores involved in iron uptake and storage (Haas et al., 2008). For example, the mutualist endophyte *E. festucae* requires a functional *sidN* siderophore gene to maintain the symbiotic relationship it has with perrenial ryegrass (Johnson, 2008), thereby, contributing insect deterrence capacities to the plant. Disruption of the *sidN* gene transforms this mutualistic fungus into an antagonist by disrupting the iron homeostasis of the relationship (Johnson et al., 2007). Given the close taxonomic relationship between *E. festucae* and several arthropod pathogens, it would not be surprising to find that one or more siderophores play critical roles in host-entomopathogen interactions.

Previously, we described how melanin served as a defensive trait for *A. fumigatus* against collembolan grazing, which increased fitness for the fungus (Scheu and Simmerling, 2004). In contrast, Jackson et al. (2009) recently demonstrated that melanin-deficient, conidial pigmentation mutants of *A. fumigatus* displayed a higher level of pathogenicity against the insect host *G. mellonella* than the wild type strain. Hence, in this system, loss of melanin production, which apparently resulted in the accumulation of intermediate metabolites that altered conidial pigmentation, appears to have increased the fitness of *A. fumigatus* in a laboratory insect model. These two studies act as examples to demonstrate that a given secondary metabolite phenotype, such as melanin production, can modulate both defensive and offensive strategies, depending on the nature of the specific insect–fungus interaction.

Understanding how arthropods resist and/or detoxify the secondary metabolites produced by entomopathogens is critical in developing fungi as effective biological control agents of agronomic pests and vectors of mammalian pathogens. Identifying the specific biological responses they elicite in susceptible hosts is also critical. The destruxin metabolites produced by members of the *M. anisopliae* complex are arguably some of the best studied secondary metabolites of fungal entomopathogens (Païs et al., 1981; Pedras et al., 2002). Their detection in pooled insect cadavers (Amiri-Besheli et al., 2000; Skrobek et al., 2008) and the broad range of biological effects they exhibit against insects (Huxham et al., 1989; Gillespie et al., 2000; Hinaje et al., 2002; Pedras et al., 2002) have suggested they may play a key role in insect disease caused by members of the *M. anisopliae* complex.

Recently, Pal et al. (2007) demonstrated that injection of destruxin A into adult *Drosophila* flies specifically suppresses the humoral immune response, mediating the down-regulation of a subset of bacterial antimicrobial peptides (AMPs), specifically cecropins, attacin, metchnikowan, and diptericin. Interestingly, they saw no measurable effects on regulation of the fungus-specific AMP drosomycin unless *Escherichia coli* was co-injected with destruxin A, which resulted in increased insect death due to bacterial infection. One possibility is that by selectively reducing bacterial AMPs with the secretion of destruxin A, *M. anisopliae* creates an environment where bacteria can proliferate and, thereby, contribute to accelerating the demise of its insect host. It is notable that destruxin A alone had no affect on cellular immune responses in vivo, i.e., melanization at the injection site and hemocyte phagocytosis were unaltered compared to the control treatment. This report is the first in vivo evidence that a fungal secondary metabolite targets an innate insect immune signaling pathway. Dumas et al. (1996) had previously demonstrated that destruxin E modulates calcium flux and protein phophorylation, first suggesting a biological role in the manipulation of host signaling (Pal et al., 2007).

Destruxins are efficiently detoxified by locusts (*Locusta migratoria*) (reviewed in Pedras et al. (2002)), which are highly resistant to this family of toxins that can cause tetanic paralysis in host insects. The *M. anisopliae* host *G. mellonella* also is capable of biotransformation of destruxins, with the ability to generate

different detoxification products than *L. migratoria*, depending on the specific structure of the destruxin starting material. Interestingly, the majority of generalist *M. anisopliae* strains capable of infecting and killing *G. mellonella* and other insects are in vitro-producers of destruxins (Kershaw et al., 1999; Moon et al., 2008), which cause tetanic paralysis in these hosts. If destruxins are confirmed by molecular genetic means as a virulence factor for *Metarhizium*, the strong correlation between destruxin production and host susceptibility/pathogen virulence might suggest that in the ongoing evolutionary arms race between *G. mellonella* and *M. anisopliae*, the destruxin detoxification mechanisms of susceptible hosts are insufficient to disable the fungus as a pathogen. In contrast, specialist strains of the *M. anisopliae* complex that are host-specific to locusts and other orthoptera, i.e., *M. anisopliae* var. *acridum* (recently renamed *Metarhizium acridum* [Bischoff et al., 2009]), generally produce little to no destruxins in vitro (Kershaw et al., 1999; Moon et al., 2008); destruxin production in orthoptera cadavers has not been reported. If the in vitro destruxin assays of orthopteran-infective strains are indicative of limited in vivo production, which requires confirmation, these correlations would suggest that destruxins are unlikely to play a key role in *Metarhizium*-mediated pathogenesis of orthoptera (Kershaw et al., 1999).

The *Metarhizium*-orthoptera pathosystem may represent an example of co-evolution whereby the highly efficient destruxin detoxification mechanisms of locust, for example, caused selection of a subset of host-specific *Metarhizium* strains (*M. acridum*) that incapacitate or kill locusts by means other than destruxin production, e.g., by production of secreted hydrolytic enzymes (Freimoser et al., 2003; Wang and St. Leger, 2005) and, possibly, secondary metabolites distinct from destruxins. Because the genetic complement of AMPs varies widely among insects, with many AMP gene families found in only a few closely related species (Lazzaro, 2008), it is not unexpected that *Metarhizium* spp. could evolve different sets of virulence factors to adapt to the diversity of AMPs found in different insects, e.g., *Drosophila melanogaster* encodes only three of the AMP classes (defensins, cecropins, and lysozymes) found in the genomes of bees, mosquitoes, and beetles (reviewed in Lazzaro (2008)). This point underscores the importance of evaluating multiple host systems in bioassays assessing the effect of gene disruption or deletion on entomopathogen virulence, as well as accounting for the source of the original host from which the pathogen was first isolated.

For the NG-391 mutagen synthesized in vitro by *M. robertsii*, it is unknown whether it is produced and detectable in insect cadavers. However, its structural similarity to the fusarins produced by *Fusarium* spp. (Gelderblom et al., 1983; Krasnoff et al., 2006) suggest it has the potential to lose mutagenic activity via degradation under high light and temperatures (Gelderblom et al., 1983). Furthermore, it is unknown whether insects are capable of biotransformation and detoxification of NG-391. However, the chemical and enzymatic interaction of fusarin C with glutathione-S-transferase (GST) causes inhibition of mutagenesis in vitro (Gelderblom et al., 1988). Since many insects use GSTs to detoxify exogenous compounds, such as pesticides (Gui et al., 2009), it is possible that NG-391 lacks toxicity, mutagenicity, and/or stability in *S. exigua* and other insects due to a highly effective capacity, mediated at least in part by GST activity, to inactivate it. Further studies of the detection, stability, and possible detoxification of NG-391, tenellin, and other entomopathogen secondary metabolites potentially produced in infected insects are warranted for both scientific, as well as, environmental safety reasons related to applications of biocontrol pathogens (Zimmermann, 2007a,b).

The medically-important immunosuppressant cyclosporin A, a fungal secondary metabolite produced by entomopathogenic *Beauveria*, *Verticillium* and *Tolypocladium* spp., is an inhibitor of a P-glycoprotein-related efflux pump involved in detoxification of insecticides and other xenobiotics (Podsiadlowski et al., 1998). The cyclosporins appear to represent yet another strategy used by entomopathogenic fungi to incapacitate the insect immune system in response to a xenobiotic threat while, perhaps concurrently, producing yet other sets of secondary metabolites to facilitate the final kill. Resistance or tolerance to cyclosporin is believed due to production of the major hemolymph-circulating lipoprotein, lipophorin. Lipophorin is a lipid body surface-binding protein secreted by the insect fat body (reviewed in Arrese and Soulages (2010)) that can bind cyclosporin A for storage or detoxification in the fat body (Vilcinskas et al., 1997a). Cheon et al. (2006) demonstrated that lipid metabolism, specifically mediated by lipophorin and its receptor, also is involved in the systemic immune response of *Aedes* mosquitoes to infection by the fungus *B. bassiana*. So lipiphorin serves a dual role in insects for both lipid transport and immune recognition (Schmidt et al., 2010). It is unclear whether the virulent *B. bassiana* strain used by Cheon and colleagues was an in vivo producer of cyclosporin A, but the extent to which various insects are capable of binding and inactivating cyclosporin A via lipophorin-mediated or other mechanisms warrants further study. Interestingly, *M. anisopliae* uses a similar kind of lipid body surface-binding protein (MPL1) of the PAT-domain family (perilipin-like) (Bickel et al., 2009) for maintenance of lipid homeostasis, which is necessary to create the appressorium turgor pressure required for penetration of the insect cuticle during infection (Wang and St. Leger, 2007). *Mpl1*-disrupted strains have reduced virulence against *Manduca sexta* larvae due to the formation of defective appressoria unable to penetrate the insect cuticle.

Ferrandon et al. (2007) have reviewed much of the knowledge to date describing the cascade of pathways induced in the *Drosophila* systemic immune response to fungi and other pathogens. With the exception of the Pal et al. (2007) report of destruxin effects on the *Drosophila* immune system, studies of the detection of fungal virulence factors by *Drosophila*, followed by immune system activation (e.g., via Toll-mediated antimicrobial defense) appear to be limited to responses induced by the serine protease PR1 (Gottar et al., 2006) of *M. anisopliae* or β-1,3 fungal glucan cell wall components (Roh et al., 2009) of *B. bassiana*. Clearly, we have much to learn about which secondary metabolites of fungal entomopathogens are critical for initiating and maintaining disease states in insects, how their presence compromises a diversity of arthropod immune systems, and the mechanisms arthropods use to defend themselves against bioactive fungal metabolites.

We can infer from the studies of secondary metabolite genes of plant pathogenic fungi (Bushley and Turgeon, 2010) that not all entomopathogen secondary metabolites should be assumed to function as "toxins" or virulence factors. It is probable that many play more subtle roles during development or survival in a range of environments (e.g., during saprophytism) for which we have yet to develop effective assays to detect their effects. We are now poised with the appropriate genetic tools in hand to elucidate how secondary metabolites of fungal entomopathogens contribute to disease processes, development, and behavior of invertebrates, as well as to better understand the evolutionary forces helping to shape these organismal interactions. In-depth studies, using multiple-host bioassays in combination with analyses of the effects of in vivo-produced fungal metabolites, or their loss, on arthropod immune function and regulatory cascades are now possible. The recently revised taxonomic organization of the *Metarhizium* complex members (Bischoff et al., 2009) and other entomopathogen taxa (reviewed in Rehner (2009)), in combination with availability of the genome sequences for these and other exemplar fungal entomopathogens (Qin et al., 2006; St. Leger and Wang, 2010) and insect hosts (http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi?p3=12:Insects&taxgroup=11:|12:Insects), will provide a wealth of opportunities to determine the contributions of entomopatho-

gen secondary metabolites to the evolutionary interplay between pathogen virulence and host resistance.

5. Towards an understanding of secondary metabolites as an evolved defense

The examples above provide supporting evidence that fungal secondary metabolites may mediate fungal resistance to animal antagonists. In the following section, we address three important topics that may help to better understand whether secondary metabolite biosynthesis is indeed part of an evolved fungal defense system that is maintained by the selective pressure of insects.

5.1. Inducibility

Attack by fungivores (or herbivores in plant–fungus symbioses) or contact with potential insect hosts can be expected to vary in space and time. Because secondary metabolite formation is an energy consuming process, fungi would be expected to synthesize the proposed chemical weapons when the ecological conditions demand their employment, viz. when fungivore attack occurs or an insect immune system is defending a host. Differentiation between constitutively expressed chemical resistance traits and those that are induced, i.e., only expressed in response to antagonistic insects, has been demonstrated for plant–herbivore interactions (Howe and Jander, 2008). Several secondary metabolites in filamentous fungi are expressed constitutively during fungal development irrespective of the impact of a fungal grazer (Calvo et al., 2002). In addition to preformed chemical barriers, fungi may mount active defense responses that elevate the production of constitutively expressed metabolites and/or stimulate the *de novo* synthesis of additional compounds at the site of tissue damage and/or systematically in undamaged tissues. This critical issue in fungal chemical ecology has remained untested. Some earlier studies (Stadler and Sterner, 1998) and, especially, recent chemical approaches by Spiteller (2008) demonstrate the ability of higher fungi to display wound-activated conversion of precursor compounds to highly toxic products; yet there is no conclusive experimental evidence that these compounds indeed serve as resistance traits to increase fungal fitness under predator attack. Although injury of fungal tissue may play a critical role in eliciting a chemical defense response, it is presumed that specific insect-derived compounds are recognized by the host fungus and thereupon activate a defensive response. Interestingly, to the best of our knowledge, no study has convincingly demonstrated that fungi possess the ability to display an induced chemical response to fungivore attack. However, some recent observations are worth noting in this regard. Toxic lectins (proteins that bind to specific carbohydrate structures) usually found in the fruiting body of *Coprinopsis cinerea* appear to be up-regulated in mycelia challenged with the mycophagous nematode *Aphelenchus avenae* (Markus Künzler, personal communication). Also, the fungal galectin CGL2 of *C. cinerea* recently has been found to kill nematodes by binding to specific glycoconjugates and may, thus, be involved in chemical defense (Butschi et al., 2010), although critical fitness assays quantifying fungal fitness are still lacking.

Given the argument that secondary metabolite biosynthesis is costly to the fungus and, consequently, that they should forego these costs of chemical defense when unnecessary, the nature of such costs needs to be determined. This, however, requires substantial knowledge about the mechanisms eliciting an induced response in order to experimentally manipulate fungi without causing any fitness-reducing harm. In analogy to plant–herbivore systems, fungal feeders may not only cause physical damage to fungal tissue but, while chewing, may unavoidably release salivary gland chemicals that could be perceived by the fungus, launching a cascade of reactions leading to the *de novo* or increased production of insecticidal chemicals. Treating fungi with such elicitors or fungus-derived communication molecules may enable us to identify the direct costs fungi pay for the proposed induced chemical defenses (see Baldwin (1998) for analyses of direct costs of chemical defenses in plants).

In entomopathogen systems, little is known about which environmental or host signals are responsible for induction of secondary metabolite pathway gene expression and natural product synthesis during the process of host infection. Donzelli et al. (2010) recently described a time course of expression of *NGS1*, a key gene responsible for synthesis of the mutagen NG-391, in beet armyworm (*S. exigua*) infected with *M. robertsii*. They demonstrated that *NGS1* expression was detectable at only low levels at 28 h post-inoculation (PI), strongly expressed by 52 h PI, and expression remained relatively high in motile, infected larvae but dropped significantly in dead larval mummies by 122 h PI. Although NG-391 does not contribute to virulence of *M. robertsii* against *S. exigua*, analyses of an *NGS1* promoter-driven GFP reporter strain indicated that in vitro *NGS1* expression may be modulated by fungal population density. Monitoring expression of the *NGS1* GFP reporter strain over time in infected host insects may help to identify physical, chemical, and genetic cues involved in fungal and/or insect development during the disease process.

5.2. Signaling

If fungi produce toxins as chemical defenses against fungivory, it is predicted that they should signal their dangerousness to potential insect predators. Like in plants (Holopainen and Gershenzon, 2010), emission of volatile organic compounds (VOCs) by fungi may be an efficient way of signaling fungal feeders about their unprofitability. Support for this hypothesis is provided by the various fungivore food choice experiments mentioned above and other studies focusing explicitly on induction of attraction or avoidance by VOCs (e.g., Bartelt and Wicklow, 1999; Drilling and Dettner, 2009; Hedlund et al., 1995; Pierce et al., 1991; Thakeow et al., 2008, and more). The powerful approach of using transgenic fungi with impaired secondary metabolite expression and/or volatile formation still awaits inclusion into chemo-ecological studies. Given the rapid response and extreme preference of the springtail *F. candida* for the chemically deficient *A. nidulans* Δ*laeA* mutant strain (see above), the study by Rohlfs et al. (2007) may point to a link between secondary metabolite formation and VOCs. Based on a preliminary analysis of variation in VOC emissions, the chemically deficient *A. nidulans* Δ*laeA* mutant strain appears also to be deficient in emitting several VOCs (Thakeow, Schütz and Rohlfs, unpublished data).

A promising approach for gaining substantial insights into fungal volatile signaling may be to better understand the role of fungal oxylipins in interactions with other organisms (Tsitsigiannis and Keller, 2007). Oxylipins represent a diverse group of metabolites that originate from the oxidation or further conversion of polyunsaturated fatty acids (PUFAs). Like in plants (e.g., jasmonates) and animals, including insects (e.g., eicosanoids), fungal oxylipins have been suggested to play important roles as signaling molecules triggering and coordinating a wide range of cellular processes, including chemical defense (Tsitsigiannis and Keller, 2007). In *A. nidulans*, for example, expression of *ppo* genes, encoding several enzymatic steps in oxylipin formation, appears to be involved in positively regulating the expression of *aflR*, a specific regulator of the biosynthetic pathway leading to the production of the cytotoxic and insecticidal mycotoxin sterigmatocystin (Tsitsigiannis and Keller, 2006). In addition to their proposed function as internal communication molecules, oxylipin formation also leads to the production of volatile compounds, such as 1-octen-3-ol (Brodhun et al.,

2010), that might act as an info-chemicals affecting the food choice behavior of fungal grazers (Bengtsson et al., 1991; Sawahata et al., 2008; Thakeow et al., 2008). The use of transgenic fungi deficient in one or more regulatory or structural genes for oxylipin biosynthesis in ecological experiments with fungivorous insects, coupled with gene expression and chemical analyses, may critically advance our understanding of oxylipin signaling in fungal chemical defense.

Oxylipin pathways have not been described in entomopathogenic fungi, but their presence would not be surprising given the number of oxylipin-forming enzymes predicted from a variety of fungal genomes (Andreou et al., 2009). Although oxylipin biosynthetic pathways are present in marine invertebrates, the genes have not been detected in insects due to their predicted evolutionary loss (Lee et al., 2008). Whereas fungi make oxylipins primarily derived from oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids, insects and other animals produce a similar group of messenger molecules, called eicosanoids, using arachidonic acid (C20:4) as the precursor. Given the suggested role of oxylipins as communication molecules between hosts and pathogens (Tsitsigiannis and Keller, 2007), it is reasonable to predict there could be interfering cross-talk between fungal entomopathogens and their invertebrate hosts via oxylipin and eicosanoid pathways. Although such activity has not yet been demonstrated, several researchers have reported that eicosanoids mediate the immune response of the lepidopteran *M. sexta* to infection by *M. anisopliae* and *B. bassiana*, specifically by inducing nodulation after detecting the pathogen in the hemolymph (Dean et al., 2002; Lord et al., 2002) and by regulating the behavioral fever response of locusts (Bundey et al., 2003). Adaptive parasite manipulation of host behavior to benefit the fungus, e.g., where diseased insects climb to the top of vegetation to clamp on, die and sporulate (Hughes et al., 2009), could be mediated by such processes. Dean et al. (2002) concluded that "Given the importance of eicosanoid signaling to the insect immune system, interference with eicosanoid metabolism would seem to be a sensible strategy, and thus a potentially important virulence factor for an entomopathogen. We predict that at least some entomopathogens will be found to inhibit eicosanoid signaling in their insect host." If this proves to be the case, such inhibition could occur via production of a variety of fungal secondary metabolites, including the predicted oxylipins. Alternatively, fungal entomopathogens may be capable of exploiting arthropod host eicosanoids to facilitate their own virulence and pathogenic development. This strategy is well described for some plant-associated fungi, which induce plant lipid metabolism to utilize plant oxylipins to promote G-protein-mediated regulation of fungal sporulation and mycotoxin production (Christensen and Kolomiets, 2010).

5.3. Evolutionary responses

An emerging tool for better understanding the trajectories and dynamics in (co-)evolutionary processes, such as arthropod–fungus interactions, is the application of experimental evolution (Conner, 2003; Fuller et al., 2005; Schulte et al., 2010). To date, however, only very few studies have used this powerful approach in fungal biology (e.g., Schoustra et al., 2009), and it is virtually absent from the study of fungus–fungivore interactions. With regard to arthropod–fungus competition on dead organic matter (see above), exposure of *Drosophila* fly larvae to competing *A. nidulans* over several insect generations led to fly populations better adapted to the fungal competitor (Wölfle et al., 2009). In line with predictions from life-history theory, the evolved adaptation to the fungal competitor is not free to the insects but extracts a cost in terms of reduced ability to resist abiotic stress (Wölfle et al., 2009). These results may explain the genetically determined variation in developmental success of insect larvae in the presence of a competing mold (Rohlfs, 2006). Interestingly, evolved protection against *A. nidulans* appears to positively correlate with resistance to sterigmatocystin, the most toxic mycotoxin produced by this fungus (Trienens and Rohlfs, unpublished data).

In an evolutionary experiment on interactions between an insect host and an entomopathogenic fungus, *Drosophila* flies evolved apparent tolerance to fungal infections with *B. bassiana* (Kraaijeveld and Godfray, 2008). It is, however, unknown whether the evolved response to the fungal pathogen involved differences in the production of antimicrobial peptides, that target fungi (Ferrandon et al., 2007; Hultmark, 2003) or whether it is due to resistance against secondary metabolites acting as virulence factors (see above). We suggest that the experimental evolution approach may constitute a crucial tool to track phenotypic (co-)evolution in arthropod–fungus interactions and to generate both adaptively diverged arthropod and fungal populations, which can then be used for in-depth molecular genetics and chemical analyses.

6. Conclusions

Natural selection seems to have favored mechanisms that allow for a tight genetic regulatory control of expression of bioactive compounds, enabling fungi to respond flexibly to various ecological challenges requiring a chemical defense. Here, we identified diverse approaches from different research perspectives on arthropod–fungus interactions, which support the hypothesis that secondary metabolites may operate as defensive or offensive chemical weapons against animals. Compared with analogous interactions, such as those between bacteria and animal hosts (e.g., Bode, 2009) or plants and herbivorous insects (Howe and Jander, 2008), we still lack conclusive knowledge about the ecological function of fungal secondary metabolites.

Our suggestions of what we need to do and which questions should be answered to achieve a better understanding of the role of fungal secondary metabolites as an evolved defense system against animal antagonists are as follows:

(i) We need to intensify collaborations between ecologists and molecular biologists to answer the basic question of whether fungi display induced responses to their natural enemies or hosts. For example, is *ppo* gene expression up-regulated in response to competitors or predators, which leads to defense-dependent 'oxylipin signatures'? Do natural enemy- or hosts-induced changes in the 'oxylipin signature' contribute to the (specific) induction of mycotoxin gene expression? If so, is the response local or systemic? What are the physical and/or chemical stimuli (i.e., fungivory associated molecular patterns, FAMPs) triggering an induced response? Is the response antagonist- or hosts-specific? Ideally, genomic approaches will be coupled with metabolic profiling studies.

(ii) Does an induced chemical response lead to enhanced protection against its antagonist (direct protection)? Do fungi signal their unprofitability or toxicity by the release of volatile organic compounds (VOCs) that differentially affect fungivore behavior?

(iii) Simultaneously, we need to investigate animal responses to fungi and their metabolites. Do animals display adaptive strategies of attacking fungi (e.g., site of attack, density-dependence, secretion of anti-fungal compounds). How do insects cope with the (increased) production of toxic chemicals by food sources and/or pathogens? To what extent are detoxification and repair mechanisms involved, and to what extent do they differ among insects, both within and

between orders? If insects are capable of displaying an induced response to toxic fungi, what are the relevant fungal signals and how are they perceived and transmitted? Is there cross-talk between fungal and arthropod signaling molecules that mediates host susceptibility? How do pathogenic fungi induce and control insect behavior to optimize fungal fitness, e.g., sporulation and spread of propagules? How do pathogenic fungi minimize insect behavioral changes, such as behavioral fevers, that potentially act to reduce fungal fitness?

(iv) Do fungi, like their antagonists, harbor genetic variation in withstanding the proposed animal resistance traits? Do insects select for enhanced production of deterrent or harmful fungal compounds, or do they favor fungal variants that synthesize qualitatively or quantitatively different compositions of chemicals with stronger synergistic effects on natural enemies? A main goal will be to establish experimental setups to study the evolutionary influence of insect pressure, which may finally allow us to investigate the consequences when we provide opportunities for the organisms to co-evolve under controlled circumstances.

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