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Fruit, flies and filamentous fungi – experimental analysis of animal–microbe competition using *Drosophila melanogaster* and *Aspergillus* mould as a model system

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In addition to their fundamental role in nutrient recycling, saprobiotic microorganisms may be considered as typical consumers of food-limited ephemeral resource patches. As such, they may be engaged in inter-specific competition with saprophagous animals feeding from the same resource. Bacteria and filamentous fungi are known to synthesise secondary metabolites, some of which are toxic and have been proposed to deter or harm animals. The microorganisms may, however, also be negatively affected if saprophagous animals do not avoid microbe-laden resources but feed in the presence of microbial competitors. We hypothesised that filamentous fungi compete with saprophagous insects, whereby secondary metabolites provide a chemical shield against the insect competitors. For testing this, we developed a new ecological model system representing a case of animal–microbe competition between saprobiotic organisms, comprising *Drosophila melanogaster* and species of the fungus *Aspergillus* (*A. nidulans*, *A. fumigatus*, *A. flavus*). Infestation of *Drosophila* breeding substrate with proliferating fungal colonies caused graduated larval mortality that strongly depended on mould species and colony age. Confrontation with conidiospores only, did not result in significant changes in larval survival, suggesting that insect death may not be ascribed to pathogenic effects. When confronted with colonies of transgenic fungi that lack the ability to express the global secondary metabolite regulator LaeA (Δ*laeA*), larval mortality was significantly reduced compared to the impact of the wild type strains. Yet, also in the Δ*laeA* strains, inter-specific variation in the influence on insect growth occurred. Competition with *Drosophila* larvae impaired fungal growth, however, wild type colonies of *A. nidulans* and *A. flavus* recovered more rapidly from insect competition than the corresponding Δ*laeA* mutants (not in *A. fumigatus*). Our findings provide genetic evidence that toxic secondary metabolites synthesised by saprotrophic fungi may serve as a means to combat insect competitors. Variation in the ability of LaeA to control expression of various secondary metabolite gene clusters might explain the observed species-specific variation in *Drosophila–Aspergillus* competition.

Competition for rich and ephemeral resource patches can be intense among saprobiotic organisms and may not only occur among closely related taxa (e.g. animal–animal or microbe–microbe competition) but has also been suggested to determine animal–microbe interactions (Janzen 1977). Since the pioneering essay by Daniel Janzen animal–microbe competition has, however, received only little attention (Diamond 1987, Hochberg and Lawton 1990). Nevertheless, there is growing awareness of the importance of this interaction between microorganisms and animals in ecology and evolution (Strohm and Linsenmair 2001, Burkepile et al. 2006, Sherratt et al. 2006, Shivik 2006, Kaspari and Stevenson 2008, Rozen et al. 2008).

Dead organic materials, e.g. decaying plant tissue, dung, carrion, etc. are resource patches where animal–microbe competition is expected to be most intense, and there is ample yet often correlative evidence that saprophagous insect larvae and noxious filamentous fungi reduce each other's fitness parameters, i.e. the organisms compete with each other (Sullivan and Sokal 1963, Lussenhop et al. 1980, Lussenhop and Wicklow 1985, Courtney et al. 1990, Hodge 1996, Hodge and Mitchell 1997, Hodge et al. 1999, Suzuki 2001, Wertheim et al. 2002, Rohlfs and Hoffmeister 2003, 2005). A prime example of the large group of saprophagous insects living in a diverse microbial world are the larval stages of many *Drosophila* species that strongly depend on the availability of decaying fruits which are concomitantly inhabited by various microorganisms. While yeasts have been described as essential food sources of *Drosophila* larvae (Begon 1982), other studies found negative correlations between the occurrence of filamentous fungi and *Drosophila* development (Hodge 1996, Wertheim et al. 2002). Because of their omnipresence, that is a high proportion of potential *Drosophila* breeding substrates may be infested with various filamentous (mould) fungi (Hodge 1996, Schatzmann 1977), this group of fungi might play an important role in the life-cycle of saprophagous insects. Indeed, a detailed characterisation of *Drosophila*–fungus competition revealed a central role of priority effects (i.e. fungal colony age) and density-dependence (i.e. insect larval

aggregation) in determining both insect developmental success and fungal growth and reproduction (Hodge et al. 1999, Rohlfs et al. 2005). In the presence of some mould species larval survival to the adult stage had a positive relationship with insect density, indicating the existence of an Allee effect (Stephens et al. 1999, Rohlfs et al. 2005). Such Allee effects may ultimately explain the adaptive value of aggregative egg-laying behaviour across food-limited resource patches (Rohlfs and Hoffmeister 2003, Lof et al. 2008), which have a crucial influence on the flies' ability to colonize a habitat (Lof et al. 2009). Moreover, larvae tend to forage in groups within resource patches and aggregate even more strongly when patches are infested with competing mould (Rohlfs 2005). Such a communal insect attack on mould fungi may enable larvae to interfere with fungal colonies, suppress fungal growth and hence improve their own survival. In turn, fungi are known to synthesise secondary chemicals which they secrete into their growing substrate, and many of these fungal secondary metabolites have been shown to have insecticidal properties (Reiss 1975, Melone and Chinnici 1986, Rohlfs 2008, Rohlfs and Obmann 2009). Thus, one prominent hypothesis states that toxic secondary metabolites protect fungi from antagonistic animals by deterring and/or harming them. In the case of competition between saprophagous insects and mould fungi which compete for space- and food-limited patches of dead organic matter, fungi may be considered as allelopathic organisms building a chemical shield that inhibits the growth of inter-specific competitors (Begon et al. 2006). Interestingly, there is only limited experimental evidence for this 'chemical shield'-hypothesis in fungi (Ruess and Lussenhop 2005, Fox and Howlett 2008, Stadler and Keller 2008, Kempken and Rohlfs 2010).

In the study presented here we hypothesise that the insecticidal properties of filamentous fungi (possibly comprising secondary chemicals) constitute a vital trait that determines the outcome of antagonistic insect–fungus interactions. We used *Drosophila melanogaster* larvae and *Aspergillus* mould as an ecological model system to mimic the obviously widespread antagonistic interactions between saprophagous insects and saprotrophic filamentous fungi. Because so little is known about the species composition and diversity of antagonistic fungi drosophilids may encounter at natural conditions, we are aware of the fact that our model only partially reflects the natural situation on decaying plant material, and the outcome of *Drosophila*–mould interactions may become more multifaceted if the microbial community in the insect environment is getting more complex (Rohlfs and Kürschner 2010). Nonetheless, we think that this model is suitable for disentangling basic principles of antagonistic insect–fungus interactions which have hitherto remained unexplored.

In detail, we confronted *D. melanogaster* larvae with both, wild type (WT) and transgenic (Δ*laeA*) strains of the filamentous fungal species *Aspergillus nidulans*, *A. fumigatus* and *A. flavus*. The transgenic strains lack the ability to express the global secondary metabolite regulator LaeA. Although the mechanism of LaeA regulation is still unknown, similarities of the LaeA protein to methyltransferases driving histone modification may indicate a role of this protein in chromatin remodelling (Keller et al. 2005). In concert with other regulatory mechanisms, this epigenetic machinery may enable various filamentous fungi to flexibly respond to changes in their environment (Shwab et al. 2007). Blocking the expression of *laeA* in Aspergilli leads to a reduction or even entire suppression of several secondary metabolites, including insecticidal mycotoxins, such as sterigmatocystin in *A. nidulans*, aflatoxin in *A. flavus*, and gliotoxin in *A. fumigatus* (Bok and Keller 2004, Perrin et al. 2007, Kale et al. 2008) (see Methods for more details).

In *A. nidulans*, blocking the expression of *laeA* induces changes in food choice behaviour and evolutionary fitness of the fungivorous springtail *Folsomia candida* (Rohlfs et al. 2007): colonies lacking the expression of *laeA* suffered more from fungivory than the WT strain that was able to express all its potential secondary metabolites. In the present study we test whether the chemical shield hypothesis has broader implications and also holds true for competitive interactions with saprophagous insect larvae. From the insect's perspective, we expected deletion of *laeA* in all competing *Aspergillus* species to result in higher survival rates of *Drosophila* larvae. Moreover, we predicted LaeA regulated fungal traits to affect density-dependent larval survival in the way that beneficial effects of high insect densities disappear in the presence of transgenic fungi (Rohlfs et al. 2005). From the fungal perspective, we expected to observe a stronger impairment of fungal growth in the transgenic than the WT strains and a positive correlation between fungal growth and insect mortality. Finally, we inspected the extent to which pathogenic effects might explain insect larval mortality rather than competitive interactions, since both *A. fumigatus* and *A. flavus* have been described as wide-spread opportunistic vertebrate and invertebrate pathogens (Brakhage and Langfelder 2002, Reeves et al. 2004, Nierman et al. 2005), with *A. flavus* additionally being a serious phytopathogen.

Methods

Insects

We established a *Drosophila melanogaster* laboratory population from flies collected in Kiel (north Germany, approx. 54°N, 10°E) in 2003. The strain was kept at 25°C as a large out-bred population in cages with >1000 individuals per adult generation. Larvae developed at moderate densities on standard *Drosophila* culture medium (DCM) containing cornmeal, yeast hydrolysate, sugar, agar, water (5.2, 5.2, 5.2, 1, 83%w), antibiotics and fungicides.

To obtain sterile larvae for all experiments described herein, we allowed flies to lay eggs on hard agar medium (standard DCM with double amount of agar) for approx. 16 h. Subsequently, we carefully washed the eggs off the medium, sterilised them with sodium hypochlorite (6%) for ten minutes, rinsed them in sterilised water and placed them on agar plates containing methyl 4-hydroxybenzoate (fungicide). The plates were incubated at 25°C and approx. 24 h later larvae hatched and could be transferred to the experimental setups by using autoclaved fine brushes.

Fungi

We used WT and transgenic strains of the filamentous fungal species *Aspergillus nidulans*, *A. fumigatus* and *A. flavus*.

Transgenic strains lacking the expression of the transcription factor LaeA were obtained by deleting the *laeA* gene (Δ*laeA*). Deletion of *laeA* reduces the expression of several secondary metabolite gene clusters, including the sterigmatocystin/aflatoxin, penicillin, lovastatin and gliotoxin clusters and other yet unknown clusters (for details see Bok and Keller 2004, Keller et al. 2005, Kale et al. 2008). While colony growth and sporulation in *A. nidulans* appears to be not affected by deleting *laeA*, the colonies have a pale appearance since they are also characterized by a loss of mycelial pigmentation (Bok and Keller 2004). Pigments also fall into the category of secondary metabolites. In addition to regulating secondary metabolite synthesis, LaeA is also involved in driving sclerotial and conidial production on different solid media and various plant seeds (Kale et al. 2008). Yet, sclerotial formation did not occur on DCM used in this study nor did we find differences in conidial production or colony growth pattern between the wild type and the Δ*laeA* mutant (data not shown). Deletion of *laeA* in *A. fumigatus* is additionally accompanied by changes in surface structure of conidia, which affects phagocytosis by human phagocytic cells and hence the development of invasive aspergillosis (Bok et al. 2005, Dagenais et al. 2010). We did, however, not check whether this morphological change is expressed on DCM. Generally, ingestion of spores does not seem influence insect development (see Test for entomopathogenic effects).

In addition to the WT and the Δ*laeA* strains, in some setups we also used complemented strains (Δ*laeA*::*laeA*), in which the *laeA* gene was reinserted from a WT strain (Bok and Keller 2004). This was done to test whether the technique used for constructing transgenic strains may itself lead to changes in fungal traits. Complemented strains should thus exhibit the same traits as WT strains. In detail, we used the following *Aspergillus* strains: *A. nidulans*: RDIT2.3 (WT), RJW46.4 (Δ*laeA*), RJW49.1 (Δ*laeA*::*laeA*); *A. fumigatus*: AF293 (WT), TJW54.2 (Δ*laeA*), TJW68.6 (Δ*laeA*::*laeA*); *A. flavus*: NRRL3357 (WT), TJW71.1 (Δ*laeA*), TJW79.13 (Δ*laeA*::*laeA*).

We cultured the fungi on malt extract agar at 25°C and a 14 h photoperiod for approx. 4–5 d. Mature conidia (asexually produced spores) were washed off with saline solution (0.9% NaCl) containing the surfactant Tween 80 (0.1%) and were stored at 4°C. Before inoculating the experimental units with conidia we adjusted a titre of 1000 conidia per µl by using a haemocytometer.

Experimental units

As experimental units we used 2 ml microtubes in which we confronted *Drosophila* larvae with fungi. As substrate suitable for growth and development of both insects and fungi we pipetted 1 ml antibiotics- and fungicide-free DCM into each microtube and autoclaved them. As standard procedure, we added 1 µl of a suspension containing 1000 conidia to each experimental tube, by placing the droplets onto the substrate surface. In the control treatment 1 µl conidia-free saline-Tween solution was added. Prior to the standard incubation regime at 25°C and a 14 h photoperiod the tubes were sealed with a sterile cotton plug.

The strength of the mould's impact on insect survival strongly depends on the timing of resource colonisation by the fungi (Rohlfs et al. 2005). To study this priority effect, we varied the time of pre-incubation of the fungal colonies in the experimental setup (one, two, three and four days) and thus created a developmental headstart of the fungus before *Drosophila* larvae were transferred on the substrate. These treatments are referred to as 'headstart conditions'. Treatments where conidia and larvae were transferred on the same day, we refer to as 'no headstart conditions'.

Egg-laying behaviour

To test whether adult *Drosophila* females avoid laying eggs on mould-infested substrate, we used choice arenas consisting of a translucent plastic container (10 × 7 × 2.5 cm). Petri dishes (35 mm ø) were filled with antibiotics- and fungicide-free DCM to provide oviposition sites. Mould-infested patches were created by inoculating 1000 conidia of *A. nidulans*, *A. fumigatus* or *A. flavus* WT strains. To obtain proliferating fungal colonies the dishes were incubated for three days at standard conditions. We covered parts of the surface area of the fungus-free control patches with a piece of sterile filter paper to mimic the situation of three day old fungal colonies covering parts of the substrate area. One fungus-infested and one control patch were placed in each choice arena (n = 10 for each fungal species). We released one mated female into each container and allowed them to lay eggs for approx. 18 h, including a dark period. Subsequently, we counted the number of eggs per patch and calculated the proportion of the total number of eggs per fly deposited on fungus-infested and fungus-free patches.

Test for entomopathogenic effects

Rather than being infected by already proliferating fungal hyphae, the infection process of insect hosts by facultative entomopathogenic fungi generally follows a specific sequence, starting with the attachment of conidia to the insects cuticle, their germination and apressorium-formation and the penetration of the insect cuticle via an infection hypha (Clarkson and Charnley 1996). To test whether *Aspergillus* fungi can behave like an entomopathogen of *Drosophila* larvae, we chose the following experimental proceedings: We prepared experimental units (n = 8 for each treatment) containing 1 × 10^6 freshly inoculated conidia each and thoroughly mixed them into the substrate to prevent larvae from avoiding to feed on uninfested medium. To exclude effects of proliferating fungal colonies and associated alterations of the medium, we transferred the larvae (ten larvae per tube at the start of the experiment) every 24 h to a new tube containing freshly prepared conidia-infested substrate. During each transfer we counted the number of larvae that had survived the previous 24 h and were thus able to record time-dependent mortality as a function of substrate treatment. If mould fungi behave like pathogens, we predicted a higher mortality in larvae confronted with conidiospores, which should resemble mortality rates found in experiments where insect were confronted with proliferating *Aspergillus* colonies.

Course of mortality during insect development

Former studies used the proportion of emerged adult flies as an indicator of fungus-borne immature insect mortality

(Rohlfs et al. 2005), leaving it unclear whether the insects die during the larval or pupal stage. To investigate this, we chose the following experimental proceedings: We confronted ten first instar larvae per experimental unit with proliferating fungal colonies (three, two and one day headstart) of all three *Aspergillus* species including the chemical deficient Δ*laeA* strains. As negative control, larvae were transferred on fungus-free substrate. In total, we prepared 50 tubes per strain and a fungus-free control. Subsequently, we randomly chose ten tubes per treatment each day, washed the larvae out of the medium and recorded the number of those still alive. With this procedure we obtained independent data on survival of the immature insect stage as a function of fungal species, strain and headstart.

Effect of fungal age and larval density

In the presence of various WT moulds, insect density and a short period between conidia germination and larval settlement have been suggested to positively affect insect development and impair fungal growth (Rohlfs et al. 2005). In a first experiment we studied the effect of variation in timing of substrate colonisation by Aspergilli on *Drosophila* development. We transferred ten sterile first instar larvae into experimental units containing either freshly added conidia, or one, two or three day old colonies. In all setups we used the WT, the Δ*laeA* or the complemented Δ*laeA*::*laeA* strains.

In a second experiment we tested whether the Allee-effect was less strong for larvae confronted with Δ*laeA* strains. We manipulated larval density by transferring one, ten or 20 insect larvae to experimental units harbouring colonies of *A. nidulans* (aged three and four days), *A. fumigatus* (aged two and three days) and *A. flavus* (aged one and two days). Again we used the WT, the Δ*laeA* or the complemented Δ*laeA*::*laeA* strains. For all treatments n = 20.

Fungal growth patterns

To test whether chemical deficient Δ*laeA* strains have lost their ability to resist the influence of competing *Drosophila* larvae, we confronted one day old fungal colonies with initially ten *Drosophila* larvae using the standard experimental unit. We inoculated the medium with the WT or the Δ*laeA* strains of all three *Aspergillus* species. After 24 h we started taking pictures of the substrates' surface every six h, which allowed us to measure the substrate area covered by fungal tissue relative to the total surface. In order to quantify the effect of insect competition on fungal growth we randomly assigned tubes containing fungi and larvae to tubes where fungi were allowed to expand without insect competitors. For each pair of experimental tubes (n = 10 for each fungal species and strain), the proportion of the substrate area covered by undisturbed colonies was subtracted from the area covered by challenged fungi. In this way we obtained data on changes in fungal growth due to insect competition as a function of the fungal species, the mutant strain and the developmental headstart of the fungus.

Statistical analysis

If not stated otherwise in the result section, proportional survival data were analysed with the Genmod function in SAS 9.0. Model fitting was carried out with a binomial error distribution and a logit link function. If deviance/DF exceeded $1 \pm 2x \sqrt{(2/DF)}$, the dscale option was used to correct for overdispersion (F statistics is shown instead of χ^2 statistics). Depending on the underlying hypothesis we conducted full model analyses, and if applicable the complete statistical output is given (see below for details). Mould growth patterns were analysed as repeated measures, for which each experimental unit was considered as a repeated subject. Since the different levels of the repeated effect represented different time steps, we fitted a time-series model by using the autoregressive 1 covariance structure, AR(1) (SAS Inst. 2005). Higher order terms of explanatory variables were added in order to test for non-linear relationships. Cox regression analysis (SAS 9.0: Proc Phreg) was applied to analyse the survival time of larvae confronted with *Aspergillus* conidia. To evaluate the proportional hazard assumption, a time-dependent explanatory variable was included in the model, as described in the SAS/STAT 9.1 User's Guide, p. 2576.

Results

Egg-laying behaviour

In neither of the choice situations did the flies display avoidance of fungus-infested substrates as oviposition sites (paired t-tests on arcsine-square-root transformed proportions of eggs laid on fungus-infested vs fungus-free substrate: *A. nidulans*, $t = 1.059$, $DF = 18$, $p = 0.304$; *A. fumigatus*, $t = -0.987$, $DF = 20$, $p = 0.336$; *A. flavus*, $t = 0.373$, $DF = 19$, $p = 0.713$). There was also no effect of the fungus on the number of eggs the females laid on both patches in each arena (one way ANOVA: $F_{2,57} = 0.278$, $p = 0.759$).

Test for entomopathogenic effects

Due to confrontation with fungal conidia only, larval mortality within single experimental units ranged from zero to a maximum of 40% during the course of our observation. At the end of the experiment 10.00% ± 0.03 SE of larvae were found dead in the control treatment, 13.75% ± 0.04 SE in the presence of *A. nidulans* conidia, 23.75% ± 0.05 SE in the presence of *A. fumigatus* conidia, and 12.50% ± 0.04 SE in the presence of *A. flavus* conidia. Despite this slight variation in survival, time-dependent mortality patterns were not different between treatments (Cox regression analysis: Wald test, $\chi^2 = 0.5973$, $DF = 1$, $p = 0.4396$).

Course of mortality during insect development

Under mould-free control conditions, there was no difference in larval mortality regarding the day on which the number of surviving larvae was recorded ($F_{1,47} = 0.40$, $p = 0.5316$). In the presence of competing fungi, larval survival was clearly reduced, independent of the fungal strain (WT or Δ*laeA*) (Fig. 1). However, as indicated by the statistically significant interactions between Day and Fungal strain (Table 1), time-dependent mortality of *Drosophila* larvae was

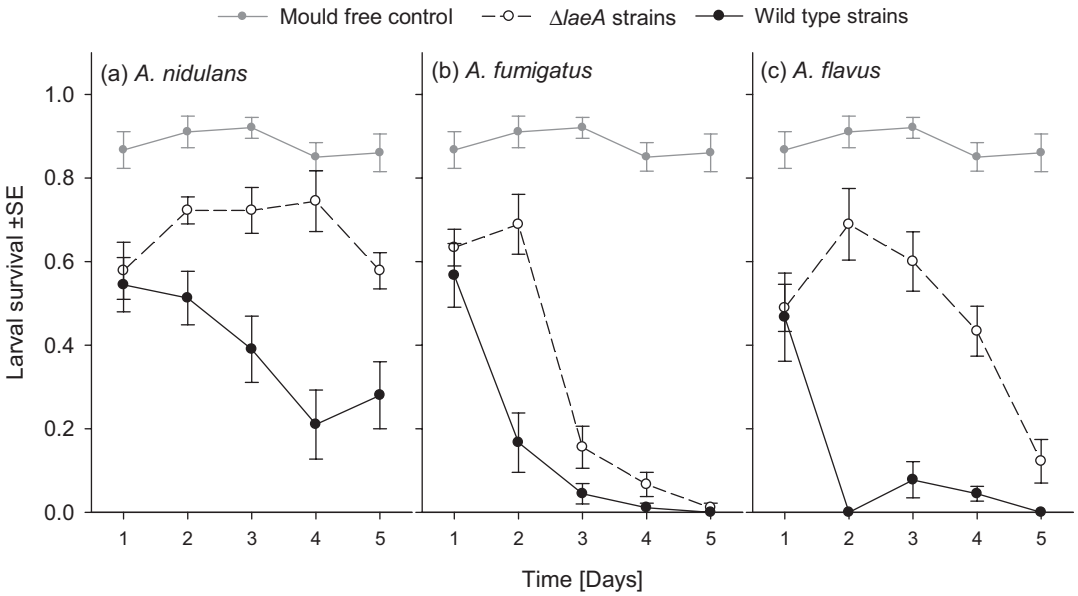

Figure 1. Daily survival of *Drosophila melanogaster* larvae in the presence of the WT or the Δ*laeA* strain of (a) *Aspergillus nidulans*, (b) *Aspergillus fumigatus* and (c) *Aspergillus flavus*.

significantly different between the WT and Δ*laeA* strains in all three *Aspergillus* species (Fig. 1). In the presence of the Δ*laeA* strains, larvae appeared to suffer less strongly from fungal competition than in the presence of the WT strains; nonetheless, confrontation with both WT and Δ*laeA* strains of *A. fumigatus* and *A. flavus* resulted in almost no surviving larvae after five days (Fig. 1b–c). A full model analysis revealed significant fungal species- and strain-specific differences in the effect on *Drosophila* survival (Day × Species × Fungal strain: $F_{2,276} = 3.47$, $p = 0.0325$; Day × Day × Species × Fungal strain: $F_{2,276} = 3.04$, $p = 0.0495$; not all results of the full model are shown).

Effect of fungal age and larval density

Timing of resource colonisation, i.e. fungal colony age at the time of larval transfer, had a dramatic impact on the probability of larval survival. Additionally, strong fungal species- and strain-specific effects became apparent (Fig. 2, Table 2). In contrast to confrontation with the WT strain of *A. nidulans*, where larval survival decreased with increasing fungal age, confrontation with the Δ*laeA* mutant did not affect survival (Fig. 2a). *Aspergillus fumigatus* had a more severe effect on *Drosophila* survival, which again depended on fungal strain and colony age: while almost no fly emerged when larvae were transferred to two day old WT colonies, the presence of the Δ*laeA* strain hardly affected insect survival (Fig. 2b). Three day old Δ*laeA* colonies, however, caused zero insect survival as did the WT strain; as indicated by the significant interaction between colony age and fungal strain (Table 2) which led to fungal strain-specific insect mortality patterns that depended on colony age. We could not statistically infer a comparable effect on *Drosophila* survival in the presence of *A. flavus*, due to the fact that no flies eclosed in the WT treatment with *A. flavus* (Fig. 2c), yet, the difference between the impact of the WT and the Δ*laeA* strain is obvious (Fig. 2c, Table 2). The effect of the WT strains and the corresponding complemented Δ*laeA*::*laeA* strains of all three *Aspergillus* species, did not differ in any of the setups (Fig. 2, Fig. 3, statistical results not shown), which indicates that genetic manipulation of the fungi per se did not influence the outcome of this antagonistic insect–fungus interaction.

Table 1. Factor influencing *Drosophila* larval survival during development in the presence of different *Aspergillus* species. Fungal strain refers to the presence of the WT or the Δ*laeA* mutant of the corresponding species. Day × Day depicts the effects of higher order terms of Day.

	A. nidulans χ^2	*A. fumigatus* χ^2	*A. flavus* χ^2
Fungal strain (FS)	5.44*	3.86*	24.11***
Day	1.83	1.77	2.99
Day × FS	11.73***	7.72**	42.95***
Day × Day	4.45*	2.33	0.01
Day × Day × FS	7.11**	4.29*	26.26***

*p < 0.05, **p < 0.01, *** p< 0.001

Since no flies emerged when larvae were forced to develop in the presence of three and two day old colonies of all *A. fumigatus* and *A. flavus* strains, we had to reduce the data set for statistical analysis accordingly. In line with the previous experiment, WT *A. nidulans* had a more negative effect on insect survival than the Δ*laeA* strain (Fig. 3a–b, Table 3). The full model analysis revealed no strain-specific density-dependent effect, but only grand effects of larval density and interaction with fungal colony age (Table 3). Despite the lack of a grand effect of fungal strain on the proportion of emerged flies in the presence of *A. fumigatus*, density-dependent larval survival depended on the fungal strain (Table 3). In the presence of the WT strain, survival had a negative relationship with larval density ($F_{1,58} = 12.05$, $p = 0.0010$), whereas survival was positively density-dependent in the presence of the Δ*laeA* strain ($F_{1,58} = 8.91$,

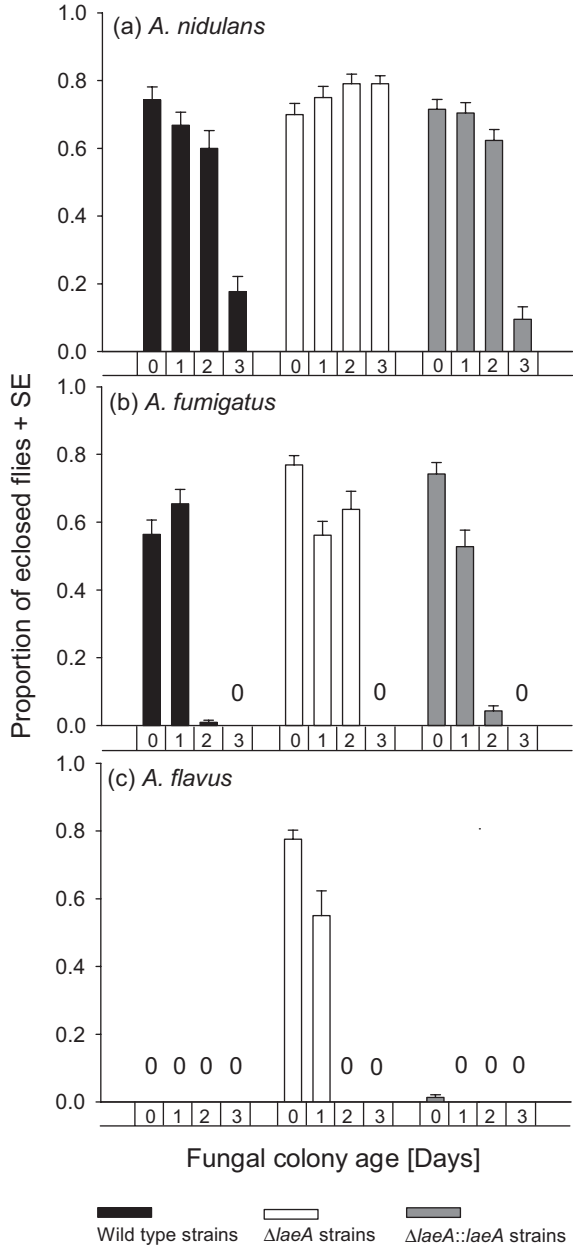

Figure 2. Survival of *Drosophila melanogaster* larvae to the adult stage in the presence of the WT, the Δ*laeA* and the complemented Δ*laeA*::*laeA* strain of (a) *Aspergillus nidulans*, (b) *Aspergillus fumigatus* and (c) *Aspergillus flavus*. Fungal colony age indicates the fungal developmental headstart prior to the transfer of ten *Drosophila* larvae.

p = 0.0042) (Fig. 3c). In contrast, confrontation with *A. flavus* led to grand strain specific effects on insect survival, but there was no significant interaction with larval density (Table 3). While there was no density-dependent effect in the presence of WT *A. flavus* ($F_{1,58}$ = 2.60, p = 0.1125), again the proportion of emerged flies was positively related to larval density in the presence of the Δ*laeA* strain, yet, this relationship is characterised by a non-linear density dependence (larval density: $F_{1,57}$ = 6.77, p = 0.0118; larval density × larval density: $F_{1,57}$ = 5.41, p = 0.0237) (Fig. 3c).

Table 2. Factors affecting *Drosophila* survival to the adult stage in the presence of different *Aspergillus* species. Colony age indicates the fungal developmental head start prior to larval transfer. Fungal strain refers to the presence of the WT or the Δ*laeA* mutant of the corresponding species. CA × CA depicts the effects of higher order terms of Colony age.

	A. nidulans⁺ $F_{1,186}$	*A. fumigatus* $F_{1,183}$	*A. flavus*‡ $F_{1,187}$
Fungal strain (FS)	3.83	5.38*	130.83***
Colony age (CA)	0.04	32.45***	–
FS × CA	69.25***	10.76**	–
CA × CA	5.07*	100.96***	–
CA × CA × FS	–	26.80***	–

⁺non-significant three-way interaction was removed from the full model to obtain the most parsimonious model; further removal of non-significant variable did not improve the model
‡ full model analysis gave no results due to only zero counts in the WT treatment
* p< 0.05, ** p< 0.01, *** p< 0.001

Fungal growth patterns

Fungal growth patterns were fungal species- and strain-specific and depended on the time span after inoculation (Table 4, Fig. 4). Statistical interactions of fungal strain and time with fungal species (Table 4) indicate complex species-specific responses to the presence of *Drosophila* larvae (Fig. 4). While impairment of young colonies of both the WT and the Δ*laeA* strain of *A. nidulans* and *A. fumigatus* was strong (Fig. 4a–b), young colonies of *A. flavus* were less severely affected by insect competition, but gradually recovered from the influence of the larvae (Fig. 4c). Interestingly, *Drosophila* larvae were able to almost exclude both the WT and the Δ*laeA* strain of *A. fumigatus* from the substrate between 54 and 78 h after inoculation, but then the colonies appeared to recover (Fig. 4b). The WT strain of *A. nidulans* was less strongly affected and started to recover earlier than *A. fumigatus* (Fig. 4a).

The *A. nidulans* Δ*laeA* strain suffered significantly more strongly from insect competition than the WT strain and both strains of *A. fumigatus* and *A. flavus* (Table 5), and was almost entirely excluded from the substrate, but started recovering after 78 h (Fig. 4a). Although *A. flavus* Δ*laeA* recovered steadily from the insects' influence, it was affected more strongly than the WT strain and could not entirely recover from insect competition during the course of our observation (Fig. 4c). In contrast, the Δ*laeA* and WT strain of *A. fumigatus* were not differently affected (Table 5) and displayed quite similar changes in growth pattern in response to the competing insect larvae (Fig. 4).

Discussion

A series of recent studies demonstrate the prevalence and diversity of competition between animals and various microbes (Hodge et al. 1999, Burkepile et al. 2006, Shivik 2006, Rozen et al. 2008) and thus highlight its importance in ecological and evolutionary processes in saprobiotic communities (Kaspari and Stevenson 2008). As

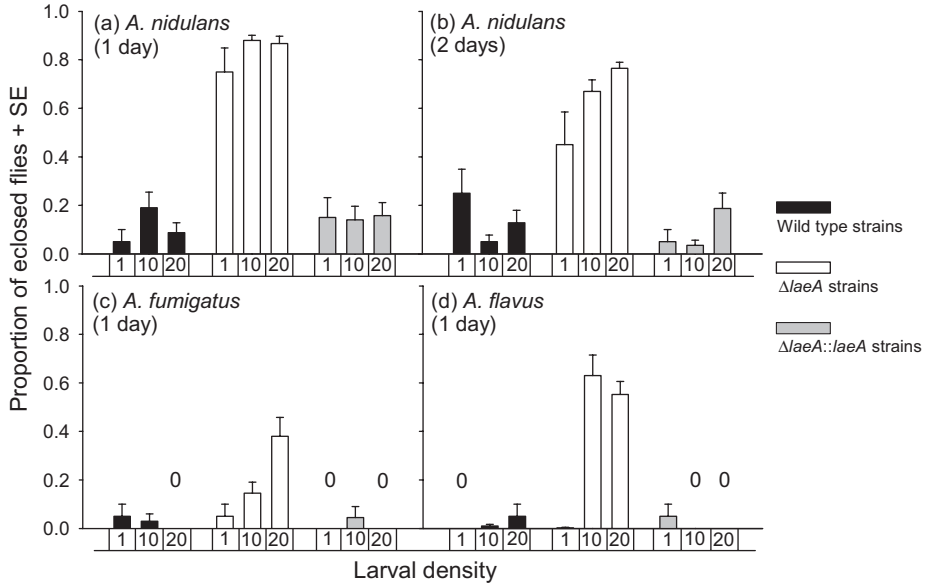

Figure 3. Density-dependent survival of *Drosophila melanogaster* larvae to the adult stage in the presence of the WT, the Δ*laeA* and the complemented Δ*laeA*::*laeA* strain of (a) three day old and (b) four day old *Aspergillus nidulans* colonies, and (c) two day old *Aspergillus fumigatus* and (d) one day old *Aspergillus flavus* colonies.

outlined in the introduction, the saprobionts *Drosophila* and *Aspergillus* may, among others and despite some limitations, constitute a suitable model system to study the evolutionary ecology and the underlying mechanisms of this insect-fungus competition under carefully controlled conditions.

By including filamentous fungi such as *A. fumigatus* and *A. flavus*, that can, in addition to their saprobiotic life style, behave like opportunistic pathogens of animals and/or plants, we could demonstrate that the negative impact of fungal growth on the developmental success of *Drosophila* is unlikely to be caused by pathogenic effects. In line with the observation that even the entomopathogenic ascomycote *Beauveria bassiana* does not infect *Drosophila* larvae (Kraaijeveld et al. 2008), our findings add further evidence to previous assertions that *Drosophila*–mould interactions fall into the category of interspecific competition.

Table 3. Factors affecting *Drosophila* survival to the adult stage in the presence of different *Aspergillus* species at varying larval densities. Colony age indicates the fungal developmental headstart prior to larval transfer. Fungal strain refers to the presence of the WT or the Δ*laeA* mutant of the corresponding species.

	A. nidulans⁺ $F_{1,234}$	*A. fumigatus*‡ $F_{1,116}$	*A. flavus*‡ $F_{1,115}$
Fungal strain (FS)	444.10***	0.57	9.23**
Colony age (CA)	10.19**	–	–
Larval density (LD)	4.49*	0.42	0.26
LD × CA	5.30*	–	–
LD × FS	–	8.24**	0.35

⁺non-significant two- and three-way interactions were removed from the full model to obtain the most parsimonious model
‡CA effects were not applicable; reduction of non-significant variables did not improve the model
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

In contrast to other systems with animal–microbe competition (Burkepile et al. 2006, Rozen et al. 2008), in the *Drosophila–Aspergillus* system *Drosophila* females did not avoid to lay eggs on fungus-infested patches, despite offspring having a higher developmental success in the absence of noxious filamentous fungi. That females do not discriminate between mould-infested and mould-free patches, might reflect the unpredictability of microbial florae and their effect on *Drosophila* offspring development on the vast array of potential breeding substrates of drosophilids (Shorrocks 1982) – yet, more field and laboratory studies that implicate the manipulation of microbial inhabitants on *Drosophila* breeding substrates are needed to better understand adult egg-laying behaviour.

Given that female *Drosophila* flies do not seem to directly protect larval offspring from the impact of mould, selection may have favoured larval strategies that mitigate fungal effects and hence determine insect–fungus competition. In addition to a possible active defence to hamper fungal growth (Rohlfs 2005), *Drosophila* larvae may possess the ability to resist fungi by other means, since larvae differ genetically in their ability to successfully develop in the presence of mould (Rohlfs 2006, Wölfle et al. 2009). As demonstrated in this study, the underlying mechanism of resistance to competing mould may be related to secondary metabolite production, because larvae developing in the presence of Δ*laeA* mutants suffered less from mould growth when compared with WT fungi. LaeA-regulated fungal traits, i.e. mainly secondary metabolism, may thus point at a central role of fungal toxins in insect–fungus competition, since fungal toxins have been demonstrated to have insecticidal properties (Reiss 1975, Chinnici and Bettinger 1984, Rohlfs and Obmann 2009). One possible mechanism of resistance against fungi might be the detoxification of mycotoxins (Foerster and Würgler 1984, Lee and Campbell 2000, Niu et al. 2008) and/or efficient

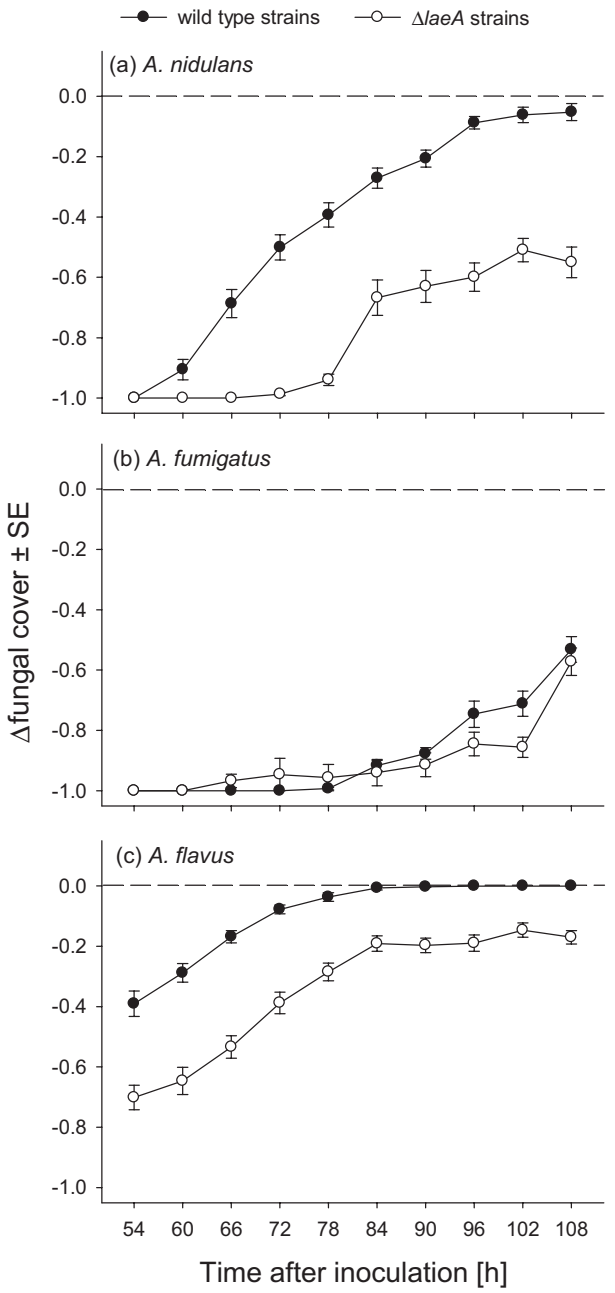

Figure 4. Changes in growth patterns of the WT and the Δ*laeA* strain of (a) *Aspergillus nidulans*, (b) *Aspergillus fumigatus* and (c) *Aspergillus flavus*, due to competition with initially ten competing *Drosophila melanogaster* larvae. The dashed line indicates the Δfungal cover value (= zero) when there are no differences in the surface covered by fungal tissue between colonies with and without insect competition (see Material and methods for details).

DNA repair mechanisms (Obana et al. 1994), because some mycotoxins such as the *A. nidulans* metabolite Sterigmatocystin has been shown to bind to DNA strands (Lukin and de los Santos 2006).

As indicated by our survival experiments, *Drosophila* larvae suffered less from the presence of transgenic fungi than that of the WT strains. However, larval mortality was still higher in the presence of Δ*laeA* than on mould free substrates; and interestingly, *A. fumigatus* and *A. flavus* Δ*laeA* strains were able to kill entire insect populations when larvae were forced to start their development on substrates where fungal colonies had been established in advance. The timing of resource colonisation by *A. nidulans* Δ*laeA* had no effect on insect survival, which indicates strong species-specific consequences of deleting *laeA*.

Table 4. Factors affecting changes in fungal growth patterns in response to competition with *Drosophila* larvae. Fungal species indicates the *Aspergillus* species (*A. nidulans, A. fumigatus* or *A. flavus*). Fungal strain refers to the WT or the Δ*laeA* mutant of the corresponding species.

	F-value
Fungal strain (FS)	$F_{1,53} = 0.01$
Fungal species (FSP)	$F_{2,53} = 45.28$***
Time (T)	$F_{1,517} = 600.89$***
FS × FSP	$F_{2,53} = 14.23$***
FS × T	$F_{1,517} = 9.35$**
FSP × T	$F_{2,517} = 17.89$***
FSP × FS × T	$F_{2,582} = 17.60$***

**p < 0.01, *** p< 0.001

In contrast to our expectation that beneficial effects of high larval density may only occur in the presence of WT but not transgenic strains, larval density hardly affected insect survival in the presence of WT *Aspergillus*; *A. fumigatus* even mediated an intensification of insect competition. Surprisingly, larvae benefited from feeding in larger groups when confronted with the Δ*laeA* strains of all *Aspergillus* species. Allee effects for larval development might only be achievable in the presence of less noxious fungi as they were used in other studies (Rohlfs et al. 2005), but not when fungi with the potential to produce highly toxic compounds (e.g. sterigmatocystin and aflatoxin B1 in *A. nidulans* and *A. flavus*, respectively) co-occur with *Drosophila* larvae. Yet, we cannot exclude the possibility of a beneficial effect of still higher larval densities than those we used in this study.

The graduation in the effect of Δ*laeA* on *Drosophila* correlated with the impact of the WT strains, with *A. flavus* having the most detrimental consequences, followed by *A. fumigatus* and *A. nidulans*. Possibly, since the diversity of known mycotoxins increases from *A. nidulans* to *A. fumigatus* and *A. flavus* (source: <www.aspergillus.org.uk>), the fraction of LaeA regulated metabolites may decrease with increasing diversity of secondary metabolite pathways, which might be due to the limited capacity of LaeA to transcriptionally

Table 5. Post hoc comparison of the effect of insect competition on strain specific changes in growth patterns of three *Aspergillus* species. Fungal strain refers to the WT or the Δ*laeA* mutant of the corresponding species.

	A. nidulans F–value	*A. fumigatus* F–value	*A. flavus* F–value
Fungal strain (FS)	$F_{1,18} = 7.96$*	$F_{1,17} = 1.36$	$F_{1,18} = 20.05$***
Time (T)	$F_{1,177} = 332.32$***	$F_{1,162} = 121.31$***	$F_{1,178} = 176.50$***
FS × T	$F_{1,177} = 32.72$***	$F_{1,162} = 1.74$	$F_{1,178} = 5.54$*

*p < 0.05, *** p< 0.001

control all genomic regions of fungal chromosomes containing clusters encoding secondary metabolite pathways (Perrin et al. 2007, Kosalková et al. 2008). Alternatively, *A. fumigatus* and *A. flavus* Δ*laeA* mutants, when confronted with competing insects, might be able to activate substitute pathways leading to the production of insecticidal mycotoxins. It is also possible that the remaining chemicals produced by the latter two Δ*laeA* species are more toxic to *Drosophila*.

The general question that arises in this context is whether fungi constitutively produce insecticidal mycotoxins or whether they are capable of regulating toxin synthesis according to ecological circumstances, i.e. presence/absence of antagonistic insects. Such an induced response has largely been approved to exist in plants challenged by herbivorous insects (Howe and Jander 2008). The evolutionary logic behind the expectation of induced chemical defences is that secondary metabolite production is energetically costly to its producer (Strauss et al. 2002). Whether this concept, mainly derived from plant–herbivore interactions, can be analogously applied to animal–fungus antagonisms remains to be seen; nonetheless, this eco-evolutionary framework may provide the conceptual basis for finding genetic evidence for adaptive changes in secondary metabolite biosynthesis, accompanied by alterations in signal transduction and identification of the means fungi use to sense changes in their environment (Georgianna and Payne 2009, Kempken and Rohlfs 2010). Distinguishing between these two modes of fungal defences will be of fundamental importance to our understanding of the ecological and evolutionary dynamics of animal-microbe competition and may ultimately explain how selfish gene clusters encoding specific secondary metabolite biosynthetic pathways (Walton 2000, Fischbach et al. 2008), such as the laeA-regulated Sterigmatocystin/Aflatoxin cluster in *Aspergillus* sp., provide a selective advantage to their fungal host.

Mould cover of decaying fruits has been suggested to predict the chance of *Drosophila* larval survival. In an earlier study, we could, however, show that mould growth patterns are only poor predictors of insect development (Rohlfs et al. 2005). Comparing the effect of the three *Aspergillus* species in this study verified this result. For instance, although both *A. flavus* and *A. fumigatus* caused a similarly high insect mortality, inhibition of *A. fumigatus* growth was severe in the beginning (probably until all larvae were dead), followed by a comparatively late recovery; contrarily, *A. flavus* rapidly recovered from initially detrimental effects of larval activity. *Drosophila* larvae suffered least from *A. nidulans*, but this fungus recovered quite rapidly even in the presence of larvae of which some reached the pupal stage. Thus, fungus species rather than fungal colony size explains insect survival.

Growth patterns of Δ*laeA* mutants principally followed that of the corresponding WT. In accordance with the chemical shield hypothesis, chemical deficient *A. nidulans* and *A. flavus* suffered more strongly from insect competition than the WT strains, capable of expressing their entire chemical arsenal. *A. fumigatus* Δ*laeA*, however, was not more strongly affected than the WT. Although a reduction of chemical diversity is the most striking effect of deleting *laeA*, we can currently not exclude a potential role of morphological changes in determining both insect survival and fungal growth response, which may accompany loss of *laeA* expression in, e.g. *A. flavus* (Kale et al. 2008) (see Methods for more details). Whether variation in secondary metabolite expression as affected by the global regulator LaeA is naturally linked via pleiotropic effects to morphological changes remains to be seen.

Conclusion

Our experimental analysis of *Drosophila–Aspergillus* interactions provides three main results that may contribute to a better understanding of animal–microbe competition: (1) we found no evidence of pathogenic effects causing *Drosophila* larval mortality in the presence of *A. fumigatus* or *A. flavus*, which hence supports the 'interkingdom competition' hypothesis. Nevertheless, when we aim at detecting animal–microbe competition, we should not dismiss the role of potential pathogenic phenomena which may also induce avoidance behavior of saprophagous animals (Burkepile et al. 2006) or antimicrobial defence (Rozen et al. 2008). (2) Insect developmental success in the presence of mould cannot be predicted via fungal growth patterns, but is driven by strong fungal species-specific effects. (3) By using chemical deficient Δ*laeA* mutants we provide genetic support for a critical role of fungal secondary metabolites in insect–fungus competition, suggesting that saprophagous insects may provide a selective force that favours the synthesis of secondary metabolites in fungi. Based on the striking species-specific effects of Δ*laeA* mutants on insect developmental success, it is yet questionable, whether LaeA is the prime target on which natural selection through insect competition may operate. Our findings nonetheless suggest that variation in fungal traits, driven by global secondary metabolism regulators, may have the potential to critically determine the flow of nutrients and energy within saprobiotic communities. Moreover, we should conceptually integrate animal–microbe competition in the analysis of ecosystem processes (Burkepile et al. 2006), since the species-specific repercussions of 'interkingdom competition' may produce yet unforeseeable dynamics.

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References

Begon, M. 1982. Yeast and *Drosophila*. – In: Ashburner, M. et al. (eds), The genetics and biology of *Drosophila*. Academic Press, pp. 345–384.

Begon, M. et al. 2006. Ecology – from individuals to ecosystems. – Blackwell.

Bok, J. W. and Keller, N. P. 2004. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. – Eukary. Cell 3: 527–535.

Bok, J. W. et al. 2005. LaeA, a regulator of morphogenetic fungal virulence factors. – Eukary. Cell 4: 1574–1582.

Brakhage, A. A. and Langfelder, K. 2002. Menacing mold: the molecular biology of *Aspergillus fumigatus*. – Annu. Rev. Microbiol. 56: 433–455.

Burkepile, D. E. et al. 2006. Chemically mediated competition between microbes and animals: microbes as consumers in food webs. – Ecology 87: 2821–2831.
Chinnici, J. P. and Bettinger, D. A. 1984. Effects of aflatoxin B1 and caffeine on viability in natural strains of *Drosophila melanogaster*. – J. Invert. Pathol. 44: 263–266.
Clarkson, J. M. and Charnley, A. K. 1996. New insights into the mechanisms of fungal pathogenesis in insects. – Trends Microbiol. 4: 197–203.
Courtney, S. P. et al. 1990. Ecology of mushroom-feeding drosophilidae. – Adv. Ecol. Res. 20: 225–274.
Dagenais, T. R. T. et al. 2010. *Aspergillus fumigatus* LaeA-mediated phagocytosis is associated with a decreased hydrophobin layer. – Infect. Immun. 78: 823–829.
Diamond, J. M. 1987. Competition among different taxa. – Nature 326: 241–241.
Fischbach, M. A. et al. 2008. The evolution of gene collectives: how natural selection drives chemical innovation. – Proc. Natl Acad. Sci. USA 105: 4601–4608.
Foerster, R. E. and Würgler, F. E. 1984. In vitro studies on the metabolism of aflatoxin B1 and aldrin in testes of different strains of *Drosophila melanogaster*. – Arch. Toxicol. 56: 12–17.
Fox, E. M. and Howlett, B. J. 2008. Secondary metabolism: regulation and role in fungal biology. – Curr. Opin. Microbiol. 11: 481–487.
Georgianna, D. R. and Payne, G. A. 2009. Genetic regulation of aflatoxin biosynthesis: from gene to genome. – Fung. Gen. Biol. 46: 113–125.
Hochberg, M. E. and Lawton, J. H. 1990. Competition between kingdoms. – Trends Ecol. Evol. 5: 367–371.
Hodge, S. 1996. The relationship between *Drosophila* occurrence and mould abundance on rotting fruit. – Brit. J. Entomol. Nat. Hist. 9: 87–91.
Hodge, S. and Mitchell, P. 1997. Inhibition of *Drosophila melanogaster* and *D. hydei* by *Aspergillus niger*. – Dros. Info. Serv. 80: 6–7.
Hodge, S. et al. 1999. Factors affecting the occurrence of facilitative effects in interspecific interactions: an experiment using two species of *Drosophila* and *Aspergillus niger*. – Oikos 87: 166–174.
Howe, G. A. and Jander, G. 2008. Plant immunity to insect herbivores. – Annu. Rev. Plant Biol. 59: 41–66.
Janzen, D. H. 1977. Why fruits rot, seeds mold and meat spoils. – Am. Nat. 111: 691–713.
Kale, S. P. et al. 2008. Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. – Fung. Gen. Biol. 45: 1422–1429.
Kaspari, M. and Stevenson, B. 2008. Evolutionary ecology, antibiosis, and all that rot. – Proc. Natl Acad. Sci. USA 105: 19027–19028.
Keller, N. P. et al. 2005. Fungal secondary metabolism – from biochemistry to genomics. – Nat. Rev. Microbiol. 3: 937–947.
Kempken, F. and Rohlfs, M. 2010. Fungal secondary metabolite biosynthesis – a chemical defence strategy against antagonistic animal? – Fungal Ecol. doi:10.1016/j.funeco.2009.08.001
Kosalková, K. et al. 2008. The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. – Biochimie 91: 214–225.
Kraaijeveld, A. R. et al. 2008. Stage-specific sex differences in *Drosophila* immunity to parasites and pathogens. – Evol. Ecol. 22: 217–228.
Lee, S.-E. and Campbell, B. C. 2000. In vitro metabolism of aflatoxin B1 by larvae of navel orangeworm, *Amyelois transitella* (Walker) (Insecta, Lepidoptera, Pyralidae) and codling moth, *Cydia pomonella* (L.) (Insecta, Lepidoptera, Tortricidae). – Arch. Insect Biochem. Physiol. 45: 166–174.
Lof, M. E. et al. 2008. The effect of chemical information on the spatial distribution of fruits flies: I model results. – Bull. Math. Biol. 70: 1827–1849.
Lof, M. E. et al. 2009. Odor-mediated aggregation enhances the colonization ability of *Drosophila melanogaster*. – J. Theor. Biol. 258: 363–370.
Lukin, M. and de los Santos, C. 2006. NMR structures of damaged DNA. – Chem. Rev. 106: 607–686.
Lussenhop, J. and Wicklow, D. T. 1985. Interaction of competing fungi with fly larvae. – Microb. Ecol. 11: 175–182.
Lussenhop, J. et al. 1980. Insect effects on bacteria and fungi in cattle dung. – Oikos 34: 54–58.
Melone, P. D. and Chinnici, J. P. 1986. Selection for increased resistance to aflatoxin B1 toxicity in *Drosophila melanogaster*. – J. Invert. Pathol. 48: 60–65.
Nierman, W. C. et al. 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. – Nature 438: 1151–1156.
Niu, G. et al. 2008. Aflatoxin B1 detoxification by CYP321A1 in *Helicoverpa zea*. – Arch. Insect Biochem. Physiol. 69: 32–45.
Obana, H. et al. 1994. Direct detection using the *Drosophila* DNA-repair test and isolation of a DNA-damaging mycotoxins, 5,6-dihydropenicillic acid, in fungal culture. – Food Chem. Toxicol. 32: 37–43.
Perrin, R. M. et al. 2007. Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. – PLoS Pathogens 3: e50.
Reeves, E. P. et al. 2004. Correlation between gliotoxin production and virulence of *Aspergillus fumigatus* in *Galleria mellonella*. – Mycopathology 158: 73–79.
Reiss, J. 1975. Insecticidal and larvicidal activities of the mycotoxins aflatoxin B1, rubratoxin B, patulin and diacetoxyscirpenol towards *Drosophila melanogaster*. – Chem.-Biol. Interactions 10: 339–342.
Rohlfs, M. 2005. Clash of kingdoms or why *Drosophila* larvae positively respond to fungal competitors. – Front. Zool. 2: 2.
Rohlfs, M. 2006. Genetic variation and the role of insect life history traits in the ability of *Drosophila* larvae to develop in the presence of a filamentous fungus. – Evol. Ecol. 20: 271–289.
Rohlfs, M. 2008. Host–parasitoid interaction as affected by interkingdom competition. – Oecologia 155: 161–168.
Rohlfs, M. and Hoffmeister, T. S. 2003. An evolutionary explanation of the aggregation model of species coexistence. – Proc. R. Soc. Lond. B (Suppl.) 270: S33–S35.
Rohlfs, M. and Hoffmeister, T. S. 2005. Maternal effects increase survival probability in *Drosophila subobscura* larvae. – Entomol. Exp. Appl. 117: 51–58.
Rohlfs, M. and Obmann, B. 2009. Species-specific responses of dew fly larvae to mycotoxins. – Mycotox. Res. 25: 103–112.
Rohlfs, M. and Kürschner, L. 2010. Saprophagous insect larvae, *Drosophila melanogaster*, profit from increased species richness in beneficial microbes. – J. Appl. Entomol. in press.
Rohlfs, M. et al. 2005. Competition with filamentous fungi and its implications for a gregarious life-style in insects living on ephemeral resources. – Ecol. Entomol. 30: 556–563.
Rohlfs, M. et al. 2007. Secondary chemicals protect mould from fungivory. – Biol. Lett. 3: 523–525.
Rozen, D. E. et al. 2008. Antimicrobial strategies in burying beetles breeding on carrion. – Proc. Natl Acad. Sci. USA 105: 17890–17895.
Ruess, L. and Lussenhop, J. 2005. Trophic interactions of fungi and animals. – In: Dighton, J. et al. (eds), The fungal community – its organization and role in the ecosystem. CRC Press, pp. 581–598.
Schatzmann, E. 1977. Früchte als natürliche Entwicklungssubstrate von Drosophiliden. – Mitt. Schweiz. Entomol. Ges. 50: 135–148.

Sherratt, T. N. et al. 2006. Why fruit rot, seeds mold and meat spoils: a reappraisal. – Ecol. Modell. 192: 618–626.
Shivik, J. A. 2006. Are vultures birds, and do snakes have venom, because of macro- and microscavenger conflict? – Bioscience 56: 819–823.
Shorrocks, B. 1982. The breeding sites of temperate woodland *Drosophila*. – In: Ashburner, M. et al. (eds), The genetics and biology of *Drosophila* 3b. Academic Press, pp. 385–428.
Shwab, E. K. et al. 2007. Histone deacetylase activity regulates chemical diversity in *Aspergillus*. – Eukary. Cell 6: 1656–1664.
Stadler, M. and Keller, N. P. 2008. Paradigm shifts in fungal secondary metabolite research. – Mycol. Res. 112: 127–130.
Stephens, P. A. et al. 1999. What is the Allee effect? – Oikos 87: 185–190.
Strauss, S. Y. et al. 2002. Direct and ecological costs of resistance to herbivory. – Trends Ecol. Evol. 17: 278–285.
Strohm, E. and Linsenmair, K. E. 2001. Females of the European beewolf preserve their honeybee prey against competing fungi. – Ecol. Entomol. 26: 198–203.
Sullivan, R. L. and Sokal, R. R. 1963. The effects of larval density on several strains of the house fly. – Ecology 44: 120–130.
Suzuki, S. 2001. Suppression of fungal development on carcasses by the burying beetle *Nicrophorus quadripunctatus* (Coleoptera: Silphidae). – Entomol. Sci. 4: 403–405.
Walton, J. D. 2000. Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. – Fungal Gen. Biol. 30: 167–171.
Wertheim, B. et al. 2002. Allee effect in larval resource exploitation in *Drosophila*: an interaction between density of adults, larvae and micro-organisms. – Ecol. Entomol. 27: 608–617.
Wölfle, S. et al. 2009. Experimental evolution of resistance against a competing fungus in *Drosophila melanogaster*. – Oecologia 161: 781–790.