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SHORT COMMUNICATION

Saprophagous insect larvae, *Drosophila melanogaster*, profit from increased species richness in beneficial microbes

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Abstract

Female fruit flies, *Drosophila melanogaster*, lay their eggs on decaying plant material. Foraging fly larvae strongly depend on the availability of dietary microbes, such as yeasts, to reach the adult stage. In contrast, strong interference competition with filamentous fungi can cause high mortality among *Drosophila* larvae. Given that many insects are known for employing beneficial microbes to combat antagonistic ones, we hypothesized that fly larvae engaged in competition with the noxious mould *Aspergillus nidulans* benefit from the presence of dietary yeast species, especially when they are associated with increasingly species rich yeast communities (ranging from one to six yeast species per community). On a nutrient-limited fruit substrate infested with *A. nidulans*, both larval survival and development time were positively affected by more diverse yeast communities. On a mould-free fruit substrate, merely larval development but not survival was found to be affected by increasing species richness of dietary yeasts. Not only yeast diversity had an effect on *D. melanogaster* life-history traits, but also the identity of the yeast combinations. These findings demonstrate the importance of the structure and diversity of microbial communities in mutualistic animal–microbe interactions.

Introduction

To make food resources accessible, provide supplemental nutrition and fend off parasites, animals, including humans, often rely on symbiotic microorganisms (Dethlefsen et al. 2007; Rosenberg et al. 2007; Klepzig et al. 2009). In contrast, many microbes, such as filamentous fungi or bacteria, render food sources unpalatable to animals (Janzen 1977; Burkepile et al. 2006; Rozen et al. 2008). Insects often face diverse antagonistic interactions with filamentous fungi, this could be one reason why they may have been selected for establishing associations with specific bacteria or yeasts that impair fungal growth, possibly on grounds of their capacity to synthesize anti-fungal metabolites (Kaltenpoth et al. 2005; Cardoza et al. 2006; Little et al. 2006; Scott et al. 2008; Haeder et al. 2009; Lam et al. 2009; Rodrigues et al. 2009).

Drosophilid flies are a prime example of the large group of saprophagous insects whose larvae develop on dead organic material, e.g. rotting plant tissue (Shorrocks 1982), that is concomitantly inhabited by various microorganisms. For a successful development the fly larvae depend on the availability of dietary yeasts (Begon 1982; Anagnostou et al. 2009) that may be transferred to the breeding site by adult flies (Wertheim et al. 2002; Rohlfs and Hoffmeister 2005), suggesting a mutualistic *Drosophila*–yeast relationship (Vega and Dowd 2005). Nevertheless, the immature insects may also encounter noxious microbes, e.g. mould fungi that can cause high mortality among the animals (Rohlfs et al. 2005). Recent pharmacological tests indicate that the interference

competition between insects and fungi may be mediated by the secretion of mycotoxins (Rohlfs and Obmann 2009).

Here, we tested the hypothesis that, in addition to their role in *Drosophila melanogaster* nutrition, dietary yeasts enhance insect survival in the presence of a competing fungus, *Aspergillus nidulans*. Insect-associated yeasts have been shown to curtail growth of antagonistic fungi in other systems (Adams et al. 2008; Rodrigues et al. 2009), and might be capable of detoxifying mycotoxins (Dowd 1992). We predicted that different yeast species and communities may have different effects on insect development, either by inhibiting mould growth or by changing the quality of the diet. Especially, we hypothesized that increased yeast species richness may positively affect the larval development of *D. melanogaster*.

Methods

Organisms

The *D. melanogaster* population originated from isofemale lines caught in Kiel, Northern Germany, in 2003 and were cultured under standard condition (see Rohlfs et al. 2005). Larvae used in the experiment originated from eggs sterilized with 50% sodium hypochlorite. The freshly hatched 1st instar larvae could then be transferred with a fine brush under sterile conditions.

The yeasts chosen were *Kluyveromyces lactis* (DSM4909), *Metschnikowia pulcherrima* (DSM70321), *Pichia toletana* (DSM70390), *Saccharomyces cerevisiae* (DSM70449), *Cryptococcus albidus* (DSM70215) and *Rhodotorula mucilaginosa* (DSM70404), which were cultured on malt extract agar at 28°C. After 4 days of cultivation, the yeast colonies were washed off with sterile Ringer solution. For the experiment, yeast suspensions comprising different combinations of yeast species were prepared. There were 63 possible yeast combinations. Independently of the yeast species richness and combination in each experimental treatment, the initial cell number was kept constant at 1 000 000 cells per μl, i.e. with increasing species richness the number of cells per yeast species decreased accordingly. A mycotoxin producing wild type strain of *Aspergillus nidulans* (RDIT2.3) was cultured under the same conditions as the above mentioned yeast species. This strain has been shown to seriously increased *Drosophila* larval mortality due to competitive but non-pathogenic interactions (Rohlfs et al. 2009). After 4 days of cultivation, the mature conidiospores were washed off with sterile Ringer solution. The desired number of 1000 conidia per μl was obtained by using a haemocytometer.

Experimental setup

We used a fruit medium (banana) to simulate natural conditions at which the insect larvae depend on the availability of yeast to reach the adult stage. Finely crushed banana mixed with the same volume of tap water and 15 g agar per litre banana-water mixture. 1 ml medium was pipetted into 2 ml micro tubes and autoclaved. Without the addition of dietary microbes, this sterile fruit substrate does not support the development of *Drosophila* larvae whatsoever (Dorsch 2007). *A. nidulans* was given a developmental head-start of 60 h by transferring 1 μl conidia suspension prior to the inoculation with the yeast cells. Ten larvae each were transferred into the tubes, the tubes were covered with sterile cotton plugs and incubated in a climate chamber at 25°C and a 16 : 8 h L : D cycle. For a randomly chosen subset (eight combinations) of the 3-spp. treatment, the full 1-spp. and the 6-spp. treatment we ran the same experiment at mould-free conditions. The development of larvae was followed until adult emergence. Since adult body weight in *D. melanogaster* has repeatedly been shown to decrease with increasing development time (Blanckenhorn 1999; Wertheim et al. 2002), we here report only on larval survival to the adult stage and development time (days from larval transfer to adult emergence). There were n = 20 replicates per treatment.

Statistical analysis

We used ANOVA models to test for the effects of yeast species richness and community composition on the survival of *Drosophila* larvae. Proportional insect survival data were square root-arcsine-transformed. Species combination was considered as a factor nested under species richness. The analyses of the nested ANOVA model were performed with SPSS 17.0.

Results and Discussion

Our experiment revealed positive effects of increasingly species-rich yeast communities on the development of *D. melanogaster* larvae that were forced to feed in the presence of the toxic mould *A. nidulans*: survival was significantly higher (ca. 20%) (species number: $F_{5,1197} = 5.013$, $P < 0.001$; species combination: $F_{57,1197} = 2.999$, $P < 0.001$) and development time significantly shorter (ca. 0.75 days) (species

number: $F_{5,887} = 2.438$, $P = 0.033$; species combination: $F_{57,887} = 2.410$, $P < 0.001$) when microbial communities were richer in species (Fig. 1a, c). Subsequent regression analyses indicated that development time had a linear relationship with yeast species richness (Fig. 1c); however, insect survival was non-linearly related to yeast species richness and appeared not to reach saturation at very high species richness (Fig. 1a). Thus, *D. melanogaster* larvae may achieve an even higher developmental success at still more diverse yeast communities.

Various, mutually non-exclusive mechanisms might explain the observed positive influence of biodiversity on insect survival. (1) Yeasts may secrete anti-fungal compounds that curtail mould growth (e.g. Liu et al. 2007). Species-rich communities may produce various, functionally different defence metabolites that, when acting in combination, may be more effective in suppressing mould development. (2) Species-rich yeast communities may provide a functionally diverse means of detoxification in the fruit substrate and/or the insect gut (Dowd 1992). Yeast-borne degradation of mycotoxins might prevent the insect detoxification system (Li et al. 2007) from being overstrained and hence allow allocation of resources and energy to larval growth. (3) A diverse microbial diet might supply the insect larvae with essential and complementary nutrients (dietary mixing, Bernays et al. 1994) that enhance specific physiological processes (e.g. mycotoxin detoxification) supporting the insects in combating the fungal competitor. In absence of the fungal competitor, however, we found no beneficial effect of yeast species enrichment on insect survival (species number: $F_{2,213} = 0.048$, $P = 0.953$; species combination: $F_{14,213} = 1.915$, $P < 0.001$) (Fig. 1b). Yet, development time was slightly accelerated (ca. half a day) (species number: $F_{2,213} = 10.07$, $P < 0.001$; species combination: $F_{14,213} = 9.512$, $P < 0.001$) (Fig. 1d), indicating subtle influences on insect performance, which might be of relevance when *Drosophila* develops on qualitatively different substrates (Starmer and Aberdeen 1990) or, as indicated by the present study, on those infested with noxious microbes.

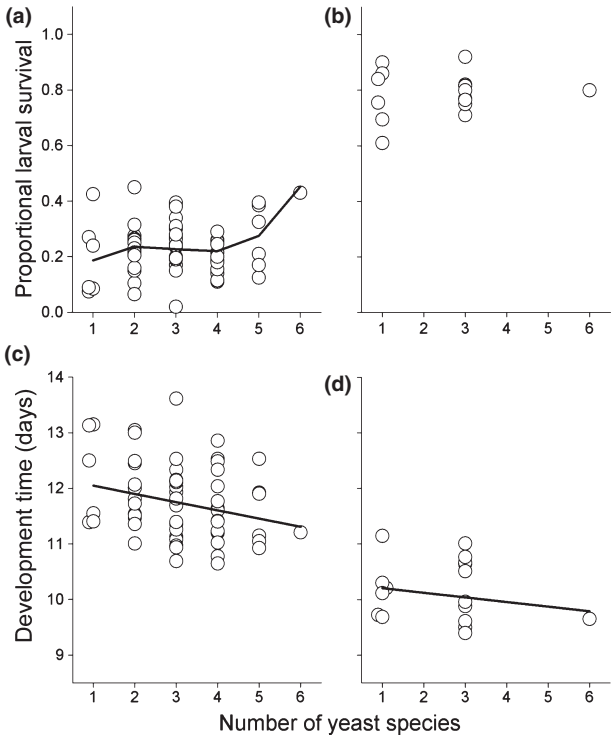

Fig. 1 Mean *Drosophila melanogaster* larval survival and development time in each yeast treatment as a function of yeast species richness under mouldy conditions (a and c) and under mould-free conditions (b and d). Regression equations: (a) $y = 0.246x - 0.089x^2 + 0.010x^3 + 0.020$; $R^2 = 0.013$, $P = 0.001$, this is the most parsimonious regression model; (b) no effect of yeast species richness; (c) $y = -0.148x + 12.195$; $R^2 = 0.012$, $P = 0.002$; (d) $y = -0.083x + 10.288$; $R^2 = 0.029$, $P = 0.010$. For clarity, the data of the one species treatment are staggered around the values on the *x*-axis. (see text for statistical details).

In addition to the positive influence of yeast species richness on *Drosophila* development in the presence of the fungal competitor, there was a strong effect of yeast community composition (see statistical results above). Thus, the impact of single yeast species or specific yeast combinations mediates significant changes in insect developmental success (see low explanatory power of regression models in Fig. 1). Without detailed analyses of changes in the yeast communities, we cannot exclude that those yeast species providing optimal conditions already in the one-spp. treatment (see Fig. 1) outcompete all other yeasts, so that species combination is in fact more important than species richness for *Drosophila* development. On the other hand, association with more diverse microbial communities may increase the probability that these beneficial species are present. Independently of the mechanism underlying the observed phenomenon, yeasts may critically mitigate the selection pressure imposed on *Drosophila* populations by competing fungi and thereby reduce the evolutionary costs that accompany evolution of protection against the fungal competitor (Wölfle et al. 2009). Moreover, being associated with species-rich microbial communities may buffer against evolutionary changes in the antagonistic ones (Rosenberg et al. 2007). How drosophilids achieve association

with beneficial microbial communities of varying diversity (Vega and Dowd 2005), however, is still an underexplored aspect of the natural ecology of this model insect.

Our analysis provides first evidence for a positive effect of dietary microbial biodiversity on saprophagous insects forced to develop in the presence of a competing toxic mould fungus. Though the underlying mechanisms of this effect remain to be investigated, our study highlights the importance of the structure and diversity of (possibly defensive) microbial communities in mutualistic animal–microbe interactions.

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