Fungal plant endosymbionts alter life history and reproductive success of aphid predators

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Endosymbionts occur in most plant species and may affect interactions among herbivores and their predators through the production of toxic alkaloids. Here, we ask whether effects of mycotoxins produced by the symbiosis of the fungal endophyte *Neotyphodium lolii* and the grass *Lolium perenne* are transmitted to the aphidophagous ladybird *Coccinella septempunctata* when feeding on cereal aphids *Rhopalosiphum padi* on infected plants. The larval development of coccinellids was extended, while their survival was reduced when feeding exclusively on aphids from infected plants. Ladybirds developing on aphids from infected plants showed reduced fecundity and impaired reproductive performance. Body size and symmetries of ladybird adults were not affected by the endophytes. Consistently strong, negative effects of endophytes on the lifetime performance of ladybirds indicates that mycotoxins are transmitted along food chains, causing significant damage for top predators. Such cascading effects will influence the population dynamics of aphid predators in the long term and could feedback to the primary plant producers.

Keywords: endophytic fungi; fecundity; micro-organisms; multi-trophic interactions; predator–prey

1. INTRODUCTION

Micro-organisms that live in close association with plants can influence primary productivity by producing or capturing limiting resources (Fitter *et al.* 2005) or by the production of toxic substances that act as herbivore defence (Schardl *et al.* 2004). Nevertheless, the role of micro-organisms in community regulation has been neglected for a long time, presumably because of their small size and hidden lifestyle (Hunter & Price 1992). Recent research describes the omnipresence in all habitats and enormous species diversity of microbial organisms (Arnold *et al.* 2000; Vandenkoornhuyse *et al.* 2002). Endophytic fungi (Ascomycota; Clavicipitaceae) that live in association with temperate grasses are able to alter processes of plant succession (Clay & Holah 1999), general relationships between species diversity and productivity (Rudgers *et al.* 2004), and insect food web interactions (Omacini *et al.* 2001). Endophytes of the genus *Neotyphodium* occur in most species of cool season grasses, and propagate vertically through seeds (Schardl *et al.* 2004). Increased herbivore resistance results for the grass host from the symbiosis with *Neotyphodium*; this is particularly well studied for agronomic grasses such as tall fescue (*Lolium arundinacea*) and perennial ryegrass (*Lolium perenne*; Breen 1994; Clay *et al.* 2005; Hunt & Newman 2005). The herbivore resistance is caused by the production of several types of alkaloid. Peramine and lolines are toxic for invertebrates, while lolitrem B and ergot alkaloids are toxic for grazing mammals (Schardl *et al.* 2004). If mycotoxins are effective against plant consumers, it is conceivable that the natural enemies of these herbivores could also be negatively affected.

Except for a few studies on specialized parasitoids, effects of endophytic fungi on higher trophic levels remain largely unexplored (Faeth & Bultman 2002; Müller &

Krauss 2005). In these studies, parasitoids showed reduced parasitization rates when their prey fed on endophyte infected grasses (Barker & Addison 1996; Goldson *et al.* 2000; Omacini *et al.* 2001; but see Barker & Addison 1997). Reduced survival rates, longer or shorter developmental time and reduced pupal body mass were reported for parasitoids (Barker & Addison 1996; Bultman *et al.* 1997, 2003), but fecundity or reproductive success was not measured.

In this study, we tested for effects of a plant–endophyte symbiosis on a specialized predator of aphids. Carnivorous ladybirds are larval and adult predators of all species of aphid and can suppress aphid populations strongly (Müller & Godfray 1997; Obrycki & Kring 1998). Some ladybird species, such as the seven spotted ladybird *Coccinella septempunctata* (Coleoptera: Coccinellidae), are commonly used in aphid pest control programmes (Obrycki & Kring 1998). Here we asked whether ladybird predators are affected by the plant–endophyte symbiosis. We predict that the produced mycotoxins move up the food chain and ladybird consumers of aphids will have fitness disadvantages when their prey is reared on endophyte infected plants. We measured developmental time, survival rates, body sizes, fecundity and reproductive success for ladybirds reared on aphids from infected plants and compared these life-history traits with those of ladybirds reared on aphids from uninfected plants. We observed that ladybirds experienced disadvantages at several life stages when feeding on aphids reared on *Neotyphodium lolii* infected grasses.

2. MATERIAL AND METHODS

The study was based on a tri-trophic interaction involving grass, asexual endophytic fungi, grass aphids and a coccinellid predator. Seeds of the English ryegrass *L. perenne* L. were provided by Brian Tapper (AgResearch, NZ). All plants we used belonged to the same cultivar *L. perenne*: Grassland

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Samson, and were either uninfected (identity number A 11104) or infected (identity number A 12038) with the specialist endophyte *N. lolii* Glenn, Bacon and Hanlin. The infection in A 11104 was lost by selection against the endophyte (B. Tapper 2003, personal communication). The uninfected cultivar showed no infection after staining of 30 seeds and microscopic control, whereas the infected cultivar showed an infection rate of 93% by the same method (Saha *et al.* 1988). Chemical analyses of the infected cultivar showed the presence of peramine ($7.5 \pm 0.7 \mu\text{g g}^{-1}$) and lolitrem B ($0.4 \mu\text{g g}^{-1}$; J. Krauss 2005, unpublished data). Bird cherry-oat aphids *Rhopalosiphum padi* L. were bought from Katz Biotech AG, Germany. This original aphid culture was kept on commercially available, uninfected barley before insects were used in the experiment. The aphid predator, *C. septempunctata* L., which is abundant in a wide range of Eurasian, African and recently North American agroecosystems (Hodek & Honek 1996) was used in the experiments. Eggs of *C. septempunctata* were bought from Katz Biotech AG, Germany, where ladybirds were kept on *Acyrtosiphon pisum* (Harris), with no experience with *R. padi* or endophyte infected grasses (P. Katz 2005, personal communication). The ladybird eggs were kept in Petri dishes at room temperature until emergence.

In the following, we will refer to 'E+' as the complex of *R. padi* aphids on grass infected with endophytes, and 'E-' as aphids feeding on uninfected *L. perenne*.

(a) Experimental design

Infected and endophyte free *L. perenne* were grown on commercially available garden compost for 15 days before use for the aphid cultures. The plants were placed in acrylic cultivation cages (60×40×40 cm) and aphids were added and left to build up large colonies. The cages were kept in an environmentally controlled climate chamber for at least 10 days until aphid density was high.

The experiment started with coccinellid larvae hatching in November 2004 (within 5 h) from seven different clutches. Ten to twelve eggs of each clutch were assigned equally between the two treatments ($n=40$ for E- and $n=40$ for E+). Newly hatched larvae were weighed within 2 h of hatching and prior to any food supply. Subsequently, single larvae were transferred to plastic Petri dishes lined with filter paper and a rough 1 cm^3 moistened sponge. The initial larval weight was not biased between the two treatments, including clutch as a random factor ($n=80$, $F_{1,72}=1.60$, $p=0.210$) and showed no influence on the main results. Therefore, it was omitted from further analyses.

All experiments took place in climate chambers with L16 : D8 h, 20–24 °C and 65% RH, with daily changes of the individual position of the Petri dishes. Larvae were fed daily with aphids from either infected or uninfected *L. perenne*. The amount of food was based on previous observations, with up to 20 aphids d^{-1} for I and II instar larvae, and more than 50 aphids d^{-1} for III and IV instar larvae, which represent sufficient amounts for *ad libitum* feeding. Larvae and pupae were individually monitored daily to record survival, and the times to moult, pupation and emergence. Newly emerged adults were weighed within 24 h, before any food supply and were then transferred to plants covered with 2 l PET bottles with ventilation windows covered with fine nylon mesh. All adult ladybirds were sexed following Baungard (1980). Seven mating pairs on E- and six on E+ were isolated for 15 days with male turnover each 1–2 days, in order to allow sexual

maturation and repeated mating with different mating partners. After this period, males were separated from the females to avoid egg cannibalism, and both sexes were kept individually on living plants to record longevity. For individual females, egg clutches were collected daily, and separated in sealed Petri dishes after counting the number of eggs per clutch. Emerged larvae were counted within 24 h of hatching. All ladybirds were surveyed daily and fresh plants with aphids were provided regularly to guarantee constant and unlimited food availability.

We distinguished between larval performance and adult life history. For the former, we recorded the larval developmental time of the four larval instars and pupation period in days and the larval survival rate. The total period of larval development consisted of the time from egg hatching to adult emergence from the pupa. We further recorded the adult body weight after emergence. Body length on their ventral side and fluctuating asymmetry in the hind tibia length (HTL) as left–right difference was measured under a stereomicroscope (12.5×), when natural death occurred, following the protocol of Palmer (1994). To remove measuring errors, each leg was measured twice, and a statistical test showed no correlation between the error and asymmetry ($p>0.2$). Reproductive success was our main fitness estimate and we recorded the lifetime egg production per female as: (i) total number of eggs produced (fecundity) and (ii) overall number of clutches. Fertility was expressed as hatching rate (number of hatched larvae per number of eggs in a clutch; Awmack & Leather 2002). Further, we tested for variation in the length of oviposition period (days between the first and the last clutch laid), and for variation in time from emergence to the production of the first clutch (see table 1).

The decreasing sample size number is a consequence of natural death of larvae and/or adults, or because we were unable to measure a specific trait for all individuals.

(b) Statistical analyses

All statistical analyses were conducted using SPSS (v. 12.0.1) or R (v. 2.1.1) for mixed effects models and Akaike information criterion (AIC) comparisons. Differences of the response variables between the treatments E+ and E- were tested with linear mixed effects models with a maximum likelihood method with treatment as fixed effect and clutch number as random effect (Pinheiro & Bates 2000). The fixed factor sex was included in the model for the response variables: larval developmental time, adult lifespan, body length, body weight and asymmetry, using type I sums of squares with sex entering the model first. Models with the random effect clutch number were compared with models without clutch number using the AIC. This showed that clutch number had to be included in the models for the dependent variables oviposition period and fluctuating asymmetry, but could be omitted in all other cases (table 1). A logistic regression was used to analyse sex as a dependent variable.

Dependent variables were transformed when necessary to meet the assumptions of normality and homoscedasticity. For the period of instar III development, we could not find a satisfactory transformation and, therefore, used the Mann–Whitney *U*-test to compare the E+ and E- treatments. Total lifespan, adult weight at emergence and fluctuating asymmetry were square-root transformed, age at first oviposition was log-transformed and clutch size and adult body length were x^2 -transformed. Differences in survival were assessed with Kaplan–Meier's approach (Breslow test-statistic; Norusis 2004).

Table 1. Mean $\pm$ s.e., test-statistic, degrees of freedom and p -values are presented for all life-history traits of the ladybirds *Coccinella septempunctata* reared either on *Neotyphodium lolii* infected ($E+$) or uninfected ($E-$) *Lolium perenne* with feeding *Rhopalosiphum padi*. (Life-history traits were separated into developmental and survival traits, and reproductive success. Sex was used as an additional fixed factor for the adult body measurements, lifespan and total larval developmental time. Model statistics and transformations, see statistical analyses.)

	$E-$ (mean $\pm$ s.e.)	$E+$ (mean $\pm$ s.e.)	test-statistic	n	p
<i>development and survival</i>					
total larval developmental time (days)	21.87 $\pm$ 0.29	23.78 $\pm$ 0.33	$F_{1,49} = 23.76$	53	<0.001
sex			$F_{1,49} = 0.13$	53	0.721
interaction			$F_{1,49} = 0.13$	53	0.722
females	22.04 $\pm$ 0.30	24.40 $\pm$ 0.48			
males	21.29 $\pm$ 0.78	23.31 $\pm$ 0.41			
length of instar II (days)	1.87 $\pm$ 0.07	2.14 $\pm$ 0.10	$F_{1,73} = 5.22$	75	0.025
length of instar III (days)	3.18 $\pm$ 0.11	4.57 $\pm$ 0.19	$U_{1,71} = 185.50$	73	<0.001
larval survival (Breslow's F)	81%	62%	$F_{1,66} = 4.14$	68	0.042
total lifespan (days)	74.62 $\pm$ 7.15	40.65 $\pm$ 5.53	$F_{1,66} = 14.04$	68	<0.001
adult lifespan (days)	92.60 $\pm$ 7.25	60.70 $\pm$ 6.35	$F_{1,36} = 7.13$	40	0.011
sex			$F_{1,36} = 9.51$	40	0.004
interaction			$F_{1,36} = 1.28$	40	0.265
females	95.29 $\pm$ 7.66	79.25 $\pm$ 12.43			
males	86.33 $\pm$ 17.25	48.33 $\pm$ 3.96			
adult body length (mm)	5.56 $\pm$ 0.08	5.38 $\pm$ 0.11	$F_{1,31} = 0.33$	35	0.572
sex			$F_{1,31} = 21.60$	35	<0.001
interaction			$F_{1,31} = 0.03$	35	0.857
females	5.67 $\pm$ 0.07	5.60 $\pm$ 0.11			
males	5.16 $\pm$ 0.12	5.12 $\pm$ 0.15			
adult weight at emergence (mg)	14.06 $\pm$ 0.29	13.33 $\pm$ 0.52	$F_{1,49} = 0.23$	53	0.637
sex			$F_{1,49} = 9.70$	53	0.003
interaction			$F_{1,49} = 0.91$	53	0.345
females	14.30 $\pm$ 0.35	14.54 $\pm$ 0.85			
males	13.24 $\pm$ 0.41	12.39 $\pm$ 0.54			
fluctuating asymmetry in HTL (mm)	0.029 $\pm$ 0.004	0.054 $\pm$ 0.017	$F_{1,17} = 1.54$	27	0.231
sex			$F_{1,17} = 0.40$	27	0.535
interaction			$F_{1,17} = 0.08$	27	0.772
females	0.029 $\pm$ 0.005	0.077 $\pm$ 0.053			
males	0.028 $\pm$ 0.004	0.043 $\pm$ 0.009			
<i>reproductive success</i>					
oviposition period (days)	47.43 $\pm$ 6.48	24.00 $\pm$ 8.46	$F_{1,6} = 17.82$	13	0.006
age at first oviposition (days)	22.43 $\pm$ 1.91	30.83 $\pm$ 1.83	$F_{1,11} = 10.86$	13	0.007
number of clutches	24.86 $\pm$ 3.31	10.00 $\pm$ 3.05	$F_{1,11} = 10.62$	13	0.008
clutch size (eggs per clutch)	20.62 $\pm$ 1.13	14.06 $\pm$ 3.30	$F_{1,11} = 3.88$	13	0.074
number of eggs per day	11.28 $\pm$ 1.39	6.50 $\pm$ 1.72	$F_{1,11} = 4.77$	13	0.051
total number of eggs	501.40 $\pm$ 58.84	175.33 $\pm$ 70.09	$F_{1,11} = 12.91$	13	0.004
number of larvae	250.00 $\pm$ 35.60	99.90 $\pm$ 54.90	$F_{1,11} = 5.59$	13	0.038
larval hatching rate (%)	53.50 $\pm$ 8.00	35.00 $\pm$ 15.50	$F_{1,11} = 1.18$	13	0.300

3. RESULTS

All life-history traits of larval and adult ladybirds feeding on $E+$ and $E-$ are summarized in table 1.

Fitness of both larval and adult seven spotted ladybirds was significantly reduced by feeding on aphids that had fed on endophyte-infected plants. This reduction in overall fitness resulted from negative effects at several life-history stages. Larvae feeding on prey reared on endophyte infected grasses ($E+$) showed longer developmental time than those feeding on $E-$ (figure 1). This was caused by a sensitive window of the second and third instar development length (L2 and L3 stage), where ladybird larvae on $E+$ took significantly longer than those grown on $E-$, while for the other instars and pupation period no significant differences in developmental time were observed (all $p > 0.1$; table 1; figure 1). The ladybirds' total lifespan was almost twice as long on $E-$ ($\bar{O} = 74.6$ days) compared to the $E+$ treatment ($\bar{O} = 40.7$ days). This result is partially due to different

larval survival rates, where only 62% (23 out of 37) of the ladybirds reared on $E+$ survived to adults, whereas on $E-$ the larval survival was 81% (30 out of 37). However, when considering only ladybirds surviving the whole larval period, adult lifespan was still significantly reduced by one-third for ladybirds feeding on $E+$ (table 1). The fresh weight at adult emergence, body length and asymmetry were not statistically different between treatments. Body length and body weight were correlated positively ($n = 35$, $R = 0.582$, $p < 0.001$). Adult lifespan, body weight and body length, were influenced by sex, with females living longer than males, and being larger and heavier. However, none of the sex-treatment interactions were significant (table 1). The $E+$ treatment significantly shifted the sex ratio towards more males (ratio females/total: $E- = 0.77$; $E+ = 0.43$; Wald_{1,51} = 5.80; $p = 0.016$).

The fitness estimates, fecundity and fertility varied between the treatments. Reproducing females on $E+$ started egg laying later, had a significantly shorter

Figure 1. Mean larval development time in days ($\pm$ s.e.), divided into the four larval stages and pupation period (L1 to L4 and pupa) for *C. septempunctata* feeding on either E+ or E- treatment. The total larval developmental time ($F_{1,49}=23.76$, $p<0.001$), the length of II instar ($F_{1,73}=5.22$, $p=0.025$) and the length of III instar ($U_{1,71}=185.50$, $p<0.001$) are significantly enhanced when *C. septempunctata* larvae feed on prey aphids on endophyte infected plants (E+) compared to prey aphids on endophyte free plants (E-).

Figure 2. The mean ($\pm$ s.e.) of (a) oviposition period (in days) of mated *C. septempunctata* ($F_{1,6}=17.82$, $p=0.006$), (b) number of eggs produced per day during the oviposition period by mated *C. septempunctata* ($F_{1,11}=4.77$, $p=0.051$), (c) total eggs produced by *C. septempunctata* females ($F_{1,11}=12.91$, $p=0.004$) and (d) number of total hatched larvae ($F_{1,11}=5.59$, $p=0.038$) on E- (prey aphids fed on endophyte free plants; grey bars) and E+ (prey aphids fed on endophyte infected plants; empty bars).

oviposition period (figure 2a), laid fewer egg clutches, and produced fewer eggs per day over the oviposition period than females on E- (figure 2b). As a consequence, ladybirds on E+ showed lower lifetime egg production (figure 2c). Further, we observed a trend for smaller clutches by females on E+. The larval hatching rate did not differ significantly between treatments. However, because of the lower egg production by ladybirds on E+, the overall number of offspring was significantly reduced on E+ (figure 2d; table 1).

4. DISCUSSION

We found that the negative effects of host plant endophyte infection (E+) on aphids permeated up to the coccinellid predators of the aphid, in accordance with our predictions.

These negative effects manifested themselves throughout the life of the coccinellid, via longer developmental times, lower survival rates and lower lifespan, and an overall reduced reproductive success, caused by later and shorter reproduction, as well as lower egg production within the reproductive period, in E+ compared with E- treatments.

Here, we report for the first time the effects of an endophyte on an herbivore predator. *Coccinella septempunctata* suffered greatly from the endophyte infection in almost every life history parameter we measured. Our results are congruent with previous studies on parasitoids, where mainly negative effects were found as well (Barker & Addison 1996; Bultman *et al.* 1997, 2003). However, such negative effects on an important generalist aphid predator

may have greater impacts on the entire community. Coccinellids play an important role in regulating aphid populations (Müller & Godfray 1997; Obyrcki & Kring 1998), and reduced fitness of coccinellids could cause herbivore outbreaks. Furthermore, predators may be more sensitive to endophyte presence than parasitoids because predators feed on insect prey during their whole life cycle, while parasitoids spend the adult life on alternative food sources (Godfray 1994). Although the endophytic alkaloid uptake by aphids and the consequent catabolic pathway are unknown, it is conceivable that, as mycotoxins move up in the food chain, predators such as ladybirds receive cumulatively higher doses by continuously feeding on aphids than do parasitoids. This would explain the detrimental effects in both larval and adult life history, resulting in reduced lifespan. According to this hypothesis, reduced predator fitness may result from herbivore adaptation, with aphids turning the endophyte-mediated plant defence into predator defence (see Richmond *et al.* 2004). However, according to previous studies on above ground systems, which demonstrated negative effects on aphids (Breen 1994; Hunt & Newman 2005; Meister *et al.* in press), it is more likely that mycotoxins move up in the trophic chain and affect negatively both herbivores and predators. Our study organism, the aphid *R. padi* is especially sensitive to the presence of endophytic fungi, and shows reduction of overall population sizes through reduced daily and total fecundity, and a shorter overall lifespan (Meister *et al.* in press). The exact effect of the endophyte on our host plant *L. perenne* is more debatable, as different environmental conditions and genetic origin of plants and fungi alter the relationship between the symbiotic partners (Cheplick 2004; Müller & Krauss 2005).

As yet, nobody has measured the occurrence or accumulation of endophyte-derived toxic alkaloids in either herbivores or natural enemies. We can only speculate how the severe negative effects on predators in our study contribute to the open discussion on the mutualistic-parasitic nature of the endophyte-plant association (Saikkonen *et al.* 1998; Müller & Krauss 2005). If predators of herbivores are viewed as allies of the plant, and the endophytes are affecting predators negatively, the plant-endophyte symbiosis will lose part of its protective nature, at least when high predator densities collapse. Such a scenario could be tested in an experimental approach with manipulated endophyte infection and predator densities under field conditions and should be considered for future research. Indeed, under field conditions, predators may feed on herbivores on infected and uninfected plants, possibly at random, if ladybirds are unable to distinguish between the two. The effect of such a 'mixed diet' on predator performance is unknown, as well as any kind of preference for aphids from endophytic and non-endophytic plants. In the field, the net effect of infection on plant fitness may depend on the natural abundance of aphids and predators, which in turn will vary strongly across years (Roschewitz *et al.* 2005). Alternating net benefits and costs of endophyte infection across different years may help in maintaining the observed mixed infections in natural plant communities of endophyte infected and uninfected individuals (Faeth 2002; Clay *et al.* 2005).

In conclusion, we recorded experimental evidence that micro-organisms do play an important role in integrated

community regulation of pest insects. Therefore, micro-organisms must be integrated in the ongoing research in multi-trophic settings and natural communities, as a potential structuring force (Rudgers *et al.* 2004). In this study, we recorded negative effects of fungal endophytes on an arthropod insect predator. As a consequence of the indirect loss of plant defence against herbivory, the plant fungus association might shift towards parasitism, within the open discussion on the real nature of endophytic fungi and grasses symbiosis.

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