Endophytic fungi decrease available resources for the aphid Rhopalosiphum padi and impair their ability to induce defences against predators

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Abstract

1. The production of winged morphs is a well known mechanism of induced defence in aphids to escape from natural enemies, and is also a reaction to poor resource quality. 2. Host plants of aphids often associate with endophytic fungi that have been shown to reduce the fitness of some species of aphids. 3. It was hypothesised that endophyte infection of host plants that represent a low quality plant resource should increase the aphid's induced response to a predator because both low plant quality and predator presence represent a stronger cue for wing production than predator presence alone. 4. In a laboratory experiment, bird cherry-oat aphids Rhopalosiphum padi L. were exposed to the factors predator threat and endophyte infection and the effects of these factors on the proportion of winged morphs produced by the aphid colonies was analysed. 5. The presence of endophytic fungi strongly decreased aphid colony sizes. When a predator threat was present, all colonies on endophyte-free grasses produced winged morphs whereas only a few colonies were able to produce winged morphs on endophyte-infected grasses. However, these few colonies produced larger proportions of winged morphs than colonies on endophyte-free grasses. Without a predator threat, no colonies on endophyte-infected grasses produced any winged morphs. 6. These results show that aphids in stressed conditions and with reduced fitness will only invest in inducible defences when predators are present but are unable to produce winged morphs in response to endophyte presence.
Endophytic fungi decrease available resources for the aphid
*Rhopalosiphum padi* and impair their ability to induce defences
against predators

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4. In a laboratory experiment bird cherry-oat aphids *Rhopalosiphum padi* L. were exposed to the factors ‘predator threat’ and ‘endophyte infection’ and the effects of these factors on the proportion of winged morphs produced by the aphid colonies was analysed.
5. The presence of endophytic fungi strongly decreased aphid colony sizes. When a predator threat was present all colonies on endophyte-free grasses produced winged morphs whereas only a few colonies were able to produce winged morphs on endophyte-infected grasses. However, these few colonies produced larger proportions of winged morphs than colonies on endophyte-free grasses. Without a predator threat, no colonies on endophyte-infected grasses produced any winged morphs.
6. These results show that aphids in stressed conditions and with reduced fitness will only invest in inducible defences when predators are present but are unable to produce winged morphs in response to endophyte presence.

**Key words.** Wing induction, pheromone, ladybird larvae, non-lethal predator, *Neotyphodium coenophialum*, poor-quality host, reproductive compensation.

Introduction
Animals and plants have evolved various traits and mechanisms to reduce risks of predation and herbivory. Inducible defences are a class of defence mechanisms that are only expressed when a threat by a predator or a herbivore is eminent. This contrasts to constitutive defences that are expressed permanently. Therefore, inducible defences have the benefit of maximising fitness because the investment in defence traits only occurs when they are needed. Inducible defence responses include several behavioural and morphological traits that increase the victim’s resistance against predator attack, reduce predator encounter probabilities and increase the escape probability after predator attack (Tollrian & Harvell, 1999). For example,
various zooplankton species such as water fleas of the genus *Daphnia* produce defensive structures, such as spines and helmets, to increase their resistance against predator attack. These defensive structures are only expressed when *Daphnia* are exposed to predators, because building the structures reduces their longevity (Harvell, 1992; Tollrian, 1995). In tadpoles of various amphibian species, inducible defences are present as behavioural or morphological changes. Tadpoles will reduce their foraging time in the presence of a predator to decrease predator encounter probability. As a second response, the tadpoles will grow a larger tail fin, which will mislead predators to attack the tail rather than the vital forepart of the tadpole, thus increasing the tadpole’s escape probability. Both induced defence mechanisms reduce resources available for tadpole growth and will delay metamorphosis (Van Buskirk & McCollum, 2000).

Life-history theory predicts a trade-off between optimal predator defence and the victim’s fitness (Stearns, 1999; Steiner & Pféiffer, 2007). If the defence would not have a cost it would be permanently expressed, thus being constitutive (Tollrian & Harvell, 1999). The intensity of the trade-off between inducible defence and fitness depends on the probability of predator encounters in a given environment and the actual costs of building the defence mechanism. If initiated quickly, inducible defences are superior to constitutive defences in environments with unpredictable predator attacks that, once initiated, are sustained long enough for the defence to become effective (Clark & Harvell, 1992; Riessen, 1992; Adler & Karban, 1994). Inducible defences are often triggered by substances secreted by predators called kairomones (Tollrian & Harvell, 1999). Alternatively, the triggering factors of inducible defences can be pheromones that are secreted as alarm signals by prey individuals sensing an imminent threat or by victims of a predator or parasite attack (Nault et al., 1973; Kunert et al. 2005).

Aphids (Homoptera: Aphididae) are cyclical parthenogenetic with asexual reproduction during most of the year and sexual reproduction in autumn, thus a colony of aphids consists of mostly clonal individuals with identical genomes (Lushai et al., 1997). Deteriorating nutritional conditions, crowding and changes in photoperiod and temperature all result in a higher proportion of winged morphs within an aphid colony (Sutherland, 1967; Dixon & Wratten, 1971; De Barro, 1992; Müller et al., 2001). This is possible because aphids can produce individuals with different morphologies asexually (Dixon, 1998). The production of winged morphs is important for aphid colonies because it enables a clone to disperse and find new resource plants when, for example, food resources deteriorate. The production of winged
morphs is also a reaction to the presence of natural enemies and thus a form of induced
defence (Dixon & Agarwala, 1999; Weisser et al., 1999).

Wing production as an inducible defence is triggered by alarm pheromones that most
aphids secrete from their siphunculi when attacked by enemies (Mondor & Roitberg, 2004;
Kunert et al., 2005). These pheromones can be perceived by other aphids as far as three
centimetres away (Nault et al., 1973). Releasing alarm pheromones within a clonal colony of
aphids is likely to increase the inclusive fitness of the signaler as the cue will reach closely
related individuals. It has been demonstrated that aphids preferably emit alarm pheromones
when surrounded by aphids of the same clone as opposed to aphids of other species
(Robertson et al., 1995). There is a cost for growing wings, because although winged morphs
have a higher chance of escaping bad conditions or a high predator risk environment, they are
less fecund (Dixon & Wratten, 1971) and develop slower than wingless morphs (Dixon,
1998). These trade-offs explain why most aphid species do not express the winged morph
type constantly.

Most plant species that accommodate aphids have evolved alliances with
microorganisms that can alter the plant’s quality (Arnold et al., 2000; Clay, 2004). In particular,
the association with endophytic fungi of the genus Neotyphodium can lead to the production of
alkaloids by the fungus which renders the grass toxic to herbivores (Clay, 1988; White et al.,
1993; Breen, 1994; Müller & Krauss, 2005). The effects of such mycotoxins also move up the
food chain and reduce the fecundity of predators and parasitoids (de Sassi et al., 2006; Härri,
S.A., unpublished data). We are not aware of any studies that investigated the effects of
endophyte presence and occurrence of mycotoxins in the plants on the induction of winged
aphid morphs although such a response to the low quality of infected plants is conceivable if
aphids perform worse on infected than on uninfected plants.

In our experiment we addressed whether wing induction as an inducible defence in
aphids against predators is altered by the presence of endophytes in the plant. We studied the
bird cherry-oat aphid Rhopalosiphum padi L., for which endophyte presence reduces lifespan
and fecundity, and thus fitness (Meister et al., 2005). We hypothesised that wing induction is
(1) generally increased on endophyte-infected plants because such plants are of lower
nutritional quality than uninfected plants and (2) that the inducible defence expressed as
increased wing production under high predation risk is also increased for aphids on infected
plants because they experience both, toxic food and predator presence. We used a crossed
factorial design with endophytes and predators either present or absent to test for possible
interactions. We predicted highest proportions of winged morphs when both a predator and the endophyte are present.

Materials and Methods

(a) Plants, aphids and ladybirds
The experiment was carried out on tall fescue (*Lolium arundinacea* Schreb.; cultivar Kentucky 31), kindly provided by Prof. Keith Clay (Indiana University, Bloomington, USA). Half of the seeds were uninfected (E-) and half were infected (E+) with the endophytic fungi *Neotyphodium coenophialum*. The infection status was confirmed after termination of the experiment as explained below.

The seeds were planted in plastic seed trays three months before the start of the experiment. At the start of the experiment the plants were cut to a length of 15 cm and 120 (60 E+ and 60 E-) randomly chosen single plants were replanted in plastic pots (Ø10 cm) filled with commercially available gardening compost. Each pot contained three single plants, resulting in 20 pots with infected and 20 pots with uninfected *L. arundinacea* Kentucky 31. *Rhopalosiphum padi* individuals were taken from a base culture that had been initiated from three clones. The base culture had been maintained in a climatic chamber for over five months on endophyte-free perennial ryegrass *Lolium perenne* L. (commercially available cultivar ARION), thus the culture was most likely dominated by one clone only.

Seven days after replanting, 15 adult *R. padi* from the laboratory culture were transferred onto each of the 40 pots. The pots were then covered with air-permeable cellophane bags (16 cm x 30 cm) that were attached to the rim of the pots with adhesive tape. The larvae of the two-spot ladybird *Adalia bipunctata* L. that represented the predation threat were bought from a commercial supplier (Biocontrol Andermatt AG, Grossdietwil, Switzerland).

(b) Experiment
For the experiment, the ladybird larvae were kept in small gauze bags (5 cm x 10 cm) together with aphids serving as food. Thus the experimental aphid colonies received all cues of a foraging predator nearby without decreasing their numbers by direct predation. Twenty bags were prepared, each containing one larvae of *A. bipunctata* and approximately 50 individuals of *R. padi* on cut grass blades (ARION). The gauze bags were sealed with pieces of thin wire and placed within half of the E- pots and half the E+ pots (P+). The other half of the pots (10 E+ and 10 E-) served as controls with empty gauze bags inside the cellophane bags (P-).
Every second day, the ladybird larvae were provided with 50 new prey aphids by opening the cellophane bags and removing the gauze bags. The gauze bags were opened, the dry grass and aphid carcasses removed and bags were restocked with new aphids on fresh grass before placing them back on the pots. As this procedure may have caused some disturbance to the experimental aphids, the same procedure was done to the control groups (P-).

The experiment was carried out in a controlled environment chamber (22°C and 16:8 light:dark cycle) with pots randomly arranged. The set of pots with predators (P+) were placed approximately one metre away from the control plots (P-). Pheromones of aphids are only transmitted over short distances (Nault et al., 1973), so the control plots (P-) could not have been affected.

After aphids in the experiment were exposed to the predators for 10 days, the first larvae of A. bipunctata reached their pupal stage and all the gauze bags were removed. After another day the grass was cut just above soil level and put in the cellophane bag that covered the pot previously to ensure minimal losses of aphids. The cellophane bags were sealed and frozen for later counting of the aphids. The number of R. padi individuals was recorded for all replicates. The developmental stage of the aphids was determined under a binocular microscope. The first to third instars were grouped as nymphs, because winged morphs cannot be determined until the aphids reach the fourth instar. Winged fourth instar R. padi can be differentiated easily from wingless fourth instars by the presence of wing buds. All fourth instars with wing buds and adults with wings were grouped as winged and all remaining fourth instars and wingless adults were grouped as wingless morphs.

After the experiment, all 120 grass plants of the 40 pots were analysed with Phytoscreen Neotyphodium Immunoblot Assays (Agrinostics Ltd., Watkinsville, USA) to confirm endophyte infection. From the parts that had been left when the grasses were cut to remove the aphids, a cross section of the base tiller was extracted with a razor blade. After carrying out the assay, the immunoblot card was photographed with a Canon EOS 350D digital camera and the tiller tissue imprints were analysed for colour intensity as described by Koh et al. (2006). The measured intensity was compared with the reference sample of Neotyphodium provided on the immunoblot card. Assuming for the reference sample an infection of 100 %, this procedure allowed us to exclude all replicates with one or more grass tillers of an infection above 20 % for E- and all replicates with one or more grass tillers with an infection of less than 20 % for E+. On E-, six pots (three on P- and three on P+) and on E+, three pots (two on P- and one on P+) had to be omitted from the analysis because their infection status was inadequate.
(c) Analysis

Statistical analyses were performed in R (version 2.3.1 for Windows XP). Data of absolute number were tested for normality of the residuals and equality of variances and had to be ln[x+1]-transformed. The number of aphids per replicate was tested by a two-way ANOVA with 'endophyte infection' and 'predator threat' as fixed effects.

For the winged morphs we tested first the influence of the explanatory variables on the occurrence (presence or absence) of winged morphs and secondly the influence on the proportion of winged morphs for the replicates with at least one winged morph present. This separation was necessary because including all replicates into the analysis of proportion of winged morphs violated the model assumption of variance homogeneity caused by the result that none of the replicates in the E+P- treatment produced any winged morphs. The proportion of winged morphs was analysed instead of absolute numbers to correct for aphid population size. Proportion of winged morphs were calculated by dividing the number of winged morphs (forth instar and adult stage) by the sum of all forth instar and adult stages. Nymphs were not included in this calculation as they may turn into either of the two morphs. In our analyses we included colony size as a co-variable to distinguish between the strong reduction in colony size caused by the endophyte and the independent, direct effect of endophytes on the occurrence and proportion of winged morphs. The interactions with colony size were not included, as model comparison tests showed no improvement of the model fit. The occurrence of winged morphs was analysed using a generalized linear model (GLM) with ‘colony size’, 'endophyte infection', 'predator threat' and the interaction between ‘endophyte infection’ and ‘predator threat’ as factors using a quasibinomial error structure to account for overdispersion (Crawley, 2002). The proportion of winged morphs for the replicates producing at least one winged morph was analysed by the same generalized model as described above. For the non-significant interaction term in the model of winged morph occurrence, we performed a Fisher’s exact test.

Results

The final size of the aphid colonies was affected by both, endophytes and predators. Overall, aphid colonies performed poorly on infected grasses. Within endophyte infection groups, aphid colonies exposed to predators reached larger colony size (Figure 1) and produced higher proportions of winged morphs (Figure 2) than those without predator threat. Both
endophyte infection and predator threat had strong significant effects on aphid colony size and there was a significant interaction between the two factors (Table 1).

*Rhopalosiphum padi* produced winged morphs in most replicates on E- (P-: 6 out of 7; P+: 7 out of 7), but on E+ winged morphs were observed in a few replicates only (P-: 0 of 8; P+: 3 of 9). This decreased probability of occurrence of winged morphs on E+ was partly caused by the smaller colony sizes but also by endophyte infection independent of the colony size (Table 1). The effect of the endophyte presence on the reduced probability of the production of winged morph was independent of the presence of a predator threat (Table 1). The non-significant interaction term was confirmed by the Fisher’s exact test on the independence of number of replicates with winged morphs present between endophyte infection and presence of a predator threat ($p = 0.25$). Also, the presence of a predator threat did not increase the probability that a colony produced winged morphs (Table 1).

Considering only colonies that produced at least one winged morph, the proportion of winged morphs per total number of adult and fourth instar aphids were significantly different in the four treatments with much higher proportions of winged morphs in the P+ treatments (Figure 2; Table 1). Larger colonies contained disproportionately higher proportions of winged morphs, but endophyte infection also led to a slight increase in the proportion of winged morphs if a predator threat was present (Table 1). The interaction between ‘endophyte infection’ and ‘predator threat’ could not be calculated because none of the replicates on E+ without predator threat (P-) did produce winged morphs.

**Discussion**

Both, the presence of endophytes and that of a predator threat influenced the production of winged morphs. Predator threat mainly increased the proportion of winged morphs within colonies that were able to produce winged morphs whereas endophyte infection reduced a colony’s ability to produce any winged morphs. This reduction in the colony’s ability to produce winged morphs was mainly but not only caused by the fact that the colonies on infected plants stayed relatively small and performed poorly independently of the presence of a predator threat. Endophyte infection also had direct effects on wing induction independent of the reduced colony size, proving that the observed pattern was not just caused by different degrees of crowding. These results contradict our initial hypothesis that cues for wing induction may be increased on infected plants with an additional predator threat, because although the proportion of winged morphs on E+P+ was slightly higher than on E-P+, only few colonies on E+P+ did produce winged morphs at all.
On endophyte-free grass, *R. padi* produced a low proportion of winged morphs of about five percent when no predator was present in six of seven colonies. This proportion might be a response to crowding but it is also possible that *R. padi* always produces small proportions of winged morphs as a form of prudent behaviour. Such low levels of winged morph production may prevent that a predator destroys a colony completely as there are always winged dispersers that can quickly initiate a colony elsewhere when a predator attack is imminent.

When *R. padi* fed on infected grasses without a predator threat, none of the colonies produced winged morphs. However, with a predator present one third of all colonies produced winged morphs with proportions slightly higher than those of the colonies on uninfected grasses. A possible explanation for this may be that most of the aphids on infected grass chose not to reproduce. Meister *et al.* (2005) showed that feeding on endophyte-infected grasses reduces lifespan and fecundity of *R. padi*, thus endophyte-infected grasses represent very poor-quality hosts and inferior resource quality for this species of aphid. In the field, these aphids may walk away from infected plants as they are able to walk as far as 180 cm to colonize new plants (Alyokhin & Sewell, 2003). Nevertheless, on-soil dispersal is risky as the aphids are exposed to a wide range of epigeic predators (Griffiths *et al.*, 1985; Sunderland *et al.*, 1986) and even one winged disperser may increase their chances of colonising new resources considerably. This might explain why the proportion of winged morphs was highest on E+P+. Aphids that feed on endophyte-infected grass and are threatened by a predator should leave their host plant immediately. In contrast, aphids on endophyte-free grass of adequate resource quality will also invest part of their resources in wingless morphs to ensure better survival of the local clone.

Our results suggest that the strong negative effects on *R. padi* colony size by endophytes in another field study (Omacini *et al.*, 2001) were unlikely to be caused by increased production of winged dispersers, as the increase in proportion of winged morphs on E+P+ was very small in absolute numbers (Figure 1). On the contrary, the low aphid densities on infected plants in the field are most likely caused by reduced survival on such plants or by emigration of wingless morphs.

A possible caveat of our experiment was that we did not control for aphid presence in bags in the control treatment (P-) but placed empty bags only. It is conceivable that aphids in bags might have produced some signals when dying that could have affected the wing induction of our target colonies. Furthermore, we did not control for clonal identity of the experimental aphids although there could be clonal variation in wing morph production.
However, as the aphids were randomly distributed over the treatments, possible differences in clonal variation would increase the overall variance and thus not distort the observed pattern.

We showed that *R. padi* can increase winged morph production in the presence of predators as has been demonstrated for the pea aphid, *Acyrthosiphum pisum* (Dixon & Agarwala, 1999; Weisser *et al*., 1999; Sloggett & Weisser, 2002; Kunert & Weisser, 2003). Little is known about the underlying molecular mechanisms that lead to wing induction in aphids but it is likely that growing wings or producing winged offspring after reception of alarm pheromones is under neural control and represents a ‘decision’ of individual aphids (Dixon, 1998). If the low proportion of winged morphs on E-P- in our experiment is indeed a result of prudent behaviour, then the lack of any winged morphs on E+P- and their presence on E+P+ would show this decision-making ability of *R. padi* as they would have to be able to assess threat level and nutritious condition.

Predator threat in our experimental colonies not only increased the proportion of winged morphs but also the total number of aphids compared to colonies without predators. This increase in colony size could be a result of increased reproduction as a response to the predator threat, a mechanism of reproductive compensation that has been demonstrated for snails exposed to trematod parasites (Minchella, 1984). Parasitized snails increase their reproduction immediately following parasite exposure. It is possible that reproductive compensation exists in aphids as well as increasing reproduction might be a good strategy to compensate for predator attacks if predators do not kill all aphids on a plant. Adult ladybirds generally leave a plant before all aphids are eaten (Minoretti & Weisser, 2000) and ladybird larvae reach their pupal stage after some time during which they do not consume any more aphids. Depending on the magnitude of the response, fecundity compensation might countervail the losses caused by a foraging predator. However, reproductive compensation must have a cost as otherwise all aphids should reproduce at a higher rate, even those that are not exposed to a predator threat. The cost may be smaller birth weight/size of the nymphs, but unfortunately our experimental design did not allow us to measure birth weight of nymphs. Reproductive compensation when exposed to a predator could explain our results of larger colony size in P+ treatments. We could detect this effect only because we used non-lethal predators, i.e. predators that did not feed on target colonies. Experiments that calculated aphids eaten by predators during the experiment might have underestimated these numbers when neglecting fecundity compensation (Weisser *et al*., 1999; Kunert & Weisser, 2003).

We suggest that besides winged morph production as a response to predator threat there may be reproductive compensation by aphids in response to predator presence. The
expression of all inducible defences depends on fitness costs and available resources. We showed that on low quality resources an inducible defence is not expressed unless a predator threat is present, and even then individuals of the same species might not all invest the scarce resources in the defence mechanism. Depending on availability of alternative strategies, a species can be very polymorphic in response to predator threat. Inducible defences may be superior to constitutive defences as they represent a way for an individual to invest in different defence strategies as they are required. They thus increase survival in harsh environmental conditions and may be a reason for their evolutionary success in many different organisms.

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References


Table 1. Results of the generalized linear models showing the effects of colony size, endophyte infection ("Infection") and predator presence ("Threat") on the occurrence of winged morphs (colonies producing winged morphs yes/no) and on the proportion of winged morphs within all colonies that produced winged morphs. From this proportion, the interaction could not be calculated, because on endophyte-infected plants without predators no winged morphs were produced at all. The total number of aphids (total colony size; \[ \ln[x+1] \] - transformed) was analysed with a two-way ANOVA with "Infection" and "Threat" as explanatory variables.

<table>
<thead>
<tr>
<th></th>
<th>Colonies producing winged morphs yes/no</th>
<th>Proportion of winged morphs</th>
<th>Total colony size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony size</td>
<td>( F_{1,26} = 123.32, p &lt; 0.0001 )</td>
<td>( F_{1,12} = 7.23, p = 0.020 )</td>
<td>( - )</td>
</tr>
<tr>
<td>Infection</td>
<td>( F_{1,26} = 8.95, p = 0.006 )</td>
<td>( F_{1,12} = 6.22, p = 0.028 )</td>
<td>( F_{1,27} = 81.08, p &lt; 0.0001 )</td>
</tr>
<tr>
<td>Threat</td>
<td>( F_{1,26} = 0.45, p = 0.507 )</td>
<td>( F_{1,12} = 15.58, p = 0.002 )</td>
<td>( F_{1,27} = 23.92, p &lt; 0.0001 )</td>
</tr>
<tr>
<td>Infection x Threat</td>
<td>( F_{1,26} = 0.00, p = 1.00 )</td>
<td>NA</td>
<td>( F_{1,27} = 4.92, p = 0.035 )</td>
</tr>
</tbody>
</table>

Figure 1. Mean (± SEM) number of aphids on endophyte-free (E-) and endophyte-infected (E+) \( L. \) arundinacea with either a predator present (P+) or absent (P-). Note the logarithmic scaled y-axis. The numbers of aphids are categorized into number of nymphs (white bars),
number of wingless (grey bars) and winged (dark grey bars) morphs ("n" indicates of the number of replicates after omitting pots with the wrong infection status).

Figure 2. Mean (± SEM) proportion of winged aphids on endophyte-free (E-) and endophyte-infected (E+) L. arundinacea, either in the presence of a predator (P+) or without a predator (P-). The proportions were only calculated for colonies that produced at least one winged individual ("n" indicates the number of replicates with winged morphs present).