DEFOILIATING INSECT IMMUNE DEFENSE INTERACTS WITH INDUCED PLANT DEFENSE DURING A POPULATION OUTBREAK

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Abstract. During population outbreaks, top-down and bottom-up factors are unable to control defoliator numbers. To our knowledge, details of biotic interactions leading to increased population density have not been studied during real population outbreaks. We experimentally assessed the strength of plant defenses and of insect immunocompetence, assumed to contribute to active insect resistance against parasitoids and pathogens, in the geometrid Epirrita autumnata during a steep increase in population density. We demonstrated rapid (same-season) induced resistance in the foliage of its host, mountain birch. The response was systemic, spreading throughout the tree, and retarded larval growth rate by ~10%. On the other hand, no direct delayed carry-over effects were found in the next season in larval growth rate, mortality, or pupal mass. Larval damage to a tree during the previous year, however, significantly (by ~13%) accelerated the advance of the immune response (measured as melanization of an implant inserted into the pupal hemocoel). The encapsulation rate correlated positively with larval mortality in trees in which larvae had been introduced the previous year, but not in control trees. Both of these observations suggest that induced plant defense was associated with an increased insect immunocompetence during the population increase.

Key words: Betula pubescens ssp. czerepanovii; direct and indirect induced plant defense; Epirrita autumnata; immune defense; insect herbivores; parasitism; population cycles.

INTRODUCTION

During the increase phase of defoliator population cycles, neither top-down factors (parasitoids or diseases) nor bottom-up ones (plant defenses) are able to control defoliator numbers. Field data indicate that a low incidence of parasitism is characteristic of growing defoliator populations, suggesting the crucial role of parasitism behind multiannual population cycles (e.g., Berryman 2002). Indirectly, the rapid increase in herbivore numbers suggests that plant defenses are relatively inefficient. At least, delayed induced defenses (the most likely plant factors leading to delayed negative feedbacks) cannot be strong during the increase phase.

The reasons why defoliators enjoy very low parasitism for several successive years at the beginning of the increase phase (e.g., Tanhuanpää et al. 2002: Fig. 2, Turchin et al. 2003: Fig. 3) have not attracted much attention. The general conclusion seems to be that parasitoids simply cannot catch up with the population growth of the defoliator. But the incidence of realized parasitism also depends, in addition to the number of parasitoids, on the ability of insects to actively resist parasitoids. Typically, insects encapsulate different types of invaders, such as viruses, bacteria, fungi, nematodes, and parasitoids, entering their hemocoel (Washburn et al. 1996, Gillespie et al. 1997, Gupta 2001). A shortage of the amino acid tyrosine and a low phenoloxdase activity are regarded as limiting factors in capsule melanization, which is necessary in successful encapsulation (Renault et al. 2002). Because defoliators obtain both amino acids and precursors of oxidases from their plant diet, it is possible that the plant’s defensive response, and the resistance of herbivores feeding on the plant to their parasitoids, interact.

Although it has been known for a long time that prevalence of parasitism may depend on host plants (e.g., Price et al. 1980), to the best of our knowledge there exist no studies assessing the efficiency of plant defenses and defoliator immune defense during the increase phase of a natural defoliator outbreak. We therefore conducted field experiments to study these factors during the increase phase of a population cycle in the geometrid Epirrita autumnata. We addressed two specific questions. First, does feeding damage from E. autumnata on the foliage of mountain birch (Betula pubescens ssp. czerepanovii) trees lead to direct rapid (current year, i.e., with no time lag) or delayed (next season, i.e., with a time lag) induced defense? Second, is the immune defense of E. autumnata modified by the host plant individual, or by the foliage damage to it?

MATERIALS AND METHODS

The experiments were conducted at the Kevo Subarctic Research Station of Turku University, in northernmost Finland (69°45’ N, 27°00’ E), in 2002–2003. E. autumnata reaches population peaks at roughly 10-year intervals in the mountain birch forests of northwest Europe, and the larvae defoliate vast forest areas (Haukioja et al. 1988, Ruohomäki et al. 2000). Para-
sitism is high in southern populations of *E. autumnata*, which do not show pronounced cyclic fluctuations in population density, and in the northern outbreak areas the collapse of a cycle peak is associated with high pupal or larval parasitism (Ruohomäki 1994, Tanhuapanä et al. 1999, 2001, Teder et al. 2000). During the early phases of an *E. autumnata* cycle the incidence of parasitism is very low, clearly <5% (Tanhuapanä et al. 2002, Klemola et al. 2004).

A rapid buildup of *E. autumnata* populations has been in progress in northern Scandinavia and Finland during the early 2000s: density indices (average numbers of larvae found per 10-minute search periods) at the Kevo station have been 0.0, 0.0, 2.0, and 4.1 larvae per period for the years 2000, 2001, 2002 and 2003, respectively (Klemola et al. 2004), and 28.3 larvae per period for 2004 (K. Ruohomäki, personal communication).

**Field experiments**

In spring 2002, we haphazardly chose 100 mountain birch trees with at least three ramets (stems) in a natural stand of mature mountain birches. One mesh bag was attached over each of two randomly chosen branches in a randomly chosen ramet in each tree. Each bag enclosed at least 50 short shoots (~150 leaves). The mesh size of the bags was 0.3 mm, small enough to prevent access of parasitoids and predators. Fifty trees were randomly assigned to the herbivory treatment, with the remaining trees serving as no-herbivore controls. At the time of leaf flush (26–27 May) we released 10 newly eclosed *E. autumnata* larvae into each bag on the herbivory trees, i.e., 20 larvae per tree. Each bag on each herbivory tree contained a similar mixture of *E. autumnata* broods (one larva from the same 10 broods in each bag). The bags on the control trees contained no larvae, and the natural density of larvae was less than half of the mean value in southern Finland (Teder et al. 2000), outside the potential outbreak area. This suggests that most of the trees must have been free of larvae. When larvae in the field were approaching pupation, the mesh bags were checked daily for larvae about to complete their last instar. These were removed from the bags and allowed to pupate in plastic vials with a lump of moss. After one week the pupae were weighed and sexed.

For the experiments in 2003, we randomly allocated the same 100 trees as in 2002 to two separate experiments. Bagged larvae were added to 60 trees that had been used in 2002, 30 each from the herbivory and control treatments. The procedure was similar to that for the herbivory trees in 2002, except that each tree had one bag, into which 15 neonate larvae were released at the time of leaf flush (22 May). The bags were on the same ramets as in 2002, but on different branches. The remaining 40 trees were left free of introduced larvae in 2003, and these trees were used to provide foliage for laboratory bioassays.

The growth of *E. autumnata* declines sharply with leaf age (e.g., Haukioja et al. 2002). An index of tree phenology, describing the progress of bud burst, was therefore recorded when the larvae were released into the bags.

**Bioassays in the laboratory**

Since bagged larvae continuously damage the leaves on their tree, they have to cope with both constitutive and possibly rapid induced plant defenses. In 2002, we therefore studied the existence of rapid induced defenses, using a bioassay in which we fed larvae in the laboratory on detached birch leaves from an intact ramet of trees with bagged *E. autumnata* (herbivory trees) or with empty bags (control trees) on another ramet. In 2003, trees in the above-mentioned group of 40 trees were similarly bioassayed, using leaves from ramets bagged in 2002. Since these trees did not harbor bagged larvae in 2003, the experiment now tested larval growth relative to possible delayed induced defense, triggered by leaf damage in the previous year. In both years the larvae were reared in 48-mL plastic vials with randomized positions in trays, one larva per vial. Before the bioassays, the larvae were reared in the laboratory on leaves from haphazardly selected trees, different from the experimental ones. Larval development was synchronized with that of larvae in the field by lowering or raising the rearing temperature as needed. When the bioassay larvae had molted to the fifth instar, they were offered one leaf from their particular experimental tree, and were allowed to feed to fill their gut (and produce the first frass droplets). Then the larvae were weighed and allowed to feed on a fresh leaf from their particular experimental tree for 24 hours at 14°C (the average summer temperature at Kevo), after which the final mass was recorded. In 2002 the rearing tubes contained a moist piece of paper to maintain leaf turgor. In 2003 leaf petioles were inserted into a 1-mL vial filled with water and sealed with parafilm.

**Immune defense**

Encapsulation is an active immune response in which insect hemocytes recognize an object as foreign and aggregate around it to form a capsule. Successful encapsulation results from a cascade of biochemical reactions, employing prophenyloxidases, and leads to the deposition of melanin and a hardening of the capsule (Gillespie et al. 1997, Renault et al. 2002). In insects, a widely used way to assay immune defense is to measure the magnitude of the encapsulation response against an artificial parasitoid “egg,” a piece of nylon monofilament, representing a novel, “passive” and standardized “antigen” (e.g., Schmid-Hempel and Schmid-Hempel 1998, Rantala et al. 2000, Cotter et al. 2004). In 2003, the branch-enclosed *E. autumnata* were studied for their immune defense at the time when they were weighed. We inserted an implant (a 2 mm long, 0.20 mm diameter piece of nylon monofilament rubbed
TABLE 1. Larval parameters in the two study years with and without herbivory in 2002.

<table>
<thead>
<tr>
<th>Year and treatment</th>
<th>Growth rate (mg·mg⁻¹·d⁻¹)</th>
<th>Pupal mass (mg)</th>
<th>Survivorship (%)</th>
<th>Sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>2.65 ± 0.039</td>
<td>63.53 ± 0.63</td>
<td>56 ± 3.46</td>
<td>0.98</td>
</tr>
<tr>
<td>Herbivory</td>
<td>2.43 ± 0.040</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.32 ± 0.052</td>
<td>58.02 ± 0.85</td>
<td>40 ± 3.87</td>
<td>0.81</td>
</tr>
<tr>
<td>Herbivory</td>
<td>3.27 ± 0.063</td>
<td>57.69 ± 0.71</td>
<td>44 ± 3.65</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Notes: Means and standard errors for dry mass growth rate in the final instar, pupal mass of surviving bagged larvae, percentage of larvae surviving per tree, and sex ratios of pupae in the two study years in trees with (herbivory) and without (control) bagged larvae in 2002. Values with different superscript letters indicate significant differences (P < 0.05) between treatments and years.

Larval fresh mass in fifth-instar growth trials were transformed into dry mass by applying the formula in Haukioja and Neuvonen (1985). In 2002 the effect of the treatment on larval growth in all 100 trees was tested with a mixed ANOVA, with dry mass growth as a dependent variable and initial larval mass as a covariate. In addition to the fixed treatment effect, tree was included in the model as a random effect. For the 40 trees tested in both experimental years, year was included in the model as a fixed effect. The effects of the 2002 treatments on pupal mass and encapsulation rate in 2003 were tested with mixed ANOVAs, with the sex of the pupae included as a fixed effect and tree as a random effect. In the analysis with encapsulation as a dependent variable, pupal mass was used as a covariate in order to ensure that the variance in encapsulation response was not just a byproduct of size. For the 30 trees with bagged larvae, and hence with data on pupal mass from both years, an additional model was applied with year as a fixed factor. All mixed models were analyzed with Proc MIXED in SAS 8.2 (SAS Institute 1999–2001). The Kenward-Roger method was used for degrees of freedom and variance components for the type of variance–covariance matrix. For differences between treatments in survival of the larvae to pupae in 2003, and for the difference in survival between years, we used a general linear mixed logistic regression model (the macro GLIMMIX iterating procedure MIXED), using binomial distribution with logit link function. In all analyses of variance, the normal distribution of model residuals was checked visually and using the Kolmogorov-Smirnov test for normal distributions. Levene’s test was used to determine the equality of variances in treatment groups. Encapsulation response was square-root transformed, as was the number of surviving larvae. In 2003 we also tested the sex ratio of the surviving pupae with the chi-square test for differences between frequencies of the sexes in the treatments.

In both years, tree-specific values were calculated for each variable to be used in the correlative analysis. The effect of sex on pupal mass was removed by transforming male mass into female mass using a formula given in Haukioja and Neuvonen (1985). For encapsulation indices, tree-specific least-square means were computed from an ANOVA model, including sex as a random factor. Pearson product-moment correlations were calculated, except in correlations including tree phenology, which were conducted with the Spearman rank correlation test.

RESULTS

In 2002, the suitability of individual birch trees for branch-enclosed E. autumnata larvae showed large variation: tree-specific pupal masses ranged from 30 to 90 mg, and larval survivorship varied between 20 and 90% (Table 1). Pupal mass correlated positively with the number of larvae surviving to pupae (r = 0.485, N = 50, P = 0.0004). Accordingly, variation in estimated productivity (survivorship × pupal mass-dependent fecundity × proportion of females) of E. autumnata per tree was almost two orders of magnitude (from <1 to ~95 eggs). Since the mesh bags prevented the action of predators and parasitoids, these values also suggest that for E. autumnata the variation in the suitability of individual birch trees was very large. The host plant...
effects consist of both constitutive and possible rapid induced defense. The bioassay demonstrated that rapid induced defense was also involved: the growth of laboratory-reared *E. autumnata* larvae was ~10% less in the 24-hour bioassay in herbivory trees than in control tree leaves in the fifth instar (Table 1). Since the bioassay leaves were clipped from other ramets (stems) rather than from the one with bags, this demonstrates that the presence of larvae on one ramet of a tree led to the systemic spread of induced defense over the whole tree, to the foliage on other ramets. Tree-specific larval growth in the laboratory correlated positively with the pupal mass ($r = 0.525, N = 50, P < 0.0001$) of bagged larvae and with their survivorship to pupae ($r = 0.546, N = 50, P < 0.0001$). Tree phenology modified larval growth rates at the beginning of the fifth instar ($r = -0.236, N = 100, P = 0.0182$), with early-leafing trees (with more mature leaves at the time of the tests) being less suitable than late-leafing trees, but showed no significant association with parameters measured at the end of the larval stage: pupal mass ($r = -0.257, N = 50, P = 0.0720$) and survivorship ($r = 0.014, N = 50, P = 0.9214$).

In 2003, among-tree variation in the success of bagged *E. autumnata* larvae was again large, but the presence of larvae on a tree the previous summer affected neither pupal mass and larval survivorship nor sex ratios in the pupal stage (Table 1). This demonstrates the absence of a carry-over effect, i.e., of direct delayed induced resistance, between the two study years. Consistent with this finding, we did not find any difference in the growth of laboratory-reared larvae on leaves from trees with a defoliation history (herbivory trees of 2002) and on control trees (Table 1). That induced rapid defense operated in 2002 but did not lead to delayed defense in 2003 was further confirmed by the significant treatment × year interaction in the growth rates of laboratory-tested larvae ($F_{1,671} = 6.08, P = 0.0139$). Tree phenology correlated negatively with pupal mass ($r = -0.370, N = 59, P = 0.039$) and the numbers of larvae surviving to pupae ($r = -0.391, N = 59, P = 0.0022$). Tree-specific growth rates of fifth-instar larvae in the laboratory in 2003, as well as pupal mass and survivorship of bag-reared larvae, correlated with values in 2002 ($r = 0.649, N = 39, P < 0.0001$; $r = 0.596, N = 30, P < 0.0005$; $r = 0.503, N = 30, P = 0.0046$, respectively).

Pupal mass and survival of *E. autumnata* in 2003 were on average lower than in 2002 ($F_{1,884} = 6.59, P = 0.0119$; $F_{1,831} = 21.60, P < 0.0001$, respectively), whereas the larval growth rate in the laboratory in 2003 was higher than in the previous summer ($F_{1,671} = 180.86, P < 0.0001$). This seemingly contradictory result may be due to the methodological difference in the treatment of leaves in laboratory bioassays (leaf petioles in water in 2003, presumably retaining a higher turgor than in 2002).

The encapsulation rate of implants in *E. autumnata* pupae varied considerably among individual trees (Wald $Z$ test with pupal mass as a covariate: $Z = 1.98, P = 0.0238$), suggesting a tritrophic level component in the immune defense of the defoliator. Furthermore, encapsulation was higher in the herbivory trees, with bagged larvae the previous year, than in the control trees ($F_{1,515} = 5.74, P = 0.0202$) (Fig. 1). The association between plant defense and the encapsulation response of the defoliator was further supported by the observation that in the among-tree correlations in 2003 encapsulation efficiency correlated negatively with current-year larval survivorship within the herbivory trees (i.e., those with bagged larvae the previous year) ($r = -0.365, N = 30, P = 0.0471$), but not within the control trees ($r = -0.188, N = 28, P = 0.338$).

Unlike pupal mass, encapsulation rate did not depend on tree phenology ($r = 0.095, N = 59, P = 0.476$). There was likewise no correlation between encapsulation and pupal mass ($r = -0.003, N = 59, P = 0.979$).

**DISCUSSION**

Our results demonstrated that a rapid, systemic induced direct defense was employed by the host plant during the increase phase of an *E. autumnata* outbreak, but seemed to be unable to curb the increase. Foliar damage did not lead to a direct, delayed induced defense between the study years, a common result in previous studies conducted in other phases of population outbreaks (e.g., Ruohomäki et al. 2000). However, we did see a significantly increased encapsulation rate by the defoliator in trees with *E. autumnata* damage the previous year. Although complex and variable tritrophic interactions between plants, defoliators, and para-
sitoids are well known (e.g., Vet and Dicke 1992, Teder and Tammaru 2002, Holton et al. 2003, Parry et al. 2003, Cattell and Stiling 2004, Karimzadeh et al. 2004, Ode et al. 2004), to our knowledge, the dependence of the immune defense of the defoliator itself on host tree quality, as well as on the delayed induced defense of the host plant, are both novel findings. The higher immune defense after previous leaf damage is not likely to be merely a chance result, since Saila Sillanpää (unpublished data) also found that, along with larval crowding, foliage from trees with bagged larvae the previous year significantly increased the phenoloxidase activity of E. autumnata. The set of trees used in that study was located ~10 km from our site; thus the experiment was completely independent from ours.

The immunocompetence in E. autumnata displayed a 13% more rapid response in herbivory trees compared to control trees during the first hour after implant insertion. The preliminary experiments had indicated that with two hours exposure time, practically all implants became fully melanized both in herbivory and control trees. Such short exposure times contrast strongly with the commonly used 24 and 48 hours at higher temperatures in other insect studies (Schmid-Hempel and Schmid-Hempel 1998, Ryder and Siva-Jothy 2000, Rennault et al. 2002). It suggests that during the increase phase, E. autumnata were very resistant against pathogens and parasitoids, and that the resistance was at least partially related to plant defense. Judging the true importance of the 13% acceleration in the rate of encapsulation during the first hour after implant insertion is at present impossible. It does not mean that parasitism would have been 13% lower in herbivory trees, just that the rate of encapsulation was significantly accelerated due to previous damage. Such an enhanced rate of encapsulation might be critical, e.g., in determining whether an insect has or does not have time to inactivate the dnaviruses, which typically are injected by ovipositing parasitoids to overcome host immunocompetence (e.g., Beckage and Reynolds 2003). Because of the scarcity of natural parasitoids in the increase populations, we have so far not been able to measure the defense of E. autumnata against real parasitoids. However, melanization is used to describe the efficiency of the active, general defense system against various enemies, and the survivorship of E. autumnata larvae to artificial fungal infection correlated significantly positively with implant melanization (M. Rantala, unpublished data). In other species it has been shown that the ability to encapsulate abiotic materials is strongly related to the ability to encapsulate true parasites (e.g., Paskewitz and Riehle 1994, Gorman et al. 1996), and that selection for parasite resistance increases the number of hemocytes, critical for encapsulation (Kraaijeveld et al. 2001).

The difference in the defoliator’s encapsulation response between herbivory and control trees might in theory result from the differential mortality of larvae in the two treatments, if for instance a high mortality rate eliminated larvae with a low encapsulation ability. However, in 2003 there was no difference between herbivory and control trees in larval survivorship to pupae or in pupal mass. Although E. autumnata females show higher indices of immune defense than males (df = 1, 343, $P = 0.0004$), sex-biased mortality was not a probable explanation either, because of the similar sex ratios of the surviving larvae in both types of trees (Table 1). We cannot exclude the possibility that E. autumnata may utilize some inducible defensive plant compounds, possibly also plant enzymes, for its own defense. The connection between insect immunocompetence and plant defense was suggested by both the higher immune response in herbivory trees compared to control trees, and the positive correlation between immunocompetence and larval mortality in herbivory trees. An alternative explanation is that the larvae may simply recognize the level of plant defense and prepare for imminent parasitism and increased transfer of pathogens and disease, which become more likely at high herbivore densities (see, e.g., Wilson et al. 2002).

The results raise the question of why the birch defends itself in ways that may promote the increase of the defoliator population. However, the situation may simply follow from individual selection. Direct birch defenses may be lethal to defoliators, as indicated by the low survivorship of E. autumnata in some trees even in the absence of parasitoids and predators. This suggests that such individual trees are likely to enjoy the benefits of strong defense, but because dispersal redistributes surviving moths with a heightened immune defense, the cost (later damage by resistant larvae) is met by the whole tree population.

These observations may have implications for the emergence of population cycles in E. autumnata (Haukioja 2005). To our knowledge, possible plant-mediated effects on the immunocompetence of defoliators have not earlier been studied in natural population cycles. On the other hand, indirect positive feedbacks, via induced plant defenses reducing defoliator susceptibility to viruses, have been reported by Hunter and Schultz (1993), but have not been incorporated into analyses of population dynamics. Altogether, these observations emphasize the importance of understanding the mechanisms contributing to the high success of defoliators during the increase phase of the population.

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**Literature Cited**


