Local adaptation of an insect herbivore to a heavy metal contaminated environment

Tapio van Ooik & Markus J. Rantala*

Department of Biology, FI-20014 University of Turku, Finland (corresponding author’s e-mail: markus.rantala@utu.fi)

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Plants show rapid adaptation towards resistance against heavy metals. In contrast, tests examining the local adaptation of insect herbivores to heavy metal pollution are lacking. Here we test whether the autumnal moth *Epirrita autumnata*, living in a heavy metal contaminated area, shows local adaptation in the growth rate and immune function. We gathered male moths from control and polluted areas for use in paternal half-sib crossings in order to ensure that we measure genotypic variation in addition to phenotypic variation. We found genotype × environment interactions in the growth of the larvae suggesting potential local adaptation in the growth of pollution-exposed moths. However, adaptation appears to incur a cost, because we observed reduced performance of the heavy metal adapted strain in a non-polluted environment. Finally, we found that pollution enhanced the immune function in female moths but not in males.

Introduction

The impacts of air pollution on vegetation have long been recognized. Since industrialization, coal burning, smelting and other activities have been releasing large amounts of pollutants, including sulfur and various metals (e.g. copper, zinc, iron), into local environments (Herrera-Estrella et al. 2001). Plants may be able to adapt to pollution to some degree. For example, it has been suggested that mountain birch (*Betula pubescens* subsp. *czerepanovii*) has adapted rapidly towards heavy metal resistance (Eränen 2008).

Genetic variation is a prerequisite for organisms to adapt to varying environmental conditions. Large genetic variation increases the probability that organisms will persist in a fluctuating environment, since it enhances the likelihood that some individuals can cope with novel conditions (Reznick et al. 1990). The forces of natural selection often vary in space, resulting in genotype × environment interactions for Darwinian fitness. In the absence of other forces and constraints, such divergent selection should allow local populations (deme) to evolve traits that provide an advantage under local environmental conditions, regardless of the consequences of these traits for fitness in other habitats (Kawecki & Ebert 2004). A well-known example of a local adaptation is industrial melanism, where pollution can cause selection pressure on the color of an organism. An increase in aerial pollution can, among other things, increase the proportion of dark moths in a population, and *vice versa* when aerial pollution decreases (Grant et al. 1996).
Adaptation to environmental conditions typically requires changes in the life history of an organism (e.g. changes in growth rate) (Reznick et al. 2001).

Heavy metals such as nickel, copper, iron, and to a lesser extent manganese and zinc can accumulate in birch leaves (Kozlov et al. 1995a). Nickel and copper accumulate similarly from the air, but only nickel can be taken up via plant roots (Kozlov et al. 2000). Nickel and copper can also accumulate in insect herbivores as they eat leaves that are exposed to heavy metals (Lindqvist 1992, Kozlov et al. 2000). Besides the obvious effects of pollution on growth rate (Warrington 1987) and mortality (Mitterböck & Fuhrer 1988), pollution can affect the immune defense of insects (Sorvari et al. 2007, van Ooik et al. 2008). Effects of pollution on the immune function in humans and other vertebrates have been studied in detail, but impacts on the immune function of invertebrates have received less attention (Galloway & Depledge 2001, Rickwood & Galloway 2004, Sorvari et al. 2007, van Ooik et al. 2008).

Although the immune system in insects is less complex than that of vertebrates and relies only on innate immune defense, many components are homologous. For example, Drosophila hemocytes synthesize the glycoprotein DS47, which is homologous to mammalian secretory proteins produced by activated macrophages (Vilmos & Kurucz 1998). Insect immunity is characterized by the inducible expression of a large array of antimicrobial peptides and by the constitutive melanization-encapsulation response. Encapsulation is a non-specific, constitutive, cellular response through which insects defend themselves against multicellular pathogens such as fungi, nematodes and parasitoids (Gillespie et al. 1997), but it also plays a role in defense against viruses (Washburn et al. 1996). In the encapsulation response, hemocytes bind to the surface of an invader and form a multilayered, overlapping sheath of hemocytes around the invader. The process, which is accompanied by blackening of the capsule due to melanization, leads finally to the death of a parasite within the capsule (Jiravanichpaisal et al. 2006). The encapsulation response has been found to vary in arthropods with genotype (e.g. Rantala & Roff 2006), quantity and quality of food (Yang et al. 2007, 2008, Ruuhola et al. 2010), physical activity (e.g. Ahtiainen et al. 2005), sex (Vainio et al. 2004), size of secondary sexual signals (Rantala et al. 2000), and heavy metal pollution (Sorvari et al. 2007, van Ooik et al. 2007, van Ooik et al. 2008).

In this study, we tested whether there is local adaptation to heavy metal pollution in the growth rate and immune defense of the autumnal moth, Epirrita autumnata (Lepidoptera: Geometridae). In a previous study, we found that the encapsulation response was stronger in moths that were fed on leaves from a heavy metal polluted area than in moths fed on leaves from a non-polluted area. However, the growth rate of moths fed on leaves from the heavy metal polluted area was lower than in moths fed on leaves from the non-polluted area (van Ooik et al. 2007). Likewise, in a laboratory study, in which we added heavy metals to food, we found that moderate levels of pollution enhanced the immune defense of moths while high levels of pollution reduced immunity (van Ooik et al. 2008). Additionally, previous studies found that acid rain may cause different immune responses in different sexes (Ruuhola et al. 2009).

Since a genotype can produce different phenotypes, we set up a paternal half-sib design. By doing so, we ensured that we are not just measuring phenotypic variation but also measure genotypic variance between the strains that we used. We examined how traits of E. autumnata varied between different broods in relation to heavy metal exposure. If there were genotype × treatment interactions between control and polluted environments, this would suggest a potential, local adaptation between the genotypes. Additionally, we examine whether there is a trade-off between immune defense and growth on these moths, since insects often trade-off between these two life-history traits (Rantala & Roff 2005).

Different genotypes (strains) of E. autumnata were used in the experiment. One strain originates from an area that has not been exposed to pollution (referred to as the non-polluted strain), while the other strain originates from an area that has been exposed to pollution since the 1940s (the polluted strain). More specifically, we tested (i) whether the life history traits in terms
of growth rate and pupal weight differ between the metal exposed and unexposed populations of *E. autumnata*, (ii) whether the moths exposed to heavy metal pollution have enhanced immune function and (iii) whether pollution has a different effect on the encapsulation rate of different sexes of *E. autumnata* (see Ruohola et al. 2009). If there is a local adaptation to heavy metal pollution we would expect that individuals whose parents originated from a polluted area would perform relatively better when fed with heavy metal polluted leaves than individuals whose parents originated from an unpolluted (control) area.

**Material and methods**

**Heavy metal polluted site**

The town of Harjavalta, in southwestern Finland, has a factory complex (61°19´N, 22°9´E) that has produced heavy metal pollution since the 1940s. Although environmental controls have caused a decrease in the emissions from the smelter there is still substantial sulfide pollution mainly in the form of SO₂ and dust containing Cu and Ni, and to a lesser extent Cd, Pb, Fe and Zn (McEnroe & Helmisaari 2001, Kiikkilä 2003). Heavy metals such as nickel, copper, iron, and less so manganese and zinc, can accumulate in birch leaves in heavy metal polluted areas (Kozlov et al. 1995b). The majority of nickel and copper in birch foliage comes from deposition of dust particles on leaf surfaces. Nickel and copper accumulate similarly from the air, but only nickel enters also via plant roots (Kozlov et al. 2000). Our experiment was performed during the spring of 2004. Thus, local populations of autumnal moths had had more than 60 generations to adapt to metal pollution.

Metal contaminated leaves used as food for larvae in the experiment were collected from five separate trees about 100 m away from the factory complex. Control leaves were collected from five trees about ten kilometers to the north from Harjavalta at a location where elevated heavy metal concentrations do not occur (Hynninen 1986). All trees were chosen so that their habitus resembled one another as much as possible.

**Study species**

The autumnal moth is a Holarctic moth with a univoltine life cycle. It is notorious for its massive outbreaks in northern Fennoscandia (Tenow 1972). The moth is a generalist over a wide variety of host plants (Seppänen 1970) and host chemical traits (Ruusila et al. 2005). The larvae hatch in spring in synchrony with birch bud break (Kaitaniemi et al. 1997) and feed on the foliage of various deciduous trees and undergrowth for about a month before pupating (Seppänen 1970).

*Epirrita autumnata* males used in this experiment were gathered from two places. Individuals of the control strain of *E. autumnata* were collected in the Turku area (about 100 km to the south of Harjavalta) from different locations that are not known to be affected by heavy metals. Individuals exposed to heavy metals were collected from different locations near the factory complex in Harjavalta. These populations will be referred to as the non-polluted and polluted strains respectively.

By using paternal half-sibs (Falconer & Mackay 1996) in the bioassays, potential adaptation of the moth was determined. *Epirrita autumnata* larvae used in bioassays were obtained by crossing four polluted strain males and five non-polluted strain males with two different females (originating from the control site) in the autumn of 2003. In total, we studied eight polluted strain broods (95 offspring) and ten non-polluted strain broods (111 offspring).

Timing of offspring emergence in the subsequent spring was made to correspond with the bursting of birch buds in early May at Harjavalta (Ruohomäki et al. 1996). Larvae were individually reared in 48-ml plastic vials at 22 °C, and were fed *ad libitum* with control birch leaves (*Betula pubescens*) until they molted into their 5th instar stage. The bioassays were performed on the last (5th) larval instar stage, since leaf consumption and weight gain of *E. autumnata* is highest during the 5th instar stage (Lempa et al. 2004).

**Growth assay**

Larval development was synchronized at the
beginning of the 5th instar: larvae were checked daily for molt, and larvae that were approaching molt were placed in +1 °C to prevent molting until the desired time. Short exposure to a low temperature does not appear to affect the larvae negatively (Lempa et al. 2000). Before the bioassay, larvae were randomly placed in control or pollution treatment groups.

Larvae were allowed to feed for 24 h to fill their gut; either on control or on pollution-exposed leaves. Then at the start of the bioassay, larvae were weighed and provided with fresh polluted or control leaves ad libitum. After 24 h, the larvae were again weighed and were given new leaves ad libitum. When larvae ceased feeding altogether, they were transferred to vials with moist moss to pupate and their masses were recorded.

**Immune assay**

The encapsulation response in insects is most easily measured defense reaction against a novel and standardized antigen such as nylon monofilament (Rantala et al. 2002, Kortet et al. 2007, van Ooik et al. 2008). In the encapsulation rate assay, a 2-mm-long piece of a nylon monofilament (diameter 0.1 mm, rubbed with sandpaper) was inserted through a puncture between two abdominal segments of one-week-old pupae. The immune system of the moths was allowed to respond to this implant for 1 hour at 22 °C. A one-hour exposure is optimal to view variation between implants in *E. autumnata* (Rantala & Roff 2007, van Ooik et al. 2007). The implants were then removed, dried and examined under a light microscope, and pictures of the implants were taken with a camera from two different angles. These images were analyzed using image analysis software ImageJ (Abramoff & Magelhaes 2004). The amount of reflective light from the implants was measured. The repeatability of this method for estimating encapsulation rate is high (Rantala et al. 2002, Rantala & Roff 2006).

**Data analysis**

All analyses were carried out with SAS 9.1 statistical software (SAS Institute Inc. 2002–2003). The effects of different variance components on larval growth and immunology were tested using ANOVA models (Proc. GLM, Type III SS). Growth rates of the larvae were measured by using initial larval mass (weight of the moths before the start of the experiment), final mass and duration of the growth experiment as covariates in the growth model (see Kause et al. 1999). Encapsulation rate values were square-root transformed to meet the assumptions of parametric tests. In the analyses of the encapsulation rate, the pupal weight of moths was taken into account by including it as a covariate. The sex × treatment interaction was omitted from the final ANOVA model (Table 1), since it was not statistically significant (p = 0.6098). The pairwise differences in the interactions were further analyzed with contrasts. Mortality of *E. autumnata* did not differ between the treatments (Logistic regression: $\chi^2 = 0.0010, p < 0.9747$).

**Results**

Female larvae showed higher growth rate than males (least squares means: 35.96 vs. 33.19, Table 1) and larvae grew better on control leaves

| Table 1. ANOVA results of the relative growth rate of *E. autumnata*. Statistically significant cases are given in boldface. $SS_{model} = 2068.1$, $SS_{error} = 7109.0$. Degrees of freedom: error = 194, total = 200. |
|-----------------|-----|-----|-----|-----|-----|
| Factor          | df  | SS  | MS  | F   | p   |
| Initial larval mass | 1   | 0.30 | 0.30 | 0.01 | 0.9286 |
| Time            | 1   | 33.42 | 33.42 | 0.91 | 0.3407 |
| Strain          | 1   | 63.68 | 63.68 | 1.74 | 0.1890 |
| **Treatment**   | 1   | 920.66 | 920.66 | 25.12 | < 0.0001 |
| **Sex**         | 1   | 374.12 | 374.12 | 10.21 | 0.0016 |
| **Strain × treatment** | 1   | 230.48 | 230.48 | 6.29  | 0.0130 |
than on polluted leaves (least squares means: 37.83 vs. 31.32, Table 1). Additionally, the strain × treatment interaction was significant as the strains differed markedly in their response to the treatments (Fig. 1). When fed with control leaves, the growth rate of the non-polluted and control strains differed significantly from one another (t = 2.633, p = 0.009). However, in the metal treatment the strains did not differ significantly from each other (t = 0.867, p = 0.387). The growth rate of both the non-polluted and polluted strains were higher on control leaves than on polluted leaves (t = 5.32, p < 0.0001; t = 2.88, p = 0.004 respectively; Fig. 1).

Pupal weight of *E. autumnata* differed in the two treatments (Table 2). The weight of the larvae on control leaves was higher than the weight of the larvae on metal contaminated leaves (least squares means: 88.98 vs. 75.91). However, the strain × treatment interaction was not significant, which means that the pupal weights of both strains responded similarly to the treatments.

The sex × treatment interaction was significant for encapsulation rate in *E. autumnata* (Table 3 and Fig. 2). Thus, encapsulation rates differed between the sexes in the two treatments. We further analyzed which treatments differed from one another by conducting contrasts within the treatments. The encapsulation rate of males did not differ between the treatments (t = 0.24, P = 0.8107). However, the encapsulation rate of females was higher in the heavy metal treatment than in the control treatment, even though the high pupal weights of females were taken into account in the model (t = 2.13, p = 0.0345). Females also had higher encapsulation rates than males in both treatments (control and metal treatment: t = 2.03 and 4.98, p = 0.0437 and 0.0001, respectively). Additionally, we looked

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**Table 2.** ANOVA results of the pupal weight of *E. autumnata*. Statistically significant cases are given in boldface. SS<sub>model</sub> = 11150.1, SS<sub>error</sub> = 9272.6. Degrees of freedom: error = 195, total = 200.

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<th>MS</th>
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<td>142.619</td>
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**Table 3.** ANOVA results of the encapsulation rate of *E. autumnata*. Statistically significant cases are given in boldface. SS<sub>model</sub> = 111.4, SS<sub>error</sub> = 539.6. Degrees of freedom: error = 178, total = 184.

<table>
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<td>0.36</td>
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<td>Strain</td>
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<td>65.46</td>
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<td>Sex × treatment</td>
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<td>12.82</td>
<td>4.23</td>
<td>0.0412</td>
</tr>
<tr>
<td>Strain × treatment</td>
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<td>0.83</td>
<td>0.27</td>
<td>0.6017</td>
</tr>
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</table>
for trade-offs between growth and immune function in different treatments but we did not find any (Pearson’s $r = -0.04$ to 0.13, $p > 0.39$).

**Discussion**

We showed that the growth rate of moths was higher on control leaves than on polluted leaves, which suggests that metal pollution in the leaves reduces the growth rate of *E. autumnata*. These results are consistent with our earlier research (van Ooik *et al.* 2007, van Ooik *et al.* 2008). We also found an indication of local adaptation, as the strain $\times$ treatment interaction was significant in case of growth. Thus, it seems that pollution may cause locally varying selection pressures among moth populations. Pollution incurs costs for the larvae, since growth is clearly poorer on polluted leaves than on control leaves. According to Kawecki and Ebert (2004), divergent selection should cause each local population to evolve traits that provide an advantage under its local environmental conditions, regardless of the consequences of these traits for fitness in other habitats.

In our experiment, moths of the non-polluted strain grew much better than moths of the polluted strain on control leaves. Thus, adaptation seems to have incurred a cost of reduced performance for the polluted strain in a non-polluted environment. In contrast, growth on polluted leaves shows an opposite response. On polluted leaves, the polluted strain showed slightly better growth than the non-polluted strain. While this difference in growth between the polluted and non-polluted strains on polluted leaves is not significant, the strain $\times$ treatment interaction is significant, as seen visually by the crossing of the growth lines between the two treatments (Fig. 1). Since only one parent (the male) of each family in the polluted strain came from Harjavalta, we could only see partial genetic variation of *E. autumnata* living in the polluted area. It seems likely that if both parents had originated from Harjavalta, the observed difference in growth rate of the moth strains on polluted leaves would have been much greater.

There was no strain $\times$ treatment effect on pupal weight; meaning that the differences in the growth rates of the two strains did not show as differences in the final pupal weight of the larvae. It may be that in order to compensate for poorer food quality, the larvae of the polluted strain consumed food for longer before pupating than the larvae of the non-polluted strain.

Previous work suggested that investment in immunity may diminish resources to be allocated to growth in insects (Rantala & Roff 2005). However, in our experiment we failed to find a significant strain $\times$ treatment effect on the encapsulation rate. This suggests that, contrary to the growth rate, there was no sign of local adaptation of the immune function. However, there were clear differences in the encapsulation rate between the sexes. The encapsulation rate of females was higher in the heavy metal treatment than in the control treatment, but treatment did not have any effect on male immunity. This supports previous findings that there are sex differences in how environmental and genetic factors affect immunity in *E. autumnata* (Rantala & Roff 2007), probably as a result of sex differences in genetic architectures in immune system.

In light of this study, it seems that not only plants but also insect herbivores may adapt rapidly to local pollution. It further appears that a locally adapted insect population may incur a cost of reduced performance in other pristine and non-polluted environments. To our knowledge this is the first study to test local adaptation of an insect to heavy metal pollution.
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