

TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. AII OSA - TOM. 224

BIOLOGICA - GEOGRAPHICA - GEOLOGICA

**EFFECTS OF HEAVY METAL POLLUTION
AND HOST PLANT LEAF CHEMISTRY
ON THE IMMUNE DEFENSE AND
LIFE HISTORY TRAITS OF
AN INSECT HERBIVORE**

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Turku 2008

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ISBN 978-951-29-3582-6 (PRINT)

ISBN 978-951-29-3583-3 (PDF)

ISSN 0082-6979

Painosalama Oy – Turku, Finland 2008

“The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" (I found it!) but "That's funny ..."

Isaac Asimov

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This thesis is based on the following articles, referred to in the text by their Roman numerals:

- I. Ruusila, V., Morin, J.-P., van Ooik, T., Saloniemi, I., Ossipov, V. & Haukioja, E., 2005. A short-lived herbivore on a long-lived host: tree resistance to herbivory depends on leaf age. *Oikos* 108 (1), 99-104.
- II. van Ooik, T., Rantala, M.J. & Saloniemi, I., 2007. Diet-mediated effects of heavy metal pollution on growth and immune response in the geometrid moth *Epirrita autumnata*. *Environmental Pollution* 145 (1), 348-354.
- III. van Ooik, T., Pausio, S. & Rantala, M.J., 2008. Direct effects of heavy metal pollution on the immune function of a geometrid moth, *Epirrita autumnata*. *Chemosphere* 71 (10), 1840-1844.
- IV. van Ooik, T., Ruuhola, T., Salminen, J.P., Haviola, S., Neuvonen, S. & Rantala, M.J., submitted manuscript. The effect of simulated acid rain and heavy metal pollution on a plant-herbivore interaction: from the leaf chemistry to the immune system of the herbivore.
- V. van Ooik, T. & Rantala, M.J., submitted manuscript. Local adaptation of the growth of autumnal moth to heavy metal environment.

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1. INTRODUCTION

1.1. Key selection pressures in the life history of an insect herbivore

An insect herbivore faces challenges along the way to adulthood and the eventual production of its own offspring. In order to become an adult, the insect has to consume a great deal of plant material in the juvenile stage. Consuming plant material, however, also means that the insect has to deal with the defense mechanisms of the plants.

The main primary defenses of plants against herbivores are plant-produced defensive compounds and nutritional traits. These can be divided into three broad classes: 1) levels of nutritive compounds in plants; 2) plant secondary compounds, such as phenols, terpenoids and alkaloids; and 3) specific defense cascades (Haukioja, 2005).

Due to the human impact, the insect faces a new challenge in addition to host plant chemistry: pollution. Metals occur naturally in the environment in small quantities (volcanoes, erosion, spring water, bacterial activity) (Florea and Büsselberg, 2006). However, anthropogenic activities such as mining, fossil fuel combustion, smelting and agricultural processes have locally increased the levels of heavy metals such as cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), arsenic (As) and nickel (Ni) in the soil up to levels that are dangerous for plants, animals and human beings (Florea and Büsselberg, 2006; Sharma and Agrawal, 2005).

Insects living in polluted areas have been shown to accumulate heavy metals, in particular Ni and Cu (Heliövaara et al., 1987; Zvereva et al., 2003). Along with the obvious effects of pollution on growth rate (Warrington, 1987) and mortality (Mitterböck and Fuhrer, 1988), pollution can have harmful effects on immune defense. The

effects of pollution on immune function in humans and other vertebrates have been studied in detail, but the effect on the immune function of invertebrates has received less attention (Galloway and Depledge, 2001; Rickwood and Galloway, 2004; Sorvari et al., 2007).

1.2. Adaptation to changing environment

The evolutionary potential of a population is determined in part by the genetic variation present within that population. If a population has a wide variation of genotypes present, there are likely to be individuals with characteristics that will be advantageous if environmental factors change: they will be more fit (Harrison, 2006).

Early on, it was noted that pollution can modify selection pressure among moths. An increase in aerial pollution can among other things increase the proportion of dark moths; correspondingly, when aerial pollution decreases the proportion of dark moths drops (Grant et al., 1996). Adaptations to environmental changes typically require changes in the life history of an organism: for instance growth rate, maturation age and/or reproduction are traits that may be altered (Clobert et al., 1998; Prati and Schmid, 2000; Reznick et al., 2001).

Populations may change on an evolutionary time-scale through many generations to be better adapted to their environment. Typical examples are the antibiotic resistance of certain bacterial strains (Baquero and Blazquez, 1997) or the development in rabbits in Australia of resistance against myxomatosis, a poxvirus (Anderson, 2004).

In the thesis I used *E. autumnata* as a model species to determine whether an insect herbivore has the potential to adapt to a moderate amount of heavy metal pollution.

1.3. Invertebrate immunology

Although the immune defense system in insects is less complex than the vertebrate immune system and relies only on innate immune defense, many components are homologous (Vilmos and Kurucz, 1998). *Drosophila* hemocytes, for example, synthesize the glycoprotein DS47, which is related to a human cartilage glycoprotein, HC gp-39, and mouse secretory glycoprotein, YM-1, identified in activated macrophages (Kirkpatrick et al., 1995).

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 whereby 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 ultimately 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 and Roff, 2006), quantity and quality of food (Yang et al., 2007), 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).

Previously, Klemola et al. (2007) have found significant heritable variation in the encapsulation rate of *E. autumnata*.

Insects are not the helpless victims of parasites and pathogens, and co-evolutionary processes have favored the evolution of an effective immune defense system.

1.4. Aims of the study

In the thesis, I address the following questions: 1) Does the autumnal moth have additive genetic variance for handling the rapidly changing food quality of its most important food plant, the mountain birch? 2) Does heavy metal pollution affect the growth and/or immune defense of the autumnal moth? 3) Is there additive genetic variance in the moth's larval growth and immune response in relation to heavy metal exposure? 4) Are the effects of heavy metals on the immune defense direct or plant-mediated, and which metals cause the effect? 5) Are there sex differences in how the heavy metals affect the immune function of the autumnal moth? 6) How does long-term moderate pollution affect growth, survival and immune defense of a moth feeding on mountain birch? 7) Does long-term pollution affect mountain birch chemistry, with consequent indirect effects on the herbivore's performance?

2. NATURAL HISTORY OF THE STUDY SUBJECT AND STUDY AREAS

Two of my substudies have been conducted at Harjavalta (II and IV), for two reasons: the Harjavalta factory complex is the heavy metal pollution source closest to Turku, and a great deal of pollution research has already been done in the Harjavalta area. Two of the substudies (I and IV) have been conducted at the Kevo Subarctic Research Institute; plant-herbivore interactions have been studied there for decades, and simulated acid rain and metal treatments have been conducted there since 1991.

In addition, one substudy (III) has been conducted at the laboratory facilities of the University of Turku.

2.1. Autumnal moth

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 feeder 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). The larvae are polyphagous, but in northern Europe their most important food plant is mountain birch due to its relative abundance. (Ruohomäki et al., 2000). The larval stage consists of five instars that feed on the foliage of various deciduous trees and undergrowth. The larvae feed for about a month around midsummer before pupating within a thin cocoon buried in the leaf litter at a depth of a few centimeters (Haukioja et al., 1988a). The pupal stage lasts for about a month, after which the winged adults hatch in the autumn. The eggs hibernate over the winter (Ruohomäki et al., 1996).

2.2. Host trees

Mountain birch (*Betula pubescens. czerepanovii*) was the host tree in substudies I and IV. It is the dominant tree species in northern Norway, Sweden and Finland, as well as in nearby areas of northern Russia. The tree is a deciduous species, which forms a tree line in the subarctic areas of Northern Europe (Atkinson, 1992). The numbers of *E. autumnata* moths increase in cycles of nine to ten years, causing severe episodic damage to mountain birch in Fennoscandia (Ruohomäki et al., 2000; Tenow, 1972).

The larvae of *E. autumnata* have to be able to handle the rapidly changing suite of nutritive and allelochemical compounds in mountain birch, which is its main host tree. At the beginning of their growth period, mountain birch leaves have higher water content and more proteins but are lower in sugars than more mature leaves. The concentration of soluble proanthocyanidins (= condensed tannins) rises steadily as the leaves mature. Concentrations of other phenolic compounds (cell-wall-bound proanthocyanidins, gallotannins, flavonoid glycosides and *p*-coumaroylquinic acid derivatives) increase at the beginning of the season and then decrease throughout most of the summer (Riipi et al., 2002).

Other common host tree for *E. autumnata* is the downy birch tree *Betula pubescens* (Seppänen, 1970). As there are no Mountain birches in southern Finland *E. autumnata* larvae were therefore fed on the leaves of downy birch At Harjavalta and in Turku (substudies II, III and V).

2.3. Population cycles of the autumnal moth and immune defense

It has been suggested that pathogens may be a driving force of autumnal moth cycles (Klemola et al., 2008; Kilpimaa et al. submitted). Two pathogens diagnosed in the autumnal moth have been a nucleopolyhedrovirus (NPV) and an entomopathogenic fungus (Tenow, 1972). Diseases have not previously been considered as drivers of autumnal moth cycles because they have only been detected during a few outbreaks (Bylund, 1995; Ruohomäki et al., 2000; Tenow, 1972); this, however, may be due to the fact that non-specialist researchers may not be able to identify these pathogens or recognize the symptoms of sick larvae.

The role of disease in host populations is a delicately balanced interplay between the pathogen's capacity to exploit a host and the host's susceptibility. Baculoviruses, such as NPV, occur widely among Lepidoptera, and in some species of forest and agricultural insects cause epizootics (disease epidemics) in outbreak populations. Baculoviruses are divided into two groups: the NPV and the granuloviruses (GV) (Cory and Myers, 2003).

Recently, Kapari et al. (2006) found that delayed induced resistance of mountain birch (*B. pubescens czerepanovii*) was associated with an increased encapsulation response against a nylon monofilament of its defoliating geometrid, *Epirrita autumnata*; this suggests an indirect positive feedback effect that might reduce the moth's susceptibility to pathogens (Rantala and Roff, 2007).

2.4. Study areas

The substudy V has been done entirely in a laboratory facility at the University of Turku. The other substudy areas are defined in the following paragraphs more in detail.

2.4.1 Harjavalta

Harjavalta in southwestern Finland (61°19'N, 22°9 'E) has a factory complex that has produced heavy metal pollution since the 1940s. Although improved environmental control methods have helped to reduce levels of emissions close to the smelter, high levels can still be detected of S (in the form of SO₂) and of dust, containing Cu and Ni and to a lesser extent Cd, Pb, Fe and Zn (McEnroe and Helmisaari, 2001). The levels of most heavy metals in birch foliage decrease clearly with increasing distance from the Harjavalta factory.

The most recent heavy metal analysis of the Harjavalta factory complex has been done by Lukkari and Haimi (2005). They measured metal concentrations in the soil ca. 800 m from the Cu-Ni smelter in Harjavalta. The measurements were performed in the same area where I collected the birch leaves for my studies. The total metal concentrations in the soil were (mg kg⁻¹ dry mass) Cu: 1590 (±50.4), Zn: 81 (±9.6), Pb: 57 (±6.3) and Cd: 1 (±0.3).

Metal-contaminated leaves for the experiments (II and IV) were collected from five trees about 100 meters from the factory complex. Control leaves for the substudy were collected from five trees ten kilometers north of Harjavalta, at a location where elevated heavy metal concentrations are not known to occur (Hynninen, 1986). All the trees for the experiment were chosen so that their habitus resembled one another as much as possible. The trees chosen for the two studies were not the same.

The area where we collected leaves for the larvae can be considered to be in a region of moderate or high pollution at Harjavalta.

2.4.2 Kevo Subarctic Research Institute

The Kevo Subarctic Research Institute is situated in northernmost Finland (69°45'N, 27°E). The experimental area for substudy IV is located near the Research Station (69° 45'N 27° 01' E). The heavy metal treatments that were used in experiment IV have been conducted in the area since 1991.

3. METHODS

Methods are discussed here only briefly. They are described in more detail in the individual substudies or in the studies cited.

3.1. Chemical analysis of birch leaves

In the first substudy (I) we sampled leaves for chemical analyses in order to determine the chemical constitution of the trees during each bioassay that we carried in the substudy. Leaves were analyzed using a mass spectrometer (for details see Ossipov et al., 2001).

Additionally, in substudy V the chemical content of birch leaves was analyzed. Dried and ground birch leaves (20 mg material per sample) were extracted three times (3×1 hr) with 500 μ l acetone/water (7/3, v/v) on a planar shaker. After extraction, the acetone was evaporated from the combined extract in vacuo with an Eppendorf concentrator. The freeze-dried aqueous phase of the extract was dissolved in 1 ml water. The supernatant of the centrifuged sample (10 min at 2500 g) was filtered through a 0.45 μ m PTFE filter and kept frozen at -20°C until analyzed with HPLC-DAD (high-performance liquid chromatography-diode array). HPLC is a form of column chromatography frequently used in biochemistry and analytical chemistry.

HPLC-DAD analysis of birch leaf extracts was performed at 280 nm and 349 nm with Merck-Hitachi's LaChrom HPLC system (Merck-Hitachi, Tokyo, Japan). Column and chromatographic conditions have been described earlier (Salminen et al., 1999), except that 0.1 M H_3PO_4 was replaced with 0.05 M H_3PO_4 . Phenolics were identified by comparing their retention times and UV spectra to those earlier reported by Salminen et al. (1999; 2001). Phenolic compounds were quantified using pedunculagin, pentagalloylglucose, catechin,

coumaric acid, chlorogenic acid and quercetin as external standards.

Several birch leaf traits have been found to correlate with growth and consumption in different instars of the autumnal moth (Haukioja et al., 2002; Kause et al., 1999).

3.2. Paternal half-sib design

In substudies I and V, we measured genetic variance in the autumnal moth.

One way of measuring genetic variation is to raise individuals from the same brood (a full-sib design). For studies of local adaptation, individuals from the same brood are allowed to grow in different environments. Full sibs, however, are likely to share common traits, and certain insect genotypes may have adapted either genetically or developmentally to certain conditions or birch genotypes. We therefore applied planned paternal half-sib designs (Falconer and Mackay, 1996) to avoid most of the temporary effects of full-sib broods, but retaining genetic homogeneity for environmental comparisons. More specifically, we examined how traits of *Epirrita autumnata* vary between different broods when they are fed on the leaves of birches that have been exposed to different levels of heavy metals.

In the half-sib design studies, multiple males were crossed in the fall with two different females. The eggs were left to overwinter; the larvae hatched the following spring, after which we were able to conduct experiments on the offspring.

3.3. Measurements of immune function

In substudies II – V we measured the encapsulation responses of the moths. The encapsulation response was measured as the

defense reaction against a nylon monofilament (Kortet et al., 2007; Rantala et al., 2002). In the assays a 2-mm long piece of nylon monofilament (diameter 0.1, 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 react to this implant for one hour. This is the length of exposure that has been found to allow optimal observation of the *extent* of variation between implants in *E. autumnata* (II, Kapari et al., 2006). The pupae were then placed in a freezer at - 80 °C prior to the analyses of immune function. The implants were then removed, dried and examined under a light microscope, and photographs of the implants were taken from different angles. These images were analyzed using the image analysis software ImageJ (Abramoff and Magelhaes, 2004). The amount of light reflected from the implants was measured.

In articles II and IV, we measured phenoloxidase (PO) activity to gain more information about immune defense in *E. autumnata*. Due to resource constraints, it was not possible to measure PO activity in the substudies III and V. In the phenoloxidase activity assay, haemolymph was collected from each pupa with a plastic micropipette. The haemolymph was diluted with ice-cold phosphate-buffered saline solution. The samples were immediately frozen at - 20 to - 25 °C to disrupt the cell membranes. PO-activity was measured using L-DOPA (Sigma) as a substrate. The increase in absorbance during 30 min at 1-minute intervals was read at 490 nm at a temperature of 20 °C with a Multilabel counter (Wallac 1420 Victor) Phenoloxidase enzyme activity was expressed as the maximum rate of the reaction (see also Rantala et al., 2003).

3.4. Artificial exposure to heavy metals (IV)

Artificial acid rain treatments have been performed at the Kevo Subarctic Research Institute for 15 years, and we made use of these experimental areas. Mountain birches and autumnal moths were exposed to four different treatments. Trees were irrigated with plain water (pH 5.5), acidified water (pH adjusted to 3.1), water containing copper and nickel sulfates (pH ca. 5.7), or a combination of the last two treatments.

4. RESULTS AND DISCUSSION

4.1. Adaptation of short lived herbivore on a long-lived host (I)

In this substudy, we examined the effects of the chemical content of tree leaves on the performance of *E. autumnata*. The effects of heavy metals were not measured in the substudy.

Our results indicate that the genetic properties of the trees did not determine leaf quality for the herbivore, since the tree did not significantly explain the variance in consumption or growth of *E. autumnata*.

Instead, instar \times tree interaction was significant for the moth's growth and consumption. This means that temporal (developmental) changes in the quality of growing mountain birch leaves explain more of the variance in leaf consumption and growth of *E. autumnata* larvae than the identity of the host tree. (Fig. 1).

We did not find evidence for larval genotype \times tree interactions. These results are consistent with the results of Ayres et al. (1987), who likewise found no effect of brood \times tree interactions on the growth of the autumnal moth. The fact that we did not find genotype \times tree interaction suggests that the digestive strategies of *E. autumnata* are not fine-tuned to individual host phenotypes. This result is supported by the fact that *E. autumnata* is a taxonomic generalist, as shown by its high number of recorded host plant species (Seppänen, 1970). This conclusion may be related to two known aspects of *E. autumnata* biology. First of all, being a growth maximizer (Kause et al., 1999) may predispose *E. autumnata* to dietary generalism; a high larval growth rate demands high nutrition, which is generally available only from young growing leaves. Second, genetic generalism in those *E. autumnata* traits that are important for the utilization of its main host

plant, the mountain birch, may be a necessary precondition for the species, which during outbreaks is able to defoliate mountain birches over huge areas.

The most drastic changes in the suitability of individual host genets for the insect traits studied here took place between the 3rd and later instars. This shift takes place in half-grown leaves, and coincides with the time when the traits that are important for the larvae also change most rapidly (Haukioja et al., 2002; Riipi et al., 2002).

In this substudy, insect traits were studied during the last three instar stages. For practical reasons only a few indices have been developed for first or second instars; thus traits of the earliest instar stages (such as relative growth rate) were not included in this substudy. However, it is likely that the quality and variation of nutrition is as important for larvae in the early stages of their development as it is later on. It is not currently known whether the host trees would show similar rankings of resistance if the first instars had been used, although some studies have indicated that different developmental phases of insects may be sensitive to different plant traits (Hare, 1983; Haukioja et al., 2002), and that insects may rank the various developmental phases of plants differently (Pilson, 1992).

The number of experimental trees in the substudy was five, due to the fact that the amount of work measuring the growth and consumption of the autumnal moth in three instars in five trees was already massive. It is possible that we would have obtained more accurate results if we had included more trees in the experiment.

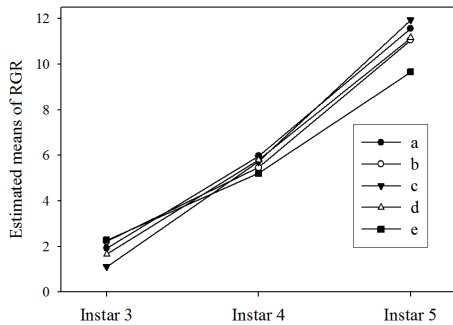


Figure 1. Estimated relative growth rates (RGR) of three instars of *E. autumnata* larvae on individual trees (a-e). Standard errors (*s.e.*) for all means were between $\pm 0.09 - 0.10$ and sample sizes (*n*) were between 64 and 126 individuals on all trees.

4.2. Diet-mediated pollution affects the growth and immune defense (II)

Metal-contaminated trees in our substudy can be considered to be in the moderate or high pollution zone at Harjavalta.

Metal contamination reduced both the growth rate (Fig 2) and the pupal weight of *E. autumnata*.

Larval growth rates of phenotypes differed between metal-contaminated and control birches, suggesting the presence of additive genetic variance in the larval growth rate.

We also found a tendency ($p < 0.0909$) toward additive genetic variation in the encapsulation rate between environments. It thus seems that some traits of the insect have the potential to respond to selection in a polluted environment.

This observed effect of pollution is characteristic of chewing insects (see meta-analysis by Koricheva et al., 1998). In general, at low concentrations pollutants have positive effects on insects, while at higher

concentrations the effects of contaminants tend to be harmful (Warrington, 1987). Low levels of pollution usually enhance the nutritional quality of plants; this may be why insects often perform well on plants, which are exposed to low levels of pollution (White, 1984). In substudy II the larvae produced more frass on contaminated leaves than on control leaves, indicating that polluted birch leaves were poorly digestible and the larvae thus could not process them as efficiently as the control leaves. As a result, larvae on polluted trees grew less and produced more frass than larvae on control trees.

Exposure to pollution enhanced immunity. Phenoloxidase enzyme activity (Fig. 3) and encapsulation rate were both enhanced on polluted leaves.

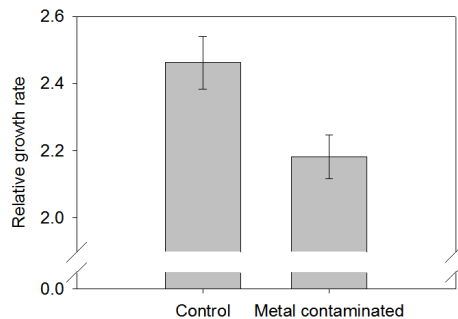


Figure 2. Relative growth rate of *E. autumnata* on control and metal-contaminated leaves (mean \pm s.e., $N = 96$ and 98 respectively).

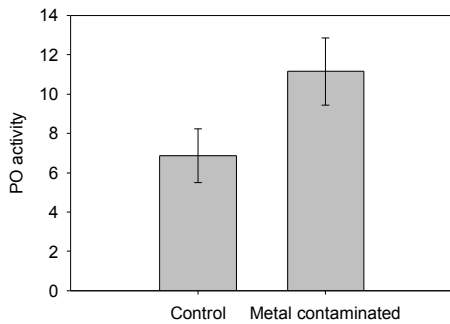


Figure 3. Phenoloxidase (PO) activity in pupae of *E. autumnata* on control and metal-contaminated leaves (mean \pm s.e., N = 91 and 90 respectively).

In the study of Kapari et al. (2006) the encapsulation response of *E. autumnata* was enhanced when larvae consumed leaves from trees which had suffered from foliar damage caused by *E. autumnata* the previous year. The heightened immune response was apparently due to larval-induced birch defenses.

Fatty acids and oxylipins in birch trees correlate negatively with the survival rate of *E. autumnata* (Haukioja, unpublished results). This suggests that birch defenses, which correlate with herbivore mortality, result from the activation of the octadecanoid pathway (see Haukioja, 2005). Furthermore, it has been shown that heavy metal stress induces plant fatty acid α -dioxygenase (DOXs), which is part of the octadecanoid pathway (Koeduka et al., 2005). A-dioxygenase is also induced by herbivore attacks (Hermsmeier et al., 2001).

It is likely that heavy metal pollution and herbivores cause a similar reaction in the octadecanoid pathway in birches, and that it was this reaction that induced the increased immune response in our substudy.

4.3. Direct heavy metal effects on immune defense (III)

After the previous experiment, we wanted to know more specifically what causes the enhanced immune reaction in moths consuming polluted leaves. Since Ni and Cu are among the most common heavy metals near a nickel-copper smelter (McEnroe and Helmisaari, 2001), we tested the direct effects of these metals on *E. autumnata*. We found that a moderate amount of Cu elevated the encapsulation response (a trend) in the moth (Fig. 4). A high dose of Cu, however, reduced the encapsulation response to the same level as the control. Additionally, in the high Ni treatment the immune response was significantly reduced far below the control level. It may be that the associated sulphate (SO_4) in the NiSO_4 and CuSO_4 had an effect on the immune response as well, since sulphates increase acidity (Saikkonen and Neuvonen, 1993).

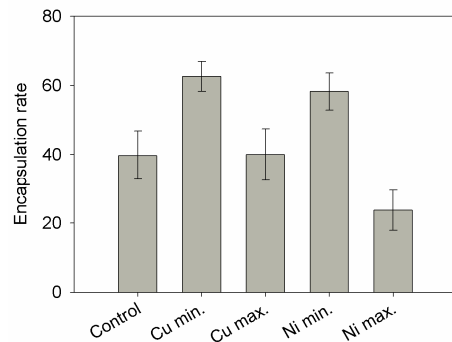


Figure 4. Encapsulation rate of pupae of *E. autumnata* on control and metal-contaminated leaves. Control: water, Cu min.: $103.5 \mu\text{g g}^{-1}$ Cu, Cu max.: $207 \mu\text{g g}^{-1}$ Cu, Ni min.: $169 \mu\text{g g}^{-1}$ Ni, Ni max.: $300 \mu\text{g g}^{-1}$ Ni (mean \pm s.e., n = 12, 14, 14, 22 and 11 respectively).

The results are consistent with Sorvari et al. (2007), who found in wild ants that the encapsulation response was enhanced at moderate heavy metal levels, while at high levels it was suppressed. Furthermore, it has been shown in marine invertebrates that immune responses may be enhanced after short-term low-level metal exposure, whereas higher concentrations or a longer exposure may inhibit the same responses. The effect of chemicals on invertebrate immune function depends on many things, including routes of uptake of chemicals, patterns of bioactivation, the biotransformation and excretion of immunotoxic compounds, and the relationship of the immunotoxic dose to other sublethal toxic effects (Galloway and Depledge, 2001). It has also been noted earlier that the accumulation of pollutants in the host plant, as well as direct exposure to pollutants, lessen some aspects of the performance of chewing insects (Heliövaara et al., 1989). This indicates that the decreased performance of chewers may be due to the direct effects of pollutants on the insects, rather than to plant-mediated effects (Koricheva et al., 1998).

4.4. Effects of prolonged pollution on plant-herbivore interaction (IV)

Neither the survival rate nor the growth of moth larvae were affected by the different treatments. However, in female moths heavy metal pollution alone and combined metal pollution \times acid treatment decreased PO activity. In males the effect of the same treatments was the opposite: PO activity was increased. The encapsulation response of the moths was not affected by the different treatments. It has been noted previously that females may be more sensitive to stress (Rantala and Roff, 2007; Ruuhola et al., 2007; Yang et al., 2007).

One must note however, that crowded *E. autumnata* larvae that are reared in aggregation may cope better with low quality food than larvae that are reared solitarily. Responses of *E. autumnata* larvae to crowding can be regarded as adaptations to reduced amount and quality of the food at high larval densities (Haukioja et al., 1988b). In this substudy, larvae were reared in aggregation. It could explain at least partially, why we did not see differences in the growth of larvae between the four treatments.

The chemistry of mountain birch leaves was markedly altered by pollutants. The levels of digalloylglucose, gallic acid and quercetin/kaempferol ratio were enhanced by acid rain. The level of (ortho-diphenolic) chlorogenic acid was doubled by the acid rain treatment, while it was unaffected by the heavy metal treatment alone. As an o-diphenolic compound, chlorogenic acid is a common substrate of phenoloxidases and the oxidation of chlorogenic acid by foliar oxidases has been shown to contribute to the resistance of plants against herbivores (Bi et al., 1997b; Bi et al., 1997a; Felton et al., 1989; Felton and Duffey, 1991). The quercetin/kaempferol ratio was also enhanced by the combined CuNi \times acid treatment.

It has also been noted earlier in a long-term study that acid rain treatment may increase the amount of phenols in mountain birch (Suomela et al., 1998). The methods used to measure phenolic compounds in birch leaves have changed considerably since then, and only rough comparisons can be drawn with regard to compounds between this substudy and the study of Suomela et al. (1998). They found only one significant difference between treatments: the content of total phenolics was higher in the acid treatments than in the irrigated controls. Of the compounds that were significantly

changed by treatments in this substudy, chlorogenic acids were also measured by Suomela et al. (1998). In their study, however, acid treatments did not have a significant effect on the level of chlorogenic acids.

The accumulation of phenolic compounds as a response to pollution has also been observed in several other studies (Diaz et al., 2001; Michalak, 2006; Öncel et al., 2000; see also Roitto, 2006). Nevertheless, this accumulation does not necessarily have an adverse effect on the performance of herbivores (Roitto, 2006) if the pro-oxidative nature of phenolics is prevented by the presence of co-oxidants such as ascorbate.

We suggest that the increased levels of phenolic compounds, especially the increased level of chlorogenic acid and ellagitannins, act as a defense against the oxidative stress promoted by pollution. We further suggest that pollution also increases the level of non-enzymic antioxidants, such as ascorbate, which may act to reduce phenolic compounds, thus enabling the antioxidative recycling of phenolics. This recycling of phenolics also keeps the pro-oxidative nature of phenolic oxidation at a low level; we did not detect any adverse effects of enhanced levels of phenolics on the performance of moth larvae.

4.5. Local adaptation (V)

We found that the growth rate of moths was higher on control than on polluted leaves (*Betula pubescens*), suggesting that metal pollution reduced the growth rate of *E. autumnata*. These results are consistent with substudies II and III. We also found indications of local adaptation, as the strain \times treatment interaction was significant for growth (Fig. 5). It thus seems that pollution may cause locally varying selection pressures among moth populations. Pollution has costs

for the larvae, since growth is clearly poorer on polluted leaves than on control ones. According to Kawecki and Ebert (2004) divergent selection should cause each local population to evolve traits that provide an advantage under local environmental conditions, regardless of the consequences of these traits for fitness in other habitats. In our experiment, the growth of the non-polluted strain was much better than the growth of the polluted strain on control leaves. Thus adaptation seems to carry a cost, in the form of reduced performance of the polluted strain in a non-polluted environment. Growth on polluted leaves seems to be contrary to growth on control leaves: the growth of the polluted strain seems to be slightly better than that of the non-polluted strain on polluted leaves. The difference in growth of the polluted vs. non-polluted strain on polluted leaves is not significant, but the interaction strain \times treatment is significant and the growth lines cross between the two treatments.

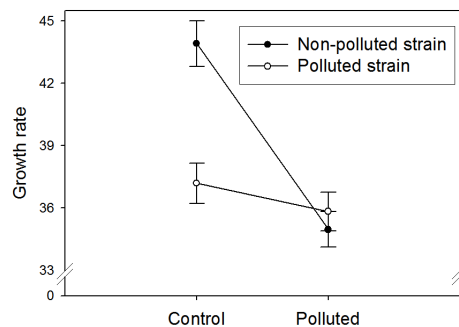


Figure 5. Relative growth rate of non-polluted and polluted strain (mean \pm SE) on control and polluted leaves. Sample sizes (n) were between 47 and 56.

There was no strain \times treatment effect on pupal weight; meaning that the differences in growth rate of the two strains do not

appear as differences in the final pupal weight of the larvae. It may be that in order to compensate for poor food efficiency, larvae in the polluted strain consume food longer before pupating than larvae in the non-polluted strain.

It has previously been shown that investment in immunity may diminish resources that are allocated to growth in insects (Rantala and Roff, 2005) and that the level of nutrition affects immunity (Kortet and Vainikka, 2008; Rantala et al., 2003). In our experiment, the strain \times treatment effect was not significant for the encapsulation rate, which means that – unlike the growth rate – there was no sign of local adaptation of immune function. However, there were clear differences in encapsulation rate between the sexes. The encapsulation rate of females was higher in the heavy metal treatment than in the controls, while male immunity was unaffected by the treatment.

5. CONCLUDING REMARKS

It is probably difficult for the autumnal moth to adapt to the chemical content of tree leaves, since the chemical content of leaves changes very dramatically during the growth season. We did not find additive genetic variation in the moth's ability to handle rapidly changing food quality of mountain birch. Instead, the moth has adapted a different strategy. It is a generalist, in the sense that it is able to consume numerous plant species. Its growth varies between leaves with different chemical content, but on average it performs well (I). Already the fact that it is a notorious outbreak species tells us that it is very resilient. During outbreaks it is able to defoliate mountain birches over huge areas.

Even the pollution arising from the factory complex at Harjavalta does not seem to affect the moth very much. Pollution decreases the growth of the larvae on *Betula pubescens* leaves, but the moth seems to be able to offset this by extending the time spent consuming leaves before pupating. Because of this long larval period, a moth feeding on inferior food has about the same pupal weight as one feeding on nonpolluted leaves. The moderate amount of pollution at Harjavalta enhances the moth's immune defense, which may actually benefit the moth: enhanced immune defense helps it to defend itself against parasites and diseases. Thus, when pollution increases the immune response in insects, the parasitism rates of insects may decrease in metal-polluted areas. This could lead to another outbreak of the moth (II and III). However, when there is a large amount of heavy metals such as nickel present, the immune defense of the moth may decrease. That is when the moth is very susceptible to pathogens and parasites (III).

The effects of Ni and Cu to the immune function of *E. autumnata* can be direct. If a

plant accumulates Ni and Cu from its environment, besides having plant-mediated effects to the plant eating herbivore, it has also direct effects (II and III).

Larval growth rates of phenotypes differed between metal-contaminated and control birches, suggesting the presence of additive genetic variance in the larval growth rate. We also found a tendency toward additive genetic variation in the encapsulation rate between environments. It thus seems that some traits of the insect have the potential to respond to selection in a polluted environment (II).

It is noteworthy that we found sex differences in the immune function of the autumnal moth. Heavy metal pollution decreased PO activity in female moths, but in males the activity increased (IV). Additionally, we found clear differences between the sexes in their encapsulation rate. The encapsulation rate of females was higher in the heavy-metal treatment than in the controls, while male immunity showed no effect of treatment (V). This supports previous findings showing sex differences in the effect of environmental and genetic factors on immunity in *E. autumnata* (Rantala and Roff, 2007). We suggest that this is probably a result of sex differences in the genetic architecture of the immune system.

In the substudy at Kevo, where we measured prolonged pollution effects on plant-herbivore interaction (IV), some of the results were contrary to those of substudies II and III. In the Kevo substudy, larval growth was not affected by the different pollution treatments. Likewise, the encapsulation response of the moths was not affected by the different treatments. However, in substudies II, III and V, which used another host tree, *Betula pubescens*, heavy metal accumulation in birch leaves was shown to decrease the growth of *E. autumnata* larvae.

Additionally, in substudies II and III the encapsulation rate was enhanced.

Since heavy-metal effects are mediated through a host plant, it is likely that the different host tree (*Betula pubescens* vs. *Betula pubescens* spp. *cserrepanovi*) plays a role in the effect of heavy metals on insect herbivores.

The variable results may also be due to the phenotypic plasticity of *E. autumnata*. Larvae may invest either in immunity or in body weight. Investments in immunity are costly, and may lead to a trade-off between immunity and another function (Rantala et al., 2007; Rantala and Roff, 2005). For *E. autumnata* females, high body weight also means high fecundity (Haukioja and Neuvonen, 1985; Tammaru et al., 1996); thus investment in a large body would be beneficial, at least in an environment with a low risk of parasitization or infection.

However, one must note, that the way the moths are reared may also have an effect on the growth of moths. Crowded *E. autumnata* larvae that are reared in aggregation may cope better with low quality food than larvae that are reared solitarily. Responses of *E. autumnata* larvae to crowding can be regarded as adaptations to reduced amount and quality of the food at high larval densities (Haukioja et al., 1988b). In the substudy IV larvae were reared in aggregation and in the substudies II and III larvae were reared solitarily. It could explain, at least partially, why we did not see differences in the growth of larvae between the four treatments in the substudy IV.

Pollution had also a large effect on the chemistry of mountain birch leaves. E.g., heavy metals had among other things an effect on the amount of phenolic compounds. However, accumulation of phenolics does not necessarily have an adverse effect on the performance of herbivores (IV).

Additionally, we found indications of local adaptation to metal pollution in *E. autumnata* growth rates at Harjavalta. With time, the potential to adapt might help the moth to adapt to locally varying pollution levels (V).

ACKNOWLEDGEMENTS

Many people have helped me in the course of this study. First of all, I wish to thank Erkki Haukioja, who introduced me to the fascinating field of the autumnal moth and its host trees. It was easy to start doing experiments under his guidance. He has taught me a lot about doing and writing science.

I further wish to thank my supervisor, Irma Saloniemi, who has been a great mentor for me. She has helped me with statistics and especially with SAS software, which was new to me since I had used SPSS during my MSc studies. I thank my other supervisor Markus Rantala. He has been an inspiring and constructive supervisor. I also have to thank him for hastening the process of finishing my thesis.

I am grateful to Julia Koricheva, who advised me on my first research plan and gave valuable comments on substudy II.

I thank all my other co-authors: Teija Ruuhola, Juha-Pekka Salminen, Sanna Haviola, Seppo Neuvonen, Sanna Pausio, Vesa Ruusila, Jean-Philippe Morin and Vladimir Ossipov. I also thank Sanna Haviola and Shiyong Yang for keeping me company during congresses abroad and Yang also for introducing me to Chinese customs.

I thank Saira Sillanpää, Kyösti Lempa and Lauri Kapari, who have worked in the same research group with me and have given me good advice about the study system.

I thank Kai Ruohomäki and Jorma Nurmi for providing me with autumnal moths for some of the experiments and Ulla Anttila for helping me in the field. I also thank Kai for lending me equipment to catch moths and giving me good advice about growing them.

Matti Ketola helped with all kind of computer-related stuff and Niina Kukko has helped with lots of practical arrangements.

I thank Ellen Valle for proofreading substudy I and this thesis, and Mirkka Jones for proofreading the revision of substudy I.

I am grateful to Outi Vesakoski for being a good friend and for her helpful comments on my manuscripts; to Riitta Koivikko, Marianna Riipi and Anni Tonteri for advice and friendship; and to Jenny Pettay, Anne Muola, Fiiia Haavisto, Laura Buggiotti, Päivi Lehtonen, Meri Lindqvist, Paula Lehtonen, Thomas Lilley, Tapio Eeva, Toni Laaksonen, Jouni Sorvari, Markus Ahola and Kalle Rainio for their friendship. I further thank all the other members of the Peggy group, which have not been mentioned before, for being such a good bunch for relaxation and fun; that way one does not have to think about experiments all the time.

When I've needed a break from thesis writing I've turned to Terhi Valtonen, Minna and Kristian for a nice chatting session.

I thank Jouni Toivola and Janne Kilpimaa for being good friends and for helping me relax with sports and conversation.

My parents have given me immense support during the time that I have studied biology in Utrecht, Jyväskylä and Turku. I am very grateful to them.

Finally, I would like to thank Reetta for her partnership during all these years and for being such a good person to lean on. She has given me much support during the writing of this thesis.

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