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HERBIVORY-RELATED VARIATION IN THE FOLIAR CHEMISTRY OF THE MOUNTAIN BIRCH (*BETULA PUBESCENS* SPP. *CZEREPANOVII*)

by

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“Toukka lähti heti paikalla etsimään syötävää. Maanantaina se söi reiän läpi omenan, mutta ei tullut vielä kylläiseksi. Tiistaina se söi reiän läpi kahden päärynän, mutta ei tullut vieläkään kylläiseksi. Keskiviikkona se söi reiän läpi kolmen luumun, mutta ei tullut vieläkään kylläiseksi. Torstaina se söi reiän läpi neljän mansikan, mutta ei tullut vieläkään kylläiseksi. Perjantaina se söi reiän läpi viiden appelsiinin, mutta ei tullut vieläkään kylläiseksi. Lauantaina se söi palan suklaakakkua, jäätelöä, kurkkua, juustoa, makkaraa, tikkukaramellia, leipää, nakkimakkaraa, leivosta ja melonia. Ja illalla sen vatsa tuli kauhean kipeäksi.”

Eric Carle ”Pikku toukka paksulainen”,
Suomentanut Kaija Pakkanen.

LIST OF ORIGINAL PUBLICATIONS

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

I Haviola S., I. Saloniemi, V. Ossipov, and E. Haukioja. 2006. Additive genetic variation of secondary and primary metabolites in mountain birch. Oikos **112**:382-391.

II Haviola S., S. Neuvonen, M. J. Rantala, K. Saikkonen, J-P. Salminen, I. Saloniemi, S. Yang, T. Ruuhola. 2012. Genetic and environmental factors behind foliar chemistry of the mature mountain birch. Journal of Chemical Ecology **38**:902-913.

III Yang S., S. Haviola, and T. Ruuhola. 2007. Temporal and spatial variation in mountain birch foliar enzyme activities during the larval period of *Epirrita autumnata*. Chemoecology **17**:71-79.

IV Haviola S., S. Neuvonen, M. J. Rantala, K. Saikkonen, J-P. Salminen, I. Saloniemi, S. Yang, T. Ruuhola: How does nitrogen fertilization affect the foliar chemistry of the mountain birch? Manuscript.

V Yang S., T. Ruuhola, S. Haviola, and M. J. Rantala. 2007. Temperature as a modifier of plant-herbivore interaction. Journal of Chemical Ecology **33**:463-475.

VI Ruuhola T., J. Salminen, S. Haviola, S. Yang, and M. J. Rantala. 2007. Immunological memory of mountain birches: effects of phenolics on performance on the autumnal moth depend on herbivory history of trees. Journal of Chemical Ecology **33**:1160-1176.

VII Haviola S., L. Kapari, V. Ossipov, M. J. Rantala, T. Ruuhola, and E. Haukioja. 2007. Foliar phenolics are differently associated with *Epirrita autumnata* growth and immunocompetence. Journal of Chemical Ecology **33**:1013-1023.

CONTENTS

1. INTRODUCTION

Trees show extensive variability on several levels, between species, populations and individuals; and there is also abundant phenotypic plasticity, involving differences between sites, seasons, age groups, branches, and even individual leaves within a tree. The foliar chemistry of individual trees has been shown to vary in several tree species like e.g. cypress (Cool et al. 1998), mountain birch (I, II), oak (Roslin et al. 2006), white birch (Laitinen et al. 2000), and willow (Nyman & Julkunen-Tiitto 2005). Foliage can also vary substantially between different branches of a single tree (Larson & Whitham 1997; Suomela et al. 1995; Fortin & Mauffette 2002). Considerable variation has been observed at scales larger than the individual tree, for example among stands growing in different habitats (Ne'eman 1993; Dudt & Shure 1994; Boege & Dirzo 2004; Yarnes & Boecklen 2005; Chacón & Armesto 2006). Likewise seasonal changes cause variation in the foliage quality (Salminen et al. 2001; Riipi et al. 2002; Ruuhola et al. 2003; Haukioja 2006; III; VI).

For insect herbivores variation makes forest foliage a patchy environment, where specializing is hard compared to the monotonic foliage of forest trees (Feeny 1975, 1976; Adler & Karban 1994; Karban 2011). Instead of relying on a single defence mechanism, such as producing only one toxin, multiple defensive mechanisms are by far more common for plants (Koricheva et al. 2004). Plants typically contain tens, if not hundreds, of secondary compounds; these compounds contribute to resistance both in themselves and through mutual interactions (Agrawal 2011).

Variation in tree foliage is the outcome of both genetic differences and environmental factors (Coley et al. 1985; Herms & Mattson 1992). Plant individuals may differ in the composition and / or abundance of compounds. Quantitative variation implies that several genes are needed to produce a trait (more specifically, to produce the observed variation in the trait) and / or that the environment affects the trait in such a way that differences arise between individuals (Falconer & Mackay 1996). In addition to genetics, there are several aspects of the environment which potentially affect a plant's foliar chemistry, including, for example soil properties; water, nutrient and light availability; wind and temperature conditions; and biotic factors such as herbivory, diseases and competition.

The observed phenotypic variation in traits, such as the chemical properties of foliage, can be divided into three categories: variation caused by environmental factors, by genetic factors, or by interaction between the two. The relative proportion of environmental and genetic variation provides a starting point for hypotheses as to how selection will and has structured the trait in a population (Falconer & Mackay 1996). The expected response to selection can be estimated by the equation $R = h^2S$, where R = response to selection, i.e.

the realized average difference between the parent generation and the next generation; h^2 = narrow sense heritability; and S = selection differential, i.e. the average difference between the parental generation mean and the mean of selected parents.

The most interesting part of genetic variation is additive genetic variance. This term refers to the quantitative change in a trait that is associated with the replacement of one allele with another. The most often used measure of genetic variation in quantitative traits is heritability, defined as the proportion of additive genetic variance scaled by total phenotypic variance. The breeding value is the deviation of an individual's phenotype from the population mean that is due to the additive effects of alleles. If an individual is mated to random individuals from the population, its breeding value for a given trait is twice the average deviation of its offspring from the population mean for that trait. Breeding value can also be seen as the heritable part of phenotypic variation (Falconer & Mackay 1996).

1.1. Plant responses to herbivory

In most cases herbivory affects plant growth negatively, but cases where the plant seems to be unaffected are also quite common. A tolerant plant has the ability to grow and reproduce, or to repair damages to a marked degree, despite the presence of herbivores (Schoonhoven et al. 2006). In some specific cases, a positive effect on plant growth has in fact been observed (Karban & Myers 1989; Schoonhoven et al. 2006).

The terms 'resistance' and 'defence' are used somewhat inconsistently in the literature. In this thesis, I restrict 'defence' to situations where the plant trait has evolved as an adaptive response in a population due to selection caused by herbivores. 'Resistance' refers to a trait that affects herbivores negatively; it is preferably used in cases where assumptions of the defensiveness of a plant trait are (as yet) unproven. Resistance includes not only active defence, where the plant is able to avoid or reduce damage due to herbivory, but also other, less straightforward changes in plants traits with a negative effect on herbivores (Karban & Myers 1989; Karban & Baldwin 1997).

The herbivory resistance of plants can be divided into direct and indirect resistance. Direct resistance reduces the herbivore's performance, while indirect resistance relies on the aid of the third trophic level (Schoonhoven et al. 2006). Indirect resistance includes cases in which plants attract the predators or parasitoids of a herbivore, or where plant traits affect the immune response of a herbivore in such a way as to make it more vulnerable to parasitoids and pathogens (Schoonhoven et al. 2006). While indirect resistance may be a significant part of plant resistance, it has as yet attracted less research attention than direct resistance (but see Paskewitz & Riehle 1994; Gorman et al. 1996; Rantala & Roff 2007; Klemola et al. 2008; Smilanich et al. 2009a-b).

Plant resistance can also be divided into constitutive and induced resistance. Constitutive resistance is always present in a plant, independent of the amount of damage, while induced resistance is exhibited only after exposure to herbivory (Levin 1976; Schoonhoven et al. 2006). Inducible resistance can be further divided into rapid and delayed inducible resistance. Rapid induced resistance (RIR) operates on a relatively short time-scale and affects the same insect generation that caused the damage; delayed induced resistance (DIR) affects the performance of the next herbivore generation (Haukioja 1991; Nykänen & Koricheva 2004), typically by affecting the next season's foliage of woody plants (Haukioja 1980; Haukioja et al. 1990; Schoonhoven et al. 2006), and may sometimes last for several years in mature trees (VI). Inducible resistance is suggested to create additional variation in the foliage of plants (Adler & Karban 1994; Pigliucci 2001; Karban 2011).

1.2. Plants as food source for herbivores

The compounds in plant leaves can be broadly divided into two categories: primary and secondary chemicals. The terms 'primary' and 'secondary' stem from a traditional dichotomy: primary chemicals include amino acids, nucleic acids, sugars and lipids, while secondary compounds are by products of plant metabolism (Taiz & Zeiger 1991). Secondary compounds can in turn be subdivided into such categories as phenolics, terpenoids and alkaloids (Taiz & Zeiger 1991; Schoonhoven et al. 2006).

Secondary compounds play multiple important roles in plants, from attracting pollinators to serving as defence compounds against a number of biotic and abiotic threats (Taiz & Zeiger 1991). The primary functions of phenolic compounds, especially flavonoids, have been suggested to lie in their anti- and pro-oxidative capacity and in the protection of plants against reactive oxygen species, ROS (Harborne & Williams 2000; Close & McArthur 2002; Salminen & Karonen 2011). ROS are harmful to membranes, DNA and other biomolecules, but in small quantities they also play a role in defence signalling (Droge 2002) and symbiotic interactions (Hamilton et al. 2012). Antioxidants suppress ROS, while pro-oxidants produce more of them. The same structural attributes that cause antioxidant capacity may also strengthen oxidative stress (Heim et al. 2002). Many other roles have also been suggested for phenolics, including participation in nutrient cycling (Kraus et al. 2003) and defence against photo-oxidation, i.e. oxidative damage to the cell induced by solar radiation (Close & McArthur 2002; Close et al. 2003).

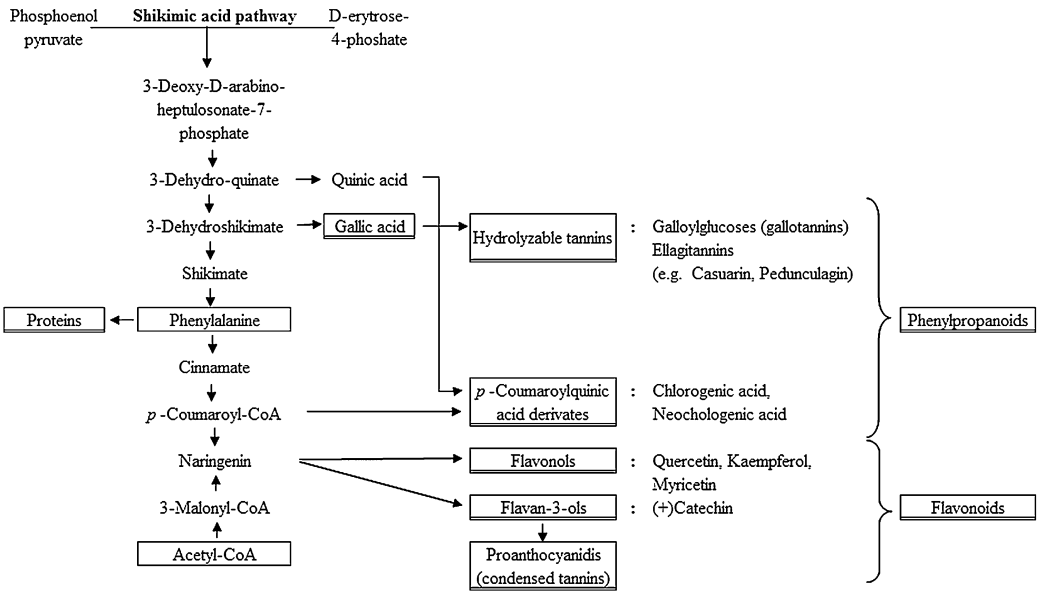

Figure 1. Biosynthetic relationships among birch phenolics (modified from Keinänen et al. 1999a, Loponen et al. 1998, Ossipov et al. 2003)

Phenolics have traditionally been considered to be defensive against herbivores (Fraenkel 1959; See Fig. 1 for groups of phenolics used in these studies). The specific mechanisms whereby phenolics provide herbivore resistance are rarely if ever known, but it is unlikely to be a simple dose effect. This is a complex issue, since individual phenolics differ in specific properties and herbivores have evolved to overcome the chemical defences of plants (Bernays et al. 1989; Bennett & Wallsgrove 1994). For example, the principal role of tannins has traditionally been thought to be the precipitation of proteins via hydrogen bonding. While this may occur under certain conditions, many insects have an alkaline midgut and / or surfactants in their gut fluid, which prevent the formation of insoluble protein-tannin complexes. An alternative idea regarding the defence mechanisms of phenolics is based on the observation that in the alkaline midgut phenolics auto-oxidize into quinones, which are highly reactive, toxic compounds (Martin et al. 1987; Appel 1993; Salminen & Karonen 2011).

The phenolics used in my studies can be divided into hydrolysable tannins, which have gallic acid as a precursor; para-coumaroylquinic acid derivates, which have quinic acid as a precursor; and flavonoids (Fig. 1; Loponen et al. 1998; Ossipov et al. 2003). All these phenolics come under the shikimic acid pathway. Ossipov et al. (2003) have suggested that in birch the active formation and accumulation of hydrolysable tannins can take place in young, actively growing birch leaves, because, in contrast to condensed tannins, the process of hydrolysable tannin synthesis is relatively short and less expensive.

Keinänen et al. (1999b) found that *B. pubescens* contains more individual flavonoids than other closely related birch species.

Primary chemicals, such as amino acids and sugars, are important actors in plant-herbivore interaction because they provide the herbivore's nutrition (Berenbaum 1995). The whole picture of the effect of different compounds on the herbivory resistance of plants is highly complex, given that different compounds are likely to interact. A herbivore feeding on highly nutritive food, for example, is more likely to tolerate the food's toxic elements than if the low nutritive status of the food is already forcing the herbivore to the limits of its capacity (Haukioja et al. 2002; Haukioja 2003). The same probably applies to other environmental stressors as well, such as non-optimal temperature, pathogens and pollution.

Besides phenolics and primary metabolites, plant oxidases, such as polyphenoloxidases (PPOs) and peroxidases (PODs), are important factors in determining the plant's suitability for herbivores. Phenoloxidases use phenolics as their substrates, converting them to highly reactive quinones that attach covalently for instance to amino acids. This reduces the nutritive value of the plant tissue for herbivores (Felton et al. 1989; Appel 1993). PPOs are located in plastids and phenolics in vacuoles (Vaughn & Duke 1981). The break-down of the cell structure, for example by a chewing insect, brings them together. The effect on the herbivore depends on the relative content of phenolics and amino acids in the plant tissues (Felton et al. 1992). The oxidation of phenolics produces toxic intermediates and ROS, which further interfere with the herbivore's ability to consume plant material, causing oxidative damage to the insect's midgut (Appel 1993). The role of plant phenoloxidases has been neglected in ecological studies of tree-insect interactions.

1.3. Heritability and implications of variation in foliar chemicals

The heritability estimates of secondary chemistry levels are typically high (Berenbaum & Zangerl 1992; Hamilton et al. 2001). Thus there seem to be plenty of opportunities for natural selection (Geber & Griffen 2003). High heritabilities among compounds of secondary chemistry have been obtained in for instance for coffee (Montagnon et al. 1998), oak (Klaper et al. 2001), aspen (Stevens & Lindroth 2005) and willow (Orians et al. 1996). On the other hand, the reported genetic variances in primary metabolite levels are generally low (Mitchell & Shaw 1993; Campbell 1996; Mitchell 2004 but see also Linhart et al. 2001; Mitchell 2004; Zangerl & Berenbaum 2004).

The presence of heritable variation in a resistance trait is a prerequisite for an evolutionary arms race between the tree and its herbivores (Futuyma 2009). However, if herbivores casued strong selection on resistance traits, heritabilities of the traits were expected to

decrease (Falconer & Mackay 1996). Therefore, high heritabilities do not necessary imply an arms race.

Most types of natural selection tend to decrease genetic variation. There are some exceptions to this rule: heterozygote advantage, a frequency-dependent selection, or some types of variable selection across habitats or time. Typically temporal fluctuation in the environment is only able to slow down the decrease of the genetic variation (Barton & Keightley 2002; Futuyma 2009).

Theoretical models allow predictions of the evolutionary trajectories of traits, if the strength of selection and the amount of genetic variation and their covariances are known (Falconer & Mackay 1996; Futuyma 2009). Classical models assume environmental constancy; this is however not true for most natural populations, which exist in variable environments. It has been found that environmental quality influences both the strength of natural selection and the genetic basis of trait variability (Price et al. 1984; Hoffmann & Merilä 1999; Hoffmann & Hercus 2000). Therefore the environmental dependence of the strength of selection and heritability may limit rates of evolution and maintain genetic variation (Wilson et al. 2006).

1.4. Mountain birch

The mountain birch, *Betula pubescens* spp. *czerepanovii* (Orlova (Hämet-Ahti) is the tree-line species and the dominant tree species in NW Europe (Kallio & Mäkinen 1978). It is adapted to live in a harsh subarctic environment, where the summers are short (the bud burst of the birch occurs in early June; senescence takes place in September) and the winters are harsh (the snow cover is around 60 cm in depth and the temperature often below -10 ºC). In addition, the subarctic soil is generally poor in nutrients due to the low rate of decomposition and rock mineralization (Karlsson & Weih 1996; Sveinbjörnsson et al. 1996). Mountain birch is a probably a hybrid subspecies of *B. pubescens* (Ehrh.) and has arisen via introgression from the dwarf birch (*B. nana* L.) (Elkington 1968; Vaarama & Valanne 1973; Anamthawat-Jónsson & Tómasson 1990).

Mountain birch has become one of the model tree species in plant-herbivore interaction studies (Haukioja & Hakala 1975; Haukioja 1980; Nykänen & Koricheva 2004). The autumnal moth is the most destructive herbivore of the mountain birch, but there are also several other herbivorous Geometridae and sawfly species that use the mountain birch as a host (Tenow 1972; Tenow 1996). Reindeer browsing is also substantial (Tenow 1972; Tenow 1996).

Tolerance is important for the long-lived mountain birch, for which successful reproduction is rare, but vegetative growth after even total loss of foliage is possible

(Haukioja 2006). However, if defoliation occurs over several consecutive years, the main trunks die. Even then, a genet has a chance to rejuvenate by sucker production (Vaarama & Valanne 1973; Kallio & Mäkinen 1978). A combination of reindeer browsing and a rot in the roots of the main trunks can destroy suckers, thus killing a large area of subarctic birch forest (Kallio & Lehtonen 1973; Lehtonen 1987; Lehtonen & Heikkinen 1995). The most common situation, however, is that herbivory does not kill the birch but reduces its biomass, thus affecting the tree's future growth and reproduction (Kaitaniemi et al. 1999).

1.4.1. Autumnal moth, the main herbivore

The most harmful invertebrate herbivore of northern mountain birch forests is the autumnal moth (*Epirrita autumnata* (Bkh.) Lepidoptera, Geometridae) (Tenow 1972). The autumnal moth is characterised by a cyclic population dynamic at intervals of circa ten years; during years of massive outbreak it can defoliate a whole birch forests, leading to the deforestation of wide areas (Haukioja et al. 1988; Tenow et al. 2007).

The overwintered eggs of the autumnal moth hatch synchronously with the birch bud burst, and all five instars feed on young and maturing leaves (Ruohomäki et al. 2000). There are several suitable deciduous host plants for the autumnal moth larvae, but the most typical host is the abundant mountain birch (Seppänen 1970). The larvae pupate in litter, and the adults emerge in the autumn. The females are relatively poor fliers and lay eggs indiscriminately (Ruohomäki et al. 2000). Many of the traits of the autumnal moth are typical of outbreaking species: polyphagous, spring-feeding larvae; poor female flying ability; eggs deposited in masses and overwintering eggs (Hunter 1995). The autumnal moth is host to several species of parasitoids (Ruohomäki 1994; Ruohomäki et al. 2000) and pathogens (Bylund 1995) which may play a role in the regulation of its population cycles (Klemola et al. 2008).

Autumnal moth larvae are specialized to young leaves. While compensatory feeding may enable larvae to survive on a sub-optimal diet of more mature leaves, an adequate growth rate can only be achieved on leaves that are sufficiently young (Ayres & MacLean 1987; Kause et al. 1999b). The growth rate of the larvae determines their pupation time and pupal mass (Tammaru 1998; Kause et al. 1999b), as well as−since adults do not feed−their fecundity (Haukioja & Neuvonen 1985b; Tammaru et al. 1996; Nykänen & Koricheva 2004).

Autumnal moth performance is affected by the host birch's genotype and by the habitat. Autumnal moth outbreaks are often locally restricted, originate from a relatively small area, and spread more widely as population densities keep rising (Haukioja et al. 1988; Ruohomäki et al. 2000). Outbreaks are most likely to be initiated in old birch forests (Ruohomäki et al. 1997). An outbreak is most likely to occur after a cold summer

(Niemelä 1980) and a (relatively) warm winter, since low winter temperatures kill the eggs (Kallio & Lehtonen 1973). The population densities of parasitoids and pathogens also need to be low to allow autumnal moth densities to rise.

1.4.2. Variation of foliar chemistry in mountain birch

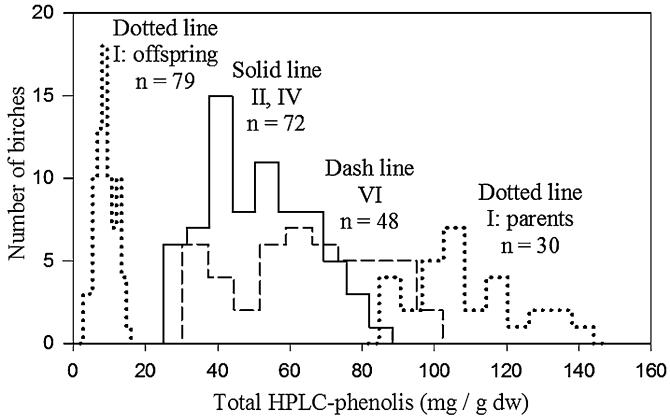

Figure 2. Amount of variation in total foliar phenolics of mountain birch in studies I, II, IV and VI. The area corresponds to the number of birches in a study.

The abundant phenotypic variation of mountain birch may slow down improvements in insect performance (Haukioja 2006). Figure 2 shows the amount of variation in total foliar phenolics of mountain birch found in my studies. Models of plant-herbivore interaction have shown that variance in plant quality can potentially influence the dynamics of herbivore population (Shelton 2004; Underwood 2004), and thus create an obstacle to herbivore adaptation to a particular host plant (Karban 2011). My studies (I; II; IV) together with earlier studies (Suomela et al. 1995; Nurmi et al. 1996; Riipi et al. 2002; 2004) have suggested that variation in mountain birch foliage is partly seasonal, partly environmental, and partly genetic. The relative importance of these different factors is not fully known. However, only heritable variation can be translated into evolutionary change, while phenotypic variation as a whole is important for herbivore performance.

Several lines of evidence support at least some genetic basis for differences in phenolics and other secondary chemistry. In studies by Haukioja et al. (1985) and Riipi et al. (2004) birch individuals showed clear differences in their leaf chemistry, while the chemical profiles of individual birch trees were consistent between years. Furthermore, data obtained from half-sibs indicated genetic variation (Ruohomäki et al. 1996). Mountain birch half-sib families had different frequencies of micro-fungi, indicating genetic differences in foliar properties (Ahlholm et al. 2002a-b). Studies with other birch species, such as the white birch, *B. pendula* Roth, also indicate a large genetic proportion

out of total phenotypic variation in foliar chemistry. The observations by Keinänen et al. (1999a) and Laitinen et al. (2000) that birches can be identified based on their foliar chemistry is compatible with underlying genetic differences.

Primary metabolites seem to display less genetic variation in the mountain birch than phenolics. Suomela (1995) found that most of the variation in amino acids and sugars occurred within birch individuals, while most of the variation in levels of phenolic compounds occurred between individuals. Riipi (2004) performed multiple chemical analyses both during a single summer and between years; she found that an individual tree was more easily identified by its phenolic profile than by its amino acid profile. It is apparent that phenotypic variation in primary metabolites is accounted for by other factors than genetic differences.

The temporal change in the quality of growing birch leaves explains a large part of the variance in herbivore performance (Ruusila et al. 2005). Carmona et al. (2011) have suggested that general plant traits, such as phenology and water content, constrain herbivores more than do plant secondary metabolites. In the subarctic, the timing of bud burst is of critical importance to the mountain birch (Aerts et al. 2006). If the buds open too early, they are susceptible to freezing; if they open too late, the brief growing season is not used efficiently and the risk of herbivory may be greater. After bud burst, the development of young maturing leaves is characterized by dynamic changes in the chemical profile of the foliage (Salminen et al. 2001; Riipi et al. 2002; Ruuhola et al. 2003; III). Seasonal variation during leaf maturation may cause most of the variation in mountain birch foliage (Haukioja 2006), making the trees heterogeneous 'moving targets' for selection over evolutionary time scales (Adler & Karban 1994; Roslin et al. 2006). Temperature affects herbivores both indirectly, for instance via phenological changes in the host plant, and directly, by influencing the growth rate of autumnal moth larvae (V).

Nitrogen is probably the major nutrient limiting the growth of mountain birches (Haukioja et al. 1985; Ruohomäki et al. 1996) since the subarctic soils are generally poor in nutrients (Karlsson & Weih 1996; Sveibjörnsson et al. 1996; Aerts et al. 2006), mountain birches have needed to adapt to low nitrogen availability. Soil properties affect the leaf chemistry of birches, and may even cause their high phenolic content (Bryant et al. 1983). Availability of nitrogen affects a plant's resistance to herbivory (Bryant et al. 1983; Herms & Mattson 1992; Jones & Hartley 1999). Thus nitrogen availability may affect both the phenolic content of birch foliage and the herbivore resistance status of mountain birches (Haukioja et al. 1985; Haukioja & Neuvonen 1985a; Ruohomäki et al. 1996; IV).

1.4.3. Defence of mountain birch against autumnal moth

The level of herbivore resistance varies between mountain birch individuals (Ayres et al. 1987; Senn et al. 1992). Herbivore growth has been consistently reported to suffer on

mountain birch trees defoliated during the previous years compared to undamaged ones (e.g. Haukioja & Niemelä 1979; Edwards & Wratten 1982; Wratten et al. 1984; Haukioja & Hanhimäki 1985; Hanhimäki & Senn 1992; Kapari et al. 2006; V). This induced resistance has been found to affect herbivores both in the same season (RIR) and in subsequent ones (DIR) (Haukioja et al. 1985; Haukioja & Neuvonen 1985a; Ruohomäki et al. 1992; Ruohomäki et al. 1996; Kaitaniemi & Ruohomäki 2001; VI). Induced resistance seems to be relatively high in mountain birches (Nykänen & Koricheva 2004). Although the chemistry of the mountain birch has been studied in detail, no individual compound by itself has been shown to be completely responsible for its herbivory resistance (Haukioja 2003; 2005; 2006).

One missing link between phenolics and birch resistance may be foliar phenoloxidases which convert phenolics into an active form, quinones. Birch PPO activity has been shown to be induced by herbivory (Ruuhola & Yang 2006; Ruuhola et al. 2008; Anttila et al. 2010; Ruuhola et al. 2011; V); this activity was negatively related to larval growth on damaged trees but not on control ones (Ruuhola et al. 2008).

Mountain birches emit large amounts of volatile organic compounds (VOCs), terpenes and green leaf volatiles. Haapanala et al. (2009) found high variation in tree-to-tree emissions, as well as variation between years. Mäntylä et al. (2008) suggest that elevated levels of emissions of many VOCs and especially terpenes in herbivory infected trees attract birds, functioning as an indirect resistance mechanism. VOCs of herbivore-damaged plants may also attract parasitoids and predatory mites.

1.4.4. Measuring the herbivore resistance of the birch

Herbivore resistance was estimated by measuring the performance of the main herbivore, the autumnal moth. I measured the pupal mass and developmental rate of larvae, which are strongly and positively correlated with the reproduction (Haukioja & Neuvonen 1985b; Tammaru et al. 1996; Tammaru 1998; Kause et al. 1999b).

In addition, the indirect herbivore resistance of mountain birch was estimated by measuring the immune response of the autumnal moth, as defined in terms of the encapsulation rate. This method has been widely used with other insect species (Paskewitz & Riehle 1994; Gorman et al. 1996; Smilanich et al. 2009a-b). In addition, Rantala & Roff (2007) found that the encapsulation rate is strongly associated with resistance against the entomopathogenic fungus, *Beauveira bassiana*; on the other hand, Klemola et al. (2008) found no association with natural parasitoid susceptibility. The indirect resistance of plants may be an important component in interactions between the plant, the herbivore and its natural enemies (Haukioja 2005; Kapari et al. 2006).

1.5. Aims of the study

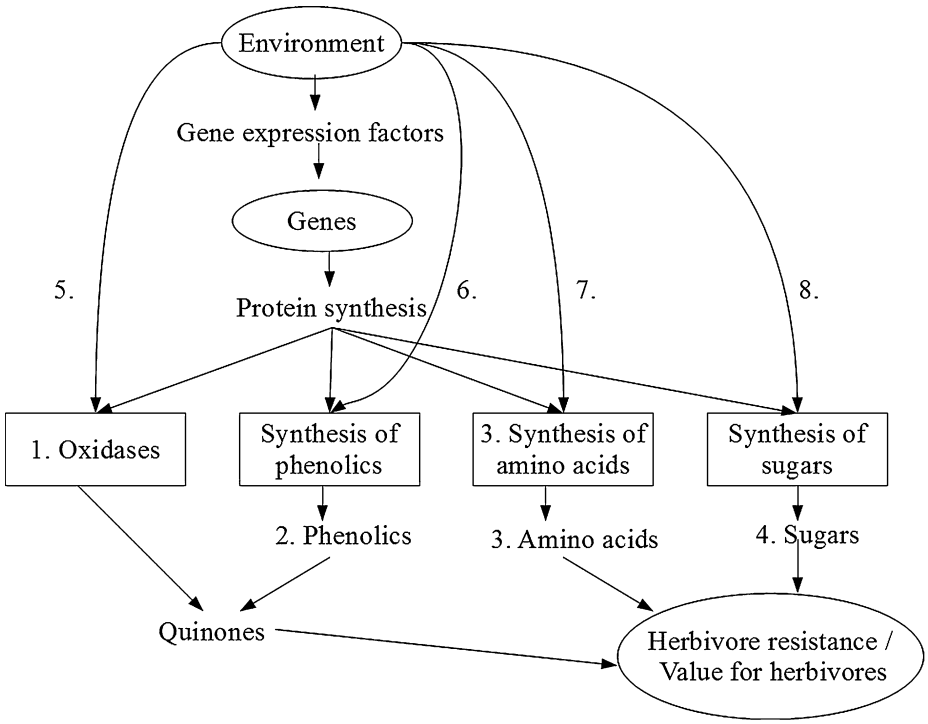

Figure 3. Relationships between genes, environment, different chemical properties and herbivore resistance of birch. In protein synthesis, amino acid chains are produced based on information in genes. Amino acid chains are further modified into functional enzymes. These enzymes produce e.g. phenolics, amino acids and sugars; some enzymes are phenoloxidases. The environmental affects which genes are turned on and off via gene expression factors. The combination of phenoloxidases, phenolics (→quinones), amino acids and sugars are the main determinants of herbivory resistance (or the nutritive value of a plant tissue for herbivores).

My aim was to study variation in chemical compounds in mountain birch foliage, and to estimate the genetic and the environment components of this variability (Fig. 3). I studied the **amount of genetic variation** in the following groups of compounds (Arabic numerals refer to Fig. 3, and Roman numerals refer to individual articles of this thesis):

1. Phenolics (I, II),
2. Phenoloxidases (II, III),
3. Amino acids (I, II),
4. Soluble sugars (I).

I also studied possible associations of different **environmental factors** (nitrogen availability, temperature, seasonal changes, presence of herbivores) with variation in the following compound groups (Numerals as above; see also Table 1):

5. Phenoloxidases: nitrogen availability (IV), temperature (V) and seasonal changes (III, VI), current (V) and previous presence of herbivores (VI);
6. Phenolics: nitrogen availability (IV), previous presence of herbivores (VI), seasonal changes (VI);
7. Amino acids: nitrogen availability (IV).

The final aim of my studies was to find out whether variation in mountain birch is of such a magnitude that it is relevant to insect herbivores. I studied **herbivore performance** in defoliated trees compared to undamaged ones (V, VI), and the association between herbivory performance and variation in the following compound groups:

phenoloxidases (II, VI),
phenolics (II, VII, VI),
amino acids (II).

Table 1. Overview of materials and methods. Number of birches in a study, sampling time of leaves for chemistry analysis, chemistry analysed, treatment of birches, type of study and presence vs. absence of autumnal moth larvae on the trees the summer the study was conducted.

study	birches	sampling of leaves	chemistry analysed	treatment	place	autumnal moth
I	79 (= progeny of 30 trees)	just matured	phenolics, amino acids, sugars	-	greenhouse	-
II, IV	72 (= 3 half-sib families)	just matured	phenolics, amino acid, oxidases	fertilization	tree-line garden	12 per birch
III	10	4 times (from young to mature)	oxidases	-	nature	-
III	10 (3 ramets / tree)	young and mature	oxidases	-	nature	-
V	10 (4 twigs / tree)	young	oxidases	temperature, current herbivory	laboratory exp.	yes
VI	24	young and mature	phenolics, oxidases	herbivory history	nature	3*12 per birch
VII	60	just matured	phenolics	-	nature	15 per birch

2. MATERIALS AND METHODS

2.1. Study area

The studies were conducted near the Kevo Subarctic Research Station of Turku University (69º45'N, 27º01'E). The area is characterised by gently sloping hills. The tops of the highest hills are treeless tundra. The valleys are covered by forest 3 to 5 m in height, dominated by mountain birch, but with rowan, willow, pine and aspen also occurring frequently. Crowberry, lingonberry and dwarf birch are also typical. The research station is at a relatively low altitude on the slope of a riverbank, where the cold kills most autumnal moth eggs during the winter (Kallio & Lehtonen 1973; Tenow & Nilssen 1990; Ruohomäki et al. 1997). Thus the foliage damage caused by autumnal moth larvae in the near vicinity of the research station is low, even during years of much higher autumnal moth population densities nearby at higher altitudes.

2.2. Methods

2.2.1. Leaf samples and foliar chemistry analyses

The birch has short shoots and long shoots. Short-shoot leaves form the majority of the foliage; long shoots are responsible for branch growth, and form new leaves as the shoot grows (Macdonald & Mothersill 1983). We used short-shoot leaves for the chemistry analysis, since they are even-aged (Macdonald & Mothersill 1983). Sampling times for leaves are shown in Table 1. The leaves intended for chemical analysis were sealed in plastic bags and taken to a laboratory in a cooler. They were weighed and freeze-dried for 72 h. The dried leaves were reweighed to determine their water content. The dry leaf samples from each tree were then homogenized to a powder and stored at -20 ºC prior to chemical analysis. For the enzymatic assays, the leaves were immediately flash-frozen in liquid nitrogen and stored at -80 ºC prior to analysis.

Phenolics were analysed by high-performance liquid chromatography with photodiode-array detection (HPLC-DAD). The phenolics were identified on the basis of their UV, mass spectra and retention times. Details of the extraction and analysis method for phenolics can be found in Salminen et al. (1999; 2001) for studies II, IV, VI and VII. The proanthocyanidin (= condensed tannin) content in studies I and VII was analysed by the method of Terril et al. (1992), with modifications as in Ossipova et al. (2001). In study I, individual phenolics were analysed by HPLC method as in Ossipov et al. (1997). The detailed method for chemistry analysis in study I can be found in Riipi et al. (2002), Ossipova et al. (2001) and Ossipov et al. (2001).

In studies I, II and IV amino acids were derivatized with 9-fluorenylmethyl chlorformat (FMOC-Cl) and analysed by the HPLC method (Bank et al. 1996). The derivatized amino acids were separated using a slightly modified ternary gradient system (Ossipov et al. 2001). The sugars in study I were measured using a gas chromatography method (Kallio et al. 1985).

Enzyme activities for studies II, III, IV, V and VI were analysed as in (Ruuhola & Yang 2006; Ruuhola et al. 2007). In study III POD and catalase (CAT) activities were measured with a spectrophotometer and changes in absorbance were followed at wavelengths of 470 nm and 240 nm respectively for 3 min. In study VI total POD activity was measured with 60 mM guaiacol (90 %, Sigma) as a substrate and 20 mM H_2O_2 as a co-substrate, and the rate of change at A470 was followed. The increase in absorbance was followed for 3.5 min (initial reaction velocity) with a spectrophotometer.

In study VI, combined acidic PPO and POD activity was measured with 200 µl 80 mM catechol (Sigma) as a substrate in (pH 5.8). The increase in absorbance over 10 min was read at 414 nm on a temperature-controlled 96-well plate reader (Multiscan Ascent, Thermo Electron, Shanghai, China). The reaction was started by adding 10 µl of the enzyme extract. Pure acidic PPO activity was measured similarly, as combined PPO and POD activity, but in the presence of a commercial catalase (Sigma, 280 U ml^{-1}), which removes H_2O_2 and inhibits POD activity. In studies II, III and IV acidic PPO activity was measured as increases in absorbance over 30 min and read at 450 nm on a Multilabel counter (Wallac 1420 Victor). In study V, pure acidic PPO activity was measured similarly at a wavelength of 420 nm and combined PPO and POD activity, but with 6.5 nM H_2O_2 as a co-substrate.

Alkaline PPO activity (II, III, and IV) was measured using 200 µl of 10 mM L-dopa (Sigma) as substrate, at pH 7.5, at 490 nm. The increase in absorbance over 30 min was read at 490 nm for L-dopa activities on a Multilabel counter (Wallac 1420 Victor) (Rantala et al. 2002).

The optima for foliar oxidase activities in study V were tested by measuring enzyme activities at temperatures ranging from +6 °C to +72 °C for PPO and from +6 °C to +58 °C for combined PPO and POD, which was measured in the presence of exogenous H_2O_2. The reaction time was 3 min.

All enzymatic measurements were performed in triplicate at +25 °C. The enzymatic activities were measured as absolute activity (dAbs min^{-1} ml^{-1}), specific activity (U mg $protein^{-1}$) and activity per fresh weight (U mg fresh $weight^{-1}$) (III, V). One unit is the amount of enzyme required to increase the absorbance by 0.001 min^{-1}.

The protein concentrations of the enzyme extracts were measured as in Ruuhola & Yang (2006) in triplicate, using the BioRad protein assay method based on the Bradford method (Bradford 1976); absorbance was measured with the Multilabel counter (Wallac 1420 Victor) at 595 nm (II, III, IV, V, VI).

2.2.2. Autumnal moth, the herbivore

Bioassays were performed both in the laboratory and in the field. At the time of leaf flush, we released newly hatched autumnal moth larvae onto the trees in mesh bags (IV, VI, VII, and see Table 1). In the laboratory experiment of V, the larvae were fed with fresh birch leaves *ad libitum* and kept in plastic vials under a regime of natural light and temperature. At the end of the larval period, when the larvae were preparing to pupate, they were placed in moist *Sphagnum* moss for pupation. After seven days the pupae were weighed and sexed and their immune response was measured (VI, VII). The strength of the pupal immune response was estimated in these studies by inserting a nylon filament into the pupal hemocoel and calculating the encapsulation rate (detailed methods in Kapari et al. 2006; Rantala et al. 2002; van Ooik et al. 2007). The larval development rate was defined as the reciprocal of the duration of the larval period (in days) (Kause et al. 1999b).

2.2.3. Genetic components of variation

Heritabilities and coefficients of additive genetic variance were calculated from the variance components in studies I and II. Heritabilities (h^2) were calculated as

$$h^2 = V_A / V_P = (4 * \text{covariance due to half-sib family}) / (\sum \text{all covariance components}).$$

Coefficients of genetic variation (CV) was calculated for additive genetic variance (CV_A) and phenotypic variation (CV_P) for easier comparison of variances (Houle 1992).

$$CV_A = [\text{sqrt}(V_A)] / \text{trait mean}$$

$$CV_P = [\text{sqrt}(V_P)] / \text{trait mean}$$

In study II, the percentages of observed variation explained by the half-sib family were calculated. In study III, I divided variance hierarchically into different levels, i.e. the time of sampling, tree (nested under time) and error term, which includes variation within trees and measurement error. The proportion of variation (%) explained by a variable was calculated as

$$[\text{covariance due to variable} / (\sum \text{all covariance components})] * 100\ \%.$$

The coefficients of variation (CV) were calculated as

CV = (sqrt variance component / trait mean) * 100 % and

CV = (sd / trait mean) * 100 %.

2.2.4. Statistics

I used linear models to estimate the components of variance for continuous birch traits in study I. I applied a binary transformation (Falconer & Mackay 1996; Roff 2001) before analyses with generalised linear models to some traits that could not be normalized with logarithmic transformation. The jackknife method (Miller 1974; Dixon 2001) was used to measure standard errors and calculate biases. In this method half-sib families are removed one by one from the data set (Knapp et al. 1989). To estimate the components of variance and the effects of family on birch traits in study II, I used a linear mixed model. Seasonal variation was analysed with repeated ANOVA, and within- and among-tree variation was analysed with hierarchical ANOVA (III). The effect of fertilization on birch characters was analysed by a linear models, I also tested the effect of herbivory, and used a mixed model to determine whether the nitrogen fertilization of birches affected the larval characters of a herbivore (IV).

Larval growth was analysed with generalized mixed linear models (V). The effect of temperature on the duration of instars was tested with the nonparametric Kruskal-Wallis test (V). The effects on enzyme activities of temperature, defoliation treatment and their interactions were tested with generalized mixed linear models (V). The effects of a defoliation treatment on larval traits were tested with mixed-model analysis or with the Kruskall-Wallis nonparametric test (VI). The effects of the defoliation treatment on the contents of phenolic compounds were tested with mixed-model analysis (VI). The effect of defoliation treatment on enzyme activity was tested with one-way analysis of variance (VI).

Phenotypic correlations were calculated as Spearman's non-parametric correlations, as the chemistry traits were not normally distributed (I, II, IV and VII) or with Pearson's two-tailed correlation test (VI). In study VII I used bootstrap analysis to estimate the 95 % confidence limits for individual correlation coefficients (Dixon 2001). Bootstrap estimates the precision of the correlation coefficient by creating random resamples from the data. With bootstrap the distribution of the data is better taken into account than with the Bonferroni correction. Before calculating the correlations I computed the tree-specific least-square mean values for birches with regard to the larval variables (II, IV and VII). The correlation coefficients were analysed with the univariate analysis of ANOVA, and the differences between correlations were tested using the Tukey test (VI).

3. RESULTS AND DISCUSSION

3.1. Birch genotype does make a difference

Half-sib family	"40"	"47"	"45"
Short shoots per birch	3900 ± 3300	3300 ± 2500	2000 ± 1600
Long shoots per birch	230 ± 370	580 ± 580	260 ± 330
Phenology of leaves	maturest	middle	youngest
Natural herbivory percentage	1.1 ± 0.4	1.9 ± 1.4	2.3 ± 2.6
Galloylglucoses (mg / g dw)	5.0 ± 2.9	11 ± 4.9	12 ± 5.6
Ellagitannins (mg / g dw)	12 ± 7.1	19 ± 10	21 ± 9.8
Hydrolysable tannins (mg / g dw)	17 ± 9.6	29 ± 15	33 ± 14
Flavonoid glycosides (mg / g dw)	14 ± 2.4	13 ± 4.5	18 ± 3.5
Phenylpropanoids (mg / g dw)	26 ± 9.8	37 ± 14	43 ± 14
p-coumader acid der. (mg / g dw)	9.7 ± 3.4	7.7 ± 3.5	9.3 ± 2.0
Total phenolics	low	middle	high
Oxidative activity	middle	low	high

Figure 4. Some differences between mountain birch half-sib families in study II. Mean values ± Standard deviation. Der. = derivate.

The findings of this thesis show considerable phenotypic variation in phenolic compounds (as shown in Fig. 2) and phenoloxidase activities of mountain birch; they also show that most of this variation has a genetic origin (I, II). Birch families are different from each other, agreeing with underlying genetic differences. Fig. 4 summarizes the differences between growth type and foliar properties of the mountain birch families in study II. Fig. 5 shows variation between mountain birch families in total phenolic content, ranked by maternal and paternal family means in study I. Along with clear differences between families, the figure also shows variation arising chiefly out of differences in family size. Study III suggests the existence of genetic differences in acidic PPO activity, which deters herbivory performance (Ruuhola et al. 2008). On the other hand, no evidence was found in genetic differences in POD, alkaline PPO or CAT activities (III). Unlike phenolics and phenoloxidases, amino acids and sugars showed almost no genetic variation (I, II).

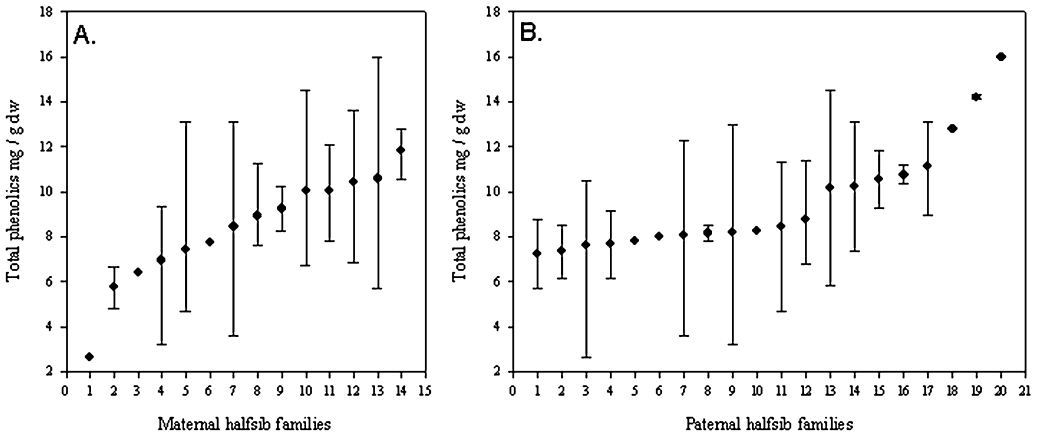

Figure 5. Mean total phenolic content of mountain birch half-sib families (based on study I). A.) Maternal half-sib families. B.) Paternal half-sib families. Minimum and maximum values of the family are also shown as error bars.

The large genetic variation in the contents of phenolic compounds and phenoloxidase activities provides the basis for natural selection against herbivory and for coping with the unpredictable subarctic environment. Phenolics and phenoloxidases have other important functions in plant cells besides herbivory resistance, such as preventing oxidative stress (Close & McArthur 2002). Slow-growing and long-living mountain birches in boreal forests are exposed to low temperatures and high light intensities. The trees thus have a high risk of photodamage, i.e. oxidative damage to the cell due to solar radiation (Close & McArthur 2002).

The basis for the high genetic variation of mountain birch has been suggested to be its likely hybrid origin (Elkington 1968; Vaarama & Valanne 1973; Anamthawat-Jónsson & Tómasson 1990). Hybridization has been shown to increase secondary metabolite variability and herbivore resistance in several other hybrid species (Cheng et al. 2011). Introgression from the dwarf birch (*B. nana* L.) to *B. pubescens* probably started during the last glacial period and early Holocene (Makela 1998; Caseldine 2001). Hybridization stores up abundant new genetic variation in a species, variation which takes many generations to prune (Arnold 1997). Not many birch generations has passed since the last glacial period, as a birch genet can live hundreds of years (Vaarama & Valanne 1973; Kallio & Mäkinen 1978). The low number of sexual generations since hybridization some thousands of years ago may have left the present-day variation far above the level predicted by theoretical models for equilibrium populations of the same size and selection pressures.

Most likely mountain birches have encountered different and varying selection pressures in different habitats and in different years. The changing parameters of natural selection

slow down the disappearance of variation from the population (Falconer & Mackay 1996; Wilson et al. 2006; Futuyma 2009). It is therefore likely that the variation stored in the mountain birch population by hybridization will remain there a long time without any specific ways to maintain variation.

The measurement of additive genetic variance and heritabilities in two separate studies with different individuals (I, II), gave very similar results, even though the birches in study II were mature trees (28 years old) growing in a tree-line garden, while those in study I were only a few months old and growing in a greenhouse. In study I, the birches were hand-pollinated; in study II, they were allowed to naturally wind-pollinate. The phenolic content was around three times higher, and the amino acid content twice as high, in the mature trees of study II, compared to the young greenhouse trees in study I. It might be assumed that birches growing for decades in the wild would show much more phenotypic variation than young saplings in the greenhouse, and than the tree-line garden experiment (II) would thus show lower heritabilities (Falconer & Mackay 1996). Contrary to this assumption, however, high heritability values were obtained in both studies (I, II).

3.1.1. Seasonal variation in foliar properties

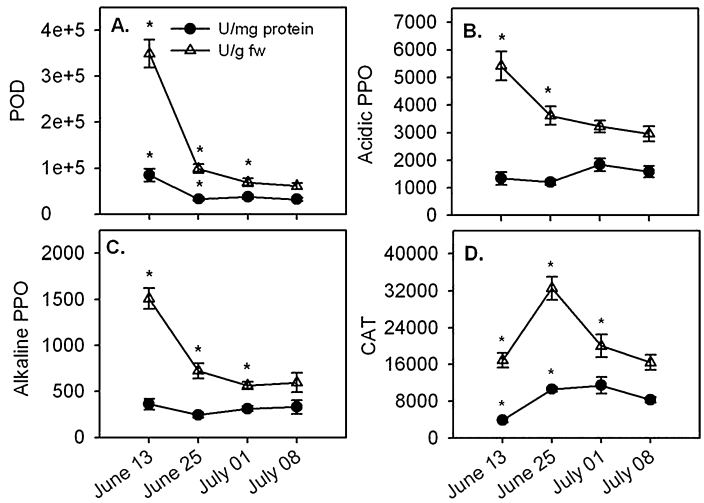

Figure 6. Seasonal variation in activities of peroxidases (PODs) (A), acidic (B) and alkaline polyphenoloxidases (PPOs) (C) and catalase (CAT) (D) in mountain birch leaves. Bars represent S.E. Sampling dates marked with asterisk differ significantly from each other.

Foliar enzyme activities changed markedly during the early summer (Fig. 6). Phenoloxidase activities (PODs, acidic and alkaline PPOs) were high in young leaves and declined with leaf maturation. This decline in POD activity was not the simple

dilution effect of leaf growth, but a down-regulated process (III). The decrease in acidic and alkaline PPO activities with leaf maturation, on the other hand, seemed to be mainly caused by tissue volume growth (III). Contrary to PPO activities, the activity of antioxidative CATs was low in young leaves but increased sharply, reaching its highest activity at the time when the leaves were full-grown and declining thereafter (III). This is to my knowledge, the first study dealing with seasonal variation in foliar phenoloxidative activities, whether in birch or in other species.

These results indicate that in the process of leaf maturation the quality of birch leaves changes dramatically. Thus autumnal moth larvae need to adapt via phenotypic plasticity and / or via evolutionary genetic changes to a continuously changing food source. It is more difficult for an insect herbivore to adapt to a changing food source than to a consistent one (Adler & Karban 1994). The timing of leaf flush (bud break) of different birch individuals varies slightly, and is thus one major source of among-tree variation in mountain birch populations (Ruusila et al. 2005; Haukioja 2006), since the quality of leaves for herbivores changes rapidly with leaf development (Salminen et al. 2001; Riipi et al. 2002; Ruuhola et al. 2003; III).

3.2. Different environmental aspects in relation to birch foliar chemistry

3.2.1. Nitrogen availability and foliar properties

According to the carbon nutrient balance hypothesis, the herbivory resistance of plants is determined by the environment, more specifically by the supply of carbon and nutrients (Bryant et al. 1983; Tuomi et al. 1988). This hypothesis postulates that the allocation of resources between plant functions (e.g. growth or resistance) is controlled by the ratio of carbon and nutrients acquired by the plant. Accordingly, fertilization or shade will decrease the plant's carbon nutrient ratio, reducing excess carbon production and consequently lowering carbon-based resistance such as phenolics.

In study IV, nitrogen fertilization had only a relatively small effect on the phenolic content of birch leaves. The levels of gallic acid, a sub-unit of hydrolysable tannins, and of one flavonoid compound, quercetin glycoside, were lower in nitrogen fertilized trees. Two compounds, (+)-catechin, a sub-unit of condensed tannins (CTs), and pedungulacin, the most abundant ellagitannin in the mountain birch leaves, were in turn higher as a response to nitrogen fertilization. Neither the total amino acid content nor the activities of leaf phenoloxidases were affected by nitrogen fertilization (IV).

The results also contradict the carbon-nutrient balance hypothesis: herbivory defences, measured in terms of the performance of the autumnal moth, were not lower after nitrogen fertilization, as predicted by the CNB hypothesis (IV). We also observed no

changes in the degree of natural herbivory damage in relation to nitrogen supply, while there was a strong positive correlation between the degree of natural herbivory and the contents of phenolic compounds (II).

Nitrogen fertilization improved the growth of birches (IV). This clearly indicates that the fertilization treatment was successful and that the nitrogen doses used were biologically relevant. Changes in chemistry would thus have been observed if they had occurred after fertilization.

While the level of total phenolics has been shown in many studies to decrease as a response to fertilization (Ruohomäki et al. 1996), nitrogen fertilization does not affect all individual phenolics the same way (Keinänen et al. 1999a; Graglia et al. 2001; Witzell & Shevtsova 2004; Keski-Saari et al. 2005). Nitrogen fertilization has been shown to reduce the level of soluble condensed tannins (proanthocyanidins) in *Betula* species (Haukioja et al. 1985; Ruohomäki et al. 1996; Keinänen et al. 1999a; Keski-Saari et al. 2005). On the other hand, insoluble, cell-wall-bound condensed tannins do not react consistently to fertilization (Henriksson et al. 2003; Keski-Saari et al. 2005).

3.2.2. Other abiotic and biotic factors

In the tree-line garden the environment was quite homogeneous, and all the trees had been planted in the same year (year 1977). The topography (or environmental factors related to it, such as temperature) nevertheless affected tree architecture: the trees were taller, and tended to have more long and short shoots, at the lower, moister end of the garden (II). We also observed some systematic trends in biotic interactions in relation to topography: natural herbivory damage, as estimated visually in terms of leaves eaten by herbivores, was most abundant at the drier, sandier and steeper high end of the garden, while at the lower, moister end pathogenic rust was more prevalent and the main insect herbivore, autumnal moth, performed better (II).

Herbivory can be seen as part of a tree's environment, since an individual tree has no ways to escape herbivores in its growing place. In study V, we found that birch foliar PPO activity was affected by both temperature and herbivory. Herbivory reduces acidic PPO activity in the cold, but in warm weather acidic PPO activity was increased by herbivory. Higher temperatures increased combined PPO and POD activity and CAT activities. POD activities were not affected by either temperature or herbivory (V).

3.3. Does variation in birch traits affect herbivorous larvae?

3.3.1. Changes in birch leaves lead to reduced growth of herbivores

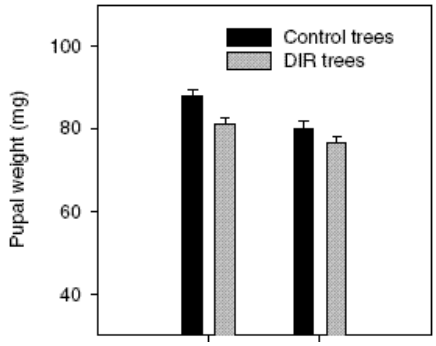

Figure 7. Pupal mass of autumnal moth on control trees and trees which also contained herbivorous larvae five years earlier (DIR). Females shown on the right, males on the left.

Herbivory induced some changes in the chemistry of birch leaves. These changes led to poorer larval growth (V, VI, Fig. 7), implying change in the quality of birch foliage for herbivores. Interestingly, we found that this response to herbivory in birch leaves can be very rapid (RIR), reducing herbivore growth as soon as twelve hours after placement of the herbivores on the leaves (V). Intriguingly, this effect is also long-lasting (DIR): trees that had experienced high herbivory five years earlier were still inferior as food for the autumnal moth compared to the control trees; the pupal mass of the autumnal moth remained 10 % lower in trees which had encountered larvae five years earlier (VI, Fig. 7). A number of studies of mountain birch have observed rapid induced resistance (RIR) (Haukioja & Niemelä 1979; Edwards & Wratten 1982; Wratten et al. 1984; Haukioja & Hanhimäki 1985; Hanhimäki & Senn 1992; Kapari et al. 2006) or delayed induced resistance (DIR) (Haukioja et al. 1985; Haukioja & Neuvonen 1985a; Ruohomäki et al. 1992; Ruohomäki et al. 1996; Kaitaniemi & Ruohomäki 2001).

The interpretation was that even small (induced) changes in foliar chemistry are able to produce a marked change in the herbivore's performance. It was hypothesized that ROS are behind the growth retardation of autumnal moth larvae on previously damaged foliage (VI). VI is possibly the first study in which herbivory has been shown to affect the ratio of dihydroxylated to monohydroxylated flavonoids (quercetin to kaemferol). (Monohydroxylated phenols are oxidatively weaker than di- or trihydroxylated phenols.) This may indicate an increase in oxidative stress in the leaves of trees that have previously encountered herbivory. Perhaps phenolics and phenoloxidases produce more reactive oxygen species in trees encountering herbivores five years earlier than in control trees. The ratio of dihydroxylated to monohydroxylated flavonoids has been reported to

increase in response to abiotic stresses, such as UV-B radiation, in many plant species; this increased ratio is suggested to be a defence against ROS (Markham et al. 1998a-b; Liu et al. 1995; Olsson et al. 1999).

3.3.2. Relationship between foliar phenolics and herbivore growth

Table 2. Correlations between autumnal moth growth or pupal mass and the most relevant birch traits for moths: phenolics, water, protein and sugar. Ossipov et al. (2001) used silver birch (*B. pendula*); the others used mountain birch. In Ossipov et al. *E.a.* = *Epirrita autumnata*. In Haukioja et al. (2002) 'young', 'normal' and 'mature' refers to the age of the leaves. In study VI, 'young' and 'mature' refers to the age of the leaves, F means female larvae, M male larvae and herbivory h. is herbivory history.

n	galloylglucoses (=gallotannins)	ellagitannins	gallic acid	*p* -coumaroylquinic acid derivates	flavonoids	catechins	soluble proanthocyanidins (=condensed tannins)	cell-wall bound proanthocyanidins (=condensed tannins)	water	proteins	sugars	treatment	study
42	-0.18						-0.21	0.18		0.62 *	0.54 *	insectiside	Kaitaniemi et al. (1998)
42	-0.41 *						0.07	-0.01		0.58 *	0.35	defoliation	
15	-0.18						-0.45	-0.20		0.42		insectiside	
15	0.25						-0.07	0.31		0.15		control	
12	-0.52			-0.39		0.08	-0.21	-0.16	-0.11	-0.03	0.42	younger leaves	Kause et al. (1999)
14	0.14			0.17		0.06	-0.52 *	-0.20	0.49	0.24	0.13	older leaves	
26	0.08			-0.03		-0.07	-0.34	-0.20	0.55 *	0.61 *	0.03	mature leaves	
40	-0.04						-0.39 *	-0.14		-0.04	0.24	pooled	Ossipov et al. (2001)
21	-0.48 *						-0.30	0.09		0.32	-0.40	good for *E. a.*	
19	-0.18						-0.09	0.35		-0.15	0.34	poor for *E. a.*	
3*30	0.25 *		0.47 *	-0.09	0.44 *	-0.12	-0.49 *	-0.09	0.75 *			pooled data	Haukioja et al. (2002)
30	0.04		-0.06	0.07	-0.12	-0.05	-0.45 *	-0.27	0.29			young	
30	-0.17		0.09	-0.02	0.13	-0.31	-0.24	-0.02	0.64 *			normal	
30	0.01		0.14	0.06	0.01	-0.03	-0.05	-0.01	0.55 *			mature	
40	-0.26			-0.64 *	-0.29	0.07	-0.70 *	-0.23	0.70 *	0.53 *	-0.36 *	pooled	Henriksson et al. (2003)
20	-0.08			-0.41	-0.03	0.04	-0.44 *	0.04	0.45 *	0.33	0.46 *	control	
20	-0.74 *			-0.30	-0.23	-0.12	-0.47 *	0.03	0.41	0.20	0.02	shade	
12	-0.25	-0.23	-0.27		0.32	0.03						young F control	VI
12	-0.17	-0.82 *	-0.10		0.28	-0.45						young F herbivore h.	
12	-0.14	-0.08	-0.44		-0.28	0.37						young M control	
12	0.04	-0.72 *	0.02		0.08	-0.49						young M herbivore h.	
12	-0.34	-0.15	0.16		0.51 *	-0.44						mature F control	
12	-0.24	-0.49	-0.28		0.28	-0.67 *						mature F herbivore h.	
12	-0.03	0.07	0.01		-0.32	-0.12						mature M control	
12	-0.15	-0.35	-0.46		0.01	-0.58 *						mature M herbivore h.	
60	0.08 *	0.08 *			0.06 *		0.09						VII
60	-0.15	-0.29	0.08	0.08	0.20	-0.14							II

Does the observed variation in foliar phenolics affect the autumnal moth? Associations tend to vary from one study to another probably due to differences in birch phenology, which drastically affects both foliar chemistry and larval performance (Table 2; Haukioja

et al. 2002). There are, however, certain consistent trends: for instance a high content of galloylglucoses (= hydrolysable tannins) and soluble proanthocyanidins (= condensed tannins) reduce autumnal moth growth (Table 2). Cell-wall-bound proanthocyanidins do not have the same effect as soluble proanthocyanidins. The biosynthetic relationships between the groups of chemicals are shown in Fig. 1.

High water and protein contents, which are traits of young leaves, are on the other hand beneficial to herbivorous larvae (Table 2). The phenology of a tree is the result of interaction between temperature and genes. Genes affecting the phenology of a tree may pleiotropically affect its herbivore resistance status. Surprisingly, flavonoids are positively associated with autumnal moth growth (Table 2); this may be caused by the good quality of young leaves as a food for the autumnal moth, even taking into account the flavonoid peak (Riipi et al. 2002).

Table 2 shows the relationships between foliar properties and herbivore performance. It summarizes the results of studies (II, VI, and VII), together with those of earlier studies (Kaitaniemi et al. 1998; Kause et al. 1999a; Ossipov et al. 2001; Haukioja et al. 2002; Henriksson et al. 2003). The table shows correlations between autumnal moth growth and the content of phenolics, water, proteins and sugars of birch foliage in studies published previously and in this thesis.

3.3.3. Are foliar phenolics associated with indirect resistance of plants?

The immune response of an insect can be used to measure the indirect resistance status of a plant. In study VII the correlations between the immune response of the autumnal moth and the hydrolysable tannins of the birch showed a negative association. There was no association between other groups of phenolics and the immune response of the autumnal moth. The results of study VI are more complex. The immune response of male moths tended to be positively related to the contents of phenolics in trees defoliated by herbivory five years earlier, but negatively in the control trees. On the other hand, female moths tended to show a negative relation in trees defoliated by herbivory five years earlier, but a positive one in the control trees. The genetic differences between males and females may lead to differences in the effects of phenolic compounds on the immunity of males and females (Rantala & Roff 2007).

The broader the scope of the research – in this case including other trophic levels within the framework of the study – the more multidimensional the resistance of plants seems to be. This is partly demonstrated by comparing the results of individual studies: minor differences in experimental setup may cause a cascade, leading ultimately to a different outcome of the experiment.

3.4. Results in an ecological context

The ecological context of birch trees is constantly changing. At the moment, the winter moth (*Operophtera brumata* L.), another harmful outbreaking Lepidopteran, is spreading to the Kevo study area, perhaps due to warming temperatures (Jepsen et al. 2008; Ammunét et al. 2011, 2012). It is not yet known whether there are differences in birch resistance to the autumnal moth and the winter moth. If the correlations between resistances against the two herbivores are positive, then the factors promoting birch resistance to one species will also provide protection against the other. Interestingly, it has been observed that birch resistance to the autumnal moth has a negative genetic correlation with resistance to birch rust (Ahlholm et al. 2002a). Thus, if autumnal moth herbivory constitutes a selection pressure on the birch, the trees will simultaneously become more vulnerable to rust. Birch rust can kill vulnerable saplings, and autumnal moth damage may lead to the death of the birch. Thus there is likely to be pressure for selection towards resistant birches regarding both threats.

Global warming has the potential to profoundly change subarctic ecosystems, including the mountain birch population. Rising temperatures will shift subarctic tree-lines and alter the distribution of plant species. It is critical for the survival of the birch in the arctic to sense timely seasonal cues: bud burst and senescence need to be optimized to the right combination of temperature and day length. Spring herbivores, the autumnal moth included, try to synchronize their larval period with the availability of young mountain birch leaves. A warming climate may affect birch bud burst and the hatching of autumnal moth eggs in a different way. Global warming or increased human interference, with longer frost-free periods, may improve nitrogen availability in a nutrient-poor soil. It is clear that selection pressures on the mountain birch can and will change on an ecological timescale.

Changes in the environment not only affect birches directly, but also affect present and potential new herbivores, and thereby, the amount of herbivory, birches have to tolerate. The environmental effects on herbivores are clearly seen during outbreaks; whether the birches are totally defoliated depends on the elevation. The lowest winter temperature forms a gradient, along which autumnal and winter moth eggs either survive or die (Jepsen et al. 2008). Since the dispersal of the larvae is restricted, this line holds even during massive outbreaks. The autumnal moth is also affected by other environmental factors, such as nitrogen availability in the soil, although less conspicuously.

If, in the future, more severe and / or frequent defoliations threaten mountain birch forest ecosystems, there might be a need for practical action / applications to conserve the mountain birch. There seems to be no gain to be achieved by transplanting birches to increase the genetic variation in populations, nor by fertilizing them with nitrogen. If

birch forests are in danger, I believe that one of the best ways to improve the situation would be to limit overgrazing by reindeers, thus enabling the rapid regrowth of birches after defoliation.

4. CONCLUSIONS

1. Additive genetic variation of birch foliar phenolics and phenoloxidase activities was high (I, II).

2. Genetic variation in the amino acid and sugar content of mountain birch leaves was low (I, II).

It is unlikely that birches would defend themselves simply by reducing the amino acid levels of their leaves, for several reasons. First, there is a very limited amount of additive genetic variation in the foliar amino acid content. Second, amino acid levels play such a pivotal role in plant metabolism, and are so tightly regulated, that newly arising mutations affecting the amino acid level would in all likelihood be detrimental to the plant. Third, in case food quality deteriorates, autumnal moth larvae are able to increase their food intake (Haukioja 2003); thus reducing the amino acid content might merely increase leaf consumption by herbivores.

It is possible that phenolics have become the most important resistance components, but not because they have the strongest effect on herbivores; in fact, amino acids seem to be more important to the insect's performance (Berenbaum 1995). Rather, phenolics may have become the most relevant resistance compounds because the constraints on their evolution are the weakest (Carmona et al. 2011).

3. Environmental factors are relevant to the foliar properties of the mountain birch. Temperature affects phenoloxidase activity (V); herbivory causes changes in phenolics (VI) and phenoloxidase activities (V). Increasing nitrogen availability resulted in a clear increase in birch growth, but only some changes in foliar chemistry were associated with extra nitrogen. Level of gallic acid and quercetin glycoside declined when nitrogen resources were added; catechin and pedunculagin levels increased (IV). There is also a pronounced seasonal dynamic to the activities of both phenoloxidase (III) and phenolics (VI).

This thesis has shown that herbivores are indeed affected by birch variation. Phenotypic variation is important, since selection always occurs at the level of the phenotype. But a herbivore can induce a selection response in the next generation of the mountain birch only if birches differ genetically in traits that are associated with herbivore performance and birch fitness.

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