Effects of variable host plant quality on

the oligophagous leaf beetle *Phaedon cochleariae*:

Performance, host plant recognition and feeding stimulation

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Synopsis

The phenotypic plasticity of plants is determined by the degree of variation of anatomical, morphological, physiological and phytochemical properties towards biotic and abiotic changes in the plants' environment. In contrast to adaptations to long-term shifts in the environment, stress rather evokes short-term responses in plants, e.g. changes in growth, development and/or metabolic profiles (Larsson, 1989). Apart from reproduction organs, tissues with a high photosynthetic capacity provide the most valuable parts of a plant, which should be protected with priority and posses a high plasticity. Therefore, plant responses towards stress may vary not only across species and populations, but also between organs and tissues of variable age.

In the present study, the phenotypic plasticity of young and old leaves of two Brassicaceae species, *Sinapis alba* versus *Nasturtium officinale,* was investigated in response to short-term exposure to ambient radiation including ultraviolet irradiances, and compared to effects of ambient radiation excluding UV (**Chapter1**). Nutrient contents were analysed and effects on chemical components of two defence systems were investigated. The storage of flavonoids provides an effective protection against harmful radiation, whereas the Brassicaceae-specific glucosinolate-myrosinase system is highly effective against generalist antagonists. Species-specific and leaf-age dependent responses of involved compounds to radiation stress treatments were expected.

On a higher trophic level, leaf-age and species-specific effects of this differently stressed plant material on the feeding behaviour and the larval performance of an oligophagous Brassicaceae specialist, the mustard leaf beetle *Phaedon cochleariae* (Chrysomelidae; Coleoptera), were studied (**Chapter 2**). According to the plant stress – insect performance hypothesis that predicts an improved performance of herbivores on stressed plants, differences in larval performance in response to differently UV-stressed young and old leaves of both Brassicaceae were expected. Feeding on leaf tissues with a high nutritional value should result in an improved larval development, whereas low leaf quality may be compensated by increased feeding rates. Flavonoids and at least some glucosinolates share the same precursor for biosynthesis. Thus, a competition between both pathways is probable that may be reflected in variable flavonoid and glucosinolate quantities in stressed leaf material, and which in turn may have different effects on herbivores.
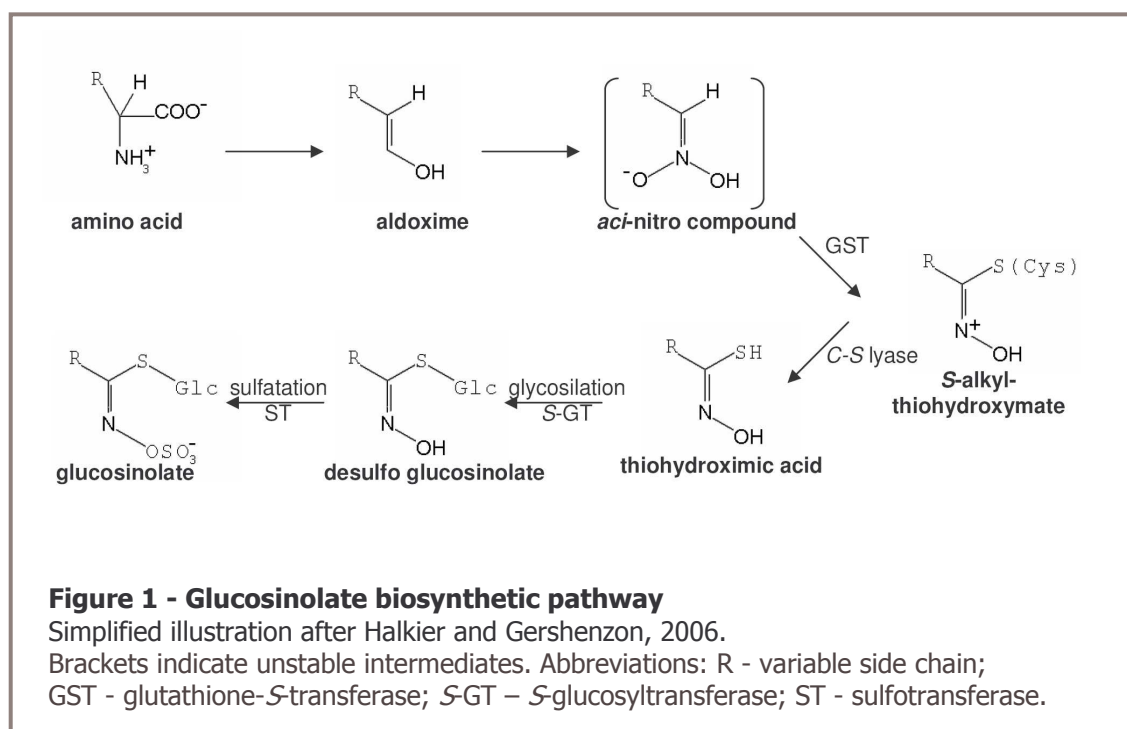
Moreover, the localisation of recognition cues of *P. cochleariae* was investigated. Earlier observations on feeding and oviposition stimulants for herbivores have led to the assumptions that glucosinolates are present on outermost waxy plant surfaces (e.g. Städler and Roessingh, 1990; Renwick et al., 1992), and that glucosinolates provide feeding stimulants for *P. cochleariae* (Nielsen, 1978a). However, the presence of glucosinolates in epicuticular waxes seemed unlikely from a physico-chemical approach regarding the plant cuticle, but also from the plant's perspective, since plants would benefit from avoiding such easily accessible stimulants for herbivores (**Chapter 3**). Therefore, the presence of glucosinolates in epicuticular waxes was re-investigated. Since we could not confirm feeding stimulation of *P. cochleariae* by the glucosinolate used in our study on the localisation of recognition cues, both, the identity of stimulating compounds and the general role of glucosinolates for this leaf beetle remained unclear. Therefore, we investigated the complexity of interacting chemical plant compounds that act as feeding stimulants of the mustard leaf beetles, particularly with regard to Brassicaceae-specific glucosinolates (**Chapter 4**).

Highly specific, exceptionally effective: The glucosinolate-myrosinase system

Plant species, which are included in the Brassicaceae and few other families of the order Brassicales, are characterised by specific secondary plant metabolites: glucosinolates (mustard oil glycosides). These are involved in host plant recognition by specialist herbivores, but acting together with the enzyme myrosinase, they form a highly effective defence against generalist insects and pathogens (Louda and Mole, 1991; Wittstock and Halkier, 2002).

Glucosinolates are β-thioglucoside N-hydroxysulfates with a sulfur-linked β-D-glucopyranose moiety and a side chain which derives from one of eight amino acids (Fahey et al., 2001; Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006). The classification of glucosinolates is determined by amino acid precursors: Aliphatic glucosinolates derive from alanine, leucine, isoleucine, methionine, or valine, whereas aromatic glucosinolates derive from phenylalanine or tyrosine, and indole glucosinolates derive from tryptophane (Halkier and Gershenzon, 2006). The amino acids are catalysed to aldoximes and subsequently to instable intermediate products, proposed as *aci*-nitro compounds or nitrile oxide compounds (Figure 1). The sulfur-

donating enzyme is not identified so far, but a glutathione-*S*-transferase-like enzyme was proposed that uses cysteine as substrate (Halkier and Gershenzon, 2006). Intermediates in the pathway from amino acids to the glucosinolate core structures are *S*-alkyl-thiohydroxymate, thiohydroximic acid and desulfo glucosinolates (Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006), enzymes involved in these processes are *C-S* lyase, *S*-glucosyltransferase and sulfotransferase (Figure 1). However, the side chains of most glucosinolates are strongly modified. Elongations by methylene moieties and/or subsequent transformations are common, e.g. hydroxylation, *O*-methylation, desaturation, glycosylation, and acylation (Fahey et al., 2001; Halkier and Gershenzon, 2006).

Figure 1 - Glucosinolate biosynthetic pathway
Simplified illustration after Halkier and Gershenzon, 2006.
Brackets indicate unstable intermediates. Abbreviations: R - variable side chain;
GST - glutathione-*S*-transferase; *S*-GT – *S*-glucosyltransferase; ST - sulfotransferase.

Glucosinolate-containing plants also comprise the myrosinase enzymes, β-thioglucoside glucohydrolases. Myrosinases occur in various isoforms (Rask et al., 2000). Among these, some are soluble under non-denaturing conditions, whereas others form complexes with one of two groups of proteins, myrosinase-associated proteins (MyAP) and myrosinase-binding proteins (MBP) (Eriksson et al., 2001). Inactive myrosinases and glucosinolates occur in the same plant tissues, but myrosinases are spatially separated in vacuoles of idioblasts (Andréasson and Jørgensen, 2003). In case of tissue damage both come into contact with the result of substrate degradation. The

cleavage of the thioglucoside linkage leads to the formation of glucose and an instable aglycone. The side chain structure and the presence of additional proteins and co-factors determines the rearrangement of the aglycone to form different products, such as the most common isothiocyanates, but also nitriles, epithionitriles, thiocyanates and oxazolidine-2-thionine (Bones and Rossiter, 1996, 2006; Halkier and Gershenzon, 2006; Figure 2), whose biological functions are not well understood.

Figure 2 – Glucosinolate degradation
Illustration from Halkier and Gershenzon, 2006.
Brackets indicate unstable intermediates. Abbreviations: ESP - epithiospecifier protein;
R - variable side chain
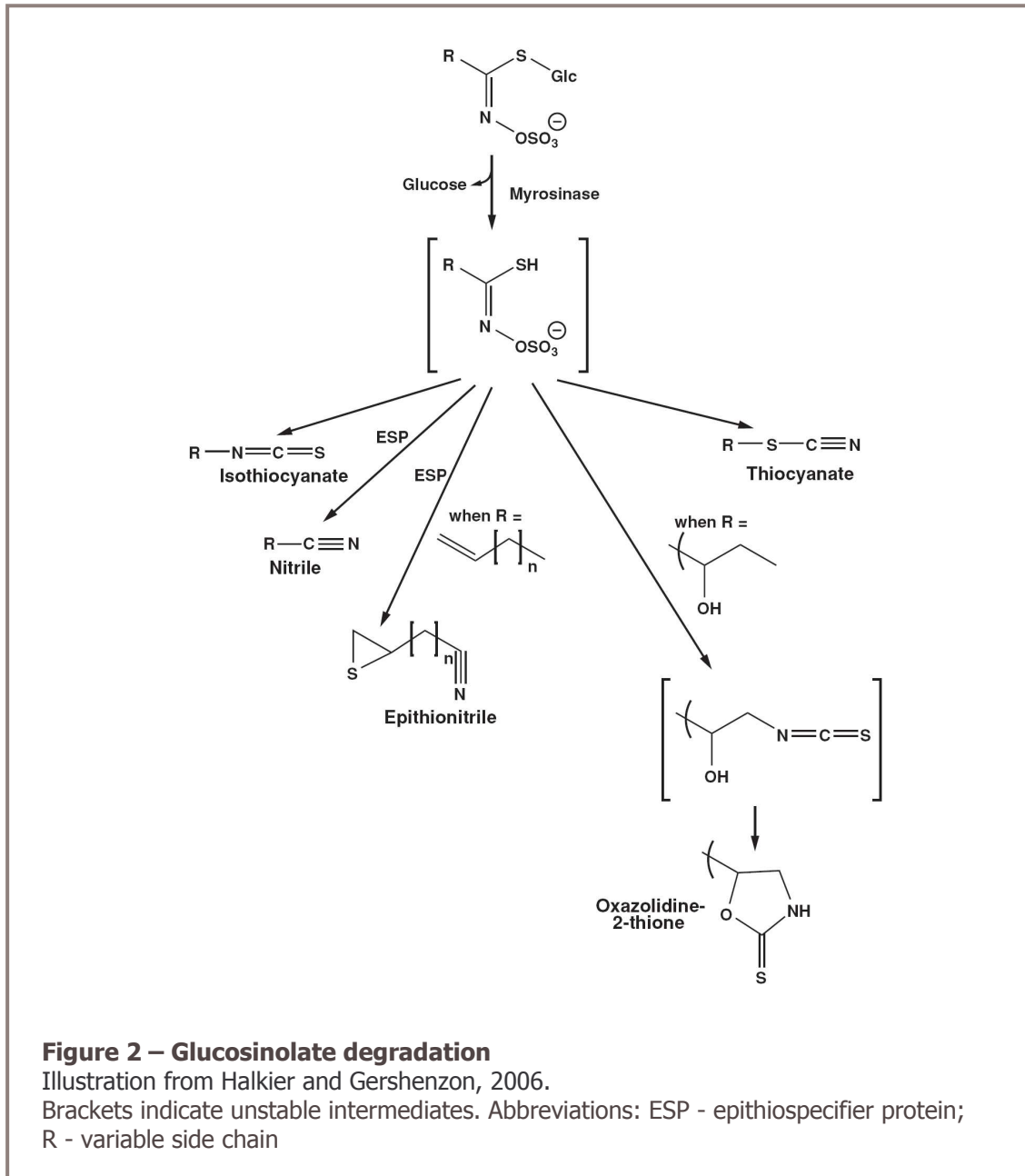
The glucosinolate-myrosinase system in Brassicaceae provides a reliable defence system, due to the toxicity of glucosinolates and the repellent effect of their volatile breakdown-products, as isothiocyanates, for many generalist insects, but also pathogens

(Louda and Mole, 1991; Wittstock and Gershenzon, 2002). The strength of the defence may depend on the quantity and quality of the available substrate and the activity of the enzyme myrosinase and other cell conditions (pH, cell iron concentration, specific proteins; Halkier and Gershenzon, 2006).

Apart from their role as a partner of this defence system, glucosinolates and their hydrolysis products were shown to play an important role as host plant recognition cues for many specialists (Larsen et al., 1992; Renwick et al., 1992, 2002, 2006; van Loon et al., 1992; Siemens and Mitchell-Olds, 1996; Barker et al., 2006). Highly specific mechanisms evolved to allow the insects to feed on these plants without toxification by circumvention of the activation of myrosinases. These mechanisms include the sequestration of glucosinolates, as observed for larvae of *Athalia rosae* (Hymenoptera) (Müller et al., 2001) and for *Brevicoryne brassicae* (Hemiptera) (Bridges et al., 2002), and detoxification, as described for *Plutella xylostella* (Lepidoptera) (Ratzka et al., 2002), *Pieris* and *Anthocaris* (Lepidoptera) (Agerbirk et al., 2006). However, Siemens and Mitchell-Olds (1996) found that the stimulation of specialists by glucosinolates can be dose-dependent. These authors observed strongest herbivory on tissues with intermediate glucosinolate levels, but decreased feeding on tissues containing high levels.

Contents of glucosinolates can vary widely within plant individuals, e.g. between leaves of different age (Shelton, 2005; Martin and Müller, 2007), organs (Brown et al., 2003), within plant populations (Agerbirk et al., 2001), between plant populations (Müller and Martens, 2005) and between species (Fahey et al., 2001). The activity of myrosinases is affected by the availability of ascorbate and other co-factors, which act as a catalytic base or inhibit the activity (Burmeister et al., 2000; Burow et al 2006). Similar to glucosinolates, myrosinase activities vary between species and organs (Bones and Rossiter, 1996) and leaves of different age (Martin and Müller, 2007) as well as between populations within a species (Müller and Martens, 2005) and across species (Travers-Martin and Müller, in preparation).

Moreover, to a certain degree, qualitative and quantitative variation of both partners of the glucosinolate-myrosinase system may be based on heritable genetic variations (Siemens and Mitchell-Olds, 1996, 1998; Kliebenstein et al., 2005, but could also be affected by biotic environmental factors. Damage of plant tissue was shown to lead to local and systemic inductions of glucosinolates (Agrawal, 1998, 2005; Karban et al., 2003), but also of myrosinase activity (Müller and Sieling, 2006; Martin and Müller,
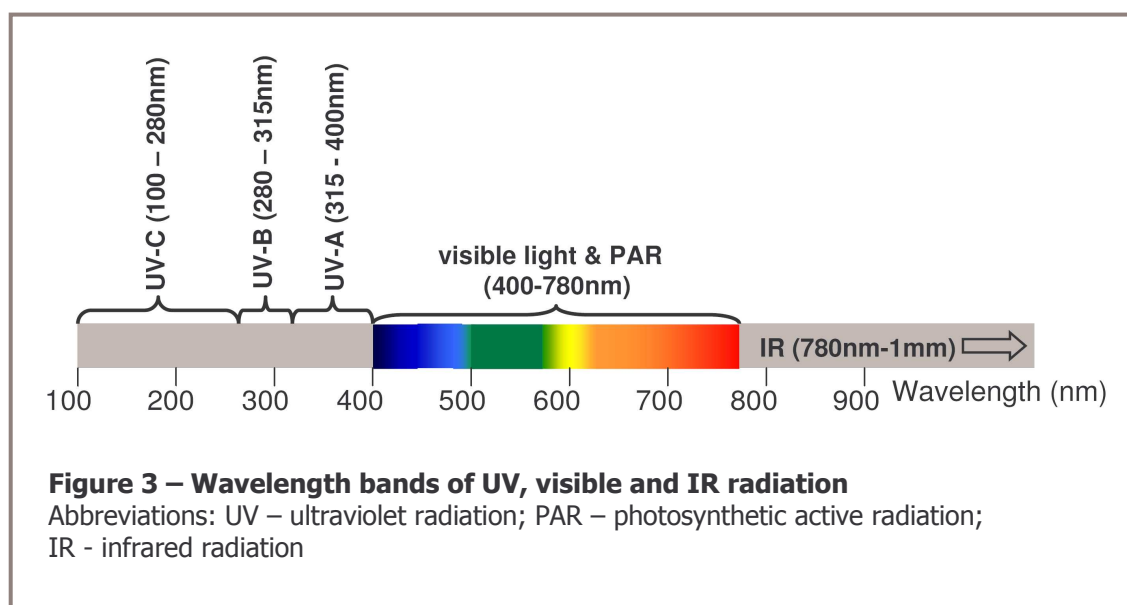
2007). The induction of glucosinolate and myrosinase levels by insect feeding was the subject of several studies (e.g. Menard et al. 1999; Agrawal and Kurashige, 2003; Martin and Müller, 2007). Thereby, the level of induction can be affected by the herbivore species, its feeding mode and the degree of feeding specialisation (Pontoppidan, et al., 2003, 2005; Mewis et al., 2006; Travers-Martin and Müller, 2007). For *Arabiodopsis thaliana* (Brassicaceae), Mewis et al. (2005) detected increases of the aliphatic methylsulfinyl glucosinolate due to sucking of the phloem-feeders *Brevicoryne brassicae* (specialist) and *Myzus persicae* (Hemiptera) (generalist) and the leaf-chewer *Spodoptera exigua* (Lepidoptera) (generalist). An increase in 8-methylsulfinyloctyl glucosinolate was detected due to feeding by *S. exigua*, whereas indol glucosinolates were not affected (Mewis et al., 2005). Feeding by the specialist aphid *Brevicoryne brassicae* leads to a down-regulation of myrosinase transcript levels in *Brassica napus,* but levels of specific myrosinase binding proteins were elevated (Pontoppidan et al., 2003).

Apart form this broad range of studies on the responsiveness of the glucosinolate-myrosinase system towards biotic influences, some studies investigated the effects of abiotic environmental factors on Brassicaceae, as sulphur fertilisation (e.g. Marazzi et al., 2004 a, b) or shading (Charron et al., 2005) and CO_2 (e.g. Schonhof et al., 2007). However, only few studies focussed on the effects of ultraviolet radiation on Brassicaceae. Among these, Olsson et al. (1998) detected specific flavonoid responses, whereas Mackerness et al. (2000) focussed on changes in gene expression. To our knowledge, none of these studies investigated leaf-age dependent stress responses of the glucosinolate-myrosinase system in Brassicaceae species that were short-term exposed to ultraviolet radiation.

An environmental threat for plants: ultraviolet radiation

The chemical composition of plants is highly responsive to variation in abiotic environmental factors, e.g. nutrient, water and light availability and irradiance intensities (Caldwell et al., 2003; Oksanen et al., 2005; Osier and Lindroth, 2006). Among these, ultraviolet radiation is known to evoke detrimental effects on plants and most other organisms living on earth. Whereas the highly energetic short-wave UV-C radiation (< 280 nm) is completely absorbed by the atmosphere, both UV-B radiation

(280-315 nm) and UV-A radiation (315-400 nm) reach the earth surface (Paul and Gwynn-Jones, 2003; Figure 3). Due to the partial depletion of the ozone layer, ambient levels of UV-B radiation on earth increased in the last decades. In higher plants, especially UV-B radiation can damage lipids, proteins and nucleic acids (Caldwell et al., 1983; Bassman, 2004) (direct effects), modify the architecture of plants and the structure of the vegetation (Rozema et al., 1997; Kakani et al., 2003a, b) and induce internal chemical responses in plants (Caldwell et al., 1989, 2003) (indirect effects). Changes in optical properties of leaves can lead to the acclimatisation of plants (Caldwell et al., 1983; Pfündel et al., 2006), whereas the storage of UV-absorbing flavonoids and hydroxycinnamic acids in the leaves' epidermis is the main response of plants to shield their photosynthetic active tissue (Caldwell et al., 1983, 1989, 2003).

Figure 3 – Wavelength bands of UV, visible and IR radiation
Abbreviations: UV – ultraviolet radiation; PAR – photosynthetic active radiation; IR - infrared radiation

Flavonoids and hydroxycinnamic acids. Flavonoids are heterocyclic compounds, consisting of two aromatic rings and one γ-pyrone ring with ether-linked oxygen. Flavonoids are based on a C_{15} skeleton, formed by three malonate units condensed with a phenylalanine-derived C_6-C_3 precursor. Phenylalanine ammonium lyase (PAL) deaminise the amino acid phenylalanine to cinnamic acid (Figure 4). Subsequent hydroxylation and methylation steps form various hydroxycinnamic acids, whereas their esterisations by coenzyme A (CoA) result in a broad range of intermediates of phenylpropanoid derivates, e.g. cumarines, lignins, and flavonoids. The chalcone synthases catalyse the condensation of three malonyl-CoA-esters and hydroxycinnamic acids under cleavage of CO_2 and CoA, followed by the cyclation of the aromatic ring by

chalcone isomerase, resulting in the formation of the base structure for various groups of flavonoids (Heldt, 2003; Figure 4).

Most common flavonoid aglycones do not differ in the oxidation level of the A-ring, but in the oxidation level of the pyran nucleus and in the number of hydroxyl groups (one to three) in the B-ring (Harborne, 1991; Figure 4). Occurring almost universally in plant leaves, kaempferol and quercetin provide the most common flavonols. Both differ in the number of hydroxyl groups in the B-ring, kaempferol with one hydroxyl group at the C_2-position and quercetin with an additional hydroxyl group in the C_3-position. In a survey of 1000 angiosperms, kaempferol flavonols were found in leaves of 48% plant species and quercetin in leaves of 56% of the species (Harborne, 1991).

Flavonoids usually occur as glycosides. The functions of these attached sugar moieties are the protection from enzymatic oxidation, stabilisation to light and the keeping of otherwise toxic materials in inactive bounds. Apart from glucose, various other kinds of sugars can be attached to one or more of the phenolic hydroxyl groups of flavonoids. Rutin, a disaccharide formed by glucose and rhamnose attached to the C_3-positioned hydroxyl group of quercetin, provides the most common leaf flavonoid. Thus, the wide variety of glycoside combinations provides a considerable complexity of flavonoids, e.g. more than 135 different quercetin glycosides were described so far for higher plants (Harborne, 1991).

However, further structural complications are caused by acyl groups attached to one or more sugar hydroxyl groups. Aromatic acyl groups derive from one of the four hydroxycinnamic acids, coumaric, caffeic, ferulic, or sinapic acid, whereas aliphatic acids acyl groups derive from hydroxybenzoic, e.g. acetic acids or malonic acid. Hydroxycinnamic acids provide the second class of phenolic compounds that is involved in shielding the leaf interior against harmful UV radiation (Burchard et al., 2000). In *A. thaliana*, flavonoids are particularly effective in absorbing UV-A radiation, whereas hydroxycinnamic acids were found to be more effective in the UV-B region (Landry et al., 1995). Such epidermal hydroxycinnamic acids provide the dominant UV-B protective compounds in early developmental stages of *Secale cereale* (Poaceae), but these are replaced by epidermal flavonoids during further leaf development (Burchard et al., 2000). In conclusion, both compound classes, flavonoids and hydroxycinnamic acids, form highly effective protection shields in plants against harmful radiation.

Shielding plant tissues against UV is one highly important characteristic of flavonoids, but these secondary plant compounds play a much broader role for plants.

Colours of many flowers and fruits which are important for attraction of pollinators and dispersers, are based on anthocyanins (Harborne, 1991; Harborne and Williams, 2000). Flavonoids can also act as antimicrobiants and protect plant against herbivory by both insects and mammals (Harborne and Williams, 2000). Moreover, for some specialist insects flavonoids are involved in host plant recognition (van Loon et al., 2002).
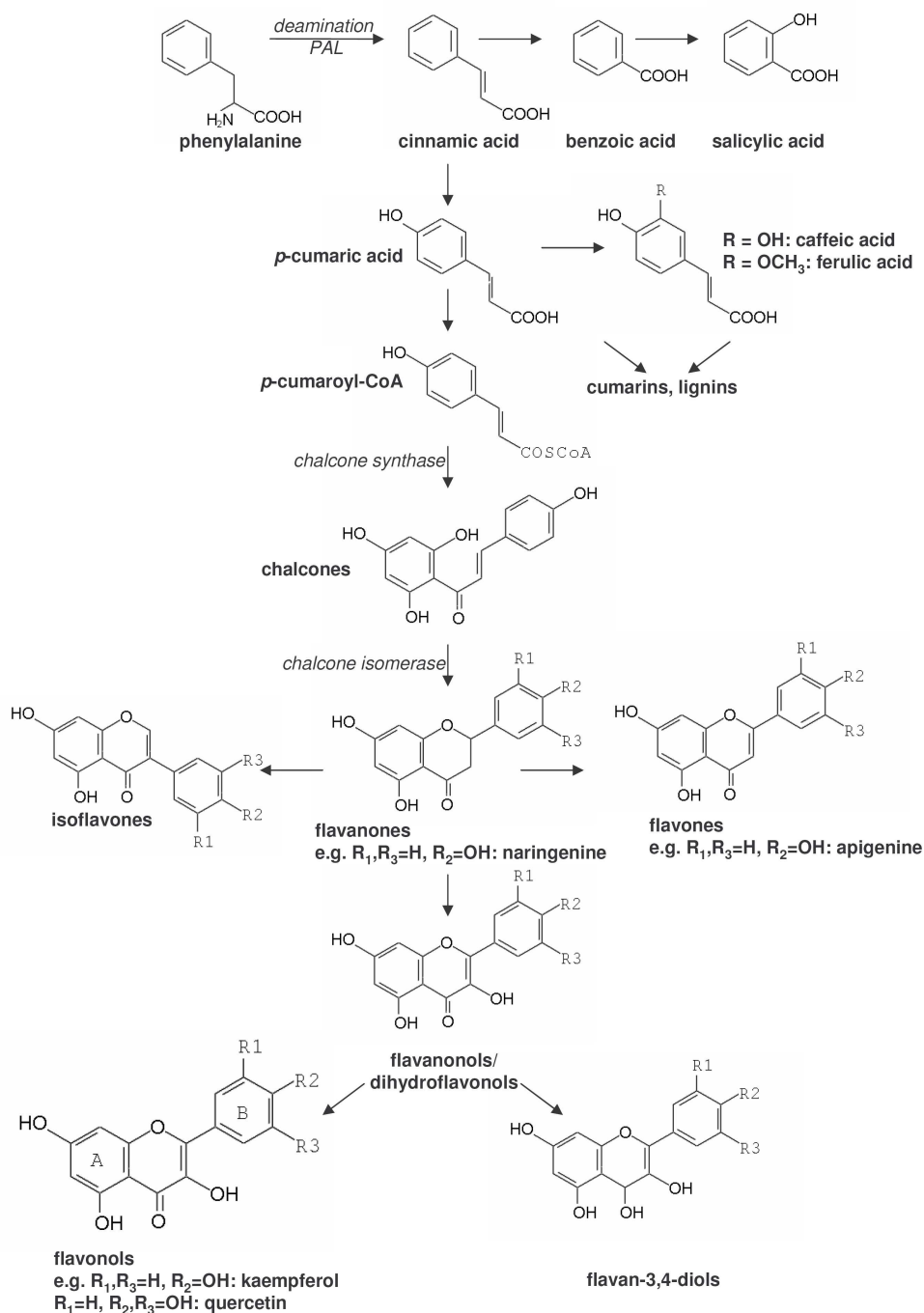
Figure 4 - Phenylpropanoid and flavonoid biosynthetic pathway
Simplified illustration.
Abbreviations: PAL – phenylalanine ammonium lyase; CoA – coenzyme A; R – variable side chains

Enhance or filtering ultraviolet radiation: lamps *versus* filtering foils. Out of a total of 25 studies on the effects of ultraviolet radiation on plants, 19 studies were carried out in controlled conditions in climate chambers or under field conditions with artificially enhanced harmful radiation by using UV-lamps, e.g. Björn et al. (1997), Levizou and Manetas (2001), Tegelberg and Julkunen-Tiitto (2001), Hofmann et al. (2003). In most studies, UV-doses used for experiments were hypothetically calculated as predictable effects of a further depletion of the ozone layer, e.g. Newsham et al. (1996), Björn et al. (1997), Levizou and Manetas (2001). Due to these calculations, plants were exposed to artificially enhanced radiations, with extraordinary high UV-levels used at least in some of these studies.

Figure 5 - UV-exposure tents
located in the Botanical Garden Würzburg. Tents are provided with two types of foils, both transmitting visible radiation. One type of foil also transmits UV-A and UV-B radiation, whereas the second foil type filters UV-B radiation.

In contrast, six studies were performed under outdoor conditions using tents provided with UV-filtering foils, (e.g. Cybulski and Peterjohn, 1999; Hunt and McNeil, 1999; Turunen et al., 1999; Caputo et al., 2006). The design of these studies allows comparisons between plants exposed to ambient radiation including or excluding natural levels of ultraviolet radiation. Compared to climate chambers, exposure conditions are less standardised in these outdoor experiments, since UV radiation does not represent the only variable environmental factor. Variation in climate, temperature, humidity, and/or shading due to cloudiness cannot be excluded during exposure periods

in these experiments. Moreover, temperature-controlled greenhouses with variable UV-filtering roofs, as recently build in the Botanical Garden Würzburg, could even improve the design of experiments with UV-filtering foils (ongoing PhD project F. Kuhlmann).

However, only exclusively the induction of variation in plant quality by ambient radiation levels allows conclusions on the plasticity of plant responses to actual environmental conditions. In our study, we used two types of foils, both transmitting visible radiation including photosynthetic active radiation (PAR). One foil type also transmits UV, whereas the second foil type filters UV radiation (Reifenrath and Müller, 2007, **Chapter 1**; Reifenrath and Müller, submitted, **Chapter 2** and **Chapter 4**; Figures 5, 6). For detailed description of the construction of the tents, foil properties and climate conditions in the tents and in the greenhouse see Reifenrath and Müller (2007, **Chapter 1**) and Reifenrath and Müller (submitted, **Chapter 2**).

Figure 6 – Transmittance spectra and spectral irradiances in exposure tents
Transmittance spectra were measured with a spectrometer (Unicam, Cambridge, UK) and spectral irradiances below foils were measured with an UV-Spectroradiometer (Optronic, Orlando, FL). Dashed line – UV-transmitting foil, straight line – UV-filtering foil
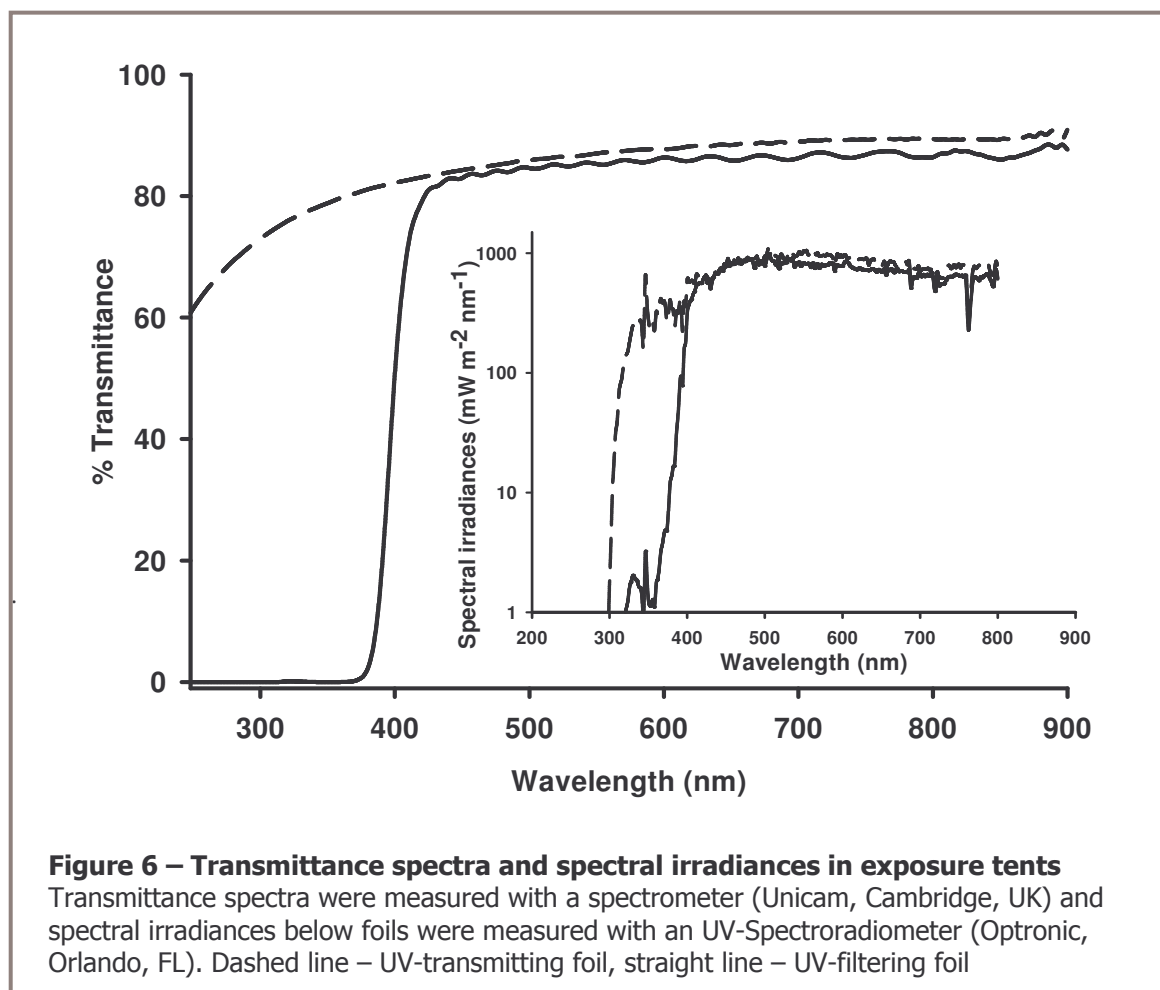
Ultraviolet radiation affects chemical leaf quality

Effects of short-term exposure to ultraviolet radiation on the chemical composition and morphological properties of plants vary across species and organs, and tissues of different age, and may thus depend on the developmental stage of the plant. For plant individuals grown in environments with low or even no UV in the radiation spectrum, a subsequent exposure to enhanced UV-conditions may evoke strong stress responses. UV-stress may change morphological characteristics of plants, e.g. leaf cuticle thickness (Semerdjieva et al., 2003), height, internode and branch lengths, leaf biomass, thickness of epidermal, palisade, and mesophyll layers (Kakani et al., 2003a, b). Moreover, primary and secondary metabolites were found to be affected, e.g. nitrogen (Hatcher and Paul, 1994; Lindroth et al., 2000), carbon (Lindroth et al., 2000), carotinoids (Cuadra et al., 1997; Feng et al., 2003), and tannins (Tegelberg et al., 2002). An inhibition of the photosystem II (Kolb et al., 2001; Bjerke et al., 2005) and a reduction in photosynthetic activity were commonly observed (Feng et al., 2003; Keiller et al., 2003). However, most studies focussed on the accumulation of phenolic shield pigments in the leaf epidermis (Cuadra et al., 1997; Markham et al., 1998; Olsson et al., 1998; Turunen et al., 1999; Kolb et al., 2001; Tegelberg et al., 2002; Caldwell et al., 2003; Feng et al., 2003; Warren et al., 2003). This increase in synthesis and storage of flavonoids can be regarded as the main defence response, resulting in the protection of the photosystem. Moreover, UV-stress induces further defence responses in plants, as the highly species-specific activation of antioxidant enzymes and hormones, e.g. jasmonic acid, salicylic acid and ethylene (Mackerness et al., 1999; Mackerness, 2000). Jasmonic acid and its methyl ester regulate gene expression affecting plant growth and development (Mackerness et al., 1999; Mackerness, 2000), but also mediate plant defence against herbivores, e.g. glucosinolate accumulation in Brassicaceae (Doughty et al., 1995). Given that environmental adaptations can be reflected in changes of primary and secondary leaf metabolites, for UV-stressed Brassicaceae not only a species-specific accumulation of flavonoids was expected, but also effects on the glucosinolate-myrosinase system and variable nutrient contents.

In our study, the specificity of stress responses of short-term exposure to ambient radiation including ultraviolet radiation on the pytochemistry of young and old leaves of two Brassicaceae species, *Sinapis alba* and *Nasturtium officinale,* were compared to

effects in UV-shielded plants, which were exposed to radiation excluding UV (**Chapter 1**: Species-specific and leaf-age dependent effects of ultraviolet radiation on two Brassicaceae; Reifenrath and Müller, 2007). Plant individuals grown in the greenhouse with a lack of UV radiation and low PAR levels, were 48 h short-term exposed in outdoor foil tents with high PAR levels and irradiances including or excluding UV. Both types of tents were provided with different types of foils, one of each filtering UV radiation or transmitting the UV spectrum (see above, Figures 5, 6).

For both plant species, the exposure to different radiation regimes revealed a strong plasticity in responses with respect to all analysed chemical components, namely water, carbon, nitrogen and soluble protein contents, glucose, flavonoid, glucosinolate and activity of free (soluble) and complexed (insoluble) myrosinase concentrations, respectively. Variation in chemical compositions in *S. alba* refer to both, UV-stress treatments and leaf-age, whereas in *N. officinale* leaf-age effects were more pronounced than UV-stress treatments (Table 1). With regard to nutrients, in *S. alba*, there was a trend for higher levels of carbon and soluble proteins in young than in old leaves, and for higher nitrogen levels in UV-exposed compared to UV-shielded leaves, whereas soluble protein and carbon contents were higher in UV-shielded than UV-exposed leaves. In *N. officinale*, young leaves showed a tendency for higher levels of carbon, nitrogen and soluble proteins than old leaves. Furthermore, there was a trend for higher levels of nitrogen, soluble proteins and glucose in UV-exposed leaves compared to UV-shielded leaves. Only carbon levels were higher in UV-shielded young leaves than in old or UV-exposed leaves.

Such leaf-age dependent shifts in nutrient levels due to short-term exposure to different UV-conditions and increased PAR may be based on different photosynthetic activities in these tissues (Teramura and Caldwell, 1981; Björn et al., 1997; Kolb et al., 2001; Keiller et al., 2003). Since nitrogen levels usually decrease with increasing leaf-age (McNeill and Southwood, 1978; Wait et al., 2002), higher nitrogen and soluble protein contents levels were expected for young leaves. These expectations were confirmed for both compounds in *N. officinale* and for soluble protein contents of *S.alba*, but not for nitrogen contents in *S. alba*. Enhanced nitrogen and soluble protein levels in UV-exposed leaves, as previously found for other species under supplemental UV (Murali and Teramura, 1985; Lindroth et al., 2000; Warren et al., 2002), were found for young *S. alba* and both leaf-ages of *N. officinale*. Such shifts in nitrogen and soluble protein contents may strongly affect the tissue quality as a food source for herbivores. In

case of low levels of both compounds, compensatory feeding may take place in order to cover the insects' requirements.

Chemical compounds	affected in *S. alba* due to…	affected in *N. officinale* due to…
Water contents	leaf-age > exposure treatment	leaf-age
Carbon contents	leaf-age	leaf-age
Nitrogen contents	exposure treatment	leaf-age
Protein contents	exposure treatment > leaf-age	exposure treatment > leaf-age
Glucose concentrations	exposure treatment	exposure treatment
Flavonoid concentrations	exposure treatment	exposure treatment > leaf-age
Hydroxycinnamic acids concentrations	-	-
Glucosinolate concentrations	leaf-age	exposure treatment
Free myrosinase activity concentration	-	leaf-age
Complexed myrosinase activity concentration	exposure treatment	-

Table 1 – Parameters affecting changes in chemical leaf components in *S. alba* or *N. officinale* leaves (effects were statistically tested with a multifactorial ANOVA, see Reifenrath and Müller, 2007; Chapter 1)

In our study, all components involved in defence systems, as glucosinolates and myrosinases and flavonoids, revealed a high plasticity in chemical responses to different radiation regimes. Both Brassicaceae species responded specifically in concentrations of glucosinolates and myrosinase activity (Table 1). In *S. alba*, total glucosinolate concentrations were higher in young compared to old leaves, but responses to UV-stress treatments were not significant. While the concentration of of free myrosinase activity was generally low, the concentration of complexed myrosinase activity was affected by UV-stress treatments and highest in UV-shielded leaves.

An optimal defence against antagonists may be obtained by a constitutive optimal ratio of myrosinase activity concentration to glucosinolate concentration. Especially in tissues that need to be protected with priority, the concentrations of both partners can be expected to correspond to this ratio or be higher. This optimal ratio is unknown so far, but we assume that it depends, among other factors on the kind of myrosinase and on the specificity of glucosinolates. Basically, an optimal defence could be obtained by the combination of intermediate or high glucosinolate levels with high myrosinase activity concentrations, resulting in rapid and highly efficient substrate degradation. Nevertheless, high substrate levels can already act deterrent on it own.

In accordance with these speculations on an optimal ratio of defence compounds, in *S. alba* young leaves may indeed be better protected against antagonists than old leaves. Moreover, due to a higher concentration of free myrosinase activity in UV-shielded leaves; those may be better defended against herbivores than UV-exposed leaves. In comparison with *S. alba*, glucosinolate levels in *N. officinale* were up to twelve-fold lower. Glucosinolates were affected by UV, but effects were stronger in old than in young leaves. Highest levels were detected in UV-shielded old leaves. Concentrations of activity of both myrosinases were less affected by different UV-stress treatments. At least free myrosinase activity concentrations varied between leaves of different age, with higher levels in young than old leaves. Consequently, in *N. officinale*, not only young leaves of both treatments should be well protected against generalist herbivores, but also old UV-shielded leaves.

The specificity of plant responses to UV-stress treatments becomes even clearer, when data on chemical analyses of another Brassicaceae species, *B. nigra,* are compared with those of *S. alba* (K. Reifenrath, unpublished data). Both species are similar in growth-form and ecological demands. In contrast to *S. alba*, each of the tested compound (carbon, nitrogen, soluble protein contents, and concentrations of glucose, glucosinolate and myrosinase activity) was higher in UV-exposed than in UV-shielded young leaves. In young *B. nigra* leaves of both treatments, lower glucosinolate levels, but up to four-fold higher levels of myrosinase activities, should result in an efficient protection against generalist herbivores. In conclusion, *B. nigra* shows strong specific responses and a phenotypic plasticity in its chemical adaptation towards environmental stresses different from *S. alba*.

Concerning the protection of leaf tissues in *S. alba* and *N. officinale* against harmful radiation, levels of hydroxycinnamic acids were not affected by UV-stress treatments. In contrast, for flavonoids, strong differences between differently treated leaves of both ages and species were detectable, with approximately 10-fold lower flavonoid quantities in *N. officinale* than in *S. alba*. Moreover, the composition of flavonoids varied strongly between leaves exposed to UV and those shielded against harmful radiation. Similar to observations by Markham et al. (1998) for *Oryza sativa* (Poaceae) and Olsson et al. (1998) for *Brassica napus*, relative quercetin glycoside contents increased at the expense of kaempferol glycoside contents in *S. alba*. Due to additional hydroxyl group at the C_2-position in the B-ring chromophore of quercetin, those flavonoids have a

potentially increased antioxidant activity compared to those without, such as kaempferol (Olsson et al., 1998). Therefore, the increase of quercetin flavonols at the expense of kaempferol may result in enhanced antioxidant activities in *S. alba*. In *N. officinale*, kaempferol flavonols were only detectable in UV shielded leaves and quercetin flavonol contents remained constant in leaves of both exposure conditions. Hence, although flavonoid levels increased under UV exposure, the efficiency of tissue protection due to specific flavonols was less pronounced in this species.

Such differences in accumulation patterns and distribution of phenolic compounds between both UV-stressed Brassicaeae may refer to ecological adaptations to the original habitats of each species. Sunny and open habitats that demand the accumulation of flavonoids with improved scavenging activities as an adaptation to high UV radiation are common for *S. alba*. In contrast, *N. officinale* grows in shady and moist places with only moderate UV radiation, where lower flavonoid concentrations are sufficient and a low plasticity towards solar radiation is needed. Moreover, these differences in flavonoid content and composition between both plant species and leaves of different age may determine their acceptability to herbivorous insects, since these compounds can be involved in stimulation or deterrence of feeding and oviposition on a host plant (e.g. Harborne and Williams, 2000; Warren et al., 2002; Caputo et al., 2006).

Effects of variable plant quality on herbivores

The level of plasticity in plant responses to UV may be closely related to the plant's requirements to adapt to its specific environment. However, in case of changing environments, plants may have to adjust their defence not only against harmful radiation, but also against feeding insects. Increasing costs for re-allocation of defensive chemicals might accompany this process of adaptation. Therefore, short-term exposure to radiation including or excluding UV does not only affect absorbing metabolites, but also the plants' morphology and other primary and secondary plant compounds. These may include components that are involved in host plant recognition, feeding and performance of members of the next trophic level (McCloud and Berenbaum, 1999; Lindroth et al., 2000; Warren et al., 2002).

The plant stress – insect performance hypothesis predicts increased growth rates and improved performance of herbivores on stressed plants (Rhoades, 1979; White, 1984;

Larsson, 1989). Accordingly, Lavola et al. (1998) and Veteli et al. (2003) observed shifts in feeding preferences towards UV-B stressed plants and increased performance on such plants. However, effects with herbivores showing lower acceptance for UV-B exposed host plants have also been observed (Grant-Petersson and Renwick, 1996; Caldwell et al., 2003). Hence, effects of variable phytochemistry on insect performance may be highly specific.

Generally, a broad range of parameters needs to be observed to allow conclusions about the performance of an insect. Developmental times, body mass increases per time and final body mass should provide suitable parameters for performance experiments, whereas short-term observations of insect growth might not be sufficient. For determination of leaf qualities as food sources, feeding choice tests can offer data about the numbers of feeding-stimulated individuals and consumption rates per individual. The design of bioassays should be carefully chosen: In choice tests, increased feeding can be expected for the preferred food source; in no-choice tests, high feeding rates could occur as compensatory feeding due to low food quality. Studies about the effects of different ultraviolet radiation regimes on two or more trophic levels should carefully distinguish between UV-effects on plants, effects of UV-affected plant material on herbivores, and effects of UV on herbivores (e.g. Caputo et al., 2006). For accurate separation of UV-effects on the chemical composition of plants and the effects of UV-affected plant material on the performance and feeding behaviour of herbivores, tests with herbivores should be carried out in UV-free environments, e.g. in greenhouses or climate chambers.

According to the plant stress – insect performance hypothesis, species-specific and leaf-age dependent effects of ultraviolet radiation on plants were also expected to affect an oligophagous leaf beetle specialised on Brassicaceae (**Chapter 2**: Effects of variable host plant quality on the performance of the oligophagous leaf beetle *Phaedon cochleariae*; Reifenrath and Müller, submitted). Plant stress treatments were carried out by 48 h short-term exposure of Brassicaceae host plants to two radiation conditions (outdoors exposure in ambient radiation including or excluding UV). Effects on the chemical composition of stressed plants were compared to those in plants that remained in the greenhouse with little UV and lower photosynthetic active radiation (PAR) levels for additional 48 h. Naïve larvae of the specialist leaf beetle *Phaedon cochleariae* were fed with young and old leaves of differently treated *Sinapis alba* and *Nasturtium*

officinale. *N. officinale* provide the natural host plant for *P. cochleariae*, which grow in shady habitats in moist meadows and shallow ponds. *S. alba* was chosen as a Brassicaceae originating from a sun exposed habitat, also accepted by *P. cochleariae* as host plant in feeding tests, but with different chemical adaptation to short-term UV-exposure treatments (Reifenrath and Müller, 2007, **Chapter 1**).

Every second day, new plants were exposed to different UV-conditions for 48 h, and individual larvae were fed with fresh leaves. Larvae were weighed every second or third day until pupation. Pupae and adult beetles were weighed within 24 h after hatch, and developmental times were recorded. In contrast to chemical analyses of short-term exposed plant material at one day of harvest (Reifenrath and Müller, 2007, **Chapter 1**), in this study, analytical data were pooled over several times of harvest during the performance experiment. Moreover, this chapter includes analyses of specific dry weights of leaves, the carbon-nitrogen ratio (C/N) and presents data on total myrosinase activity concentrations, where complexed and free forms were measured together.

Variable flavonoid and glucosinolate concentrations across plant species, leaf-age and UV-stress treatments were analysed with regard to possible competition for precursors and between two or more biosynthetic pathways for variable defence compounds, since at least some glucosinolates and flavonoids share the same precursor, phenylalanine. Flavonoid and glucosinolate concentrations differed significantly between both plant species and due to leaf age, and, except glucosinolate levels in *S. alba*, between UV-stress treatments. In comparison with greenhouse-kept leaves, UV-exposed young leaves showed highest flavonoids concentrations. Increased levels were also found in plants exposed to outdoor conditions lacking UV, but with increased PAR levels. Changes in flavonoid compositions were similar to those described in Reifenrath and Müller (2007, **Chapter1**). Highest glucosinolate and total myrosinase activity concentrations, both partners of the Brassicaceae-specific defence system, were found for young leaves. In conclusion, flavonoid shield pigments were accumulated in both plant species stressed by different radiation conditions, but not at the expense of general plant defences against leaf feeding insects. Since flavonoids and glucosinolates were both found to be involved in feeding stimulation of adult *P. cochleariae* (**Chapter 4**), these quantitative and qualitative changes of both compounds could also affect the development of the leaf beetles' larvae. However, correlations between these compounds and performance parameters were not obvious.

In this study, the high variation found in primary and secondary chemistry of young and old leaves of *S. alba* and *N. officinale*, was similar or even more pronounced compared to results of previous chemical analyses that were based on one set of exposed plants (Reifenrath and Müller, 2007, **Chapter 1**). Water, carbon/nitrogen ratio and soluble protein contents as well as glucose concentrations differed strongly between plant species. In *S. alba*, all parameters were significantly altered by leaf-age and UV-stress treatment. Specific dry weights and soluble protein contens were higher in UV-shielded than in UV-exposed leaves, and lowest in greenhouse-kept plants. In *N. officinale*, all analysed parameters were affected by leaf-age and plant stress treatment. In both plant species, nutrient quantities and specific dry weights were generally higher in young and outdoor-exposed plants compared to old and greenhouse-kept plants, and water contents were higher in old compared to young leaves, whereas C/N ratios were lower in greenhouse-kept plants than in outdoor exposed plants, but largely unaffected by leaf-age. Stressed plants commonly have high levels of available nitrogen and amino acids (White, 1984), and an optimal ratio of proteins and carbohydrates is assumed to turn a plant into a highly suitable feeding source (Bernays and Chapman, 1994).

Developmental parameters	Effects of variable host plant quality?	Due to significant factors?
Developmental times larvae	Yes	Species > leaf-age > UV-stress plant treatment
Developmental times pupae	No	
Body mass larvae	No	
Body mass pupae	No	
Body mass adult	No	
Relative growth rate	Yes	Species > leaf-age, larval instar > UV-stress plant treatment
Relative consumption rate	Yes	Species > leaf-age, larval instar > UV-stress plant treatment
Growth efficiencies	Yes	leaf-age > larval instar > species, UV-stress plant treatment

Table 2 – Developmental parameters investigated for *P. cochleariae* raised on UV-stressed and greenhouse-kept host plants (effects were statistically tested with a multifactorial ANOVA, see Chapter 2)

However, despite this strong variation in host plant phytochemistry, the larval development of *P. cochleariae* was poorly affected. Moreover, there was no obvious correlation between plant nutrient levels and any of the development parameters

measured in this study. Although young *S. alba* leaves contained comparably high levels of soluble proteins and glucose, larvae developed significantly slower on these than on conspecific old leaves or on *N. officinale* leaves. The reduced quality of old leaves as feeding source could be a result of dilution of leaf nutrients, since higher water contents were detected in old compared to young leaves. Compensatory feeding by herbivores may be a consequence. However, retard in developmental times is accompanied by a longer exposure period to potential enemies, and therefore an enhanced risk of mortality (Slow-growth-high-mortality hypothesis; Clancy and Price, 1987).

In our study, relative growth and consumption rates and growth efficiencies of larval mustard leaf beetles within 24 h differed across larval instars, plant species and leaf-age, as larvae consumed more, but growth efficiencies were lower on old than on young leaves. However, increasing consumption rates or higher efficiencies of food conversion due to variable plant quality were compensated during development until the pupal phase was reached. Moreover, larval developmental times were affected by plant species, leaf-age and treatment, whereas the duration of the pupal stage, and body mass of larvae, pupae and adults measured during development remained similar. These observations demonstrate a weak plasticity in variation of developmental parameters probably due to a strong genetic fixation of life history traits in *P. cochleariae*.

However, other leaf parameters than the chemical compounds we measured here, may be also important for larval development, e.g. proteinase-inhibitors, tripsin-inhibitors, tannins, as well as structural leaf characteristics. Leaf toughness was shown to retard the growth of *P. cochleariae* (Tanton, 1962). Estimated as specific dry weight of leaves, leaf toughness varied strongly between leaves of different age and UV-stress treatments in our study, but these differences did not obviously correlate with larval development of mustard leaf beetles either. Generally, in our study, differences in the beetles' performance primarily corresponded to species-specific variation and leaf-age specific effects, whereas different exposure treatments of plants had only weak effects. Thus, the plant stress – insect performance hypothesis does not hold for our system.

Similar to our results, stronger effects of leaf-age than variable UV-stress of plants on the performance of herbivores were also found by McCloud and Berenbaum (1999). The degree of variation in insect performance as response to stressed host plants might depend on the feeding guild a herbivore belongs to (Larsson, 1989). Focussing on the

strength of effects of stressed host plants on the performance of insects, Larsson (1989) proposed a ranking among insect feeding guilds with cambium feeders > sucking insects > mining insects > chewing insects > gall-forming insects. Hence, stronger responses to plant stress treatments might have been expected for sucking insects compared to the leaf feeder *P. cochleariae*. Moreover, effects of variable food quality on insect performance may also depend on the degree of specialisation of the herbivore (McCloud and Berenbaum, 1999; Cornell and Hawkins, 2003). For a strictly monophagous leaf beetle, *Cassida canaliculata*, higher larval, pupal and adult body mass of individuals were observed for individuals raised on UV-exposed host plants, compared to those feed on UV-shielded plants. Moreover, the duration of the larval phase was extended, whereas the pupal phase was shorter on UV-exposed than on UV-shielded plants (Diploma thesis S. Ulmann, 2007). For larvae of *Athalia rosae* (Hymenoptera), which are also oligophagous specialists on Brassicaceae, feeding preference for UV-exposed plants were observed, as well as a higher body mass and an improved food conversion of larvae raised on UV-exposed host plant material compared to those raised on UV-shielded plants, but developmental times were not affected (Diploma thesis C. Öller, 2006). Similar to our study, McCloud and Berenbaum (1999) observed faster larval growth on old than on young leaves, unaffected by UV-B altered plant quality. In the same study, a generalist, *Trichoplusia ni* (Lepidoptera) showed shorter larval developmental times on old leaves and accelerated growth on UV-B stressed plants.

Under natural environmental conditions, generalist herbivores may generally have the opportunity to switch their host plant in case of decreasing quality. However, in no-choice performance tests, variable chemical planty quality may strongly affect the feeding behaviour and the performance of generalist herbivores. At least for certain plants or habitats, the abundance of host plants for monophagous and oligophagous specialists could be limited in the field. In case of changes in host plant quality, e.g. due to changes in the environment, specialist herbivores may have to cope with a strong variation of specific chemical plant compounds. Hence, specialist herbivores may generally be characterised by a high plasticity in food utilisation that allows feeding on chemically variable specific host plants, combined with a weak variation in developmental parameters due to a strong genetic fixation.

Consequently, effects of variable host plant quality on the feeding behaviour and the development of herbivores are highly species-specific, and may depend on different

degrees of specialisation, and the feeding mode of the insects. Performance experiments with differently specialised herbivores belonging to various feeding guilds on UV-stressed *S. alba* and *N. officinale* may provide a further basis for the interpretation of the connection between the degree of specialisation and the plasticity of feeding and performance responses of insects.

Host plant recognition cues:
Are they present on the leaf surface?

Concerning the interaction between plants and herbivores, two main aspects are crucial: The localisation of stimulants and the identification of the chemical compound or the combination of compounds involved. For many insects specialised on Brassicaceae, glucosinolates were assumed to act as the sole recognition cue. Indeed, some herbivores were shown to be stimulated by glucosinolates to oviposit, e.g. *Pieris rapae* (Renwick et al., 1992), *P. brassicae* (van Loon et al., 1992), *A. rosae* (Liston, 1995; Barker et al., 2006), or to feed, e.g. weevils (Larsen et al., 1992) and various leaf beetles (Nielsen, 1978a; Nielsen et al., 1979).

Many studies on the localisation of feeding or oviposition stimulants focussed on the outermost leaf surface, the plant cuticle, which serves as a multifunctional interface between plant and abiotic and biotic environments (Riederer and Schreiber, 2001; Bargel et al., 2004), and provide the first contact zone between insect and plant (Müller, 2006). Physico-chemical surface properties vary between plant species, whereas the structure of the cuticle follows a general *bauplan*. The plant surface is composed of the cuticle layer and the cuticle proper, a polymer matrix of hydroxyl and hydroxyepoxy fatty acids. The cutin fraction is covered by epicuticular waxes, a mixture of long-chain aliphats primarily including alkanes, alkylesters, aldehydes, primary alcohols, and fatty acids (Walton, 1990), often supplied by cyclic compounds, like phenolics, flavonoids and triterpenoids (Jetter and Riederer, 1996; Jetter and Schäffer, 2001; Hare, 2002; Jetter et al., 2006). The morphology of epicuticular waxes is highly variable, but plant species-specific (Ensikat et al., 2000; Jetter et al., 2000; Jetter and Schäffer, 2001). The highly lipophilic composition of the cuticle determines the hydrophobic quality of the plant surface (Riederer and Schreiber, 2001; Müller and Riederer, 2005).

Figure 7 – Schematic cross-section of the the plant cuticle
(Illustration from Müller and Riederer, 2005)
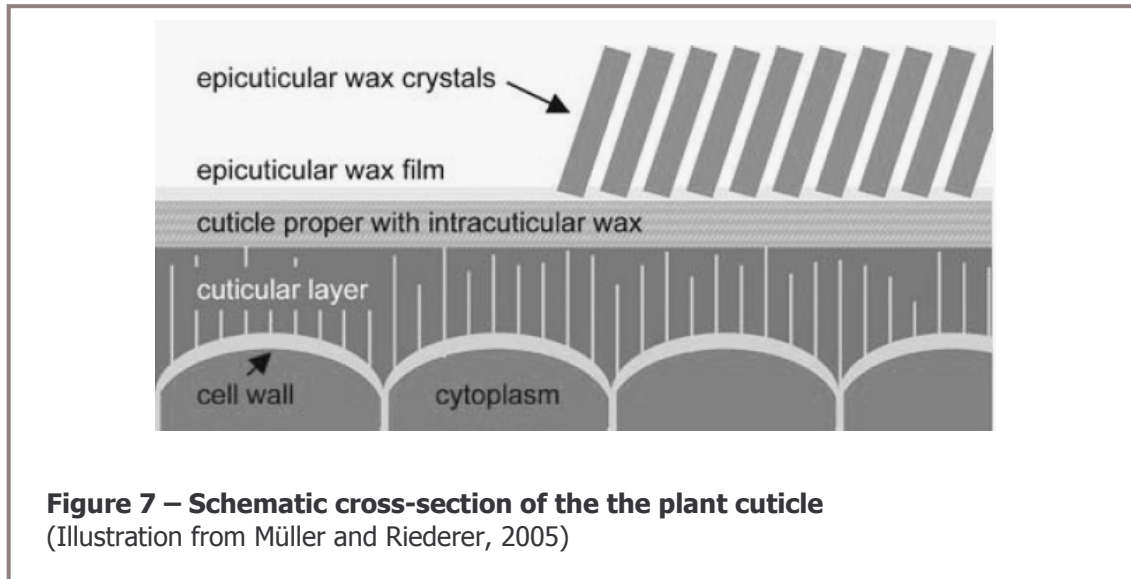
In order to localise feeding and/or oviposition stimulants in plants, earlier studies used crude extracts of Brassicaceae leaf surfaces containing high concentrations of glucosinolates. Results from bioassays based on these extracts led to the suggestion that glucosinolates occur in the outermost wax layer of Brassicaeae and are perceived by insects when they walk over the leaf surface (Städler and Roessingh, 1990; Renwick et al., 1992; Roessingh et al., 1992; van Loon et al., 1992; Baur et al., 1996; Hopkins et al., 1997; Roessingh et al., 1997). However, these extracts were prepared by immersion of Brassicaceae leaves in chloroform two times for 5 seconds, in order to dissolve the cuticular wax layer, and subsequent dipping in methanol for glucosinolate extraction (Städler and Roessingh, 1990; Roessingh et al., 1992; Hopkins et al., 1997; Hurter et al., 1999; Griffith et al., 2001). Since dipping leaves in chloroform is a common method for surface wax extractions (Jetter et al., 2000; Jetter and Schäffer, 2001), we assumed characteristic compounds of epi- and intracuticular waxes to be present in the chloroform extract of the two-phase extraction. The subsequent dipping in methanol may damage epidermal cells, resulting in the extraction of the mesophyll. Interestingly, pure water or methanol extracts did not contain glucosinolates and did not stimulate specialised *Delia radicum* (Städler and Roessingh, 1990; E. Städler, personal communication), although these solvents should be suitable for extractions of polar glucosinolates.

In our study, we used more sophisticated techniques in order to investigate whether stimulants for the mustard leaf beetle *P. cochleariae* are indeed present on the surface of two species of Brassicaceae, *Brassica napus* 'Martina' and *Nasturtium officinale*

(**Chapter 3**: Leaf surface wax layers of Brassicaceae lack feeding stimulants for *Phaedon cochleariae*; Reifenrath et al., 2005). We focussed on the general role of epicuticular waxes and glucosinolates on the feeding behaviour of this leaf beetle, and used two highly selective methods for surface extraction. In epicuticular waxes of lower and upper leaf surfaces that were mechanically removed with gum arabic (Jetter and Schäffer, 2001), no glucosinolates were detectable (analysed as desulfoglucosinolates by HPLC). In threefold short rinse extractions (3 × 20 sec) of leaf surfaces with chloroform/methanol/water (2:1:1 v/v/v) variable glucosinolate concentrations were detectable, depending on leaf-side and the light conditions under which plants were kept hours prior to extraction.

For both species, in the first extraction of plants that were kept in light conditions before extraction, minor glucosinolate concentrations were detectable. In the second and third extraction of plant surfaces concentrations increased. In plants that were kept in darkness prior the extraction procedure, glucosinolates were almost absent in the first extraction, but again, increasing concentrations were detected in subsequent extractions. Concentration differences detected in the various extraction steps suggests that glucosinolates are washed out of the mesophyll and/or epidermis when stomata are open and organic solvents are applied. According to this assumption, glucosinolate concentrations were higher in extracts of lower leaf sides where stomatal densities were highest. The higher glucosinolate concentration found in *N. officinale* compared to *B. napus* surface extracts correspond not only to higher glucosinolate contents in the leaf interior, but also to a 1.5-fold higher stomatal density on the lower compared to the upper surface (Reifenrath, unpublished data). Total glucosinolate concentrations gained from all three surface extractions were still less than 1/10 (*B. napus*) and 1/20 (*N. officinale*) of those detected in total leaf extracts of both plant species. Moreover, total glucosinolate concentration of surface extractions of both species were dramatically lower than those found in surface extracts in *Brassica oleracea* var. *fimbriata* and *Brassica napus* ssp. *rapifera* (Hopkins et al., 1997) or *Lepidium sativum* (Griffith et al., 2001). However, glucosinolate concentrations detected in total leaf extracts were similar to those obtained by Roessingh et al. (1992) for *B. oleracea* convar. *botrytis*.

We concluded that the high glucosinolate concentrations reported by other authors can be attributed to the extraction of leaf parts other than the outermost surface, thus layers that are inaccessible to the alighting insects. Moreover, the extraction method used by Städler and colleagues does not provide a suitable tool for analyses of polar

chemical compounds localised in or on epicuticular waxes. Considering the physico-chemical properties of both, glucosinolates and the plant cuticle, transportation of glucosinolates through the lipophilic cuticle to the leaf surface is highly unlikely. Polar glucosinolates are water soluble and negatively charged in aqueous solution. This reduces the probability of glucosinolates to penetrate or partition into lipid phases like the plant cuticle or waxes to negligibility. With a logarithm of the 1-octanol/water partition coefficient (log K_{OW}, Schönherr and Riederer, 1989) of -2.8 for sinigrin (Brudenell et al., 1999), the equilibrium concentration of this glucosinolate in a lipid phase comparable to 1-octanol (biomembranes, cuticular matrix) is by a factor of 630 lower than that in the adjacent aqueous phase (for further details on the calculation of the log K_{OW}, see Müller and Riederer (2005) and Riederer and Friedmann (2006)). Moreover, a transport of glucosinolates with a molecular volume of 268.4 cm^3 mol^{-1} (sinalbin) through cuticular wax layers via the polar pathway, generally restricted to polar compounds smaller than xylose (molar volume 110 cm^3 mol^{-1}, shown for *Hedera helix*; Popp et al., 2005), is improbable, but cannot be completely excluded (M. Riederer, C. Popp, pers. com.).

In bioassays, *Phaedon cochleariae* preferred leaves from which the wax layer was removed over intact surfaces. This preference was found for both upper and lower host plant leaf surfaces. Feeding was exclusively evoked by total leaf extracts of host plants, but not by leaf surface wax extracts of *N. officinale*. Sinigrin (2-propenylglucosinolate) was also not stimulating when offered in concentrations corresponding to those detected in total leaf extracts. Moreover, sinigrin combined with leaf waxes of *N. officinale* did not evoke feeding behaviour. In conclusion, leaf waxes of *N. officinale* do not only lack feeding stimulants for the mustard leaf beetle, but they may also prevent direct contact with stimulating compounds. Feeding behaviour of *P. cochleariae* could be evoked by glucosinolates only in combination with other compounds than surface waxes.

Generally, plant surfaces may provide chemical stimulants other than glucosinolates: Soluble carbohydrates and sugar alcohols were shown to leak from leaf tissues to surfaces (Mercier and Lindow, 2000). Volatile hydrolysis products of glucosinolates that may also stimulate crucifer specialists for feeding and/or oviposition (van Loon et al., 1992; Bartlet et al., 1994; Mewis et al., 2002) might evaporate through the leaf surface or stomata. Moreover, various glucosinolates might differently affect specialist

herbivores, and additive or synergistic effects of glucosinolates and other chemical (surface) compounds cannot be excluded.

The first contact between insects and plants occurs at leaf surfaces. Brassicaceae specialist leaf feeders might obtain information about the quality of a host plant by contacting leaf surface waxes, when compounds other than glucosinolates are involved. For the cabbage root fly, thia-triaza-fluorenes (TTF, 1,2-dihydro-3-thia-4,10,10b-triaza-cyclo-penta[.a.]fluorine-1-carboxylic acid; Hurter et al., 1999) and glucosinolates were identified as oviposition stimulants (Roessingh et al., 1997; Hurter et al., 1999). However, the combination of data on physico-chemical properties of leaf surfaces with results from chemical analyses of surface extracts and bioassays reveals that it is more likely for TTF to occur on outermost leaf surfaces than it is for glucosinolates (K. Reifenrath, unpublished data). The probability of glucosinolates and other substances to penetrate leaf cuticles may vary strongly across molecule properties, such as load and size, and may additionally depend on the structure of the leaf surface, the thickness of the cuticle and the number and density of stomata.

In conclusion, the waxy coverage of leaf surfaces provide an important protective layer against abiotic stress (Riederer and Schreiber, 2001) and, at least to some extent, against some feeding specialists, as previously suggested by Juniper (1995). For *P. cochleariae*, the wax cover of plant surfaces discourages from feeding due to a lack of stimulation, furthermore waxes may hinder the initiation of biting or the attachment of the beetle's tarsi (Stork, 1980; Bodnaryk, 1992). For information on glucosinolate contents and quality of host plants, herbivores may have to scratch the waxy surface to come into contact with chemical compounds of the inner plant tissue.

Host plant recognition cues:
A single or numerous compound class(es) involved in feeding stimulation?

Although polar glucosinolates are known to be involved in host plant recognition of several herbivores feeding on Brassicaceae (Chew, 1988a, b; Nielsen, 1988; Louda and Mole, 1991; Halkier and Gershenzon, 2006), their role for stimulation of adult mustard leaf beetles remained unclear. Many insects do not use only a single compound for host plant recognition, but two or even more compounds that act in concert by additive or synergistic effects (e.g. Bartlet et al., 1994; van Loon et al., 2002). Different compounds

may be even inactive when offered individually, e.g. glucosinolates and sugars for *Psylliodes chrysocephala* (Coleoptera) (Bartlet et al., 1994), or flavonoids and sinigrin for *Plutella xylostella* (Lepidoptera) (van Loon et al., 2002). Earlier studies (Nielsen, 1978a, b) on feeding stimulation of chrysomelid beetles evoked by glucosinolates, could not be confirmed for *Phaedon cochleariae* when sinigrin was offered in concentrations corresponding to those detected in leaf extracts (Reifenrath et al., 2005, **Chapter 3**).

Therefore, we re-investigated the cues responsible for feeding stimulation of *P. cochleariae* (**Chapter 4**: Multiple feeding stimulants in *Sinapis alba* for the oligophagous leaf beetle *Phaedon cochleariae*; Reifenrath and Müller, submitted). Whole leaf extracts of *S. alba* of different polarity were used to narrow down the range of potential feeding stimulants. Beetles responded stronger to polar methanol leaf extracts than to hexane leaf extracts, but not to dichloromethane extracts. The strength of feeding responses was estimated by the number of beetles feeding and net consumption rates per individual. Further bioassay-guided fractionation of the highly active methanol extracts led to the isolation of six fractions, among these one mainly contained flavonoids, the other contained glucosinolates. These both fractions evoked feeding behaviour of mustard leaf beetles to various degrees, whereas all other fractions were not stimulating. However, the combination of both fractions evoked significantly higher feeding responses than individual fractions. Additive stimulatory effects of both fractions or at least of one compound of each fraction were suggested. Within the glucosinolate fraction, sinalbin (4-hydroxybenzylglucosinolate) was identified as the main compound. The flavonols were at least roughly classified. However, in subsequent bioassays with pure metabolites, sinalbin indeed stimulated the beetles, but feeding responses were comparably weak and did not fully match the results found for fractions obtained from methanol leaf extracts.

For volatile recognition cues, specific ratios of key compounds were found to be of primary importance (Bruce et al., 2005). However, the effects of variable quality and quantity of contact cues for *P. cochleariae* remained unclear. In order to modify plants in their qualitative and quantitative composition of compounds involved in feeding stimulation, we used the exposure to different radiation conditions including and excluding UV (Reifenrath and Müller, 2007, **Chapter 1**; **Chapter 2**). The comparison of greenhouse-kept and outdoor-exposed *S. alba* plants, revealed strong differences in the ratios of glucosinolate to flavonoid concentrations. This ratio was about three times higher in greenhouse-kept plants compared to outdoor exposed plants. Sinalbin contents

were similar in both plants treatments, and benzyl glucosinolate contents differed only marginally. Stronger shifts were detected in flavonoid contents, but see Reifenrath and Müller (2007, **Chapter 1).** However, in tests with individual and combined fractions of these differently treated plants, variation in phytochemistry did not affect the strength of feeding of the mustard leaf beetle. An additional test with fresh leaves of both radiation conditions offered in a dual choice assay did not reveal any different responses by the beetle either. In conclusion, composition and content of flavonoids and variation in levels of glucosinolates and potential further unidentified metabolites that might be additionally involved are of minor importance for feeding stimulation, at least within the range of variation tested in our study.

The mere presence or absence of certain chemical compounds acting as contact cues of the mustard leaf beetle may be of higher importance than specific ratios of compounds, corroborating other studies on herbivorous insects (Larsen et al., 1992; Müller and Renwick, 2001; Heisswolf et al., 2007). Since methanol extracts had a higher stimulatory activity than combined fractions gained from those extracts, additional chemical leaf compounds only present in extracts may also be active. Among other polar metabolites, glucose or other sugars might be involved in feeding stimulation, as shown for other herbivores (Hsiao, 1985; Bartlet et al., 1994).

Feeding stimulation of the mustard leaf beetle *P. cochleariae* demands a complex interaction of several chemical plant compounds, with unidentified nonpolar compounds being less effective than the combination of several polar compounds, including sinalbin. In contrast to our previous results on the lack of feeding stimulation by sinigrin (Reifenrath et al., 2005, **Chapter 3**), sinalbin evoked feeding behaviour of the mustard leaf beetle. We conclude that various glucosinolates may differently affect the behaviour of this leaf beetle. In a natural environment, chemical plant profiles are highly variable (Reifenrath and Müller, 2007, **Chapter 1**). Therefore, it might be advantageous for a mono- or oligophagous herbivore to respond only to a combination of two or even more chemical plant compounds as feeding stimulants, but not to depend on certain quantitative ratios of involved individual compounds or compound classes. The avoidance of unsuitable host plants may be a consequence.

Conclusions and future prospects

Effects of short-term exposure of *Sinapis alba* and *Nasturtium officinale* (Brassicaceae) evoke species-specific and leaf-age dependent stress responses. UV-stress induces strong changes in nutrients and secondary metabolite contents and qualities, which include species-specific responses of two defence systems against harmful radiation and herbivore attacks. However, the underlying mechanisms of these plant responses to environmental stress are only poorly understood. Since UV-effects differed strongly between *S. alba* and *N. officinale*, the regulation of gene expression induced by UV in both species may be of highest interest. Both defence systems investigated in our study, the glucosinolate-myrosinase systems against generalist herbivores, and the accumulation of flavonoids for the protection against harmful radiation, share the same precursor, phenylalanin. Although our data on shifts of levels of all included compounds in UV-exposed plants demonstrates that there is no competition of both systems, analyses of gene expression levels may provide further information on competing biosynthetic pathways in chemically variable plants. Moreover, more controlled UV-treatments of plants, now possible in UV-transmitting greenhouses in the Botanical Garden Würzburg, narrow the range of abiotic factors accidentially affecting the experimental setup.

The performance of larvae of the oligophagous specialist leaf beetles *P. cochleariae* raised on this stressed plant material is barely affected by phytochemical variation. The lack of variation in larval development on UV-altered host plant quality may correspond to a strong genetic fixation of life history traits in *P. cochleariae*. Moreover, a high plasticity towards variable food quality may be determined by its degree of specialisation. For a better understanding on the factors determining the plasticity in feeding and performance responses of herbivores towards variable food quality, insects belonging to different feeding guilds and herbivores with different degrees of feeding specialisation (generalists, monophagous specialist) should be included in future studies. Such investigations on the reponses in plant quality towards abiotic and biotic factors, and effects on the next trophic levels, herbivores, could lead to a better understanding of effects of abiotic and biotic environmental changes.

Concerning investigations on the presence of glucosinolates on plants surfaces, we suggested an improved method for the extraction of leaf surfaces, compared to earlier studies. Low levels of glucosinolates which were detectable in our extracts were

concluded to be artefacts, indicating that solvent extractions run the risk of damaging the leaf epidermis or washing out chemical compounds of leaf interior through open stomata. The investigation of glucosinolates in epicuticular waxes provides a highly sensible research area that demands highly accurate extraction and detection methods. So far, their occurrence on leaf surfaces seems highly unlikely. However, plant species and substrate specific investigations are needed. Experiments on transport properbilities of glucosinolates and other compounds involved in host plant recognition via isolated leaf cuticles provide promising experiments. Finally, detailed structural analyses of leaf surface properties, guided by bioassays, are needed for clarification on how insects perceive information on plant qualities that may be covered by leaf waxes. The knowledge on the localisation of feeding stimulants in a plant could offer new opportunities for investigations on plant protection against herbivores.

Although fractions containg 4-hydroxybenzylglucosinolate were found to act together with flavonoid containing fractions as feeding stimulants in *S. alba* for *P. cochleariae*, the variable effects of different glucosinolates as recognition cues and feeding stimulants are not well understood for *P. cochleariae*, or other Brassicaceae specialists. Electro-physiological investigations and observations on dose-dependent feeding responses of herbivores to variable glucosinolates could lead to a more detailed understanding of their role in specialist herbivore - Brassicaceae interactions. Moreover, for our system, the identification of flavonoids involved in feeding stimulation of *P. cochleariae* may provide another step to clarify their function in feeding stimulation. Concentrations of compounds involved in feeding stimulation may have to exceed a certain threshold value to evoke feeding responses. Again, the identification of feeding stimulants of herbivores is an important step for further research on plant protection.

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Chapter 1

Species-specific and leaf-age dependent effects of ultraviolet radiation on two Brassicaceae

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Abstract. Ultraviolet (UV) radiation affects the chemical composition of a plant. Since young leaves are of higher value due to their increased photosynthetic activity, for these a more efficient protection and thus stronger responses to a short-term exposure to natural radiation including or excluding UV-A plus UV-B radiation ("+UV" vs. "–UV") were expected than for old leaves. Nutrients and characteristic secondary metabolites of two species of Brassicaceae were analysed after two days exposure in foil-tents with different UV filtering qualities. Contents of water, carbon, nitrogen and soluble protein were found to be affected by both UV and leaf-age in *Sinapis alba* L. but mainly by leaf-age in *Nasturtium officinale* L. Glucosinolates and myrosinases, both partners of the defence system of Brassicaceae, responded highly species-specific to UV-exposure. Moreover, leaf-age mainly affected total glucosinolate concentrations in *S. alba*, but myrosinase activities in *N. officinale*. The most pronounced response to UV was found in the accumulation of flavonoids which are needed to shield the leaf interior against UV. In *S. alba*, relative contents of quercetin flavonols increased at the expense of kaempferols in +UV-exposed leaves. In *N. officinale*, total flavonoid quantities were 10-fold lower in –UV-exposed young leaves compared to *S. alba*, and flavonoid accumulation was induced by UV specifically in old leaves. Hydroxycinnamic acid concentrations were not affected in both species. In total, these herbaceous species showed a highly species-specific and age-dependent plasticity in response to short-term exposure to UV which is discussed with respect to their defence strategies.

1.1 Introduction

Plants have to regulate the protection of their photosynthetic active tissue when exogenous factors or other conditions change in the environment or in new habitats. Moreover, the endogenous constitution affects the chemical composition of a plant or plant organs. Even within an individual plant, the quality and quantity of metabolites may differ between young and old leaves, sun exposed parts and organs that remain in shade (Raupp and Denno, 1983; Collinge and Louda, 1988; Kursar and Coley, 2003). Since tissue damage should be minimised especially in photosynthetic active tissues, young leaves can be regarded as more valuable than old leaves and should be better protected against biotic and abiotic harms (Iwasa et al., 1996; Lambdon et al., 2003). Shifts in quality and quantity of metabolites may affect a plant's susceptibility to abiotic stresses, and thus in turn also the attractiveness to herbivorous insects (Feeny, 1976; Cornell and Hawkins, 2003; Kursar and Coley, 2003).

In order to investigate the adaptation of plants to their environment, the influence of ultraviolet-A and -B radiation (UV-B 280-315 nm, UV-A 315-400 nm) became the subject of many studies. Enhanced UV-B radiation reaches the earth's surface due to a depletion of the ozone layer, severely affecting plant life, since this radiation is strongly absorbed by proteins, nucleic acids, and macromolecules (Bassman, 2004). The number of studies on the effects of UV-B radiation, especially on plants and insects, increased in the last twenty years, with most studies artificially enhancing UV-B radiation using UV-B lamps under controlled conditions, e.g. in greenhouses or climate chambers (e.g. Liu et al., 1995; Feng et al., 2003; Warren et al., 2003), or in field experiments (e.g. Rinnan et al., 2003; Bjerke et al., 2005) in order to test the effects on plants and their potential for adaptation and/or self-protection. Only few studies performed outdoor experiments using UV-filtering foils to expose plants to natural irradiation including or lacking the UV-portion of the spectrum (e.g. Turunen et al., 1999; Kolb et al., 2001; Caputo et al., 2006).

Since UV-B induces general stress responses in plants, the syntheses of a broad variety of metabolites regarding growth, development and defence may be affected (Doughty et al., 1995; Paul et al., 1997; Mackerness et al., 1999; Mackerness, 2000). The accumulation of flavonoids and hydroxycinnamic acids in epidermal cells is the main mechanism of plants to build up protection against UV (Caldwell et al., 1983; Olsson et al., 1998; Harborne and Williams, 2000; Kolb et al., 2001).

Brassicaceae are characterised by a binary chemical defence system against herbivores and pathogens, the glucosinolate-myrosinase system (Halkier and Gershenzon, 2006). The non-volatile glucosinolates are composed of a thioglucoside moiety and an amino acid derived side chain of varying character (Andréasson et al., 2001). In case of tissue damage, the enzyme myrosinase (β-thioglucoside glucohydrolase) which is stored in separate compartments, can hydrolyse the substrate to various toxic volatile break-down products (Rask et al., 2000). Myrosinases occur in various isoforms and can be either soluble or complexed with myrosinase-binding or -associated proteins (insoluble) (Rask et al., 2000; Eriksson et al., 2001). Glucosinolates and their volatile hydrolysis products can deter or repel several organisms but also serve as host plant recognition cues for specialists (Levin, 1976; Louda and Mole, 1991; Siemens and Mitchell-Olds, 1996). Concentration and accumulation of glucosinolates differ between plant organs and depend on the developmental stage of the plant (Agerbirk et al., 2001; Brown et al., 2003). Both, levels of glucosinolates and their hydrolysing enzymes can increase or decrease due to insects feeding (Siemens and Mitchell-Olds, 1998; Pontoppidan et al., 2003; Martin and Müller, 2006; Müller and Sieling, 2006), and JA- and SA-mediated pathways have been shown to be involved in glucosinolate induction (van Dam et al., 2004; Mewis et al., 2005). However, it remains unclear in how far shifts in both partners of the binary defence system are affected by UV irradiance.

Our goal was to study effects of short-term exposure to ambient visible and UV radiation on the chemical composition of two species of Brassicaceae taking into account the specific leaf-age. As young leaves are more valuable for the plant than old leaves, young leaves should build up protection mechanisms against harmful radiation with priority. Therefore, we expected stronger responses in young than in old leaves. Differences in quality and quantity of several primary and secondary plant compounds were studied, with special regard to the glucosinolate-myrosinase defence system. The plasticity of responses of young and old leaves to short-term +UV and –UV exposure were discussed with respect to the defence strategies of both species.

1.2 Results and discussion

1.2.1 Exposure conditions

Plants of *S. alba* and *N. officinale* were grown from seeds in the greenhouse for 19 days and then transferred to outside positioned foil-tents to investigate the plants' adaptations to different UV-conditions within 48 h exposure.

Irradiance spectra in the greenhouse lacked UV-B and had reduced levels or photosynthetic active radiation (400-700 nm). In +UV-tents spectral irradiances included UV-B and UV-A radiation from 280 to 400 nm, whereas in –UV-tents UV-B radiation was excluded, and the transmission of UV-A radiation was low between 320-360 nm, increasing slightly to full transmission at 400 nm (Fig. 1.1).

Throughout the exposure period of 48 h, the maximum day time temperature in the tents did not exceed 33 °C and did not fall below 13 °C at night time, and the temperature was minutely higher in +UV-tents compared to –UV-tents (mean deviation 0.06 °C). The relative humidity in the tents was highest in the early morning (98.0%), and lowest in the late afternoon (44.8%), with almost equal relative humidity in +UV-tents compared to –UV-tents (mean deviation of 0.8%).
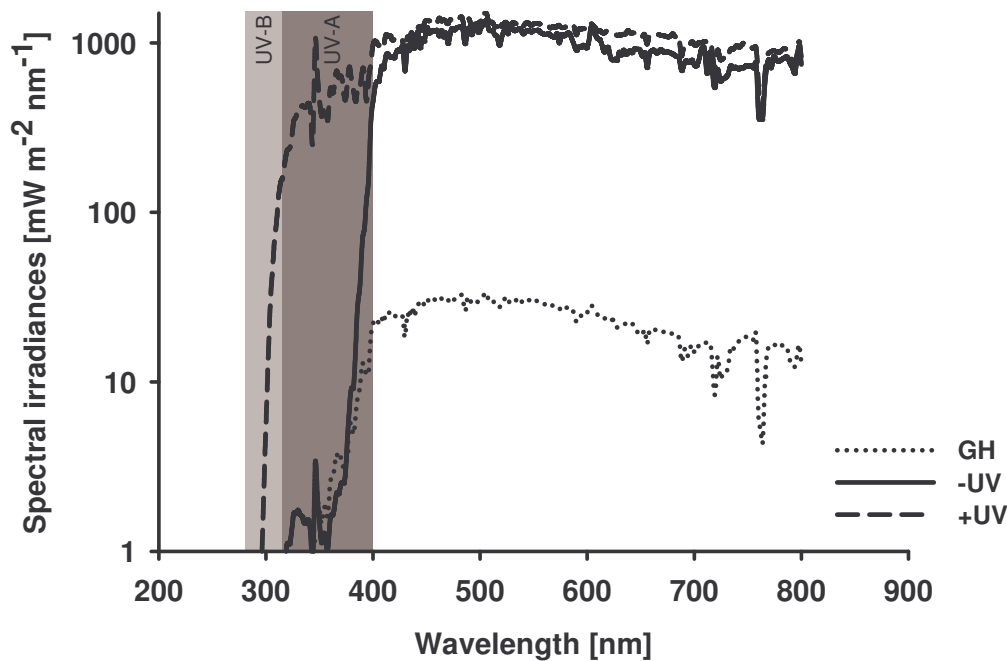
Figure 1.1 Spectral irradiances in the greenhouse and in the exposure tents transmitted by both foil types, measured under cloudless sky in the early afternoon. Wavelength sections indicating UV radiation are highlighted in light grey for UV-B radiation (280-315 nm) and dark grey for UV-A radiation (315-400 nm). Note the logarithmic y-axis.

The duration of plant exposure to UV was determined as the exposure period that is needed to achieve likely highest differences in chemical composition between –UV and +UV-exposed plants, expressed as UV-A screening capacity of the leaf epidermis and measured with a UV-A-PAM chlorophyll fluorometer. In *S. alba*, highest differences were found after 48 hours of exposure, while for *N. officinale*, differences were higher after 3 d exposure (Table 1.1). However, as in *S. alba* after 72 hours shielding capacity already converged in leaves of –UV and +UV-treatment, a 48 h exposure was used consistently for experiments. In order to study species-specific short-term exposure effects on young vs. old leaves, second youngest completely unfolded leaves and oldest non-cotyledon leaves (primary leaves) were taken for analyses.

		0 h	24 h	48 h	72 h
S. alba	-UV	33.7 ± 3.2	54.4 ± 1.0	62.5 ± 1.5	71.5 ±1.7
	+UV	33.7 ± 5.1	72.8 ± 2.4	84.7 ± 1.0	84.1 ± 1.0
N. officinale	-UV	2.2 ± 1.3	4.9 ± 0.6	7.6 ± 2.7	14.7 ± 1.9
	+UV	5.5 ± 2.5	12.9 ± 4.4	26.7 ± 3.7	44.3 ± 3.0

Table 1.1 Epidermal UV-shields (%) in young leaves of *Sinapis alba* and *Nasturtium officinale* measured after 3 time points of exposure to ambient radiation lacking UV (-UV) and ambient radiation including UV (+UV). Data were obtained with a UV-A-PAM chlorophyll fluorometer. N = 6 (mean ± SE).

1.2.2 Water and nutrients

Between young and old leaves exposed to –UV and +UV-conditions, highly plant species-specific quantitative differences in water and nutrient levels (based on dry weights) were detectable. In *S. alba*, water, nitrogen and soluble protein contents and glucose concentrations were significantly affected by UV-treatment, whereas leaf-age dependent effects were detected for water, carbon and soluble protein contents. Water contents were significantly higher in +UV than in –UV-exposed young leaves, and higher in old than in young leaves. Soluble protein contents were higher in –UV than in +UV-exposed young leaves and higher in young than in old leaves. Significant differences for glucose concentrations were only found between –UV and +UV-exposed old *S. alba* leaves (Table 1.2, Fig. 1.2).

In *N. officinale*, soluble protein contents and glucose concentrations were significantly affected by UV-treatment, whereas water, carbon, nitrogen and soluble

protein contents were affected by leaf-age. In contrast to water contents and glucoses concentrations in +UV-exposed leaves, which were higher in old than in young leaves, carbon, nitrogen and soluble protein contents and glucose concentrations in –UV-exposed leaves were on average higher in young than in old leaves (Table 1.2, Fig. 1.2).

Decreases in nitrogen contents with increasing leaf-age are characteristic for many plant species (McNeill and Southwood, 1978; Wait et al., 2002) and were more pronounced in *N. officinale* than in *S. alba*. Higher nitrogen contents in +UV-exposed plants compared to –UV-exposed plants had been also found in other plant species under supplemental UV-B (Murali and Teramura, 1985; Lindroth et al., 2000; Warren et al., 2002). Similarly, higher soluble protein levels in +UV-exposed leaves were detected in *N. officinale.* In contrast, in *S. alba* levels of soluble proteins were lower after +UV-exposure, as earlier detected in pea leaves under elevated UV-B conditions (He et al., 1993). Drastic shifts in water and nutrient levels due to different UV-exposure conditions that are furthermore leaf-age dependent must be expected as a direct result of different photosynthetic activities in these tissues (Teramura and Caldwell, 1981; Björn et al., 1997; Kolb et al., 2001, Keiller et al., 2003). These shifts may affect the plant's susceptibility to herbivorous insects, which might adapt their consumption rates to specific nitrogen contents (Feeny, 1976; Cornell and Hawkins, 2003; Kursar and Coley, 2003; Lambdon et al., 2003). However, whereas primary compounds determine the nutritional value of plant parts, the accumulation of secondary compounds, which might differ in quality and quantitiy not only between young and old leaves but also due to UV-exposure, might also strongly influence the susceptibility to harmful radiation and to herbivore attacks.

1.2.3 Secondary metabolites

One of the main mechanisms of protection against UV is the accumulation of flavonoids and hydroxycinnamic acids in epidermal cells (Caldwell et al., 1983; Olsson et al., 1998; Harborne and Williams, 2000; Kolb et al., 2001), driven by an increase in the activity of phenylalanin ammonia lyase (PAL) and other genes required for flavonoid biosynthesis (Mackerness, 2000). The UV dependent regulation of these gene expressions varies between tissues of different age and developmental stages (Kubasek et al., 1992).

In our study, in *S. alba* and *N. officinale* total flavonoid concentrations were significantly affected by UV-treatment (higher concentrations in +UV than –UV-exposed leaves, based on µmol g^{-1} DW) and leaf-age (higher concentrations in young compared to old leaves), and by the interaction between UV-treatment and leaf-age in *S. alba*. Within young –UV-exposed leaves and within old +UV-exposed leaves total flavonoid concentrations in *S. alba* were ten-fold higher compared to *N. officinale*. In old –UV-exposed *N. officinale* leaves flavonoid concentrations were below the detection limit (taken as zero µmol g^{-1} DW).

Both plant species differed in their qualitative and quantitative composition of three groups of glycosylated flavonols quercetins, kaempferols and unidentified flavonols. In *S. alba*, relative contents of all flavonols were affected by UV only, whereas in *N. officinale*, highly significant effects were evoked by UV-treatment and the interaction between UV-treatment × leaf-age (quercetin, kaempferol and unidentified flavonol glycosides) and by leaf-age (kaempferol and unidentified flavonol glycosides) (Table 1.2, Fig. 1.2). In *S. alba*, +UV-exposed leaves showed an approximately seven-fold higher relative quercetin glycoside content than –UV-exposed leaves (young leaves: –UV 6.7%, +UV 47.8%; old leaves: -UV 7.6%, +UV 46.6% of total flavonoid content), whereas relative contents of kaempferol glycosides were approximately two-fold higher in –UV-exposed than in +UV-treated leaves (young leaves: –UV 84.3%, +UV 48.3%; old leaves: -UV 83.6%, +UV 48.6% of total flavonoid content). Consequently, relative quercetin glycoside contents highly increased at the expense of relative kaempferol glycoside contents in +UV-exposed leaves compared to –UV-leaves. This flavonol shift in ratios is based on approximately 22-fold higher concentrations of glycosylated quercetins in young +UV-exposed leaves and approximate 14-fold higher concentrations in old +UV-exposed leaves compared to those exposed to –UV. Concentrations of kaempferol flavonols were hardly affected by UV-treatment in leaves of both age classes.

In *N. officinale* equally low contents of relative quercetin glycosides were found for both –UV and +UV-exposed young leaves (–UV 28.5%, +UV 23.4%), whereas the absolute quercetin glycoside concentration showed an approximate 5-fold increase between –UV and +UV-exposed young leaves. A high relative quercetin glycoside content was found for old +UV-exposed leaves (42.6% of total flavonoid content), whereas kaempferol glycosides were only detectable in young leaves exposed to +UV (13.9% of total flavonoid content).

Olsson et al. (1998) detected relatively lower kaempferol but higher quercetin glycoside levels in *Brassica napus* L. leaves exposed to supplemental UV-B radiation than in control plants under natural radiation conditions, similar to the flavonol shift we found in *S. alba*. An accumulation of 3'4'-dihydroxyflavonoids at the expense of 4'-hydroxyflavonoid synthesis was also observed for *Oryza sativa* (Poaceae; Markham et al., 1998). This might result in an increased antioxidant activity of 3'4'-dihydroxyflavonoids due to an additional ortho-dihydroxyl group in the B-ring of the flavonoid skeleton. Therefore, quercetin flavonols, prominent flavonols in *S. alba* and *N. officinale*, might play a more important role in free radical scavenging than kaempferol flavonols (Markham et al., 1998, Olsson et al., 1998, Harborne and Williams, 2000).

Hydroxycinnamic acids support the protection of the plant tissue against harmful radiation. In both plant species investigated, their concentrations were neither affected by UV-treatment, nor by leaf-age or the interaction between UV-treatment × leaf-age. Concentrations of hydroxycinnamic acids in *N. officinale* (young leaves: -UV 14.2 ± 3.0 µmol g^{-1} DW, +UV 11.3 ± 1.3 µmol g^{-1} DW; old leaves: -UV 9.6 ± 0.5 µmol g^{-1} DW, +UV 12.7 ± 1.3 µmol g^{-1} DW; mean $\pm$ SE, N = 6; Table 1.2) were about eight-fold higher compared to those in *S. alba* (young leaves: -UV 1.2 ± 0.3 µmol g^{-1} DW, +UV 1.5 ± 0.3 µmol g^{-1} DW; old leaves: -UV 1.3 ± 0.1 µmol g^{-1} DW, +UV 3.0 ± 0.9 µmol g^{-1} DW; mean $\pm$ SE, N = 6;. Table 1.2). Kolb et al. (2001) observed a high increase in the accumulation of hydroxycinnamic acids in leaves of greenhouse grown *Vitis vinifera* L. cv. Silvaner (Vitaceae) after placing them outside under different radiation regimes. These shifts started at the second day of outdoor exposure and were highest after seven days in conditions lacking UV irradiance. Increased levels of hydroxycinnamic acid might be also found in *S. alba* and *N. officinale* after longer exposure periods.

The different patterns of accumulation and distribution of phenolic compounds within both Brassicaeae due to variable UV-conditions might represent responses to ecological adaptations to the original habitats of each species. Naturally, *S. alba* grows in sunny and open habitats, and thus individuals might be potentially well adapted to high UV radiation, reflected by accumulation of large quantities of flavonols with improved scavenging activities.

Table 1.2 ANOVA *P*-and *F*-values for water, carbon and nitrogen contents (%) and soluble protein contents (µg mg^{-1} DW), glucose concentration (nmol mg^{-1} DW), total flavonoid concentration (µmol g^{-1} DW) and relative contents of individual glycosylated flavonols (% of total flavonoid concentrations, DW; not detectable concentrations were taken as zero %) and hydroxycinnamic acid concentrations (µmol g^{-1} DW), total glucosinolate concentrations (µmol g^{-1} DW) and relative contents of individual glucosinolates (% of total glucosinolate concentrations, DW; and activities of soluble and insoluble myrosinases [nmol (mg FW*min)$^{-1}$] in *Sinapis alba* and *Nasturtium officinale* leaves of different age and UV-treatments.

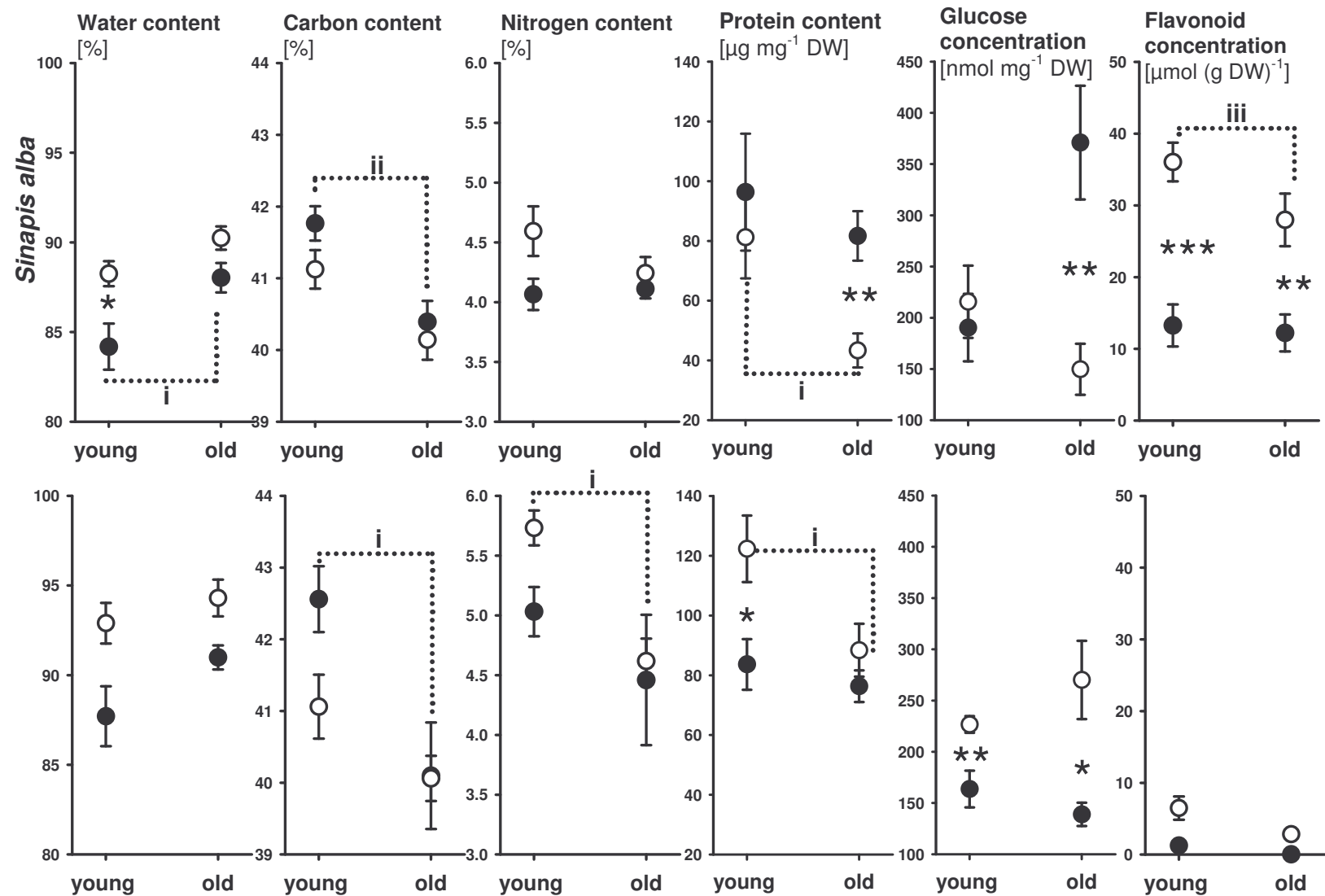|	*S. alba*			*N. officinale*		
	UV	**Leaf-age**	**UV × age**	**UV**	**Leaf-age**	**UV × age**
Water content	*P* = **0.005**, *F* = 10.5	*P* = **0.003***, *F* = 12.2	*P* = 0.32, *F* = 1.1	*P* = 0.08, *F* = 3.9	*P* = **0.007***, *F* = 13.0	*P* = 0.45, *F* = 0.6
Carbon content	*P* = 0.20, *F* = 2.7	*P* < **0.001***, *F* = 18.6	*P* = 0.48, *F* = 0.5	*P* = 0.13, *F* = 2.9	*P* = **0.003***, *F* = 13.6	*P* = 0.14, *F* = 2.4
Nitrogen content	*P* = **0.029**, *F* = 5.6	*P* = 0.29, *F* = 1.2	*P* = 0.17, *F* = 2.0	*P* = 0.08, *F* = 3.8	*P* = **0.002***, *F* = 14.5	*P* = 0.24, *F* = 1.5
Protein content (lo,*S.alba,N.officinale*)	*P* = **0.014**, *F* = 7.2	*P* = **0.023**, *F* = 6.1	*P* = **0.010**, *F* = 2.9	*P* = **0.009**, *F* = 8.5	*P* = **0.028**, *F* = 5.6	*P* = 0.14, *F* = 2.4
Glucose concentration (lo,*N.officinale*)	*P* = **0.046**, *F* = 4.5	*P* = 0.46, *F* = 0.6	*P* = **0.012**, *F* = 7.7	*P* < **0.001***, *F* = 23.5	*P* = 0.89, *F* = 0.0	*P* = 0.09, *F* = 3.2
Flavonoid concentration	*P* < **0.001***, *F* = 47.4	*P* < **0.001***, *F* = 25.0	*P* = **0.004**, *F* = 10.3	*P* < **0.001***, *F* = 19.7	*P* = **0.014**, *F* = 7.3	*P* = 0.181, *F* = 1.9
Quercetin glycosides (sqrt,*S.alba*)	*P* < **0.001***, *F* = 137.5	*P* = 0.94, *F* = 0.0	*P* = 0.63, *F* = 0.3	*P* < **0.001***, *F* = 15.5	*P* = 0.34, *F* = 0.97	*P* < **0.001***, *F* = 25.1
Kaempferol glycosides (†,*N.officinale*)	*P* < **0.001***, *F* = 83.0	*P* = 0.96, *F* = 0.0	*P* = 0.90, *F* = 0.0	*P* < **0.001***, *F* = 43.8	*P* < **0.001***, *F* = 43.8	*P* < **0.001***, *F* = 43.8
Unidentified glycolsylated flavonols	*P* = **0.003**, *F* = 11.1	*P* = 0.80, *F* = 0.1	*P* = 0.70, *F* = 0.2	*P* < **0.001***, *F* = 33.6	*P* < **0.001***, *F* = 83.7	*P* < **0.001***, *F* = 62.3
Hydroxycinnamic acids (†,*N.officinale*)	*P* = 0.10, *F* = 3.0	*P* = 0.20, *F* = 1.8	*P* = 0.25, *F* = 1.4	*P* = 0.63, *F* = 0.2	*P* = 0.36, *F* = 0.9	*P* = 0.09, *F* = 3.3
Glucosinolate concentration (lo,*S.alba*)	*P* = 0.26, *F* = 1.4	*P* < **0.001***, *F* = 36.2	*P* = 0.72, *F* = 0.1	*P* < **0.001***, *F* = 16.7	*P* = 0.93, *F* = 0.0	*P* = **0.036**, *F* = 5.0
4-OH-benzyl GS (sqrt,*S.alba*)	*P* = 0.57, *F* = 0.3	*P* < **0.001***, *F* = 47.4	*P* = 0.74, *F* = 0.1	-	-	-
Arom. GS (9 min) (†,*S.alba*)	*P* = **0.009**, *F* = 8.4	*P* = 0.19, *F* = 1.8	*P* = 0.19, *F* = 1.8	-	-	-
Arom. GS (19 min)	*P* = 0.43, *F* = 0.7	*P* = 1.00, *F* = 0.0	*P* = 0.17, *F* = 2.0	-	-	-
Arom. GS (28 min)	*P* = 0.85, *F* = 0.0	*P* = **0.003***, *F* = 11.2	*P* = 0.40, *F* = 0.7	-	-	-
Benzyl GS (sqrt,*S.alba*)	*P* = 0.06, *F* = 4.1	*P* < **0.001***, *F* = 20.6	*P* = 0.12, *F* = 2.6	-	-	-
Aliph. sulfonyl GS (24min) (sqrt,†,*S.alba*)	*P* = 0.09, *F* = 3.1	*P* < **0.001***, *F* = 49.5	*P* = 0.73, *F* = 0.1	-	-	-
2-PE (lo,†,*N.officinale*)	-	-	-	*P* = **0.020**, *F* = 6.5	*P* = 0.80, *F* = 0.1	*P* = 0.35, *F* = 0.9
4-Pentenyl GS	-	-	-	*P* = 0.51, *F* = 0.5	*P* = **0.004***, *F* = 10.5	*P* = 0.60, *F* = 0.3
8MSOO (†,*N.officinale*)	-	-	-	*P* = 0.75, *F* = 0.11	*P* = **0.001***, *F* = 13.5	*P* = 0.75, *F* = 0.1
I3M	P = 0.57, *F* = 0.3	P = 0.31, *F* = 1.1	P = 0.93, *F* = 0.0	P = 0.18, *F* = 1.9	*P* = **0.007**, *F* = 8.9	P = 0.44, *F* = 0.6
4MOI3M (at,†,*N.officinale*)	*P* = **0.006**, *F* = 9.5	*P* = 0.06, *F* = 4.1	*P* = 0.27, *F* = 1.3	*P* = **0.003***, *F* = 11.1	*P* = **0.004***, *F* = 10.9	*P* = 0.40, *F* = 0.8
Activity of soluble myrosinases	*P* = 0.41, *F* = 7.1	*P* = 0.34, *F* = 1.0	*P* =**0.040**, *F* = 5.0	*P* = 0.37, *F* = 0.8	*P* < **0.001***, *F* = 17.4	*P* =0.15, *F* = 2.3
Activity of insoluble myrosinases	*P* < **0.001***, *F* = 18.0	*P* = 0.12, *F* = 2.8	*P* = **0.049**, *F* = 4.7	*P* = 0.86, *F* = 0.0	n.t.	n.t.

Abbreviations for glucosinolates (GS): 2PE = 2-phenylethyl GS; 8MSOO = 8-metylsulfinyloctyl GS; I3M = indol-3-yl-methyl GS; 4MOI3M = 4-methoxy-indol-3-yl-methyl GS; arom. GS (9 min, 19 min, 28 min; minutes refer to retention times) = glucosinolates with aromatic side chains, not further identified; aliphat. sulfonyl GS (24 min): glucosinolate with an aliphatic sulfonyl side chain, not further identified. Data were square root -transformed (sqrt), arcsin-transformed (at) or log-transformed (lo) to achieve homogeneity of variances (data are marked with (†) where homogeneity of variances could not be achieved, but data were transformed to approximate homogeneity). N = 4-7, n.t. = not tested due to sample sizes < 4. Boldface indicates *P* < 0.05. Asterisks denote significant *P* values after a sequential Bonferroni correction for each effect (UV-treatment, leaf-age) per plant, respectively.

In contrast, *N. officinale* with comparably low flavonoid concentrations naturally grows in shady and moist places with only moderate UV radiation. However, variable flavonoid contents and compositions between different plant species and different plant organs might also determine their acceptability by herbivorous insects, since these compounds can be involved in stimulation or deterrence of feeding and oviposition on a host plant (e.g. Harborne and Williams, 2000; Warren et al. 2002; Caputo et al., 2006).

Apart from an induction and accumulation of flavonoids, UV induces further defence responses. A highly species-specific activation of sets of antioxidant enzymes and of hormones, e.g. jasmonic acid, salicylic acid and ethylene due to UV-exposure has been described (Mackerness et al., 1999; Mackerness, 2000, and references therein). Jasmonic acid and its methyl ester are known to regulate gene expression affecting plant growth and development (Mackerness et al., 1999; Mackerness, 2000), but also mediate defence responses against antagonists, such as an accumulation of glucosinolates (Doughty et al., 1995). In Brassicaceae, the glucosinolate-myrosinase system plays an important role in defence against herbivores and pathogens (Rask et al., 2000; Halkier and Gershenzon, 2006). Its effectiveness might depend on the quality and quantity of glucosinolates as well as on the activity of myrosinases.
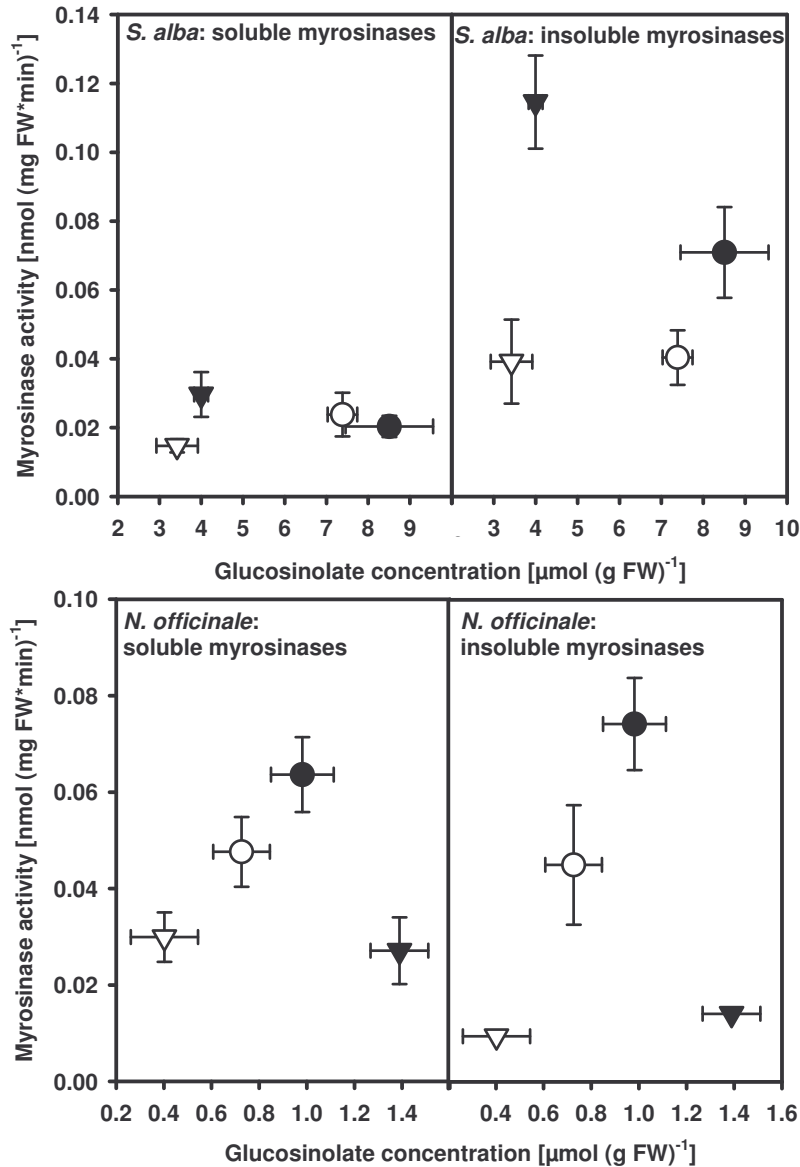
In *S. alba*, total glucosinolate concentrations (based on µmol g^{-1} DW) were significantly affected by leaf-age, with higher concentrations in young compared to old leaves (-UV-exposed young vs. old leaves: $P = 0.006$, t = 4.0, +UV-exposed young vs. old leaves: $P < 0.001$, t = 6.5; Welch-t-test). Eight main glucosinolates (GS) were identified in *S. alba*. The relative contents of some minor GS were significantly affected by UV-treatment [unidentified aromatic GS (at 9 min retention time), 4-methoxy-indol-3-yl-methyl GS], whereas others were affected by leaf-age [main GS 4-hydroxybenzyl GS, an unidentified aromatic GS (28 min), benzyl GS, an aliphatic sulfonyl GS (24 min)] (Table 1.2). In combination, the activity of soluble myrosinases [nmol (mg FW*min)$^{-1}$] in young and old *S. alba* leaves were rather low. The activity of insoluble myrosinases was strongly affected by UV-treatment with highest activity in –UV leaves, but differences were statistically significant only for old leaves ($P = 0.010$, t = 4.5; Welch t-test; Table 1.2, Fig. 1.3). The interaction between UV-treatment × leaf-age significantly affected the activity of both, soluble and insoluble myrosinases.

Figure 1.2

Metabolite quantities in young and old leaves of *Sinapis alba* and *Nasturtium officinale* after 48 h short-term exposure to natural radiation +/-UV (N = 4-7, mean ± SE). Open circles = +UV-exposed leaves, filled circles = -UV-exposed leaves. Statistical comparisons for differences in chemical compositions of leaves within each leaf-age class due to xposure to – UV and +UV, and within each UV-condition due to leaf-age, were performed with Student's t-tests (since flavonoid contents of –UV-exposed old leaves of *N. officinale* were below detection limit, this data set could not be statistically tested). For UV-affected differences asteriks indicate * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, for leaf-age differences i indicate $P < 0.05$, ii $P < 0.01$, iii $P < 0.001$. After a sequential Bonferroni-correction, only significances $P < 0.001$ remain.

Figure 1.3 Glucosinolate concentrations (µmol g^{-1} FW) and activities of soluble and insoluble myrosinases [nmol (mg FW*min)$^{-1}$] in young and old leaves of *Sinapis alba* and *Nasturtium officinale* after 48 h exposure to natural radiation +/-UV (N = 5-7, mean ± SE). Myrosinase activities were quantified as nmol glucose released per minute and mg fresh weight. Open circles = +UV-exposed young leaves, filled circles = -UV-exposed young leaves, open triangles = +UV-exposed old leaves, filled triangles = -UV-exposed old leaves.

In *N. officinale* the pattern was rather different: glucosinolate concentrations were significantly affected by UV-treatment and the interaction between UV-treatment × leaf-age. Compared to *S. alba*, glucosinolate quantities in *N. officinale* were 3 to 9-fold lower in young leaves and 10 to 12-fold lower in old leaves. Whereas in young leaves differences between differently UV-treated leaves were only weak, in old leaves significantly lower glucosinolate concentrations were measured in +UV than in –UV-exposed leaves ($P = 0.001$, t = 4.2; Welch t-test). Five glucosinolates were identified;

their relative content was affected by UV-treatment [main GS 2-phenylethyl GS, 4-methoxy-indol-3-yl-methyl GS] or leaf-age (4-pentenyl GS, 8-metylsulfinyloctyl GS, indol-3-yl-methyl GS, 4-methoxy-indol-3-yl-methyl GS]. The 4-methoxy-indol-3-yl-methyl GS could neither be detected in old leaves nor in –UV-treated young leaves, but was found in +UV young leaves (Table 1.2). The activities of soluble and insoluble myrosinases were not strongly affected by different UV-treatments, but differences in soluble myrosinase activities were significant between –UV-treated young and old leaves, with a higher activity in young leaves ($P = 0.004$, t = 4.0; Welch t-test; Table 1.2, Fig. 3). In a few leaf samples, enzyme activities were under the detection limit due to small tissue sizes (< 15 mg FW).

Strong plant species-specific and leaf-age dependent differences in the quantitative and qualitative composition of glucosinolates might variably affect the host plant recognition of specialist, whereas the combination with hydrolysing myrosinases determines the defence against generalist herbivores. Thereby the quantity of glucosinolate hydrolysis products was shown to depend on the reaction duration of active myrosinases (Lazzeri et al., 2004). However, an optimal defence against plant enemies should be obtained by a constitutive optimal ratio of myrosinase activity to glucosinolate concentration. In any plant part that needs to be protected with priority the concentrations of both partners should correspond to this ratio or be higher. This optimal ratio is unknown so far, but probably depends among others on the kind of myrosinase (soluble vs. insoluble) and on the substrate, namely specific glucosinolates. Nevertheless, high substrate levels can act already deterrent in itself.

In leaves of *S. alba* and *N. officinale* UV-treatments affected both partners of the defence system. In *S. alba*, the activities of myrosinases were similar in leaves of both age, but glucosinolate concentrations were quite different. Rather high levels of the substrate in young leaves might provide an efficient protection. In contrast, in *N. officinale* differences between young and old leaves were more pronounced in myrosinase activities than glucosinolate concentrations (Fig. 3). High myrosinase activities in young leaves might be the most important factor in the defence system of *N. officinale*. However, extraordinary low substrate levels might also weaken it independent of myrosinase activities, since a threshold value of break-down products might be necessary for effective enemy defence.

The different responses of glucosinolates and myrosinases to UV-treatments between both plant species might be again related to contrasting ecological requirements of the

species' original habitats. To our knowledge, our study is the first that investigated effects of UV on both, glucosinolates as well as myrosinase activities in leaves, taking also the leaf-age into account.

1.3 Conclusions

Responses to an environmental stress such as short-term exposure to different light regimes with increased photosynthetic active radiation levels and including or excluding UV-A plus UV-B radiation were shown to be highly species-specific and leaf-age dependent in the two investigated Brassicaceae. UV induces probably a plethora of key enzymes transmitting a general stress response. Thereby not only UV absorbing metabolites are affected but also other primary and secondary metabolites including several compounds that might influence members of the next trophic level by affecting host plant recognition, feeding and oviposition behaviour of herbivores and their performance to various degrees (e.g. McCloud and Berenbaum, 1999; Lindroth et al., 2000; Warren et al., 2002). The level of plasticity in response to UV might be closely related to the species' needs for adaptation to its specific environment, where plants might have to adjust their defence not only against harmful radiation, but also against feeding insects. Increasing costs for chemical production or for reallocation of defence chemicals might accompany this process of adaptation, depending on the abundance and sensitivity of generalist insects (Iwasa et al., 1996). Generally, old leaves were found to be less nutritious than young leaves (e.g. Raupp and Denno, 1983; Lambdon et al., 2003), and optimal levels of chemical defence were also found to decrease with leaf-age (Iwasa et al., 1996).

The feeding behaviour of insects could be affected by the ratio of certain chemical plant compounds. Young leaves of *Brassica* cultivars with a high ratio of glucosinolates to nitrogen were found to be well defended against herbivores despite a high nutritional value (Lambdon et al., 2003). Similarly, with high levels of nitrogen and soluble proteins young +UV-exposed *S. alba* leaves should provide a nutritive feeding source, but due to corresponding high flavonoid and glucosinolate levels and comparably low myrosinase activities these leaves might be efficiently defended against both harmful radiation and herbivore attacks. In contrast, old +UV and –UV-exposed leaves of *S. alba* with low nutritional values, low flavonoid and glucosinolate contents and at least variable activities of insoluble myrosinases could be regarded as less defended and

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might be preferential attacked by leaf feeders. Low nutritional quality might be thereby compensated by increased consumption rates. Old *N. officinale* leaves also contain low nutrient levels, low myrosinase activities and particularly low flavonoid levels which were even not measureable in –UV-exposed leaves, but at least –UV-exposed leaves could be efficiently defended against herbivores due to high glucosinolate contents. In young *N. officinale* leaves comparably low flavonoid and glucosinolate levels in combination with mediate myrosinase activities might indicate a low defence of this tissue despite high nitrogen and soluble protein contents. Therefore, young +UV-exposed *N. officinale* leaves should be preferred by leaf feeders among all these differently treated young and old leaves of both plant species.

Finally, the interaction between qualitative and quantitative shifts of primary and secondary metabolites of plants and their role for host plant perception of insects should be analysed in order to estimate the variable susceptibility to herbivore attacks in different plant parts and plant species exposed to different radiation regimes. Studies of herbivore performance and preferences using *S. alba* and *N. officinale* after different UV-treatments as food will provide a further base for the interpretation of the high phenotypic plasticity of leaves due to UV-treatment in the two investigated Brassicaceae.

1.4 Experimental

1.4.1 Plant species

White mustard (*Sinapis alba* L. cv. Silenda, Kiepenkerl, Norken, Germany) and water cress (*Nasturtium officinale* L., Rühlemann's, Horstedt, Germany) were grown from seeds in unfertilised soil (peat, pH 6) in the greenhouse (20: 15 °C, 16: 8 h L: D, 70% r.h.). In the greenhouse irradiance spectra lacked UV-B, whereas low levels of UV-A could be detected starting at 320 nm, but not exceeding 21 mW m^{-2} nm^{-1}. Photosynthetic active radiation (400-700 nm) was approximately $10.3 \cdot 10^{-6}$ mW m^{-2} nm^{-1}. Irradiance spectra were measured in the early afternoon under cloudless sky with a portable high accuracy UV-visible spectroradiometer configured with 0.25 mm entrance and exit slits that result in a half bandwidth of ≤ 2 nm, detected at 400 nm (OL 754, Optronic Laboratories, Orlando, USA; Fig. 1). At an age of 19 days (4-5 leaf stage for *S. alba*, multiple leaf stage for *N. officinale*) individuals plants were transferred

outdoors in two types of foil-tents to allow adaptations to two different radiation regimes including and excluding ultraviolet radiation. A minimum of 12 plant individuals of *S. alba* and six pots with *N. officinale* were exposed in each tent, resulting in 24 *S. alba* individuals and 20 *N. officinale* pots per exposure condition. Samples of the second youngest completely unfolded leaves and the primary and thus oldest non-cotyledon leaves of each plant individual were collected after short-term UV-exposure; harvest time was at about 10 o'clock in the morning. Exposure was performed in July 2005.

1.4.2 UV-exposure and climate conditions

Four exposure tents provided with two types of foils were situated outdoors in the Botanical Garden of Würzburg. Tents (3 × 1 m ground area) were constructed of wooden frames (beam width: 5 cm) with roofs and walls of foils, and aligned with the longer axis in an east-west direction. The roof sloped from 1.8 m (north) to 1.2 m (south), and the northern wall remained open for ventilation; diffuse radiation from the north was minimised by shielding the open end with the appropriate foil (3.5 × 1.5 m) mounted at 45° and 20 cm from the top of the tent (Kolb et al., 2001). Two tents were provided with a teflon foil (Nowofol, Siegsdorf, Germany) transmitting the complete visible light spectrum plus UV-A (315-400 nm) and UV-B (280-315 nm) radiation. Spectral irradiances in "+" -tents ranged from 280-900 nm; henceforth termed "+UV-treatment". The two other tents were provided with a "Lee 226 UV" foil (FFL-Rieger, Munich, Germany) cutting off UV-B, transmitting visible light, and transmitting low levels of UV-A between 320-360 nm, increasing slightly to full transmission at 400 nm. Spectral irradiances in "-UV" -tents ranged approximately from 400-900 nm; henceforth termed " –UV-treatment" (Fig. 1.1). Transmitted radiation was measured in the early afternoon under cloudless sky with a spectroradiometer (see above). Tents of both foil types were situated alternating in a distance of 3 m to each other, in order to avoid shading. Outside the tents radiation parameters (UV-A and UV-B-radiation, global radiation) were recorded by a meteorological station (Thies Clima, Göttingen, Germany) in 25 m distance from the tents. A maximum UV-A radiation of 61 W m^{-2}, UV-B radiation of 1.12 W m^{-2} and global radiation of 815 W m^{-2} were recorded. Furthermore, in both types of foil-tents, humidity and temperature were measured (Tiny Tag Ultra, Gemini Data Loggers, UK) over the whole exposure period.

1.4.3 Physical analysis of plant material

The UV-A screening of the leaf epidermis was measured with a UV-A-PAM chlorophyll fluorometer (Gademann Meßinstrumente, Würzburg, Germany). By use of light-emitting diodes, a quasi-simultaneous excitation of chlorophyll fluorescence at 375 nm, F(UV-A), and 470 nm (blue-green light), F(BL), is achieved. The resulting UV-shield (%) is calculated from $100 \times [1 - F(UV-A)/F(BL)]$ and gives a measure for the content of screening pigments, mainly phenolic compounds like flavonoids, and hydroxycinnamic acids (Bilger et al., 1997). Maximum differences in epidermal UV-shields between individual plants reflect highest differences in pigment accumulation and were taken as indicators for additional differences in chemical composition of differently treated plants. UV-shields of adaxial leaf sides of youngest leaves of six individual plant species were measured per species daily over four days of exposure at 10 o'clock in the morning in a preceding experiment. Per type of foil-tent three plant species were investigated in each of both tents, but no differences in UV-shields were detectable between these tents. Therefore, data were pooled per type of foil-tent. The optimum exposure time was determined and used for UV-treatment of plants for the main experiment.

1.4.4 Chemical analyses

For chemical analyses, leaf samples were taken from the second youngest and the oldest leaf of each plant individual exposed in the tents for 48 h, and frozen in liquid nitrogen right after harvest. Plants exposed in both tents provided with the same type of foil were haphazardly taken for leaf samples; per plant individual only one leaf sample was used for each analysis. Number of replicates varied between 4 and 7. All samples were stored at -80 °C until analysis. Leaf samples collected for analyses of glucosinolate concentration and myrosinase activity were collected from corresponding leaf halves of longitudinally sectioned leaves, respectively, in *S. alba*. As far as leaf size allowed, further samples for analysis of flavonoid concentration and C/N content were also taken from the same leaf. Otherwise, and in *N. officinale*, these were taken from additional plant individuals of the same treatment. Since *N. officinale* leaves are of small size, three or four leaves of identical age were pooled and longitudinally sectioned, in order to ensure two chemically identical samples for analyses of

glucosinolates and myrosinases, respectively. Glucose concentration and soluble protein contents were analysed from samples taken for myrosinase analysis.

Water content. Leaf samples were taken from freshly collected leaf material, weighed and lyophilised. Dry weights were measured, and weight differences of fresh and dry samples were taken as water content (in percent).

Carbon and nitrogen content. Carbon and nitrogen content of lyophilised leaf samples were determined by quantitative decomposition of substances via oxidative combustion (CHN-O-Rapid, Elementar, Hanau, Germany).

Soluble protein content. Frozen leaf discs were ground and extracted three times with buffer (200 mM Tris, 10 mM EDTA, pH 5.5). The protein content of the resulting extracts was determined with Bradford reagent (Sigma, Taufkirchen, Germany) by using bovine serum albumin (1.4 mg ml^{-1} in extraction buffer) as a standard. The absorbance was measured with a photometer (Multiskan EX, Thermo Labsystems, Vantaa, Finland) at 595 nm. Obtained absorption values were used for calculation of soluble protein content using a cubic polynomial standard curve.

Flavonoid classification and quantification. The frozen leaf material was lyophilised, weighed, ground, and extracted in aqueous 80% MeOH with catechin (Phytoplan, Heidelberg, Germany) as internal standard. In order to remove chlorophyll, petrolether (Fluka, Taufkirchen, Germany) was added to the extracts and the resulting upper phases were discarded. These purified extracts were analysed by HPLC (1100 Series, Hewlett-Packard, Waldbronn, Germany) with a quaternary pump and a 1040M diode array detector. Gradient separation of flavonoids was achieved on a Supelco C-18 column (Supelcosil LC-18, 250 × 4.6 mm, 5 μm, Supelco) with an eluent gradient (solvent A: 0.1% formic acid in aqua bidest, solvent B: acetonitril, Rotisol > 99.9%, Roth, Karlsruhe, Germany) of 8-15% B (5 min), 15-22% B (15 min), 22% B (5 min hold), 22-27% B (5 min), 27-40% B (5 min), followed by a cleaning cycle (40-100% B in 5 min, 5 min hold, 100 to 8% B in 1 min, 10 min hold). Glycosylated flavonols and hydroxycinnamic acids were classified by comparison of retention times and UV spectra to those of purified standards. Compounds were quantified by calculating the peak area at 254 nm (bandwidth 4 nm) relative to the area of the internal standard peak. In order to determine response factors of flavonol compounds and hydroxycinnamic acids to the internal standard catechin, known concentrations of the internal standard and quercetin-3-*O*-glucoside (Phytoplan, Heidelberg, Germany), kaempferol-7-*O*-neohesperidoside (Extrasynthèse, Genay, France), and ferulic acid (Fluka, Taufkirchen,

Germany), respectively, were repeatedly injected. Relative ratios of integrated peak areas were calculated and used as response factors for quantification of quercetin glycosides (response factor 43), kaempferol glycosides (36.9), other unidentified glycosylated flavonols (40, estimated) and hydroxycinnamic acids (13).

Glucosinolate identification and quantification. The frozen leaf material was lyophilised, weighed, ground, and extracted in aqueous 80% MeOH with 2-propenyl glucosinolate ($\geq$ 99%, Fluka, Taufkirchen, Germany) as internal standard. Glucosinolates were converted to desulfoglucosinolates using purified sulfatase [E.C. 3.1.6.1, 'type H-1, from *Helix pomatia*, 15100 units (gram solid)$^{-1}$; Sigma, Taufkirchen, Germany] (Graser et al., 2001). The resulting desulfoglucosinolates were analysed by HPLC and identified by comparison of retention times and UV spectra to those of purified standards, which were further confirmed by LC-ESI-MS as described earlier (Reifenrath et al., 2005). Desulfoglucosinolates were quantified by calculating the peak area at 229 nm (bandwidth 4 nm) relative to the area of the internal standard peak, corrected by the response factors as in Brown et al. (2003). A few aromatic glucosinolates of *S. alba* could not be further identified. For these, a response factor of 0.5 was assumed. For another glucosinolate of *S. alba* with an aliphatic sulfonyl side chain at 24 min retention time, a response factor of 1 was taken.

Myrosinase activity and glucose concentration. The activity of soluble and insoluble myrosinases was determined by photometric quantification of released glucose from externally added substrate. For *S. alba*, 4-hydroxybenzyl glucosinolate (obtained from glucosinolates.com, Denmark) and for *N. officinale*, 2-phenylethyl glucosinolate (Phytoplan, Germany) were used as substrates, which are the most dominant glucosinolates in both species, respectively. The extraction and determination of myrosinase activity followed slightly modified protocols of Müller and Martens (2005) and Martin and Müller (2006). Frozen leaf samples were extracted three times in 500 µl extraction buffer (200 mM Tris, 10 mM EDTA, pH 5.5) on ice and supernatants applied to Sephadex A25-columns (Sigma, Taufkirchen, Germany) to remove internal glucosinolates. Extracts were eluted with further 500 µl buffer. 150 µl of each extract were added to four individual cells on a 96-cell microplate with 25 µl of 1.9 M glucosinolate/phosphate buffer in two cells as substrate. The other two cells served as background controls and 25 µl phosphate buffer were added instead of glucosinolate. Released glucose was measured by addition of 50 µl freshly mixed colour reagent, including glucose oxidase, peroxidase, 4-aminoantipyrine and phenol (Siemens and

Mitchell-Olds, 1998) and determined by measuring the absorbance at 490 nm on a photometer for 90 min at room temperature. Means of the two replicate measurements were calculated after subtraction of the mean of the background control. A glucose standard curve was included for each assay. Maximum rates of colour production were observed between 5 and 15 min. Glucose concentrations were calculated by relating sample maximum rates to those of the standard curve (Martin and Müller, 2006). The resulting enzyme kinetics of myrosinases were analysed for a linear range of enzyme activity (usually between 30 and 70 min). Myrosinase activity was quantified as nmol glucose released per minute and mg fresh weight. To detect activity of insoluble myrosinases in the remaining pellets, these were dissolved in 500 µl buffer, and measured in the same way as the supernatants, with two background controls per sample. Under our assay conditions, rates of colouration were not affected by ascorbate (data not shown).

1.4.5 Statistical analyses

Multifactorial ANOVA were used to determine the effects of UV-exposure, leaf-age and the interaction term UV-treatment × leaf-age (Table 1.2). Data were square root -, arcsin- or log-transformed to achieve homogeneity of variances. For further analyses Students t-tests for independent variables were used instead of post hoc tests following the ANOVA, since only comparisons of chemical composition within leaf-age classes due to UV-exposure and within UV treatments due to leaf-age classes, respectively, were of interest (Fig. 1.2). In case of heterogeneity of variances, Welch correction was used, and two-tailed P-Values are given. Data were analysed with Statistica 7 (StatSoft, Tulsa, USA).

1.5 Acknowledgements

1.6 References

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Chapter 2

Effects of variable host plant quality on the performance of the oligophagous leaf beetle *Phaedon cochleariae*

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Abstract. 1. Short-term stress responses of plants can lead to shifts in metabolic profiles which may not only differ between plant species, but also between young and old leaves within the same plant individual. According to the plant stress - insect performance hypothesis, such changes of host plant quality result in an improved performance of herbivores.

2. Short-term exposure of two Brassicaceae species, *Sinapis alba* and *Nasturtium officinale*, to three different radiation regimes (greenhouse vs. outdoors conditions including or excluding ambient ultraviolet radiation) was performed to induce variable chemical leaf qualities.

3. Naive larvae of the oligophagous mustard leaf beetle *Phaedon cochleariae* were raised on young and old leaves of differently stressed plants under standardised conditions. Effects of different food qualities on the development of larvae were investigated.

4. In contrast to larval developmental times, the duration of pupal phases as well as mean larval, pupal and adult body masss were not affected by different host quality. However, within 24 h of development of first and second instar larvae, increases in body mass, growth and consumption rates varied significantly in dependence of plant species and leaf-age, but not with regard to plant stress treatments. Differences may be compensated by increases in larval consumption rates or growth efficiencies, or by extension of larval phases.

5. The lack of variation in phenotypic responses to stressed plant tissues could be explained by a high plasticity of mustard leaf beetles to variable food quality. Plasticity of herbivores may depend on the degree of specialisation.

2.1 Introduction

The plant stress - insect performance hypothesis predicts improved insect performance and higher densities of herbivores on stressed plants (Rhoades, 1979; White, 1984; Larsson, 1989). Stress rather evokes short-term responses in plants than adaptations to long-term shifts in the environment (Larsson, 1989), and can be expressed as change in growth and development and/or changes in metabolic profiles. Variation in radiation quality, specifically in the ultraviolet range, can provide an important stress factor. Common plant responses to UV-B stress include changes in photosynthetic activity, pigment composition and enzyme activities (Mackerness, 2000) and morphological variation (Caldwell et al., 2003). In higher plants, the accumulation of flavonoids and hydroxycinnamic acids in the plants' epidermal cells functions as protection shield against UV-B radiation (wavelength 280 - 315 nm) that otherwise damages lipids, proteins and nucleic acids (Caldwell et al., 1989; Bassman, 2004). However, increased flavonoid concentrations might also affect the feeding behaviour of herbivores (Harborne and Williams, 2000).

The biosynthetic pathway for UV-B screening flavonoids may compete for precursors needed for other metabolites used for anti-herbivore-protection (Tegelberg and Julkunen-Tiitto, 2001; Casati and Walbot, 2003), e.g. tannins or glucosinolates. In Brassicaceae, glucosinolates and their hydrolysing enzymes, myrosinases, are both partners of a highly specific defence system (Halkier and Gershenzon, 2006). These compounds are stored in separate compartments in plant cells; they only come into contact in case of tissue damage. Myrosinases hydrolyse glucosinolates to various toxic breakdown products, that can repel generalist herbivores, e.g. as nitriles and isothiocyanates (Rask et al., 2000). In contrast, for specialists, glucosinolates and their volatile break-down products can serve as host plant recognition cues (Renwick, 2002), whereby effects of glucosinolates may be dose-dependent (Siemens and Mitchell-Olds, 1996). In case of UV-exposure of Brassicaceae, the accumulation of flavonoid shield pigments may occur at the expense of herbivore protection via glucosinolates, since biosynthesis of at least some flavonoids and glucosinolates share the same precursor,

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phenylalanine (Harborne, 1991; Halkier and Gershenzon, 2006). Herbivores may respond to such strong chemical variation of their host plants by increased or decreased acceptance and/or variable performance (Caldwell et al., 2003; Cornell and Hawkins, 2003). Increases in soluble nitrogen and amino acids contents (White, 1984) in UV-exposed host plants have been shown to cause shifts in feeding preferences towards UV-B stressed plants and increased performance on such plants (Lavola et al., 1998; Veteli et al., 2003) which is in accordance with the plant stress-insect performance hypothesis.

Furthermore, the chemical composition may also vary across organs and tissues of different age within plant individuals. Young leaves have been found to respond with priority to abiotic impacts such as altered radiation conditions, including increased photosynthetically active radiation (PAR) and exposure to UV radiation (McCloud and Berenbaum, 1999; Reifenrath and Müller, 2007). Such within-plant variation in susceptibility to radiation conditions should also determine feeding preferences of herbivores for specific plant parts exposed to or shielded against UV and/or the performance of leaf feeders raised on differently treated leaves.

Our goal was to study the impact of changes in chemical composition of stressed host plants on the performance of an oligophagous Brassicaceae specialist, the mustard leaf beetle *Phaedon cochleariae*, with regard to three sources of variation: plant species, leaf-age and stress treatment. Greenhouse grown plants of *Sinapis alba* and *Nasturtium officinale* were stressed by short-term exposure to ambient outdoor radiation including or excluding UV-A and B. Naïve larvae were raised on either young or old leaves of differently treated plants until adulthood. Furthermore, relative growth rates, relative consumption rates and growth efficiencies for first and second larval instars were measured over a 24 h-feeding period on different host plant tissues. Since the insects were kept under standardised conditions lacking UV radiation, developmental effects could be directly devoted to plant quality. Higher nutritional values were expected for young leaves and for UV-exposed plant material and primary and secondary metabolites were quantified in the different leaf tissues of both plant species. According to the plant stress – insect performance hypothesis, feeding mustard leaf beetle larvae on stressed plant tissues was expected to lead to an improved performance expressed as shorter developmental times, increased growth rates and higher growth efficiencies.

2.2 Materials and methods

2.2.1. Plants and UV-treatment

White mustard, *Sinapis alba* L. cv. Silenda (Kiepenkerl, Norken, Germany), and water cress, *Nasturtium officinale* L. (Rühlemann's, Horstedt, Germany), were grown from seeds in the greenhouse (20: 15°C, 16: 8h L: D, 70% r.h.) in pots in unfertilised soil (peat, pH 6). For stress treatment by short-term exposure to different irradiance spectra, two third of 19 days old plants (4-5 leaf stage for *S. alba*, multiple leaf stage for *N. officinale*) were transferred in tents provided with two types of foils (see below) transmitting or excluding UV-A+B radiation ("+UV-treatment", "–UV-treatment") . The remaining plants were kept under greenhouse radiation conditions ("GH treatment").

The foil tents (base 3×1 m, height 1.20 m in south direction, sloping to 1.80 m in north direction, open at the northern side for ventilation but provided with an appropriate foil mounted at 45° and 20 cm from the frame for minimisation of diffuse radiation) were placed outdoors in the Botanical Garden Würzburg. For further details of the construction see Kolb et al. (2001). Relative humidity and temperature were similar in both tents over the whole exposure period (Tiny Tag Ultra Gemini Data Loggers, UK). For details on climate measurements in the tents and spectral irradiances measured in the tents and in the greenhouse, see Reifenrath and Müller (2007).

"GH treatment": Greenhouse grown plants remained in the greenhouse for additional 48 h. In the greenhouse irradiance spectra lacked UV-B, while UV-A started at 340 nm, without exceeding approximately 30 mW(m²·nm)$^{-1}$; and photosynthetic active radiation (PAR) was about $10.3 \cdot 10^{-6}$ mW(m²·nm)$^{-1}$.

"–UV-treatment": Greenhouse grown plants were exposed for 48 h in foil tents provided with a "Lee 226 UV" foil (FFL-Rieger, Munich, Germany). This foil filtered off UV-B radiation, but transmitted a low UV-A radiation level between 320-360 nm, slightly increasing to full permeation at 400 nm; the PAR was about 934 mW(m²·nm)$^{-1}$.

"+UV-treatment": Greenhouse grown plants were exposed for 48 h in foil tents provided with a teflon foil (Nowofol, Siegsdorf, Germany). This foil transmitted the complete visible light spectrum plus UV-A and UV-B radiation with irradiance spectra of 280-900 nm and a PAR of approximately 1117 mW(m²·nm)$^{-1}$.

After 48 h short-term exposure, the second youngest completely unfolded leaves ("young leaves") and the primary, non-cotyledonous leaves ("old leaves") were

harvested and used for performance experiments and chemical analyses. Over the whole period of the experiment, every other day new plants were transferred in the tents (or kept in the greenhouse) and plant material renewed in the insect performance trials (see below). Experiments were conducted in May/June 2005 and 2006.

2.2.2 Insect bioassays

Larvae of *Phaedon cochleariae* (F.) (Chrysomelidae; Coleoptera) used for performance experiments originated from a laboratory colony reared on chinese cabbage and maintained in a climate chamber at 20 °C, 70% r.h., and a photocycle of 16: 8h L: D. Freshly emerged larvae with an initial weight between 0.06 and 0.1 mg were placed individually in Petri dishes (∅ 5 cm) and fed with young or old leaves of differently treated plants (GH, -UV, +UV). Per plant species, plant stress treatment and leaf-age twelve larvae were tested (twelve parallel rearings, 144 larvae in total). Petri dishes were equipped with moist filter paper and leaves that were water supplied by placing the petioles in water filled Eppendorf cups. Petri dishes were sealed with Parafilm in order to assure constantly high humidity and were kept in the climate chamber.

Larvae were weighed every second or third day, and pupae and adult beetles were weighed within 24 h after hatching. Mean body mass increase of every single larva until pupation was defined as the factor β in the exponential growth function $m_t = \alpha\, e^{\beta\, t}$, where m_t was the body mass at larval age t, α was taken as the average parameter across all larvae for the entire dataset, whereas β was fitted for each larva. Moreover, total developmental times of larval and pupal stages were recorded. Effects of different host plant quality on larval body mass increases β and on the developmental times of larval and pupal stages (days) were analysed with reference to plant species (analysed with Student's t-test), leaf-age (Student's t-test) and differently stress treated plant material (one-way ANOVA).

During first instars (day 3 to day 4) and second instars (day 7 to day 8) development, initial and final larval body masses after 24 h feeding were measured and consumption of host plant leaf material within this period per larva was calculated. Leaf discs of *S. alba* (∅ 1.5 cm) and total leaves of *N. officinale* were offered, and afterwards remaining leaf areas measured with a leaf area meter (Winfolia 2004a, Régent Instruments, Canada). Consumption rates were calculated as mg fresh weight (FW) from reference

leaves for plant species, leaf-age and stress treatment, respectively. Insect performance indices were determined following Waldbauer (1968). Relative growth rate (RGR) was calculated as [final larval body mass (mg FW) – initial larval body mass (mg FW)] × initial larval body mass (mg FW)$^{-1}$, relative consumption rate (RCR) was obtained as leaf consumption (mg FW) × initial larval body mass (mg FW)$^{-1}$, and growth efficiency (= efficiency of conversion of ingested food to body substance, ECI) as [final larval body mass (mg FW) – initial larval body mass (mg FW)] × leaf consumption (mg FW)$^{-1}$. The effects of plant species, leaf-age, plant treatment and larval instars and related interaction terms on RGR, RCR, and ECI of larvae were analysed by multifactorial ANOVA.

2.2.3 Phytochemical analyses

For chemical analyses, leaf samples were frozen in liquid nitrogen right after harvest. Per plant species, leaf-age, plant stress treatment and date of harvest the number of replicates varied between 5 and 10, taken from individual plants, respectively. Leaf samples for analyses of specific dry weights, water and protein contents, glucose, glucosinolate, flavonoid and myrosinase activity concentrations were collected at three different time points during the insect bioassay, samples for analysis of the carbon/nitrogen ratio were collected at a single time point (N = 4-6). All samples were stored at -80°C until analysis.

Specific dry weights were taken for comparisons of leaf toughness and calculated as dry weight of a leaf disc divided by the corresponding area of the leaf disc. Water content was calculated by weighing leaf discs of freshly collected material before and after lyophilisation. Ratios of carbon/nitrogen content of lyophilised leaf samples were determined with an elemental analyser (CHN-O-Rapid, Elementar, Hanau, Germany). Determination of soluble protein concentration followed the protocol by Bradford (1976).

For flavonoid quantification frozen leaf material was lyophilised, weighed, ground, and extracted in 80% MeOH. Purified extracts were analysed by HPLC and glycosylated flavonols were classified and quantified as described earlier (Reifenrath and Müller, 2007). Total flavonoid concentrations were evaluated by adding concentrations of individual glycosylated flavonols per leaf sample.

For glucosinolate quantification frozen leaf material was lyophilised, weighed, ground, and extracted 80% MeOH. Glucosinolates were converted to desulfoglucosinolates using purified sulfatase [E.C. 3.1.6.1, 'type H-1, from *Helix pomatia*, 15100 units (gram solid)$^{-1}$; Sigma, Taufkirchen, Germany]. Resulting desulfoglucosinolates were analysed by HPLC and identified and quantified as described earlier (Reifenrath et al., 2005; Reifenrath and Müller, 2007). Total glucosinolate concentrations were calculated by adding concentrations of individual glucosinolates per leaf sample.

Myrosinases occur in various isoforms and can be either soluble or complexed with myrosinase-binding or -associated proteins (Rask et al. 2000; Eriksson et al., 2001). The activity of soluble (present in supernatants of plant extracts) plus insoluble myrosinases (present in remaining pellets) was determined by photometric quantification of released glucose from externally added substrates, namely 4-hydroxybenzyl glucosinolate (obtained from glucosinolates.com, Denmark) for *S. alba*, and 2-phenylethyl glucosinolate (Phytoplan, Heidelberg, Germany) for *N. officinale*, the most dominant glucosinolates of each species, respectively. The extraction and determination of myrosinase activity concentration followed slightly modified protocols of Müller and Martens (2005) and Martin and Müller (2007). Frozen leaf discs were extracted in extraction buffer (200 mM Tris, 10 mM EDTA, pH 5.5) on ice and supernatants applied to Sephadex-columns (Sigma, Taufkirchen, Germany) to remove internal glucosinolates. In order to determine the activity of both soluble plus complexed myrosinases, the remaining pellets were dissolved in supernatants. These extracts were added to four individual cells on a 96-cell microplate with additional glucosinolate/phosphate buffer in two cells as substrate. The other two cells served as background controls with phosphate buffer added instead of glucosinolate. Released glucose was measured by addition of freshly mixed colour reagent (Siemens and Mitchell-Olds, 1998) and determined by measuring the absorbance at 490 nm with a photometer (Multiskan EX, Thermo Labsystems, Vantaa, Finland) for 90 min at room temperature. The resulting enzyme kinetics were analysed for a linear range of enzyme activity (usually between 30 and 70 min). A glucose standard curve was included for each assay. Myrosinase activity concentration was quantified as nmol glucose released per minute and mg fresh weight. Under our assay conditions, rates of colouration were not affected by ascorbate (data not shown).

Glucose concentrations were measured from supernatants only, using the same protocol as described above. Glucose concentrations of each sample were calculated by

relating sample maximum rates to those of the standard curve (Martin and Müller, 2007).

2.3 Results

2.3.1 Insect performance

Feeding on differently stressed plant material had no significant effects on larval body mass increases of *P. cochleariae* during development (all $P \geq 0.20$, one-way ANOVA; Fig. 2.1). However, body mass increases of larvae were affected by leaf-age (*S. alba* young vs. old GH kept leaves, t = 2.3, $P = 0.040$; *S. alba* young vs. old –UV-exposed leaves, t = 2.4, $P = 0.028$; *N. officinale* young vs. old +UV-exposed leaves, t = 2.7, $P = 0.015$; all other comparisons between body mass increases on old vs. young leaves $P \geq 0.07$; Student's t-test for unpaired samples). Moreover, larval body mass increases differed significantly with regard to food plant species, with higher body mass increases for larvae raised on *N. officinale* than on *S. alba* (young GH kept leaves of *N. officinale* vs. *S. alba*, t = 4.5, $P < 0.001$; young –UV-exposed leaves, t = 4.7; $P < 0.001$; young +UV-exposed leaves, t = 6.1; $P < 0.001$; old +UV-exposed leaves t = 2.2; $P = 0.044$; all other $P \geq 0.06$; Student's t-test for unpaired samples). Pupal and adult masses did not differ significantly in response to plant stress treatment (pupal masses all $P \geq 0.23$; adult masses all $P \geq 0.32$; one-way ANOVA), leaf-age (pupal masses all $P \geq 0.25$; adult masses all $P \geq 0.28$; Student's t-test) and plant species (pupal masses all $P \geq 0.33$; adult masses all $P \geq 0.40$; Student's t-test for unpaired samples; Fig. 2.1). The duration of larval development was not affected by plant stress treatment (all $P \geq 0.71$; one-way ANOVA), but by leaf-age of *S. alba*. Developmental times of larvae reared on *S. alba* were shorter on old than on young leaves (GH kept leaves, t = 3.4, $P = 0.004$; –UV-exposed leaves, t = 3.9, $P = 0.001$; +UV-exposed leaves, t = 2.7, $P = 0.018$; all other $P \geq 0.14$, Student's t-test for unpaired samples). Moreover, the duration of larval development differed between both food plant species: larvae raised on *N. officinale* leaves developed faster than larvae fed with *S. alba* (young GH kept leaves, t = 4.4, $P = 0.002$; young –UV-exposed leaves, t = 4.8; $P < 0.001$; +UV-exposed leaves, t = 5.4; $P < 0.001$; Student's t-test for unpaired samples, Welch corrected, all other $P \geq 0.08$). The

mean pupal duration was consistently about 6 days long regardless of host plant species, leaf-age and plant stress treatment.

	Relative growth rate	Relative consumption rate (log)	Growth efficiency (sqrt, †)
Plant species	17.6, **< 0.001**	32.0, **< 0.001**	2.7, 0.10
Plant stress treatment	1.8, 0.17	1.7, 0.19	1.8, 0.17
Leaf-age	20.8, **< 0.001**	26.5, **< 0.001**	31.1, **< 0.001**
Larval instar	55.0, **< 0.001**	61.4, **< 0.001**	6.1, **0.015**
Species × plant stress treatment	0.8, 0.47	2.0, 0.14	1.0, 0.37
Species × leaf-age	6.3, **0.013**	8.1, **0.005**	3.2, 0.08
Plant stress treatment × leaf-age	2.0, 0.15	1.6, 0.21	2.3, 0.11
Species × larval instar	13.1, **< 0.001**	26.2, **< 0.001**	17.9, **< 0.001**
Plant stress treatment × larval instar	0.1, 0.91	0.2, 0.86	4.6, **0.011**
Leaf-age × larval instar	7.1, **0.008**	7.7, **0.006**	4.5, **0.035**
Species × stress treatment × leaf-age	0.8, 0.46	1.9, 0.16	12.7, **< 0.001**
Species × treatment × larval instar	0.1, 0.95	0.6, 0.56	2.3, 0.11
Species × leaf-age × larval instar	3.9, 0.051	3.4, 0.07	8.1, **0.005**
Treatment × leaf-age × larval instar	0.1, 0.95	0.1, 0.93	2.9, 0.06

Table 2.1 F- and *P*-values of multifactorial ANOVA for relative growth rates, relative consumption rates and growth efficiencies (number of replicates varied between 5 and 10) of first and second instar larvae of *Phaedon cochleariae* raised on young and old leaves of *Sinapis alba* and *Nasturtium officinale* exposed to different radiation conditions [greenhouse (GH), outdoors in foil tents filtering ambient UV radiation (-UV) or transmitting ambient UV radiation (+UV)]. For calculation of indices see Material and Methods. Data were arcsin-transformed (at) or log-transformed (log) to achieve homogeneity of variances (data are marked with (†) where homogeneity of variances could not be achieved, but data were transformed to approximate homogeneity). Boldface indicates $P < 0.05$.

Relative growth rates and relative consumption rates of first and second larval instars of *P. cochleariae* within 24 h were significantly affected by larval instars, leaf-age and food plant species, but not by plant stress treatment, respectively (Tables 2.1, 2.2). Moreover, these parameters were also affected by all two-way interactions between plant species, leaf-age and larval instars (Table 2.1). Effects of larval instars and leaf-age on RGR and RCR were variable, but both RGR and RCR were consistently higher for first larval instars and mostly also for second larval instars fed with *N. officinale* than those fed with *S. alba* (Table 2.2). In contrast, growth efficiencies of *P. cochleariae* larvae were significantly affected by larval instars and leaf-age, but not by plant species or plant stress treatment. ECI of larvae varied due to larval instars, but were consistently higher for larvae raised on young leaves than those raised on old leaves, especially for first instar larvae. Furthermore, ECI were affected by all two-way

interactions with larval instar and three-way interactions with plant species and leaf-age (Table 2.1).

2.3.2 Phytochemistry

Contents of water, C/N, and soluble protein, and concentrations of glucose, flavonoids and glucosinolates differed significantly between plant species (calculated for fresh weights, Table 2.3a). In *S. alba*, all parameters except myrosinase activity concentration, were significantly affected by either leaf-age and/or plant treatment, but not by the interaction between leaf-age and plant stress treatments (Table 2.3b). With the exception of water contents and C/N ratio, metabolite quantities and specific dry weights were higher in outdoor exposed plants compared to greenhouse-kept plants (clear patterns for specific dry weights, glucose and flavonoid concentrations), and higher in young than in old leaves (clear patterns for specific dry weights, protein contents, flavonoid and glucosinolate concentrations; Fig. 2.2). Highest values in –UV-exposed leaves were detected for specific dry weights, soluble protein contents and glucosinolate concentrations.

In *N. officinale*, all parameters analysed were significantly affected by leaf-age, and, with the exception of myrosinase activity concentrations, also by plant stress treatment (Table 2.3c). Furthermore, flavonoid and glucosinolate and myrosinase activity concentrations were affected by the interaction between leaf-age and plant treatment. Again with the exception of water contents and C/N ratio, quantities of some metabolites were higher in outdoor exposed plants compared to greenhouse-kept plants (clear patterns for soluble protein contents, glucose and flavonoid concentrations), and higher in young than in old leaves (clear patterns for protein contents, glucose, flavonoid, glucosinolate and myrosinase activity concentrations; Fig. 2.2). Higher water contents in old compared to young leaves were found in both plant species.

Due to variable water contents in leaves of different age and plant stress treatment, for some metabolites, dry weight (DW) concentrations differed from fresh weight calculations, like for soluble protein contents (for DW: no significant differences between stress treatments; in *S. alba* leaf-age specific differences remain significant for +UV-exposed leaves only; in *N. officinale* higher contents in old than in young GH kept leaves, but leaf-age differences persist for GH and +UV-exposed plants) and glucose concentrations (for DW: in *S. alba* no differences between plant stress treatments, leaf-

age differences persist in GH kept leaves only; in *N. officinale* lower concentrations in old than in young leaves).

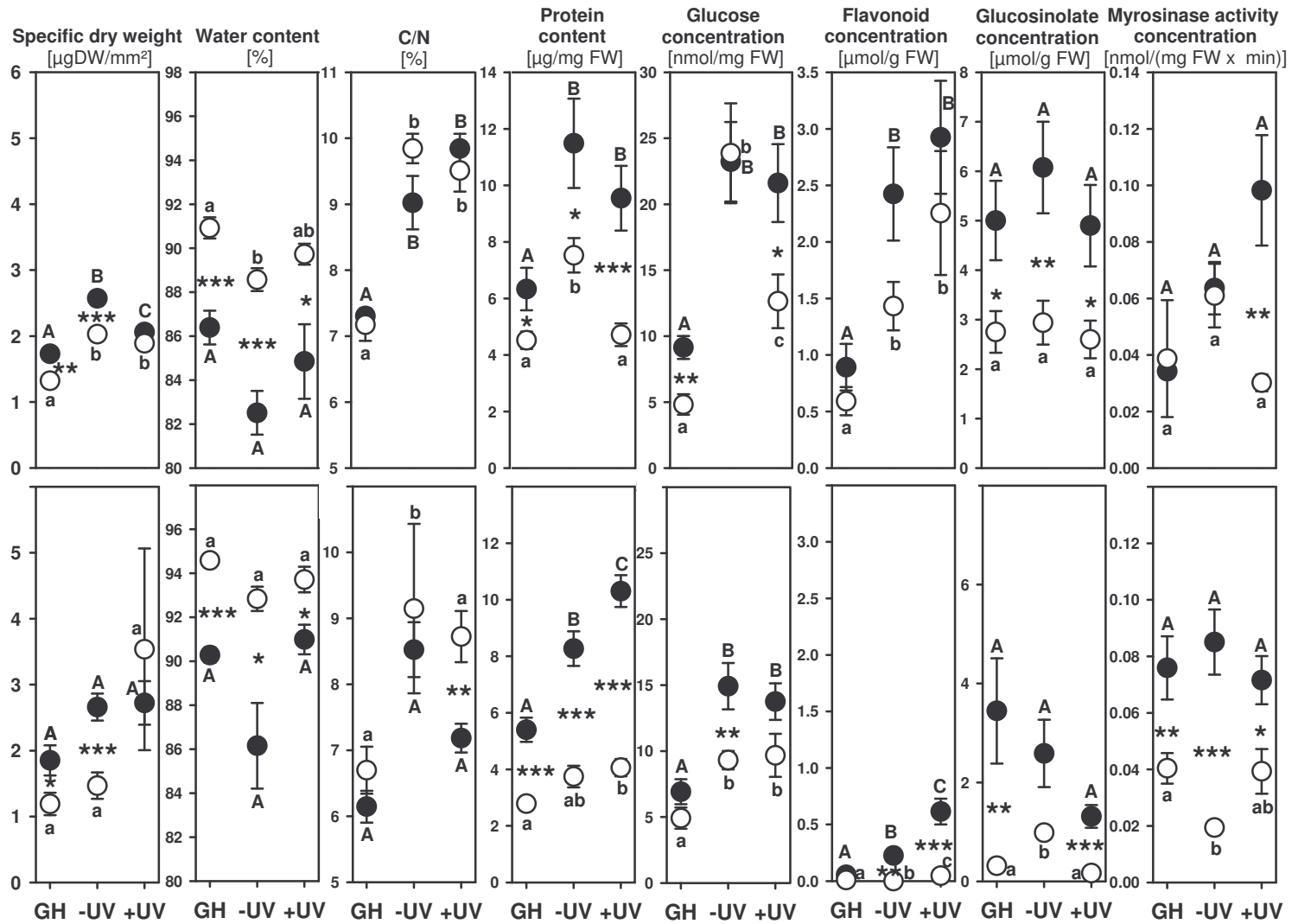|	Specific dry weight	Water content	C/N content	Protein content	Glucose concentration	Flavonoid concentration	Glucosinolate concentration	Myrosinase activity concentration
	†				log, †	sqrt, †	sqrt	
(a) Species	1.0	36.3	12.6	6.6	14.2	111.3	64.1	0.0
	0.31	**< 0.001**	**< 0.001**	**0.011**	**< 0.001**	**< 0.001**	**< 0.001**	0.85
(b)								
S. alba	log	arc, †		log	log	log	log	
Stress treatment	34.0	8.2	44.3	14.4	23.9	21.2	0.3	1.1
	< 0.001	**< 0.001**	**<0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.71	0.36
Leaf-age	21.5	59.2	0.0	29.9	11.4	7.5	8.9	2.3
	< 0.001	**< 0.001**	0.86	**< 0.001**	**0.001**	**0.008**	**0.004**	0.14
Stress treatment × leaf-age	1.1	0.2	1.3	2.1	2.2	0.0	0.4	3.4
	0.33	0.79	0.28	0.13	0.11	1.0	0.67	0.05
(c)								
N. officinale	log	arc		log	log	†	log, †	log
Stress treatment	4.6	9.6	16.3	16.5	11.9	8.5	15.2	2.7
	0.013	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.08
Leaf-age	13.1	61.6	7.0	129.9	6.3	20.6	59.7	44.9
	< 0.001	**< 0.001**	**0.016**	**< 0.001**	**0.014**	**< 0.001**	**< 0.001**	**< 0.001**
Stress treatment × leaf-age	0.8	2.1	0.9	0.6	0.1	6.2	8.2	5.4
	0.45	0.15	0.42	0.53	0.89	**0.004**	**< 0.001**	**0.006**

Table 2.3 F- and *P*-values of one-way ANOVA for differences in specific dry weights, water contents and metabolite quantities between *Sinapis alba* and *Nasturtium officinale* (a). F- and *P*-values of multifactorial ANOVA for specific dry weights, water contents and metabolite quantities of young and old leaves of *Sinapis alba* (b) and *Nasturtium officinale* (c) exposed to different radiation conditions [greenhouse (GH), outdoors in foil tents filtering ambient UV radiation (-UV) or transmitting ambient UV radiation (+UV)] (number of replicates varied between 10 and 15). Data were log- (log), square root- (sqrt) or arcsin-transformed (arc) to achieve homogeneity of variances (data are marked with (†) where homogeneity of variances could not be achieved, but data were transformed to approximate homogeneity). Boldface indicates $P < 0.05$.
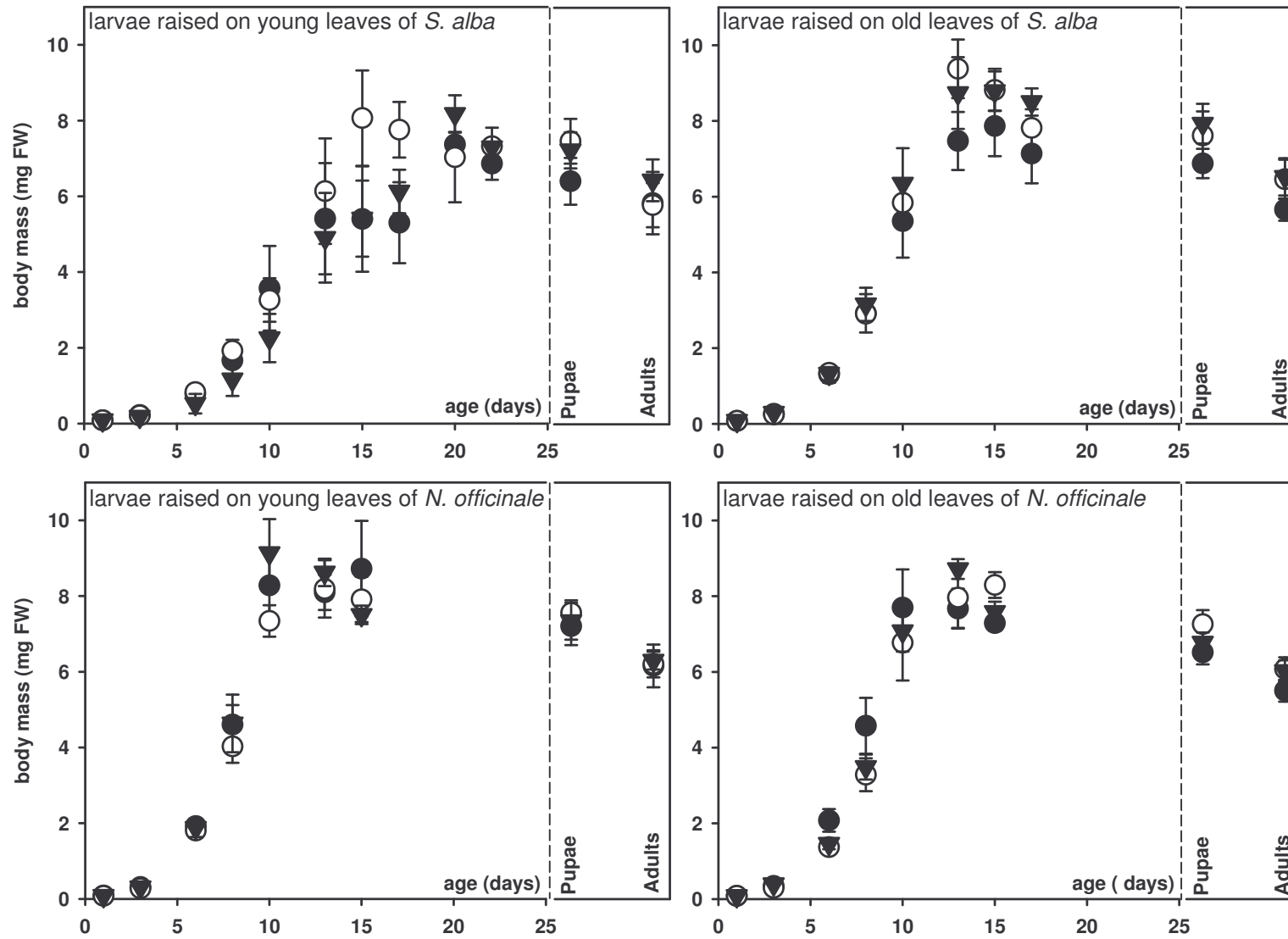
Figure 2.1 Body masses of larvae, pupae and adults (mg FW) of *Phaedon cochleariae* raised on young and old leaves of *Sinapis alba* and *Nasturtium officinale* exposed to different radiation conditions in the greenhouse (GH) or outdoors in foil tents filtering ambient UV radiation (-UV) or transmitting ambient UV radiation (+UV). Body masses of larvae are plotted against the age of the larvae (days). Pupal and adult masses were taken within 24 h after molt. Mean ± SE, number of replicates varied between 4 and 12 due to mortality. Filled circles = individuals raised on greenhouse plants, open circles = individuals raised on –UV-exposed plants, filled triangles = individuals raised on +UV-exposed plants.

	Leaf-age	Larval instar	**Relative growth rate (RGR)** Plant stress treatment			**Relative consumption rate (RCR)** Plant stress treatment			**Growth efficiency (ECI)** Plant stress treatment		
			GH	-UV	+UV	GH	-UV	+UV	GH	-UV	+UV
S. alba	young	1st	0.53 ± 0.08	0.43 ± 0.09	0.43 ± 0.04	1.40 ± 0.30	1.41 ± 0.25	0.53 ± 0.10	0.33 ± 0.04	0.34 ± 0.03	1.09 ± 0.23
		2nd	0.28 ± 0.06	0.21 ± 0.03	0.21 ± 0.04	1.20 ± 0.20	2.44 ± 0.78	1.25 ± 0.24	0.23 ± 0.03	0.11 ± 0.02	0.17 ± 0.02
	old	1st	0.13 ± 0.04	0.15 ± 0.03	0.18 ± 0.08	0.57 ± 0.11	0.61 ± 0.14	1.93 ± 0.32	0.18 ± 0.04	0.31 ± 0.08	0.08 ± 0.02
		2nd	0.15 ± 0.04	0.16 ± 0.04	0.11 ± 0.04	0.62 ± 0.14	3.80 ± 0.75	3.17 ± 0.68	0.49 ± 0.20	0.05 ± 0.01	0.03 ± 0.01
N. officinale	young	1st	0.68 ± 0.07	0.59 ± 0.07	0.44 ± 0.05	3.46 ± 0.51	2.87 ± 0.35	2.98 ± 0.57	0.23 ± 0.04	0.21 ± 0.02	0.15 ± 0.02
		2nd	0.29 ± 0.06	0.27 ± 0.03	0.14 ± 0.06	1.59 ± 0.45	1.05 ± 0.19	2.10 ± 0.93	0.48 ± 0.19	0.36 ± 0.09	0.14 ± 0.03
	old	1st	0.48 ± 0.04	0.48 ± 0.03	0.49 ± 0.04	3.87 ± 0.34	5.76 ± 1.19	4.07 ± 0.53	0.13 ± 0.02	0.16 ± 0.07	0.13 ± 0.01
		2nd	0.20 ± 0.05	0.22 ± 0.05	0.22 ± 0.05	2.96 ± 0.77	1.77 ± 0.31	2.59 ± 0.66	0.13 ± 0.05	0.20 ± 0.07	0.23 ± 0.11

Table 2.2 Data for relative growth rates (RGR), relative consumption rates (RCR) and growth efficiencies (= efficiency of conversion of ingested food to body substance, ECI) (mean ± SE, number of replicates varied between 5 and 10) of first and second instar larvae of *Phaedon cochleariae* raised on young and old leaves of *Sinapis alba* and *Nasturtium officinale* exposed to different radiation conditions in the greenhouse (GH) or outdoors in foil tents filtering ambient UV radiation (-UV) or transmitting ambient UV radiation (+UV).

Figure 2.2 Specific dry weights, water contents and metabolite quantities of young and old leaves of *Sinapis alba* and *Nasturtium officinale* exposed to different radiation conditions in the greenhouse (GH) or outdoors in foil tents filtering ambient UV radiation (-UV) or transmitting ambient UV radiation (+UV). Data were pooled from different dates of harvest (except data for C/N contents). Mean ± SE, number of replicates varied between 10 and 21. Filled circles = young leaves, open circles = old leaves. Differences in levels between young and old leaves are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA, data were log-or arcsin-transformed whenever necessary to achieve homogeneity of variances), for radiation effects capital letters indicate differences within differently treated young leaves, small letters indicate differences within old leaves ($P < 0.05$; Student's t-test for unpaired samples, Welch-corrected whenever necessary to achieve homogeneity of variances).

2.4 Discussion

Short-term responses of plants to environmental stress can substantially change the composition of nutrients and secondary metabolites of plants (White, 1984; Reifenrath and Müller, 2007), and thereby affect their suitability as feeding source for herbivores. An improved insect performance on stressed plants is predicted by the plant stress – insect performance hypothesis (Rhoades, 1979; White, 1984; Larsson, 1989). Plants stressed by UV-exposure respond by adapting their photosynthetic activity, accumulating shield pigments in the epidermis and by induction of stress-related signalling cascades (Caldwell et al., 1989; Harborne and Williams, 2000; Mackerness, 2000; Kolb et al., 2001; Reifenrath and Müller, 2007). Thus, effects on primary metabolites, important as nutrients for herbivores, and on secondary compounds, involved in plant defence or feeding stimulation by insects, are expected.

Stressed *S. alba* and *N. officinale* plants that were short-term exposed outdoors to ambient spectral irradiances including or excluding UV differed significantly in most chemical compounds from plants that were kept in the greenhouse at low photosynthetic active radiation levels and lacking UV radiation. All chemical compound classes belonging to the nutrients tested here differed quantitatively across plant stress treatments, but also between plant species and leaves of different age (Fig. 2.2). Higher levels of available nitrogen and amino acids, as commonly found in stressed plants (White, 1984), and an optimal ratio of proteins and carbohydrates (Bernays and Chapman, 1994) can turn a plant into a highly suitable feeding source. In case of C/N imbalance, severe nutrient deficits or increased metabolic costs for catabolism of proteins as an energy source may considerably affect the development of an herbivore (Noseworthy and Despland, 2006). In our study, carbon/nitrogen ratios, soluble protein contents and glucose concentrations varied between young and old leaves of *S. alba* and *N. officinale*, with generally higher contents in outdoor exposed young leaves than in old or greenhouse-kept leaves.

However, variable C/N ratios of leaves did not correlate with developmental times of larvae of the mustard leaf beetle. Despite comparably high soluble protein contents and glucose levels in young *S. alba* leaves, larvae developed significantly slower on these than on conspecific old leaves or on *N. officinale* leaves. Thereby, higher water contents in old leaves, as detected for both plant species, may result in dilution of available nutrients with strong effects on leaf quality as feeding source for herbivores as

a consequence. Leaf-age indeed affected the performance and the feeding behaviour, namely relative growth and consumption rates and growth efficiencies of first and second larval instars (Table 2.1). At least for some treatments, higher mean consumption rates on old than on young leaves, combined with lower mean growth efficiencies were observed (Table 2.2).

Different consumption rates and performance parameters could be also affected by secondary metabolites which are susceptible in their qualitative and quantitative presence towards changes in environmental conditions. In both investigated Brassicaceae, highest flavonoid concentrations were detected in short-term +UV-exposed plants, but increased flavonoid levels were also found in plants exposed to –UV outdoor conditions with increased PAR levels compared to greenhouse-kept plants. Accumulations were more pronounced in young than in old leaves. Next to flavonoids, levels of glucosinolates and myrosinase activities are inducible by abiotic environmental factors (Bidart-Bouzat et al, 2005, Reifenrath and Müller, 2007). However, in *S. alba* and *N. officinale* the different plant stress treatments had only minor effects on glucosinolate and myrosinase activity concentrations which are important traits in plant defence. Thus, in both plant species stressed by different radiation conditions, flavonoid shield pigments were accumulated, but not at the expense of general plant defences against leaf feeding insects, although synthetic pathways of at least some flavonoids and glucosinolates share the same precursor (Harborne, 1991; Halkier and Gershenzon, 2006). Among other chemical leaf compounds, glucosinolates and flavonoids have been found to additively evoke feeding stimulation of the mustard leaf beetle (Reifenrath and Müller, submitted). Consequently, the feeding behaviour and the performance of *P. cochleariae* might be directly affected by variable quantities of both compounds; however, no clear correlation was obvious.

Other leaf parameters than those related to internal chemical leaf quality may affect larval development as well, such as epicuticular leaf wax cover that lack any nutrient value and at least discourage the adult beetles from feeding (Reifenrath et al., 2005). Leaf toughness has already been shown to retard the growth of *P. cochleariae* (Tanton, 1962). Calculated as specific dry weights of leaves, this leaf characteristic varied strongly between leaves of different UV-treatments in our study. Higher values for young than old leaves were found for greenhouse-kept and UV-shielded leaves of both plant species, whereas UV-exposed leaves of both age had similar values (Fig. 2.2).

However, these patterns did also not correlate with larval development of mustard leaf beetles.

Whereas developmental times of larvae responded to plant species, leaf-age and treatment, the duration of the pupal stage, and body masses of larvae, pupae and adults measured during development remained similar. Hence, any differences in larval development due to variable plant qualities were compensated until the pupal phase was reached. Relative growth and consumption rates and growth efficiencies of larvae within 24 h differed across larval instars, plant species and leaf-age. Higher relative growth and consumption rates were detected for *N. officinale* fed larvae compared to those fed with *S. alba* leaves, and higher growth efficiencies for first over second larval instars (Table 2.2). Values measured for all other parameters showed no consistent pattern. Increasing consumption rates or higher efficiencies of food conversion for compensation of lower food quality may take place during larval development, expressed as variable feeding activity and efficiency or an extension of the larval phase.

However, an explanation for the lack of responses in the development of mustard leaf beetles towards strong variation in leaf quality due to UV-exposure, as investigated in this study, may be found in the high plasticity of these oligophagous specialist herbivores. Thereby, the degree of specialisation may determine the strength of effects of changed food quality on the performance and feeding behaviour of herbivores (McCloud and Berenbaum, 1999; Cornell and Hawkins, 2003). Similar to our study, McCloud and Berenbaum (1999) found stronger effects of leaf-age than of UV plant treatment on herbivore performance. Larvae of the specialist *Precis coenia* Hbn (Lepidoptera) developed faster on old than on young leaves, but were unaltered by feeding on foliage exposed to enhanced UV-B compared to feeding on foliage exposed to ambient UV-B. In contrast, larval development of the generalist *Trichoplusia ni* Hbn (Lepidoptera) was clearly shorter on old than on young leaves and accelerated on UV-B stressed plants. Under field conditions, one can assume that generalist herbivores generally have the opportunity to change their host plant in case of decreasing quality of a certain species. In contrast, the abundance of a specialists' host plant may be usually limited in the field and a high plasticity might be necessary for specialist herbivores to cope with variable levels of specific plant compounds. The meaning of the degree of herbivore specialisation for the effect of stressed host plant quality on insect performance may provide an important aspect to be considered in studies testing the plant stress – insect performance hypothesis.

2.5 Acknowledgements

2.6 References

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Chapter 3

Leaf surface wax layers of Brassicaceae lack feeding stimulants for *Phaedon cochleariae*

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Abstract. Numerous reports indicate that glucosinolates are important stimulants for specialist herbivores feeding on Brassicaceae, and that these metabolites might be present on the plant surface and thereby detectable by an alighting insect. We investigated the outermost layer of leaves of two species of Brassicaceae, *Brassica napus* L. var. 'Martina' and *Nasturtium officinale* R. Br., using two highly selective extraction methods. When the epicuticular wax layer was mechanically removed with gum arabic, no glucosinolates were detectable in lower and upper leaf surfaces. Extracting leaf surfaces with a threefold short rinse with chloroform/methanol/water (2:1:1 v/v/v) led to varying results, depending on the light conditions under which plants had been kept in the period prior to extraction. In plants kept under light, glucosinolates were detectable in a first extraction in minor concentrations, with increasing amounts in a second and third extraction. In plants kept in darkness, glucosinolates were almost absent in the first extraction. We postulate that the polar glucosinolates are washed from inner leaf tissue through open stomata to the outside during solvent extraction but are not naturally present in the outermost wax layer. The response of the crucifer specialist *Phaedon cochleariae* (F.) (Coleoptera: Chryso-melidae) to leaf surfaces of the host plants *B. napus* and *N. officinale* and to a glucosinolate was tested. Adults preferred both the adaxial and abaxial leaf surfaces of host plants that had been treated with gum arabic in order to remove the epicuticular

waxes over intact surfaces. Waxes may therefore prevent direct contact with stimulants. Sinigrin (allyl glucosinolate) and/or surface extracts of *N. officinale* leaves applied on *Pisum sativum* leaf discs did not evoke feeding, but feeding did occur when total leaf extracts of *B. napus* or *N. officinale* were applied on this non-host. We conclude that glucosinolates might only act as feeding stimulants for *P. cochleariae* in concert with compounds other than surface waxes.

3.1 Introduction

Host plant recognition in phytophagous insects is determined by a complex of physical and chemical stimuli. The orientation of an insect towards a plant is mainly triggered by visual and olfactorial cues (Björkman et al., 1997; Schoonhoven et al., 1998; Kanno and Harris, 2000a, b). Once an insect alights on a plant, the outermost leaf surface constitutes the zone of initial contact. The structural properties and/or the chemical composition of this surface layer may determine the insect's acceptance of this plant, and within a given plant individual, a preference for specific localities such as the abaxial or adaxial leaf surfaces. However, the nature and significance of such surface cues for host plant detection are only partly understood.

With regard to structural properties, the first evidence for the role of surface layers for host acceptance was revealed from studies of plant mutants showing different surface characteristics. Phenotypes with reduced densities of epicuticular wax crystals (glossy type) of several plant species are less accepted (Kanno and Harris, 2000a; Steinbauer et al., 2004). On the other hand, many insect species prefer, for feeding and oviposition, glossy leaves or leaves with waxes removed over leaves with normal or heavy epicuticular wax loads (Stork, 1980; Bodnaryk, 1992; Brennan et al., 2001; Cervantes et al., 2002). Further evidence for the importance of the surface layer for host acceptance was gained in experiments in which the leaf waxes were mechanically removed by stripping with an adhesive (cellulose acetate). Female leaf beetles of *Cassida stigmatica* Suff. (Coleoptera: Chrysomelidae) preferred intact to de-waxed abaxial leaf surfaces for oviposition (Müller and Hilker, 2001).

With regard to chemical composition, leaf cuticular waxes typically comprise a mixture of long-chain aliphatic components primarily including alkanes, alkylesters, aldehydes, primary alcohols, and fatty acids (Walton, 1990). In addition, cyclic compounds such as phenolics, flavonoids and triterpenoids can be present (Jetter and

Riederer, 1996; Jetter and Schäffer, 2001; Hare, 2002). In general, the chemical composition of the surface components is species-specific and shows a high degree of diversification (Jetter et al., 2002). Many insects gather information about the plants' quality by probing the leaf surface with antennae or tarsi, but without biting into the leaf tissue (Roessingh et al., 1992; Isidoro et al., 1998; Schoonhoven et al., 1998). Thus, at least some crucial chemical compounds contained in the waxy layer may be detectable by chemoreception. The effect of various wax compositions on host finding of herbivorous insects seems to be highly diverse and system-specific, but only few studies have been carried out thus far (van Loon et al., 1992; Eigenbrode and Espelie, 1995; Morris et al., 2000; Cervantes et al., 2002).

Most studies on plant-insect relationships have focussed on secondary plant compounds that might not, however, necessarily be present on the plant surface. One of the best studied groups of allelochemicals is produced in the Brassicaceae. A common feature of this plant family is the presence of glucosinolates, sulfur-rich-β-thioglucosides with side chains of varying structure, including aliphatics, benzylic structures or indols (Wittstock and Halkier, 2002). While glucosinolates act as deterrents against generalist herbivores, several specialist species use them for host plant detection (Chew, 1988; Renwick, 2002). Tanton (1977) and Nielsen (1978) found that the mustard leaf beetle, *Phaedon cochleariae* (F.) (Coleoptera: Chrysomelidae), responded to a variety of mustard oil glycosides (glucosinolates) in feeding bioassays, with distinct preferences for sinigrin (allyl glucosinolate) over sinalbin (*p*-hydroxybenzyl glucosinolate) over glucotropaeolin (benzyl glucosinolate). A methanol extract of cabbage leaves (*Brassica oleracea* L.) that included an indol glucosinolate was found to stimulate the oviposition of cabbage white butterflies (*Pieris brassicae* L., Lepidoptera: Pieridae) (van Loon et al., 1992), but no stimulation was observed when the pure glucosinolate was offered. Similar observations were made for the cabbage root fly (*Delia radicum* L., Diptera: Anthomyiidae): although three glucosinolates could be detected in a stimulating cabbage surface extract, bioassay-guided fractionation of this extract revealed the highest stimulatory activity in the glucosinolate-free fraction (Roessingh et al., 1992). Nevertheless, cabbage root flies were found to have tarsal chemoreceptors that can perceive glucosinolates (Roessingh et al., 1992; Hurter et al., 1999). Consequently, it was suggested that glucosinolates are present at the leaf surface. Extractions for glucosinolate analysis were done in these studies by immersing the leaves in chloroform for five seconds and subsequently twice in methanol (Städler and

Roessingh, 1990; Roessingh et al., 1992; Hopkins et al., 1997; Hurter et al., 1999; Griffiths et al., 2001).

In several studies using also other plants than Brassicaceae, raw extracts were prepared from the plant surface for use in insect behavioural bioassays by dipping the leaves in different organic solvents for various lengths of time (Brooks et al., 1996; Hare, 2002; Green et al., 2003). However, this crude procedure might be prone to artefacts, as the extracts may include compounds derived from locations other than the outermost leaf surface (Roessingh et al., 1992; Riederer and Markstädter, 1996; Jetter et al., 2000). Furthermore, the reasons for the preferences of insects for adaxial or abaxial surfaces based on differential chemical cues (Müller and Hilker, 2001) cannot be detected in this way.

Recently, better methods have been developed for mechanically and chemically probing the epicuticular wax layer in a highly selective manner (Ensikat et al., 2000; Jetter et al., 2000; Jetter and Schäffer, 2001). In this study, we investigated whether glucosinolates were indeed present in the outermost leaf surface of two species of Brassicaceae (*Brassica napus* L. 'Martina' and *Nasturtium officinale* R.Br.) by using these more sophisticated techniques. In bioassays we examined the influence of epicuticular waxes and of a glucosinolate on the feeding behaviour of the mustard leaf beetle, *Phaedon cochleariae*, which uses *B. napus* and *N. officinale* as host plants (Nielsen, 1978).

3.2 Materials and methods

3.2.1 Insects

Mustard leaf beetles collected from *Armoracia rusticana* G.M. Sch. in Berlin, Germany, were reared in the laboratory at 70% r.h. and 25 °C with a photo cycle of L16:D8 for several generations. Larvae and adults were fed with commercial Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr.). Adult beetles were used for bioassays 5-10 days after hatching.

3.2.2 Plants

Brassica napus var. 'Martina', *Nasturtium officinale* and *Pisum sativum* L**.** var. 'Rheinperle' were grown from seeds in plastic pots in the green house (25 °C, 70% r.h., L16:D8). Developing plants of *N. officinale* were transferred to a shady place outside. Young, fully expanded leaves were used for wax removal, extractions and bioassays.

3.2.3 Adhesive leaf wax removal by gum arabic

For selective preparation of epicuticular waxes, leaves of potted plants were treated with gum arabic (Jetter and Schäffer, 2001). Prior to use, gum arabic (Roth, Karlsruhe, Germany) was purified by a soxhlet extraction with hot chloroform. Approximately 0.1 ml of a 50% (w/w) aqueous solution of gum arabic was applied per cm^2 of leaf surface using a small paintbrush. After one hour, epicuticular waxes could be removed together with a dry film of gum arabic, leaving the leaves physically intact (without damaging epidermal and mesophyll tissue). To test for presence of glucosinolates in the epicuticular layer, the polymer films removed from a total area of approximately 25 cm² were pooled and dissolved in aqua bidest and analysed as described below. Half-side treated leaf discs were offered in behavioural assays (see below).

3.2.4. Solvent leaf wax extracts

In order to remove compounds of different polarity from epicuticular waxes with solvents, a mixture of chloroform (RostisolV, 99.9%, Roth, Germany), methanol (RostisolV, 99.9%, Roth, Germany) and aqua bidest (2:1:1, v/v/v) was applied to adaxial and abaxial leaf surfaces, respectively. Intact leaves were placed on a flexible rubber mat, a glass cylinder (13 mm diameter) gently pressed onto the exposed surface, and the solvent mixture agitated for 20 seconds by pumping with a Pasteur pipette and then removed. This procedure was repeated twice. The three subsequent extracts were pooled separately from a total area of approximately 26 cm² of individual leaves and reduced to dryness under a gentle air flow. For analyses of glucosinolates, leaf wax extracts were taken (1) from plants that had been kept under normal light conditions and whose stomata were expected to be open, and (2) from plants which had been kept in darkness for 3-4 hours prior to the experiment in order to ensure maximum stomatal

closure. For bioassays, extraction was done only once on leaf surfaces from plants kept in light.

3.2.5 Total leaf extraction

For use in bioassays, total leaf extracts were prepared with a Soxhlet apparatus. Lyophilised leaf material (*B. napus* 'Martina': 3.5 g; *N. officinale*: 3.4 g) was extracted in 100 ml dichloromethane (Rotipuran, $\geq$ 99.5%, Roth, Germany) for 8 h at 50 °C and extracts were subsequently reduced to 4 ml under vacuum.

3.2.6 Glucosinolate analysis

In order to obtain the whole spectrum of glucosinolates of *B. napus* and *N. officinale*, respectively, fresh leaf material was collected, frozen at -80 °C, and lyophilised. Dried material was ground. These total leaf samples, the dissolved epicuticular waxes gained with gum arabic, and the solvent surface extracts (see above), respectively, were dissolved in boiling aqueous 70% methanol with sinigrin (allyl glucosinolate; $\geq$ 99%, Fluka, Taufkirchen, Germany) in aqueous solution added as internal standard. Glucosinolates were converted to desulfoglucosinolates using sulfatase (E.C. 3.1.6.1, 'type H-1, from *Helix pomatia*, 15100 units/gram solid; Sigma, Taufkirchen, Germany), following the protocol by Agerbirk et al. (2001). The resulting desulfoglucosinolates were analysed by HPLC (1100 Series, Hewlett-Packard, Waldbronn, Germany) with a quaternary pump and a 1040M diode array detector. Gradient separation of desulfoglucosinolates was achieved on a Supelco C-18 column (Supelcosil LC-18, 250 × 4.6 mm, 5 µm, Supelco) with an eluent gradient (solvent A: aqua bidest, solvent B: methanol, RotisolV, 99.9%, Roth, Germany) of 0-5% B (10 min), 5-38% B (24 min), followed by a cleaning cycle (38-100% B in 0.5 min, 3 min hold, 100 to 0% B in 0.5 min, 7 min hold). The desulfoglucosinolates were quantified by calculating the peak area at 229 nm (bandwidth 4 nm) relative to the area of the internal standard peak, corrected by the response factors as in Brown et al. (2003). Desulfoglucosinolates were identified by a comparison of retention times and UV spectra to those of purified standards and further confirmed by LC-ESI-MS as described in van Dam et al. (2003).

3.2.7 Design of behavioural assays

In order to investigate the role of epicuticular waxes and of glucosinolates for feeding preferences of adult *P. cochleariae*, dual choice tests were performed. Beetles were starved for 24 h prior to the experiments. Samples (see below) were offered in Petri dishes (5.5 cm diameter) on a moistened filter paper. All tests were carried out in a dark room at approximately 22 °C where the beetles showed the highest feeding activity. Each test lasted for at least 4 h, except in experiments where beetles did not show any response within this time; here tests were prolonged by further 20 h (Table 1). In some tests, leaves of *Pisum sativum* were used as substrate. Although *P. sativum* is not a host plant for *P. cochleariae*, the beetles accepted the leaves when a stimulant was applied to the leaf surfaces. When extracts were offered, solvents were allowed to evaporate sufficiently prior to the start of an assay. At the end of a bioassay, remaining leaf fragments were photocopied and scanned, and consumed areas determined (using Adobe Photoshop 6.0). Feeding amounts at test and control leaves were compared by t-test for paired samples using Statistica 6.1 (StatSoft, Tulsa, USA).

3.2.8 Tests for activity of total leaf extracts

Leaves of *P. sativum* were treated with 10 µl each of the total leaf Soxhlet extracts of *B. napus* 'Martina' and *N. officinale*, respectively (1, Table 3.1). Leaves treated with dichloromethane only served as control. The concentrations used corresponded to the concentrations detected in host plant leaf material of the same area.

3.2.9 Tests for activity of epicuticular waxes

Half of a side of a leaf disc (13 mm diameter) of *B. napus* 'Martina' and *N. officinale*, respectively, was treated with gum arabic as described above in order to remove epicuticular waxes. The other half of the disc surface remained untreated (2, Table 3.1). To prevent beetles from feeding on the leaf margins and to test their response to adaxial or abaxial surfaces exclusively, a metal ring (10 mm inner, 20 mm outer diameter) was placed onto each disc thus covering the disc's margin.

In a further approach, leaves of the non-host plant *P. sativum* were treated with surface waxes of a host plant. Solvent extracts were prepared from adaxial and abaxial leaf surfaces of *N. officinale* as described above and dissolved in dichloromethane or

chloroform (3, Table 3.1). Ten µl were applied to the surface of a *P. sativum* disc, the concentration of the extract corresponding to the extracted area of a host leaf of the same area. Control leaves were treated with 10 µl of the solvent only. Test and control leaves were offered pair-wise.

3.2.10 Tests for activity of epicuticular waxes combined with glucosinolate

Leaves of *P. sativum* were offered as in 3 (Table 3.1), but sinigrin was added together with the wax solvent extracts in a concentration appropriate to total glucosinolate concentrations detected in leaf material of the same area (approximately 5 nmol cm^{-2}). Various solvents were tested for applying the glucosinolate (4, Table 3.1).

3.2.11 Tests for activity of glucosinolates

Leaves of *P. sativum* were treated with sinigrin dissolved in various solvents or in 0.05% sodium dodecyl sulphate (SDS ultra pure $\geq$ 99%, Roth, Germany) in H_2O (H_2O-detergent) (5, Figure 3.1) the latter following the protocol of Nielsen (1978). In contrast to Nielsen who had immersed *Pisum* leaf discs in a 2 mM and a 8 mM sinigrin-detergent solution, we applied approximately 5 nmol cm^{-2} glucosinolate to the upper leaf side. Control leaves were treated with H_2O-detergent only.

In a last bioassay, whole leaves of the host plant *N. officinale* were treated with a solution of sinigrin in H_2O-detergent (approximately 5 nmol cm^{-2}), and control leaves with H_2O-detergent alone (6, Table 3.1). After the H_2O-detergent had evaporated, the leaves were cut into half and a sinigrin-treated plus a control-treated leaf half were added to form a complete disc. The leaf margins of such leaves were covered with a metal ring and the sample offered to the beetles.

3.3 Results

3.3.1 Glucosinolate contents

No glucosinolates could be detected with the equipment used in epicuticular wax samples obtained by treatment with gum arabic, either from adaxial (*B. napus* n = 4, *N. officinale* n = 6) or from abaxial leaf surfaces (*B. napus* n = 3, *N. officinale* n = 6) (detection limit 0.2 nmol). In total leaf samples of *B. napus* 'Martina' (total

glucosinolate content (mean ± SE) 298.3 ± 65.2 nmol g^{-1} FW; n = 3), the predominant glucosinolates were progoitrin (*R*-2-hydroxy-3-butenyl glucosinolate), glucobrassicanapin (4-pentenyl glucosinolate), followed by gluconapin (3-butenyl glucosinolate) and glucoallysin (5-methylsulfinylpentyl, 5MSOP). Apart from these, three indol glucosinolates, 4-methoxyglucobrassicin (4-methoxy-indol-3-yl-methyl glucosinolate, 4MOI3M), glucobrassicin (indol-3-yl-methyl glucosinolate, I3M) and 4-hydroxyglucobrassicin (4-hydroxy-indol-3-yl-methyl glucosinolate, 4OHI3M), were detected in lower concentrations (Figure 3.1a).

In total leaf samples of *N. officinale* (total glucosinolate content 1064.8 ± 102.0 nmol g^{-1} FW; n = 3), the main glucosinolate was nasturtiin (2-phenyl-ethyl glucosinolate, 2PE), followed by the two indols 4-methoxyglucobrassicin and glucobrassicin. Several other glucosinolates were only present in minor quantities and were therefore not identified (Figure 3.2a).

In the first solvent extraction of *B. napus* 'Martina' leaf surfaces, minor concentrations of only a few glucosinolates could be detected in the upper surface of plants that had been kept in darkness in comparison with plants kept in light. Glucobrassicin and 4-methoxyglucobrassicin were less pronounced in the latter. Only small differences could be found in qualitative composition and quantities of glucosinolates between lower leaf surfaces of plants kept in light and in darkness (Figures 3.1c, d). In the first solvent extraction of the upper leaf surfaces of *N. officinale*, only minor concentrations of one indol glucosinolate, 4-methoxyglucobrassicin, could be detected when the plants had been kept in darkness. Higher concentrations and a broader spectrum of glucosinolates were found in the second and third extraction of the leaf surface on both abaxial and adaxial sides (Figures 3.2c, d). Overall, a substantially higher concentration of glucosinolates could be detected in surface extracts of light-exposed leaves than in leaves kept in darkness prior to extraction, with concentrations being higher on the abaxial side (Figures 3.1b, 3.2b). In extracts from abaxial leaf surfaces of *N. officinale*, about four-fold higher glucosinolate amounts were found than in extracts of *B. napus*. However, glucosinolate concentrations of all three surface extractions combined accounted for less than 1/10 (*B. napus*) or 1/20 (*N. officinale*) of the concentration of total leaf samples in both plant species.
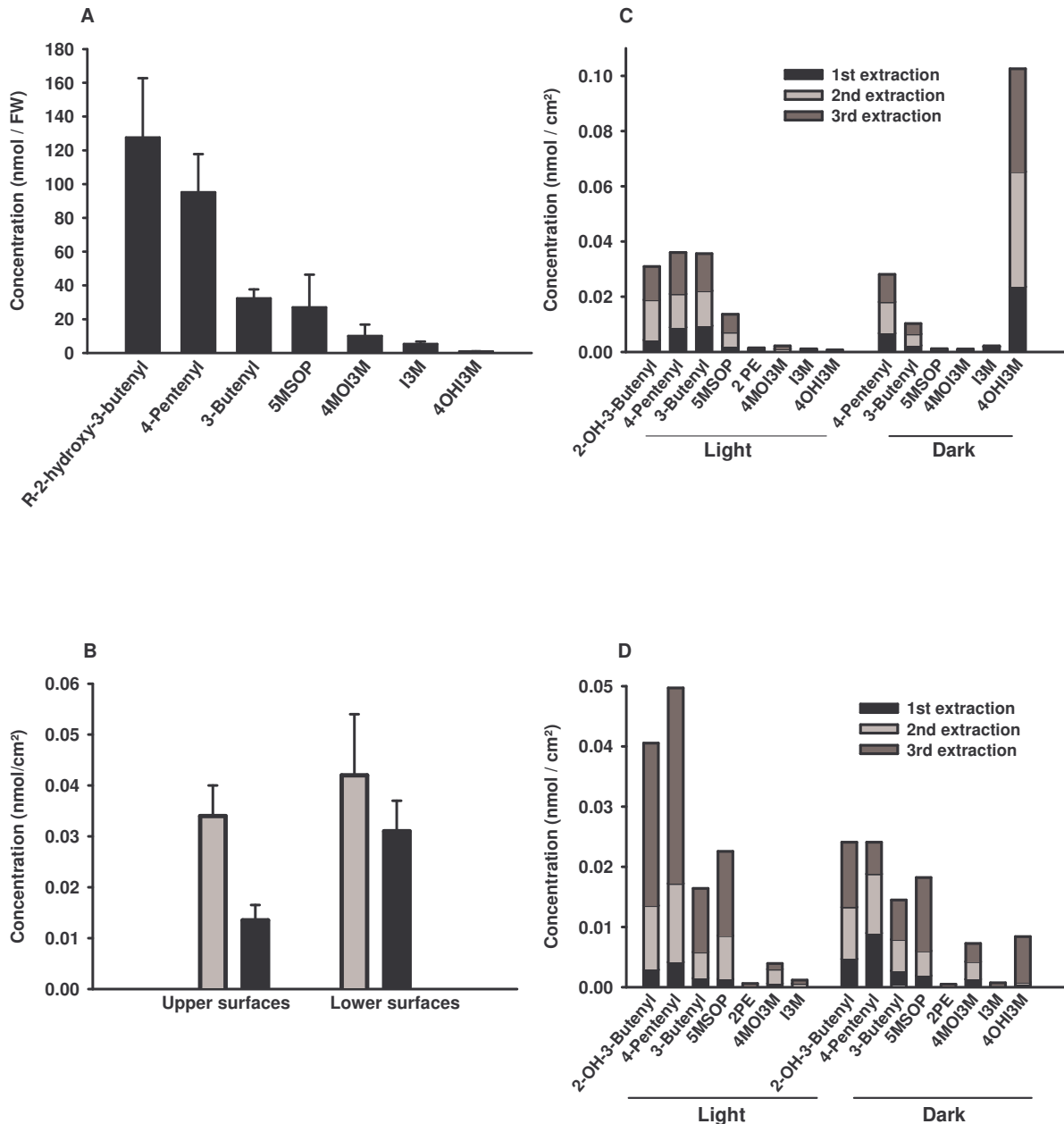
Figure 3.1 Composition of glucosinolates in leaves of *Brassica napus* 'Martina'. Glucosinolate profile (mean ± SE; n = 3) of a complete leaf **(a)**. Total glucosinolate concentration (mean ± SE; n = 3) of a three-fold solvent extraction of upper and lower surfaces of leaves kept at light (light bars) or in darkness (black bars) 3-4 h prior to extraction **(b)**. Mean concentration of glucosinolates in upper **(c)** and lower **(d)** leaf surfaces after a first, second and third solvent extraction (n = 3 for each extraction procedure, respectively). Abbreviations for R-groups (side chains) of glucosinolates: 2-OH-3-butenyl = *R*-2-hydroxy-3-butenyl; 5MSOP = 5-methylsulfinylpentyl; 2PE = 2-phenylethyl; 4MOI3M = 4-methoxy-indol-3-yl-methyl; I3M = indol-3-yl-methyl; 4OHI3M = 4-hydroxy-indol-3-yl-methyl.
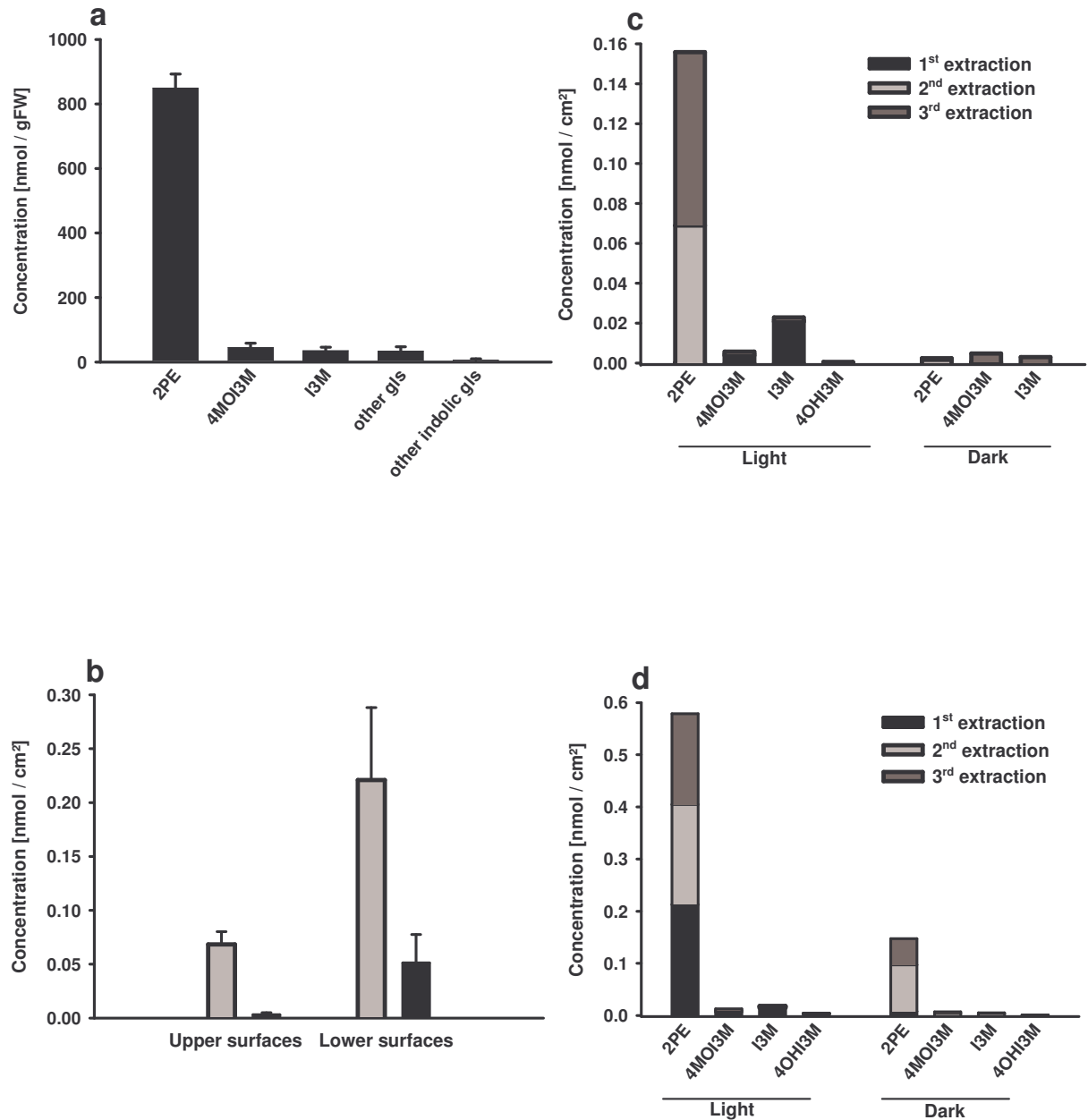
Figure 3.2 Composition of glucosinolates in leaves of *Nasturtium officinale*. Glucosinolate profile (mean ± SE; n = 3) of a complete leaf **(a)**. Total glucosinolate concentration (mean ± SE; n = 3) of a three-fold solvent extraction of upper and lower surfaces of leaves kept at light (light bars) or in darkness (black bars) 3-4 h prior to extraction **(b)**. Mean concentration of glucosinolates in upper **(c)** and lower **(d)** leaf surfaces after a first, second and third solvent extraction (n = 3 for each extraction procedure, respectively). Abbreviations for R-groups (side chains) of glucosinolates: 2PE = 2-phenylethyl; 4MOI3M = 4-methoxy-indol-3-yl-methyl; I3M = indol-3-yl-methyl; 4OHI3M = 4-hydroxy-indol-3-yl-methyl.

4.3.2 Behavioural assays

Activity of total leaf extracts. Leaves of *P. sativum* were accepted by beetles of *P. cochleariae*, when Soxhlet extracts of *B. napus* leaves (consumption (mean ± SE) 7.3 ± 1.3 mm²; n = 13) or *N. officinale* leaves (consumption 10.4 ± 1.9 mm²; n = 8), respectively, were applied (1, Table 3.1). Control leaves treated with the solvent only were not eaten.

Activity of epicuticular waxes. In feeding preference tests with *B. napus* 'Martina' leaf discs partly treated with gum arabic, beetles significantly preferred de-waxed over intact surfaces of both upper and lower leaf sides (2, Table 3.1; *P* < 0.01, Figures 3.3). Moreover, de-waxed leaf parts of upper sides of *N. officinale* leaves were significantly preferred (*P* < 0.01, Figure 3.4). In choice tests with *P. sativum* leaves covered with upper- or lower-surface solvent extracts of *N. officinale* against solvent controls (3, Table 3.1), mustard leaf beetles did not feed on either test or control leaves.
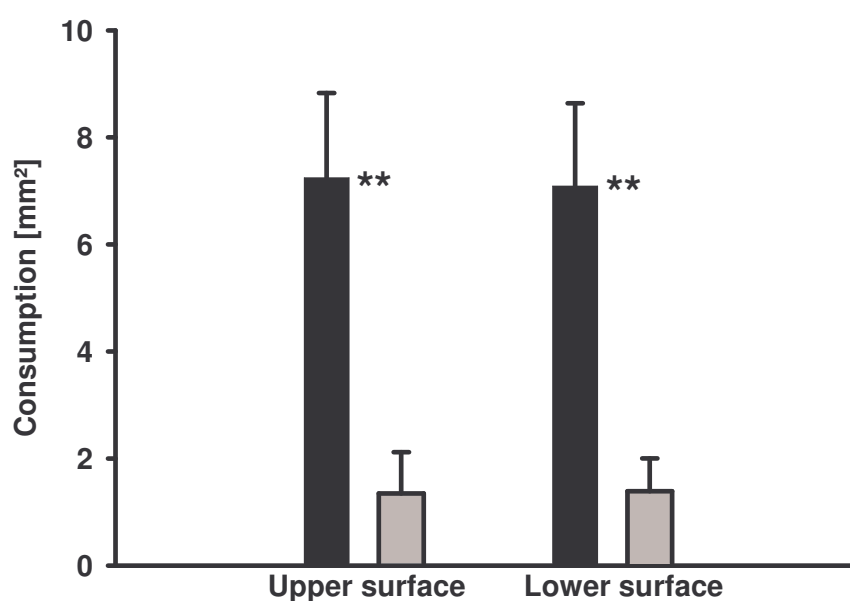
Figure 3.3 Consumption by adult *Phaedon cochleariae* (mean ± SE) on *Brassica napus* 'Martina' leaf discs offered in a dual choice test for 4 h. Wax from one half of a leaf disc was removed using gum arabic on both the upper (n = 11) and the lower surface (n = 14), respectively. Black bars = treated surface, grey bars = untreated surface.
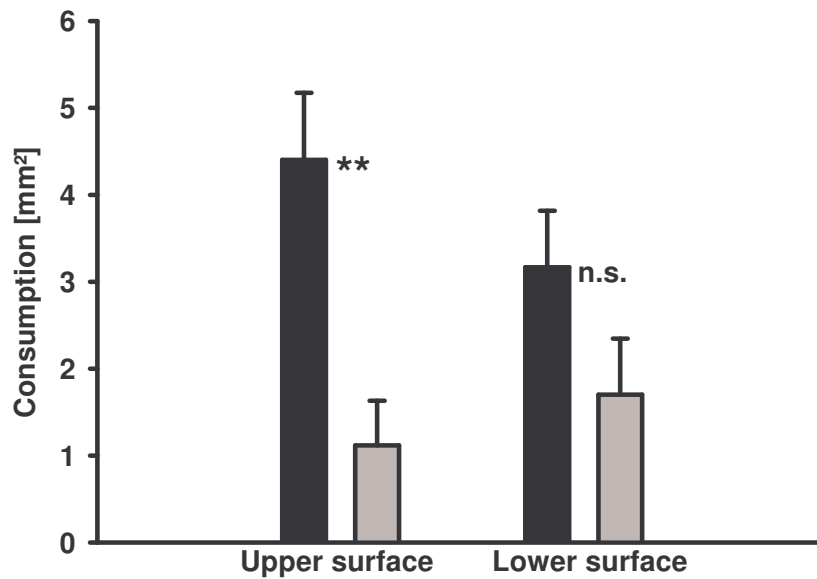
Figure 3.4 Consumption by adult *Phaedon cochleariae* (mean ± SE) on *Nasturtium officinale* leaf discs offered in dual choice test for 4 h. One half of a leaf disc was treated with gum arabic for wax removal on both the upper (n = 14) and the lower surface (n = 16), respectively. Black bars = treated surface, grey bars = untreated surface.

Activity of epicuticular waxes combined with glucosinolate. In choice tests with *P. sativum* leaves treated with upper and lower surface solvent extracts of *N. offi*cinale leaves mixed with sinigrin and offered against control leaves (4, Table 3.1), no feeding of mustard leaf beetles was initiated.

Activity of glucosinolates. Mustard leaf beetles did not accept any of the *P. sati*vum leaves with sinigrin applied in different solvents (5, Table 3.1). They showed a weak but not significant preference for *N. officinale* leaf parts treated with sinigrin dissolved in detergent over control leaf parts (6, Table 3.1) (mean consumption ± SE on leaves with sinigrin: 7.2 ± 2.6 mm²; on control leaves: 5.3 ± 1.9 mm²; *P* = 0.6; n = 17).

	Leaf discs	Test treatment	Time (h)	Design	n	Solvents	Consumption
(1)	*P. sativum*	*B. napus* leaf extract	24	✕✕	16	CH_2Cl_2	+
	P. sativum	*N. officinale* leaf extract	24	✕✕	8	CH_2Cl_2	+
(2)	*B. napus* (upper surfaces)	gum arabic	4	✕	11		+
	B. napus (lower surfaces)	gum arabic	4	✕	14		+
	N. officinale (upper surfaces)	gum arabic	4	✕	14		+
	N. officinale (lower surfaces)	gum arabic	4	✕	16		+
(3)	*P. sativum*	USW of *N. officinale*	24	✕✕	20	$CHCl_3$	−
	P. sativum	USW of *N. officinale*	24	✕✕	20	CH_2Cl_2	−
	P. sativum	LSW of *N. officinale*	24	✕✕	20	$CHCl_3$	−
	P. sativum	LSW of *N. officinale*	24	✕✕	20	CH_2Cl_2	−
(4)	*P. sativum*	USW of *N. officinale* + Sinigrin	24	✕✕	20	$CHCl_3$	−
	P. sativum	USW of *N. officinale* + Sinigrin	24	✕✕	20	CH_2Cl_2	−
	P. sativum	LSW of *N. officinale* + Sinigrin	24	✕✕	20	$CHCl_3$	−
	P. sativum	LSW of *N. officinale* + Sinigrin	24	✕✕	20	CH_2Cl_2	−
(5)	*P. sativum*	Sinigrin	24	✕✕	20	$CHCl_3$	−
	P. sativum	Sinigrin	24	✕✕	20	CH_2Cl_2	−
	P. sativum	Sinigrin	24	✕✕	20	MeOH 100%	−
	P. sativum	Sinigrin	24	✕✕	20	MeOH 70%	−
	P. sativum	Sinigrin	24	✕✕	20	H_2O + detergent	−
(6)	*N. officinale*	Sinigrin	24	✕✕	17	H_2O + detergent	+

Table 3.1 Treatments for testing feeding stimulation and preferences of *Phaedon cochleariae*. Leaf discs of *Pisum sativum*, *Brassica napus* 'Martina' or *Nasturtium officinale* were offered in dual-choice assays in two designs: ✕ = test area and control area on one leaf disc, ✕✕ = two discs offered simultaneously (one treatment and one control); Control samples were treated with solvent only (1., 3.-6.) or untreated (2.); USW - upper surface waxes, LSW - lower surface waxes.

3.4 Discussion

The application of two highly selective methods for removing epicuticular compounds from leaves of two cruciferous species shed new light on studies of crucifer-insect interactions. A purely mechanical removal of the epicuticular layer circumvents the use of solvents that may penetrate the cuticle and thus extract compounds from beneath the epicuticular layer or the inner leaf tissue. The samples obtained by this method contained no detectable amounts of glucosinolates. In a second approach, we simultaneously applied polar and nonpolar solvents to the intact plant surface for a short period in order to remove waxes and potentially present polar compounds.

We found that leaf surface extracts contained almost no glucosinolates when the plants were kept in darkness, when stomata were expected to be at maximum closure. In contrast, quantitatively and qualitatively more glucosinolates were present in leaves kept in the light, with stomata open during extraction. Only in a second and third extraction of the same surfaces, could most glucosinolates be detected both in plants kept in darkness and in light, with total concentrations being much higher in the latter case (Figures 3.1 b-d; 3.2 b-d). Glucosinolate concentrations gained from all three surface extracts were still less than 1/10 (*B. napus*) and 1/20 (*N. officinale*) of the concentrations found in total leaf samples, which is in accordance with the results obtained by Roessingh et al. (1992) for *B. oleracea* L. convar. *botrytis* CC Cross. The difference in concentrations found from the various extraction steps suggests that glucosinolates are washed out of the mesophyll and/or epidermis when the stomata are open and organic solvents are applied. This notion was supported by the fact that glucosinolate concentrations were higher in extracts of the lower leaf sides where stomatal densities were higher (Figures 3.1b, d; 3.2b, d). In addition, the higher glucosinolate concentration found in *N. officinale* compared to *B. napus* surface extracts may be due not only to the higher glucosinolate content in the leaf interior, but also to a 1.5-fold higher stomatal density on the lower compared to the upper surface (Reifenrath, unpublished data).

In many earlier investigations, high amounts of glucosinolates were reported on the leaf surfaces of *Brassica napus* and other crucifers (Roessingh et al., 1992; Hopkins et al., 1997; Griffiths et al., 2001). These surface extracts were obtained by dipping leaves in chloroform and subsequently in methanol, with no information given on whether the plants were kept in darkness prior to extraction. The levels of glucosinolates found in

these leaf surface extracts ranged from 0.07 µmol gram^{-1} leaf equivalent (gle) (*Brassica oleracea* L. var. *fimbriata*) and 0.14 µmol gle^{-1} (*Brassica napus* L. var. *rapifera*; Hopkins et al., 1997) to 3.08 µmol gle^{-1} in *Lepidium sativum* L. (Griffiths et al., 2001). In our investigations we found similar levels for total leaf extracts: 0.4 ± 0.1 µmol gle^{-1} in *B. napus* 'Martina' and 1.39 ± 1.2 µmol gle^{-1} in *N. officinale*. We suggest that the high glucosinolate concentrations reported by other authors can be attributed to the extraction of leaf parts other than the actual surface accessible to the alighting insects. Furthermore, when dipping whole leaves in a solvent, the occurrence of compounds cannot be differentiated between the adaxial and abaxial surfaces. However, wax composition can differ on both leaf sides and thereby influence the insects' behaviour (Müller and Hilker, 2001).

To summarize, a rapid extraction procedure of leaves with maximum stomatal closure from plants that have been kept without light several hours before extraction will minimize the risk of finding glucosinolates as artefacts in surface extracts. With a mechanical removal of the epicuticular wax layer, however, artefacts can be completely avoided. The lack of glucosinolates in the epicuticular wax layer is not surprising when the physico-chemical properties of both are considered. Glucosinolates have a high water solubility and are negatively charged in aqueous solution, and thus have only a negligible tendency to penetrate or partition into lipoid phases like the plant cuticle or waxes. The logarithm of the 1-octanol/water partition coefficient for sinigrin is -2.8 (Brudenell et al., 1999) which indicates that the equilibrium concentration of this glucosinolate in a lipid phase comparable to 1-octanol (biomembranes, cuticular matrix) is a factor of 630 lower than that in the adjacent aqueous phase. Given this pronounced polarity of glucosinolates, it is highly unlikely that these substances can be transported across the lipophilic cuticle to the leaf surface.

In behavioural assays, we were not able to stimulate feeding in *P. cochleariae* when applying the glucosinolate sinigrin and/or surface extracts of *N. officinale* in realistic concentrations on leaves of the non-host *P. sativum* (3-5, Table 3.1). This plant was used as a substrate to show preferences for different glucosinolates for *P. cochleariae* and other chrysomelid species in earlier assays (Nielsen, 1978). However, in our study *P. sativum* leaves were only accepted when total leaf extracts of host plants were applied (1, Table 3.1). Because many *P. sativum* varieties are known to differ both in amount and composition of surface waxes (White and Eigenbrode, 2000), the variety used by Nielsen (1978) might have had other characteristics from ours.

Nevertheless, chemical stimulants other than glucosinolates may occur on the plant surface of host plants. Some compounds like soluble carbohydrates and sugar alcohols were shown to leak from leaf tissues to surfaces (Mercier and Lindow, 2000). The volatile hydrolysis products of glucosinolates might evaporate through the leaf surface and/or stomata. Such metabolites can be involved in feeding and oviposition stimulation, as shown for other crucifer specialists (van Loon et al., 1992; Bartlet et al., 1997; Schoonhoven et al., 1998; Mewis et al., 2002). For some crucifer specialist herbivores, additive and/or synergistic effects between glucosinolates and certain surface compounds were found to stimulate oviposition and feeding behaviour of Lepidoptera (Spencer, 1996; Renwick, 2002; van Loon et al., 2002). We assume that *P. cochleariae* might also respond to glucosinolates when these are combined with other stimulatory compound(s) yet to be detected. The total leaf extracts we found to stimulate feeding of beetles must contain such compounds.

Obviously, for host plant searching insects, glucosinolates are not available as feeding and oviposition stimulants on the outermost leaf surface of *B. napus* and *N. officinale*, and at least *P. cochleariae* prefers de-waxed over wax-covered leaves (Figure 3.3, 3.4). Thus, the beetles might have easier access to information about the host plant quality, such as the content of glucosinolates or other stimulatory compounds, when waxes are removed. Insects may attain such information by scratching the leaf surface before feeding (Städler, 2002). The waxes might also hinder the initiation of biting or the attachment of the beetle's tarsi (Stork, 1980; Bodnaryk, 1992). *Phaedon cochleariae* has been shown to have the best adhesion to glossy leaves (Stork, 1980).

The surface layer of leaves can be considered as an important protective layer, not only against abiotic stress (Riederer and Schreiber, 2001), but also to some extent, against feeding specialists, as previously suggested by Juniper (1995). For *P. cochleariae* it might be a mechanical as well as a chemical barrier. Epicuticular waxes that cover leaf surfaces in different quantities but do not contain any feeding stimulants could be regarded as an adaptive trait for plants' defence against herbivores.

3.5 Acknowledgements

3.6 References

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Chapter 4

Multiple feeding stimulants in *Sinapis alba*

for the oligophagous leaf beetle *Phaedon cochleariae*

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Abstract   In earlier investigations on host plant discrimination of leaf beetles glucosinolates were described as feeding stimulants for the Brassicaceae specialist *Phaedon cochleariae*. However, since these findings could not be confirmed in later studies offering 2-propenylglucosinolate in concentrations corresponding to those detected in host plant leaf material, the identification of cues for feeding stimulation of this leaf beetle species remained unclear.

In order to investigate chemical compounds of the host plant *Sinapis alba* acting as feeding stimulants, leaf extracts of different polarities were tested in bioassays with *P. cochleariae*. Number of feeding beetles and net consumption rates were highest for polar extracts, whereas little feeding responses were also detectable for nonpolar extracts. In subsequent bioassay-guided fractionations of methanol extracts with semi-preparative high performance liquid chromatography, two distinct fractions, one containing glucosinolates and another containing flavonoids, were found to stimulate beetles for feeding to variable degrees. The other collected fractions had zero activity. The combination of both active fractions evoked significantly higher consumption rates and larger numbers of beetles feeding than fractions tested individually. Additive effects of at least one compound of each fraction, among these the main glucosinolate of *S. alba*, 4-hydroxybenzylglucosinolate, were concluded. Effects of different ratios of

glucosinolates and flavonoids on the strength of feeding responses were investigated with two sets of host plants. One set was outdoors-exposed, whereas the second set was kept in the greenhouse. However, although both plant sets differed significantly in their relative composition of both compound classes, the feeding behavior of *P. cochleariae* was not affected.

In conclusion, mustard leaf beetles use a complex composition of host leaf compounds as feeding stimulants, whereby the mere presence of these stimulants, but probably not the ratio of involved compounds, determines the feeding responses of these beetles.

4.1 Introduction

Chemical plant metabolites serve as important host recognition cues for herbivorous insects. They may act as volatile repellents or attractants, or as contact cues that insects only perceive after biting the tissue and that stimulate or deter further feeding or oviposition. For a first classification of such stimulants or deterrents, tissue extracts can be prepared with different solvents or solvent mixtures. The behavioral responses of herbivorous insects to such extracts offered in bioassays may provide information about the polarity of compounds acting as host recognition cues (Nielsen, 1978a, b; Honda et al., 1997; Müller and Renwick, 2001; Endo et al., 2004). Subsequent bioassay-guided fractionation can lead to the isolation of individual stimulating compounds. Using this method, Honda et al. (1997) identified for example pyrrolizidine alkaloids in a fraction of a methanol extract from the host plant *Parsonia laevigata* (Apocynaceae) as oviposition stimulants for danaid butterflies (*Idea leuconoe* Erichson, Lepidoptera: Nymphalidae).

For several herbivores feeding on Brassicaceae, polar glucosinolates are involved in host plant recognition (Chew, 1988a, b; Nielsen, 1988; Louda and Mole, 1991; Halkier and Gershenzon, 2006). Glucosinolates are characteristic secondary metabolites of Brassicaceae that are hydrolyzed by the enzyme myrosinase to various break-down products, e.g. nitriles and isothiocyanates, upon tissue disruption (Rask et al., 2000). Glucosinolates and their break-down products were shown to deter and repel generalists (Louda and Mole, 1991; Halkier and Gershenzon, 2006), whereas herbivorous insects specialized on Brassicaceae were found to use glucosinolates as stimulants for oviposition, e.g. *Pieris rapae* L. (Lepidoptera: Pieridae) (Renwick et al., 1992; van

Loon et al., 1992) and the sawfly *Athalia rosae* L. (Hymenoptera: Tenthredinidae) (Liston, 1995; Barker et al., 2006), or for feeding, e.g. weevils (Larsen et al., 1992) and various leaf beetles (Nielsen, 1978a; Nielsen et al., 1979). Larvae of the oligophagous mustard leaf beetle *Phaedon cochleariae* (F.) (Coleoptera: Chrysomelidae) can be attracted to the hydrolization product of 2-propenylglucosinolate (sinigrin), 2-propenylisothiocyanate (Tanton, 1977). The use of intact glucosinolates, among others 4-hydroxybenzylglucosinolate (sinalbin), as recognition cues was shown for adult mustard leaf beetles (Nielsen, 1978b). However, in this study (Nielsen, 1978b) extraordinary high concentrations of glucosinolates were offered. In similar bioassays, a feeding stimulation of mustard leaf beetles could not be achieved when 2-propenylglucosinolate was offered in concentrations corresponding to those detected in host plants (Reifenrath et al., 2005). Therefore, the responsible cues for feeding stimulation of the Brassicaceae specialist *P. cochleariae* remained unclear.

For many insects more than one stimulating compound was detected within one host plant. Müller and Renwick (2001) found that *Manduca sexta* (Johan) (Lepidoptera: Noctuidae) and *Leptinotarsa decemlineata* L. (Coleoptera: Chrysomelidae), both specialist feeders on solanaceous plants, use in part different feeding stimulants; for both insects at least two compounds of different polarity provoked feeding behavior. Two or even more compounds might act in concert by additive or synergistic effects. However, the same compounds can be inactive when offered individually, e.g. glucosinolates and sugar for *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae) (Bartlet et al., 1994), or flavonoids and 2-propenylglucosinolate for *Plutella xylostella* L. (Lepidoptera: Plutellidae) (van Loon et al., 2002).

Little information is available on necessary concentrations of individual cue compounds or ratios of additively or synergistically acting compounds that are essential for feeding or oviposition stimulation. Whereas for many herbivores volatile recognition cues are based on specific ratios of key compounds (Bruce et al., 2005), qualitative contact cues for specialist beetles might rather depend on the mere presence of a certain compound or a mixture of compounds (Larsen et al., 1992; Müller and Renwick, 2001; Heisswolf et al., 2007).

In order to test the stimulating effects of chemical compounds on herbivores, a neutral substrate is needed to which pure substances or leaf extracts can be applied and offered to the insects. Artificial diets (e.g. Tanton, 1977; Bartlet et al., 1994; Abe et al., 2000; Nagasawa and Matsuda, 2005) or wax coated paper leaves (Degen and Städler,

1997; Honda et al., 1997) can provide useful substrates to add or remove certain compounds, but many insects do not accept such artificial substrates. In these cases other substrates are needed, e.g. wheat flour based wafers (Müller and Renwick, 2001) or non host plant leaves (Nielsen, 1978b). Nielsen Nielsen (1978b) found that *P. cochleariae* will only feed on pea leaves (*Pisum sativum*, Fabaceae) if stimulating compounds, e.g. extracts of host plant leaves, are added to the surface. Therefore, pea leaves can provide a suitable substrate for feeding bioassays with the mustard leaf beetle.

In the present study, we re-investigated the responsible cues for feeding stimulation for *P. cochleariae*. We tested the response of these leaf beetles to host plant extracts of different polarity offered on pea leaf discs, and used bioassay-guided fractionation for the isolation of compound classes involved in feeding stimulation. Moreover, we studied the role of quantitative ratios of compound classes acting additively as feeding stimulants. In order to modify the qualitative and quantitative composition of compounds involved in feeding stimulation, we exposed greenhouse-kept plants shortly to outdoor conditions. We analyzed both groups of plants (greenhouse-kept and outdoor-exposed) for their chemical profiles and tested their stimulatory activity in feeding assays.

4.2 Materials and methods

4.2.1 Insects and Plants

Mustard leaf beetles [*Phaedon cochleariae* (F): Chrysomelidae, Coleoptera] were reared in the laboratory at 70% r.h. and 25 °C with a photo cycle of L16:D8 for several generations. Larvae and adults were fed with greenhouse-grown Chinese cabbage [*Brassica pekinensis* (Lour.) Rupr.]. Adult beetles were used for bioassays 8-15 days after hatching.

White mustard, *Sinapis alba* L. cv. Silenda and pea, *Pisum sativum* L. cv. Rheinperle (Fabaceae; Kiepenkerl, Norken, Germany), were grown from seeds in the greenhouse (20: 15°C, 16: 8h L: D, 70% r.h.) in 12 cm $\varnothing$ pots in unfertilized soil (peat, pH 6). Pea plants and half of the *S. alba* plants were kept in the greenhouse (greenhouse-kept plant treatment). Irradiance spectra in the greenhouse lacked UV-B, while UV-A was detected starting at 340 nm, without exceeding approximately 30 mW (m²·nm)$^{-1}$. The

photosynthetic active radiation (PAR) was about $10.3 \cdot 10^{-6}$ mW $(\text{m}^2 \cdot \text{nm})^{-1}$ (Reifenrath and Müller, 2007). In order to induce chemical changes, the other half of *S. alba* plants was exposed to higher ultraviolet and photosynthetic active radiation levels. At an age of 19 days, these plants were placed in an open foil tent in the Botanical Garden Würzburg and exposed to outdoor conditions for 48 h, but shielded against wind and rain (outdoor-exposed plant treatment). The foil tent [base 3×1 m, height 1.20 m in south direction, sloping to 1.80 m in north direction; for further details of the construction see Kolb et al. (2001)] was provided with a teflon foil (Novofol, Siegsdorf, Germany) transmitting the complete visible light spectrum plus UV-A and UV-B radiation. The total wavelength range measured in the tent was 280-900 nm; the PAR was approximately $1.1 \cdot 10^3$ mW $(\text{m}^2 \cdot \text{nm})^{-1}$ (Reifenrath and Müller, 2007). For further details on the outdoor exposure condition compared to greenhouse conditions see Reifenrath and Müller (2007).

At an age of 21 days, *S. alba* plant material was harvested. Second youngest unfolded leaves of 15 greenhouse-kept and 15 outdoor-exposed plant individuals were freshly used for feeding choice tests. Moreover, leaf material of each of six plant individuals was pooled, frozen at -80°C, lyophilized and taken for preparation of leaf extracts for use in bioassays (number of extracts: greenhouse-kept plants N = 10, outdoor-exposed plants N = 4). For chemical analyses, two leaf discs were collected from the second youngest unfolded leaf of individual plants, frozen at -80°C and lyophilized. One of both leaf discs was taken for glucosinolates analyses (N = 15), and the second one for analyses of flavonoids (N = 11). Pea leaves were taken as a substrate for bioassays.

4.2.2 General bioassay design

To investigate feeding stimulation, test leaf extracts, fractions or metabolite solutions of *S. alba* were applied on leaf discs of the non-host *P. sativum* and offered together with *P. sativum* discs treated with equal amounts of the corresponding solvents as controls in dual-choice assays to mustard leaf beetles. In all feeding choice assays, two leaf discs (1 cm diameter) were offered in Petri dishes (5.5 cm diameter) on a moistened filter paper in a distance of approximately one centimeter. Concentrations of extracts, fractions and metabolite solutions applied to the pea leaf discs corresponded to concentrations detected in host plant leaf material of the same area. Solvents of tests and

controls were allowed to evaporate sufficiently prior the start of an assay. One adult beetle was added to each Petri dish and allowed to feed for 20 h (8-20 replicates per assay).

Bioassays ended before total pea leaf discs were consumed. At the end of each bioassay, remaining leaf fragments were photocopied and consumed areas were determined with a leaf area meter (Winfolia 2004a, Canada) and calculated as mg FW from reference pea leaf discs. Feeding stimulation by test substances was determined as net consumption rate per leaf beetle on test discs which was calculated as consumption on test discs (mg FW) – consumption on control discs (mg FW). The number of beetles that were stimulated to feed on test discs was determined for each treatment and expressed as percentage of total number of beetles tested within this treatment. The number of beetles that fed on control discs was counted.

4.2.3 Determination of the polarity of feeding stimulants

In order to investigate the polarity of feeding stimulants, leaf extracts of hexane, dichloromethane or methanol were tested against the corresponding solvents in the above described dual-choice assays. Dried leaf material of greenhouse-kept plants (~ 3.5 mg DW per solvent) was ground, extracted in a Soxhlet apparatus (~ 100 ml) for 8 h in either one of the solvents, and extracts were subsequently concentrated to smaller volumes (~ 4 ml) in a rotary evaporator. At least two extracts from different plant sources were prepared for each solvent and offered at random to the beetles.

4.2.4 Bioassay-guided fractionation via high performance liquid chromatography (HPLC)

Since the methanol extract elicited the highest response in P. cochleariae, this was selected for further fractionation in order to narrow down the identity of stimulating compounds. Four separate methanol extracts of greenhouse-kept and outdoor-exposed leaves of *S. alba*, respectively, were fractionated by semi-preparative reverse-phase HPLC (1100 Series, Hewlett-Packard, Waldbronn, Germany) with a quaternary pump and a 1040M diode array detector. Gradient separation was achieved on a semi-preparative Supelco C-18 column (Supelcosil LC-18, 250 × 10 mm, 5 μm, Supelco) with an eluent gradient (solvent A: aqua bidest, solvent B: acetonitril) of 0-4% B (8 min), hold at 4% B (4 min), 4-7% B (8 min), 7-10% B (6 min), 10-100% B (14 min),

followed by a cleaning cycle, detecting wave lengths at 229 and 254 nm (bandwidth 4 nm) (Fig. 4.1). Within each sequence several fractions were collected separately, at 0-2 minutes, 2-6 min, 6-20 min, 20-30 min, 30-36 min, and 36-40 min. Fractions were reduced in a rotary evaporator and subsequently resolved in 80% methanol.
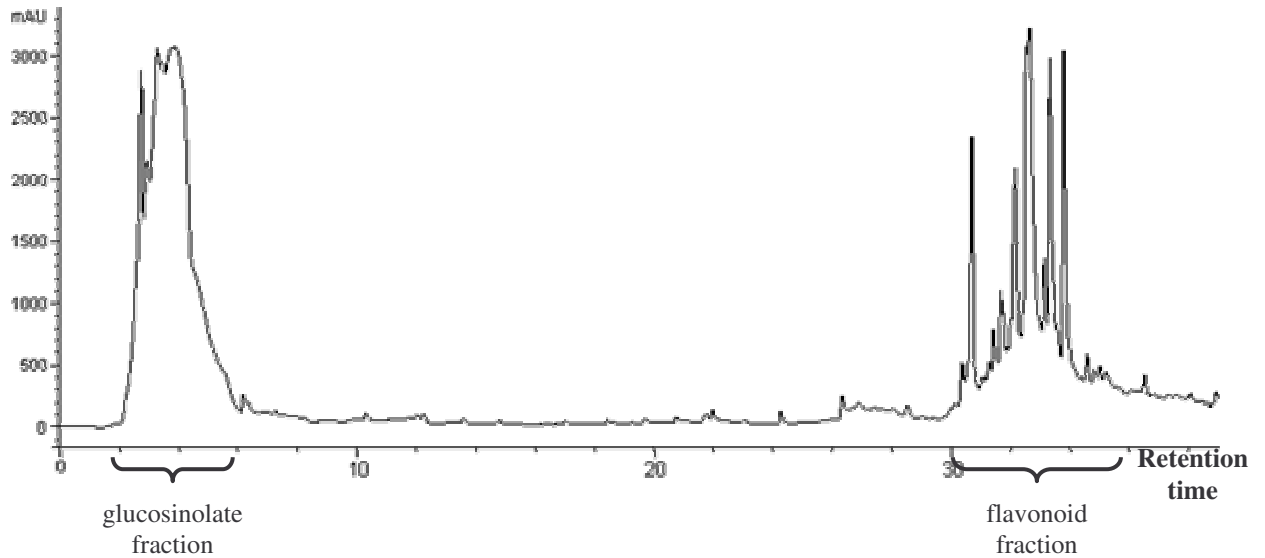
Figure 4.1 Chromatogram of a methanol leaf extract of greenhouse-kept *Sinapis alba* (reversed-phase HPLC, 229 nm) with peak regions mainly containing glucosinolates (3-6 min) and flavonoids (30-36 min). mAU - arbitrary units.

Comparisons of retention times and UV spectra to those of purified standards led to the classification of glucosinolates in fractions collected at 2-6 min and flavonoids in fractions at 30-36 min. Those fractions are henceforth termed "glucosinolate fraction" and "flavonoid fraction", although further unidentified metabolites occur in these fractions. Ratios of the glucosinolate containing peak areas to flavonoid containing peak areas were roughly calculated for both plant treatments. Glucosinolate and flavonoid containing fractions were quantified by summing up the peak areas at 229 nm for peaks in the glucosinolate containing fraction and at 254 nm for peaks in the flavonoid containing fraction (bandwidth 4 nm).

Each of the six individual fractions gained from two greenhouse-kept plants extracts and three outdoor-exposed plant extracts, respectively, were separately tested in feeding choice bioassays on pea discs against the control solvent. Fractions gained from different extracts within one plant treatment were offered at random. Furthermore, glucosinolate containing fractions and flavonoid containing fractions were combined and tested as a mixture against the control for feeding stimulation of *P. cochleariae*. To

test whether different concentrations or ratios might influence feeding activity by *P. cochleariae*, stimulation by the glucosinolate containing fraction, the flavonoid containing fraction and the combined fraction of the greenhouse-kept plant extracts were each tested against the corresponding fraction gained from outdoor-exposed plant extracts (one extract per plant treatment was fractionated).

4.2.5 Feeding stimulation by purified glucosinolates and flavonoids

The feeding stimulation by 4-hydroxybenzylglucosinolate (sinalbin) (obtained from glucosinolates.com, Denmark), the main glucosinolate in *S. alba* host plants, was tested in a dual choice assay. *P. sativum* leaf discs were treated with 4-hydroxybenzylglucosinolate dissolved in 70% methanol in a concentration appropriate to total glucosinolate concentrations detected in leaf material of the same area (approximately 5 nmol/cm²) and offered against control discs. Due to the extraordinary high diversity of flavonol glycosides, individual flavonoids of *S. alba* could not be identified. However, a mixture of two common flavonol glycosides, quercetin-3-glucoside (Phytoplan, Heidelberg, Germany) and kaempferol-3-rutinoside (Phytoplan, Heidelberg, Germany) (each approximately 5 nmol/cm²) were tested as they were assumed to occur in *S. alba*. Furthermore, the combination of 4-hydroxybenzyl-glucosinolate with both flavonoids were tested against the solvent control.

4.2.6 Analyses of glucosinolates and flavonoids in host-plants

For glucosinolate identification and quantification of greenhouse-kept and outdoor-exposed *S. alba* plants dried leaf material was weighed, ground, and extracted in aqueous 80% methanol with addition of 2-propenylglucosinolate ($\geq$ 99%, Fluka, Taufkirchen, Germany) as internal standard. Glucosinolates were converted to desulfoglucosinolates using purified sulfatase [E.C. 3.1.6.1, 'type H-1, from *Helix pomatia*, 15100 units (gram solid)$^{-1}$; Sigma, Taufkirchen, Germany; purification after Graser et al. (2001)]. The resulting desulfoglucosinolates were analyzed by HPLC, individually identified and quantified by calculating the peak areas at 229 nm (bandwidth 4 nm) relative to the area of the internal standard peak and corrected by the response factors, as described earlier (Reifenrath et al., 2005). Total glucosinolate concentrations were evaluated by adding concentrations of individual glucosinolates per gram leaf sample.

For classification and quantification of flavonoids the dried leaf material was ground, and extracted in aqueous 80% methanol with addition of catechin (Phytoplan, Heidelberg, Germany) as internal standard. In order to remove chlorophyll, petrol ether was added to the extracts and the resulting upper phases were discarded. These purified extracts were analyzed by HPLC and flavonol glycosides classified and quantified by calculating the peak areas at 254 nm (bandwidth 4 nm) relative to the area of the internal standard peak and corrected by the response factors, as described earlier (Reifenrath et al., 2005). Total flavonoid concentrations were evaluated by adding concentrations of individual flavonol glycosides per gram leaf sample. Flavonoid concentrations of pea leaves were analyzed in the same way.

### 4.2.7 Feeding stimulation by fresh host plant leaves

Discs (1.3 cm diameter) of fresh second youngest *S. alba* leaves of greenhouse-kept *versus* outdoor-exposed plants were offered in dual choice bioassays. Bioassays ended before total leaf discs were consumed. At the end of each bioassay, remaining leaf fragments were photocopied and consumed areas were determined with a leaf area meter and calculated as mg FW from reference leaf discs.

4.3 Results

4.3.1 Polarity of feeding stimulants

In assays with leaf extracts from greenhouse-kept plants *versus* controls, beetles showed a highly different response to extracts of different polarities. 7% of the beetles fed on leaf discs treated with dichloromethane leaf extracts, whereas 67% were stimulated to feed on hexane extracts and 87% on leaf discs treated with methanol extracts (N = 15 each). Differences were significant between bioassays with hexane and dichloromethane extracts (Chi-square test of homogeneity, $\chi^2 = 13.9$, $P < 0.001$), and between methanol and dichloromethane extracts ($\chi^2 = 19.3$, $P < 0.001$), but not between hexane and methanol extracts ($\chi^2 = 0.8$, $P = 0.36$). Feeding on control discs was observed for no more than one beetle per bioassay. *P*-values of < 0.001 remain after a sequential Bonferroni-correction.

Moreover, net consumption rate per leaf beetle differed significantly between the three extracts (one-way ANOVA followed by Tukey HSD, methanol vs. dichloromethane $P < 0.001$, methanol vs. hexane $P < 0.001$, dichloromethane vs. hexane $P = 0.041$, Fig. 4.2). Thereby, data were log-transformed in order to achieve approximate variance homogeneity.
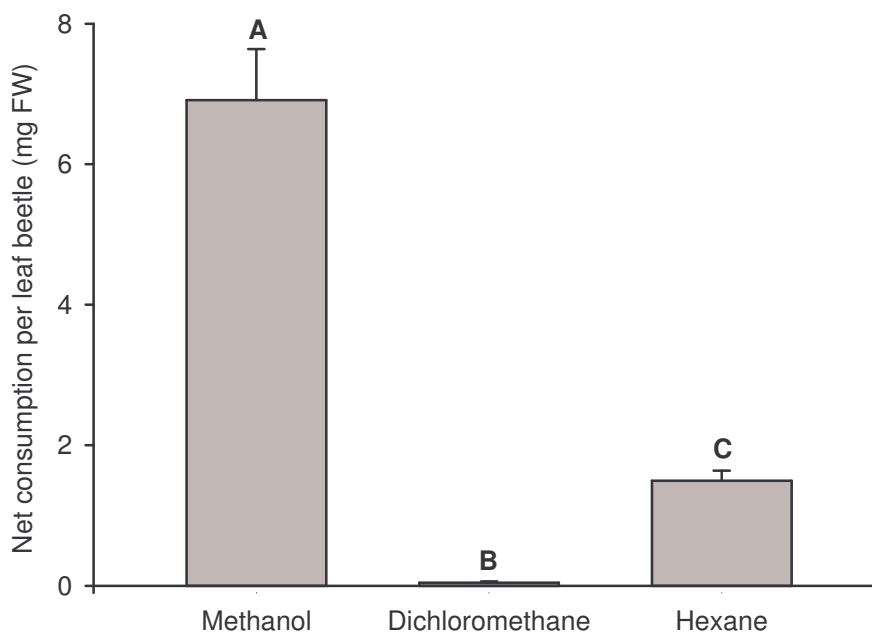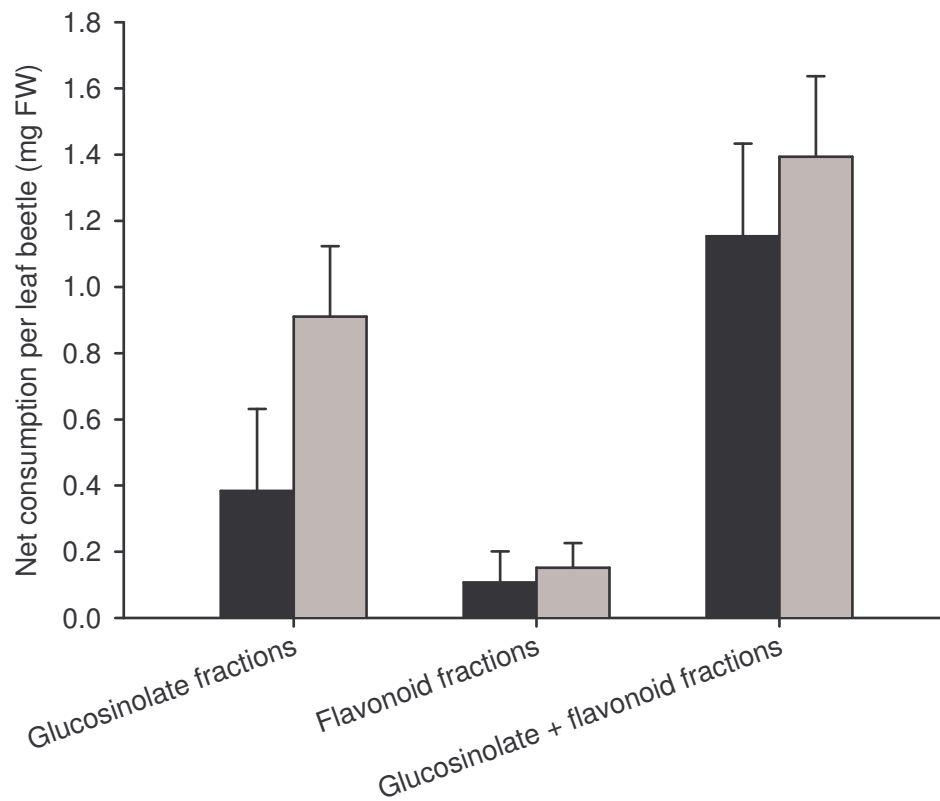

Figure 4.2 Net consumption (mg FW amount of feeding on test discs – amount of feeding on control discs) of *Phaedon cochleariae* adults on pea leaf discs treated with extracts of greenhouse-kept *Sinapis alba* leaves. Test extracts of different polarities were offered against pure solvents as controls in dual choice assays; mean ± SE, N = 15. Capital letters indicate significant differences with $P < 0.05$ (one-way ANOVA followed by Tukey HSD).

4.3.2 Feeding stimulation by fractions from methanol extract

No feeding stimulation was observed on leaf discs treated with the fractions collected at 0-2 minutes retention time, 6-20 min, 20-30 min, and 36-40 min of both plant sources. In contrast, for greenhouse-kept leaf extracts 40% of the leaf beetles were stimulated to feed on test discs treated with glucosinolate fractions (feeding was also observed on three control discs), 10% on flavonoid fractions (no beetle fed on control discs), and 60% on the combined fractions (feeding was also observed on four control discs) (N = 20 for each fraction). Differences in the number of feeding beetles were significant between glucosinolate and flavonoid fractions (Chi-square test of homogeneity, $\chi^2 = 4.8$, $P = 0.028$), flavonoid and combined fractions ($\chi^2 = 11.0$, $P <$

0.001), but not between glucosinolate and combined fractions ($\chi^2 = 1.6$, $P = 0.21$). *P*-values < 0.001 remain after a sequential Bonferroni-correction. In bioassays with outdoor-exposed plant extracts 53% of the leaf beetles fed on test discs treated with glucosinolate fractions (four beetles fed on control discs), 23% on flavonoid fractions (two beetles fed on control discs), and 70% fed on combined fractions (two beetles fed on control discs) (N = 30 for each fraction). Like for fractions of greenhouse-kept plant extracts, differences in the number of beetles feeding on tests were significant between glucosinolate and flavonoid fractions ($\chi^2 = 5.7$, $P = 0.017$), flavonoid and combined fractions ($\chi^2 = 13.1$, $P < 0.001$), but not between glucosinolate and the combined fractions ($\chi^2 = 1.8$, $P = 0.18$). *P* -values of ≤ 0.017 remain after a sequential Bonferroni-correction.

Figure 4.3 Net consumption (mg FW amount of feeding on test discs – amount of feeding on control discs) of *Phaedon cochleariae* adults on pea leaf discs treated with fractions of methanol leaf extracts of greenhouse-kept *Sinapis alba* leaves. Fractions mainly containing glucosinolates and fractions mainly containing flavonoids were obtained from semi-preparative HPLC fractionation and offered either individually or combined against controls in dual choice assays; mean ± SE, N = 20 for fractions of greenhouse-kept plant extracts, and N = 30 for fractions of outdoor-exposed plant extracts. Black bars indicate greenhouse-kept plants; gray bars indicate outdoor-exposed plants.

In total, net consumption rates (mg FW) on test leaf discs were not significantly affected by plant treatment, but varied significantly between fractions (multifactorial

ANOVA, plant treatment $F = 2.5$, $P = 0.12$; fractions $F = 14.9$, $P < 0.001$; plant treatment × fractions $F = 0.7$, $P = 0.51$; N =150). Thereby, variance homogeneity was confirmed for net consumption rates on glucosinolate fractions and combined fractions, but not for flavonoid fractions. However, conclusions about significant differences in net consumption rates of fractions were unaffected when data for flavonoid fractions were removed from the analysis and when flavonoid fractions were compared with the other two by Welch-corrected t-tests. Since consumption rates were not significantly affected by different plant treatments, data of both treatments were pooled for further analyses. The net consumption rates per leaf beetle were higher on leaf discs with glucosinolate fractions compared to those with flavonoid fractions, but highest on leaf discs with the combined fractions containing glucosinolates and flavonoids (Fig. 3). Differences were significant between glucosinolate and flavonoid fractions and between each fraction and the combined fractions (Tukey HSD, glucosinolate vs. flavonoid fractions $P = 0.016$, glucosinolate vs. combined fractions $P < 0.010$, flavonoid vs. combined fractions $P < 0.001$). In order to test whether additive effects may explain the consumption rates on combined fractions, we compared the net consumption on the combined fractions (mean 0.13 ± 0.18 SE mg FW) with the sum of net consumption on glucosinolate fractions and the mean value for net consumption on flavonoid fractions (0.07 ± 0.02 mg FW). Differences between both were not significant (Student's t-test, t $= 1.8$, $P = 0.07$, N = 100), indicating that additive effects are very likely.

No differences in feeding stimulation were detected when fractions of greenhouse-kept plants were offered against corresponding fractions of outdoor-exposed plants. 50% beetles fed on glucosinolate fractions of greenhouse-kept plant extracts and 37.5% on those of outdoor-exposed plant extracts ($\chi^2 = 0.3$, $P = 0.61$), 12.5% beetles fed on flavonoid fractions of greenhouse-kept plant extracts and none on those of outdoor-exposed plant extracts ($\chi^2 = 1.1$, $P = 0.30$), and 100% beetles fed on combined fractions of greenhouse-kept plant extracts and 75% on those of outdoor-exposed plant extracts ($\chi^2 = 2.3$, $P = 0.13$; N = 8). Also, consumption rates did not differ between corresponding fractions of both plant treatments when offered together (glucosinolate fractions: greenhouse-kept plants 0.13 ± 0.01 mg FW, outdoor-exposed plants 0.06 ± 0.03 mg FW, Student's t-test for paired samples, t $= 1.4$, $P = 0.20$; flavonoid fractions: greenhouse-kept plants 0.04 ± 0.04 mg FW, outdoor-exposed plants 0 ± 0 mg FW, t $= 1.0$, $P = 0.35$; combined fractions: greenhouse-kept plants 0.29 ± 0.04 mg FW, outdoor-exposed plants 0.20 ± 0.1 mg FW, t $= 1.7$, $P = 0.13$).

The mean ratio of compounds in the fraction containing glucosinolate to those in the flavonoid containing fraction differed significantly between greenhouse-kept plants (mean ratio 2.0 ± 0.3 SE; N = 4) and outdoor-exposed plants (0.7 ± 0.1) (Mann-Whitney U-test, $Z = 2.3$, $P = 0.021$).

4.3.3 Feeding stimulation by purified metabolites

In bioassays with purified metabolites offered against controls, 47% of the mustard leaf beetles were stimulated by 4-hydroxybenzylglucosinolate (feeding was also observed on three control discs), 7% fed on a mixture of the flavonols quercetin-3-glucoside and kaempferol-3-rutinoside (feeding was also observed on two control discs) and 60% on a mixture of 4-hydroxybenzylglucosinolate with both flavonols (feeding was also observed on three control discs) (N = 15 for each bioassay). Similarly to fractions of greenhouse-kept and outdoor-exposed plant extracts (see above), differences in the number of beetles feeding on test discs were significant between 4-hydroxybenzylglucosinolate and the flavonol mixture ($\chi^2 = 6.1$, $P = 0.013$), the flavonol mixture and the combination of 4-hydroxybenzylglucosinolate and the flavonol mixture ($\chi^2 = 9.6$, $P = 0.002$), but not between 4-hydroxybenzylglucosinolate and the combination of 4-hydroxybenzylglucosinolate with the flavonols ($\chi^2 = 0.5$, $P = 0.46$). P-Values of ≤ 0.013 remain after a sequential Bonferroni-correction. In contrast to the assays with fractions of leaf material, net consumption rates did not vary significantly among the three bioassays with purified metabolites (one-way ANOVA, $F = 2.5$, $P = 0.09$). Variance homogeneity was confirmed for the glucosinolate and the mixture of glucosinolate and flavonol glycosides, but not for flavonol glycosides *versus* the other two. However, conclusions about significant differences in stimulation among fractions were unaffected when the flavonoid fraction was compared with the other two by Welch-corrected t-tests.

4.3.4 Chemical composition of host plant material

In selective analyses of leaf material total glucosinolate concentrations were similar between greenhouse-kept and outdoor-exposed plants (Student's t-test, t = 0.1, $P = 0.93$; N = 14; Fig. 4.4a). Also, between the following individual glucosinolates no significant differences were found (Student's t-test, all $P \geq 0.08$): 4-hydroxybenzylglucosinolate as major compound, benzylglucosinolate, sum of three further minor aromatic

glucosinolates (at 9 min, 19 min, and 28 minutes retention times, not further identified) and one minor aliphatic sulfonyl glucosinolate (24 min).
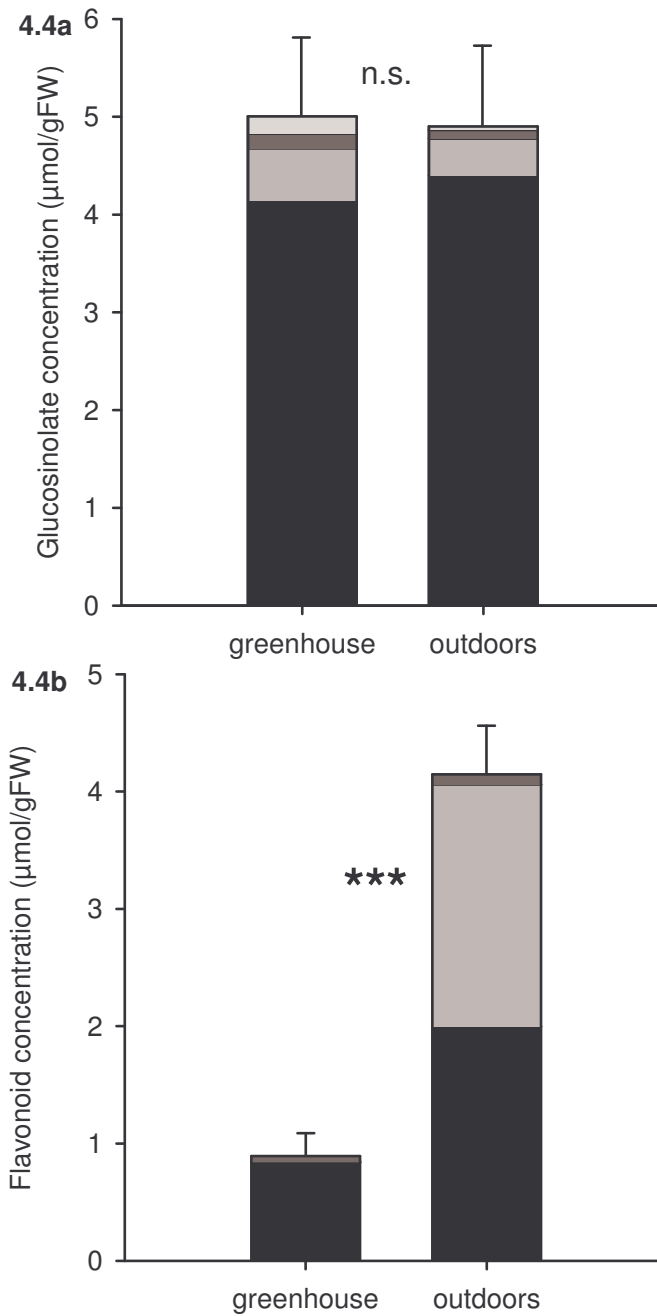
Figure 4.4 Glucosinolate and flavonoid concentrations of methanol leaf extracts of *Sinapis alba* plants either kept in the greenhouse or short-term exposed outdoors. 4.4a) Glucosinolate (GS) compositions: black bars indicate 4-hydroxybenzylGS, light gray bars indicate benzylGS, dark gray bars indicate other GS with aromatic side chains, white bars indicate other GS with indolic and aliphatic side chains. Mean ± SE of total amounts, N = 14. 4.4b) Flavonoid compositions: black bars indicate kaempferol glycosides, light gray bars indicate quercetin glycosides, and dark gray bars indicate unidentified glycosides. Mean ± SE of total amounts, N = 11. Asterisks indicate significant differences *** $P < 0.001$, n.s. = not significant; Student's t-test.

Significant differences in concentrations were detected for two indolic glucosinolates, indol-3-yl-methyl-glucosinolate and 4-methoxy-indol-3-yl-methyl-glucosinolate (sum of both, t = 2.4, *P* = 0.022) (Fig. 4.4a). In leaves of both plant treatments 4-hydroxybenzyl-glucosinolate presented 82% of the total glucosinolate content. Benzylglucosinolate concentrations made up 10% in greenhouse-kept plants, and 7% in outdoor-exposed plants, but differences were not significant (*t* = 9.0, *P* = 0.49).

Flavonoid concentrations were more than four times higher in outdoor-exposed plants than in greenhouse-kept plants (Student's t-test, t = 7.2, *P* < 0.001; N = 11; Fig. 4.4b). Three groups of flavonol glycosides were classified and quantitatively compared between both plant treatments: quercetin glycosides (*t* = 8.5, *P* < 0.001), kaempferol glycosides (*t* = 3.9, *P* < 0.001), and unidentified flavonol glycosides (*t* = 1.4, *P* = 0.17). Whereas kaempferol glycosides made up 89% of total flavonol contents in greenhouse-kept plants, but only 48% in outdoor-exposed plants (*t* = 10.5, *P* < 0.001), quercetin glycoside contents were only 2% in greenhouse-kept plants and 50% in outdoor-exposed plants (*t* = 21.0, *P* < 0.001, Welch corrected; Fig. 4.4b). Mean flavonoid concentrations in pea leaves were 7.11 ± 1.5 SE µmol/g FW (N = 5), composed of 94% unidentified glycosylated flavonols and 6% quercetin and kaempferol glycosides.

4.3.5 Feeding stimulation by fresh leaves from differently treated plants

Beetles were stimulated to feed on fresh leaves of outdoor-exposed and greenhouse-kept plants in a similar proportion. The number of stimulated beetles did not differ between both plant treatments, since feeding was observed on all leaf discs (N = 20). Also, the net consumption rates (Student's t-test, t = 1.4, *P* = 0.19; N = 20) did not differ significantly between both plant treatments.

4.4 Discussion

Glucosinolates and their breakdown products provide feeding and/or oviposition cues for many herbivorous insects that are specialized on Brassicaceae (e.g. Nielsen, 1978a, b, 1988; Nielsen et al., 1979; Chew, 1988a, b; Louda and Mole, 1991; Renwick, 2002; Barker et al., 2006; Halkier and Gershenzon, 2006). Whether leaf-feeders come into

contact with intact glucosinolates might depend on the velocity of glucosinolate degradation. The hydrolization of glucosinolates by myrosinases might start immediately upon tissue disruption, but might also depend on the composition of glucosinolates and myrosinases (Bones and Rossiter, 2006). Therefore, herbivores likely also come into contact with remaining intact glucosinolates. For the specialist mustard leaf beetle *Phaedon cochleariae* feeding stimulation by glucosinolates was described in an early study (Nielsen, 1978b), however, in this study extraordinary high concentrations of glucosinolates were used. Therefore, feeding stimulation of the mustard leaf beetle was reinvestigated.

In the present study, *P. cochleariae* showed strong responses to polar methanol leaf extracts of its host plant *S. alba* in feeding bioassays, expressed as a high percentage of feeding beetles and high net consumption rates per individual, whereas non-polar hexane leaf extracts stimulated also a large number of beetles, but consumption rates were significantly lower (Fig. 4.2). In contrast, the extract of intermediate polarity, dichloromethane, had no stimulatory activity for the beetles. Thus, compounds of contrasting polarities are involved in the feeding stimulating of the mustard leaf beetle to various degrees.

Further bioassay-guided fractionation of the highly active methanol extracts of differently treated plants (Fig. 4.1) led to the isolation of fractions that evoked different feeding responses. Within these fractions, flavonoids and glucosinolates were identified. The fraction containing flavonoids had only very little stimulatory activity, but offered together with the clearly stimulating glucosinolate containing fraction, an increased net consumption rate was observed. The sum of net consumption rates on glucosinolate fractions and the mean consumption on flavonoid fractions was on average lower, although not statistically different, from the net consumption rate on the combined fractions. Therefore, we suggest additive effects of both fractions, or at least of one compound of each fraction, acting in concert as feeding stimulants.

Bioassays with pure metabolites could not fully resemble this pattern. Similar to glucosinolate containing fractions, pure 4-hydroxybenzylglucosinolate evoked feeding behavior within half of the tested individuals. Hence, this glucosinolate might provide the active stimulant in the glucosinolate containing fraction. As 2-propenyl-glucosinolate, tested in an earlier study (Reifenrath et al., 2005), did not stimulate *P. cochleariae*, glucosinolates with variable side chains obviously differently affect the mustard leaf beetle. Different effects on host selection behavior due to glucosinolates

with varying side chains and their resulting hydrolysis products were also shown for other Brassicaceae specialists (Nielsen, 1978b; Giamoustaris and Mithen, 1995; Barker et al., 2006; Renwick et al., 2006). The two pure flavonol glycosides tested here had very little stimulatory activity on the mustard leaf beetles. In contrast to the tests with the isolated fractions, consumption rates differed significantly between the flavonol mixture and the mixture of 4-hydroxybenzylglucosinolate and both flavonols, but not between 4-hydroxybenzylglucosinolate and mixture of 4-hydroxybenzylglucosinolate and both flavonols. Thus, the additive effects visible with the isolated fractions of both plant treatments could not be reproduced with the purified metabolites. Among the high diversity of flavonol glycosides (Harborne, 1991), quercetin-3-glucoside and kaempferol-3-rutinoside were only assumed to occur, but not identified, in these host plants. Further flavonoids and other compounds likely occur in the HPLC-fractions of the *S. alba* methanol extracts, at least some of them might also be involved in feeding stimulation of the mustard leaf beetle.

Since it remained unclear whether variable ratios of chemical compounds differently affect host plant perception and stimulation of the mustard leaf beetle, two sets of chemically different *S. alba* plants were used, namely greenhouse-kept and outdoor-exposed plants. Glucosinolate concentrations were similar in both plant treatments, with slight quantitative differences being restricted to relative contents of benzylglucosinolate (Fig. 4.4a). Flavonoid concentrations were significantly higher in outdoor-exposed plants compared to greenhouse-kept plants, with profound differences in quercetin and kaempferol glycoside contents (Fig. 4.4b). Therefore, the ratio of glucosinolate to flavonoid concentrations strongly differed between both plant treatments. However, despite these variations the strength of the feeding response of the mustard leaf beetle was not significantly affected by plant treatments and did not differ towards fractions obtained from differently treated plant material. Furthermore, also in choice tests with fresh leaves of greenhouse-kept and outdoor-exposed plants neither the number nor the consumption rates of beetles that were stimulated to feed differed. In these plants, many more metabolites than just glucosinolates and flavonoids might have been altered.

In conclusion, the variation in quality and quantity of glucosinolates and flavonoids as well as potential shifts in further unidentified metabolites that might be additionally involved, are of minor importance for feeding stimulation, at least in the tested range. Contact cues of the mustard leaf beetle might be primarily based on the mere presence

or absence of certain chemical compounds of different polarities, as was also found for other herbivorous insects (Larsen et al., 1992; Müller and Renwick, 2001 Heisswolf et al., 2007). This is in contrast to some volatile recognition cues, where ratios can be of prior importance (Bruce et al., 2005). Nevertheless, also for certain non-volatile compounds threshold concentrations might determine whether herbivores feed or not. Van Loon et al. (2002) tested a series of 2-propenylglucosinolate concentrations on the feeding stimulation of *Plutella xylostella* larvae and detected a dose-dependency with preferred concentrations of > 3 µM, whereas each of four tested acylated flavonoids evoked significant feeding preferences at 1mM or higher concentrations. The mixture of all four flavonoids plus 2-propenylglucosinolate led to a significant preference for this mixture over the glucosinolate alone (van Loon et al., 2002).

In the present study, the percentage of stimulated beetles and consumption rates per mustard leaf beetles were highest on test discs treated with methanol leaf extracts. The combined fractions containing glucosinolates and flavonoids did also stimulate most beetles for feeding, but consumption rates were comparably lower. Hence, methanol extracts might contain additional chemical leaf compounds that were absent in both additively stimulating fractions gained from the same extract by HPLC. Potential polar metabolites include glucose or other sugars which have been shown to play a role as feeding stimulants for many other herbivores (e.g. Hsiao, 1985; Bartlet et al., 1994). They may also act synergistically or additively with glucosinolates for specialists on Brassicaceae (see Bartlet et al., 1994, and references therein). Moreover, in bioassays with extracts and fractions, glucosinolate-free pea leaves were used as a putative neutral substrate for tests of feeding stimulation of extracts and fractions. Interacting effects of flavonoids and unknown chemical compounds of these leaves with test substances applied to the surface on the feeding behavior of leaf beetles can not be totally excluded.

In conclusion, feeding stimulation of the mustard leaf beetle *P. cochleariae* represents a complex interaction of several chemical plant compounds. Unidentified nonpolar compounds were found to be less effective than a combination of several polar compounds, including 4-hydroxybenzylglucosinolate. In a natural environment, mustard leaf beetles are confronted with chemical profiles of plants that can vary drastically. Therefore, it might be advantageous for a mono- or oligophagous herbivore to utilize two or even more chemical plant compounds as feeding stimulants, but not to depend on certain quantitative ratios of involved individual compounds or compound classes.

4.5 Acknowledgements

4.6 References

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Summary

Abiotic environmental stress, as evoked by short-term exposure of greenhouse-grown plants to ambient ultraviolet radiation (UV), induces chemical and morphological adaptations of plants. Responses depend on the strength of stress and differ between species and tissues of variable age. In two Brassicaceae, *Sinapis alba* and *Nasturtium officinale*, stress responses towards short-term exposure to ambient radiation including or excluding UV reveal a high phenotypic plasticity, with strong differences their chemical composition compared to plants that remained in the greenhouse. Changes in nutrient contents, induced by exposure treatments, varied strongly between young and old leaves of both plant species, probably corresponding to different photosynthetic activities in these tissues. The most pronounced defensive response against UV, the accumulation of flavonoid pigments, was strongest in young UV-exposed leaves, with an increase of the more effectice flavonol quercetin on the expense of less effectice kaempferol. Glucosinolates and myrosinase enzymes showed highly species-specific responses to UV-stress. Acting together, both compounds provide a highly efficient defence system against generalist herbivores in Brassicaceae. In both plant species, the combination of detected glucosinolate quantities and concentrations of myrosinase activities suggests that young and UV-shielded leaves are more efficiently protected against antagonists than old and UV-exposed leaves, or greenhouse-kept plants.

However, feeding behaviour and larval performance of the oligophagous Brassicaceae specialist, *Phaedon cochleariae* (Chrysomelidae; Coleoptera) were poorly affected by these differently UV-exposed host plants. The plant stress – insect performance hypothesis predicts an improved performance of herbivores on stressed plants. In our study, effects of plant stress on larval development were restricted to a minor variation in body mass due to variable food conversion of certain larval instars, which were compensated until pupation. Moreover, larval developmental times were unaffected by UV-exposure, but varied between species and leaves of different age. For *P. cochleariae*, this lack of variation in larval and pupal development towards UV-altered phytochemistry may suggest a strong genetic fixation of life history traits. In combination, the high plasticity towards variable food quality may correspond to the beetles's specialisation on a narrow range of chemically highly variable host plants. Apart from being involved in plant defence against generalist herbivores, glucosinolates may also act as recognition cues and feeding stimulants for specialist insects. The localisation of such chemical cues provides an important aspect in herbivore-plant

interactions. In earlier studies, glucosinolates were assumed to stimulate feeding by *P. cochleariae*, and they were suggested to be present on outermost leaf surfaces. However, since these findings were based on crude extraction methods, the presence of feeding stimulants in epicuticular waxes of Brassicaceae was re-investigated. In our study, glucosinolates were not detectable in mechanically removed waxes in *Brassica napus* and *N. officinale*, whereas substrate concentrations in solvent leaf extracts corresponded to densities and closure of leaf surface stomata. Therefore, glucosinolates that originate from the mesophyll may have been washed out through open stomata. Neither leaf waxes, nor leaf waxes combined with sinigrin (2-propenylglucosinolate) or pure sinigrin evoked feeding. Moreover, in choice tests, these leaf beetles clearly preferred to feed on de-waxed surfaces. Finally, the presence of feeding stimulants in epicuticular waxes is highly unlikely considering the physico-chemical properties of the plant cuticle. The lack of stimulants on the outermost surface corresponds to the plant's perspective, which should avoid easily accessible feeding stimulants.

Nevertheless, the role of glucosinolates for feeding stimulation of *P. cochleariae* remained unclear. Therefore, *S. alba* leaf extracts of different polarities were tested in bioassays in order to identify which chemical leaf compounds act as stimulants. Polar extracts evoked the highest feeding responses. In subsequent bioassay-guided fractionations of the methanol extracts by semi-preparative HPLC, two distinct fractions with stimulating activity were detected, whereas other fractions were not effective. Flavonoids were identified as main component in one stimulating fractions, the second fraction mainly contained glucosinolates, including sinalbin (4-hydroxybenzyl-glucosinolate). The combination of both fractions was significantly more stimulating than each individual fraction, indicating additive effects of at least one compound of each fraction. However, since the combined fractions were less effective compared to the original extracts, other compounds may additionally be involved in the complex composition of leaf compounds acting as feeding stimulants for *P. cochleariae*. Finally, fractionated extracts of UV altered plants were used to test whether the strength of feeding responses depend on different ratios of glucosinolates and flavonoids. However, since the feeding behavior of this leaf beetle was not affected, such quantitative variations were concluded to be less important. The initiation of feeding behaviour may solely depend on the presence of stimulating compounds.

Zusammenfassung

Abiotische Umwelteinflüsse induzieren chemische und morphologische Adaptionen in Pflanzen. Nachdem Pflanzen im Gewächshaus in Lichtverhältnissen ohne ultraviolette Strahlung (UV) aufgezogen wurden, zeigten diese bei Einwirkung von UV zeigen artspezifische Veränderungen, die sich außerdem zwischen Blättern unterschiedlichen Alters unterschieden. *Sinapis alba* und *Nasturtium officinale*, zwei Vertreter der Brassicaceae zeigten dabei eine ausgeprägte phänotypische Plastizität. Die art- und blattalterspezifische Variation im Nährstoffgehalt basiert vermutlich auf unterschiedlicher Photosyntheseleistung. Die Akkumulation von Flavonoiden in der Epidermis stellt die wirksamste pflanzliche Schutzreaktion gegen UV dar. Die höchste Flavonoidkonzentration lag in jungen UV-exponierten Blättern vor, verbunden mit einem Anstieg hochwirksamer Quercetine auf Kosten der weniger wirksamen Kämpferole. Weiterhin wurden artspezifische Veränderungen der Konzentration von Glukosinolaten und Myrosinase-Enzymen festgestellt. Das Zusammenwirken beider Komponenten resultiert in einer hochwirksamen Abwehr gegen generalistische Herbivore. Aufgrund der gemessenen Konzentrationen beider Komponenten kann für beide Arten für junge und UV geschützte Blätter ein effizienterer Schutz angenommen werden als für alte Blätter, UV exponierte oder im Gewächshaus verbliebene Pflanzen.

Die Larvalentwicklung von *Phaedon cochleariae* (Chrysomelidae; Coleoptera), einem oligophagen Spezialisten auf Brassicaceae, wurde kaum von der veränderten Qualität der unterschiedlich exponierten Pflanzen beeinflusst. Die *plant stress – insect performance* Hypothese sagt eine bessere Entwicklung von Herbivoren auf gestressten Pflanzen voraus. In dieser Studie hingegen beschränkten sich Auswirkungen von Pflanzenstress auf die Larvalentwicklung des Blattkäfers allein auf geringe Gewichtsveränderungen durch unterschiedliche Futterverwertung bei bestimmten Larvenstadien, die aber noch vor der Verpuppung kompensiert wurden. Die Dauer der Larvalentwicklung unterschied sich zwischen Pflanzenarten und bei unterschiedlichem Blattalter, nicht aber in Folge der UV-Behandlung des Pflanzenmaterials. Dass die Entwicklung von Larven und Puppen trotz der durch UV hervorgerufenen Unterschiede der Wirtpflanzenchemie nicht beeinflusst wurde, könnte in der strengen genetischen Festlegung der Wachstumsparameter des Käfers begründet sein. Die hohe phänotypische Plastizität des Blattkäfers in Bezug auf unterschiedliche Futterqualität, könnte mit seiner starken Spezialisierung auf wenige Wirtspflanzen zusammenhängen.

Neben ihrer Funktion im Abwehrsystem gegen generalistische Herbivore nehmen Glukosinolate außerdem eine wichtige Rolle bei der Wirtspflanzenerkennung und Fraßstimulanz von Spezialisten ein. Die Lokalisierung solcher Schlüsselkomponenten in der Pflanze stellt somit einen wichtigen Aspekt der Interaktion zwischen Herbivoren und Pflanzen dar. In früheren Studien wurde angenommen, dass Glukosinolate auf *P. cochleariae* fraßstimulierend wirken und auf Blattoberflächen vorhanden sind. Die in diesen Studien angewandte Methode zur Extraktion von Blattoberflächen scheint jedoch ungeeignet und wurde in unserer Studie modifiziert. In mechanisch abgelösten Blattwachsen von *Brassica napus* und *N. officinale* (Brassicaceae) konnten keine Glukosinolate nachgewiesen werden. In Lösungsmittelextrakten hingegen stieg die Glukosinolatkonzentration mit dem Öffnungszustand und der Anzahl der Stomata deutlich an. Offenbar werden demnach Glukosinolate aus dem Mesophyll durch geöffnete Stomata extrahiert. In Biotests konnte *P. cochleariae* weder durch Blattwachse, noch durch ein Gemisch aus Wachsen und Sinigrin (2-Propenyl-Glukosinolat) oder reines Sinigrin stimuliert werden. Darüber hinaus präferierten sie entwachste Blätter gegenüber bewachsten Blättern. Schließlich sprechen auch physikochemische Eigenschaften von Glukosinolaten und pflanzlicher Kutikula gegen das Vorkommen von Glukosinolaten in epikutikulären Wachsen.

Somit blieb die Rolle von Glukosinolaten für die Fraßstimulation von *P. cochleariae* unklar. Um fraßstimulierende Komponenten zu identifizieren, wurde die Wirkung von *S. alba* Blattextrakten unterschiedlicher Polarität auf das Verhalten der Käfer untersucht. Die stärkste Reaktion wurde durch Methanolextrakte verursacht. Durch Fraktionierungen mittels HPLC, begleitet durch Biotests, wurden zwei stimulierende Fraktionen aus diesen Extrakten isoliert, während die übrigen Fraktionen unwirksam waren. Eine der aktiven Fraktionen enthielt hauptsächlich Flavonoide, die zweite Glukosinolate, darunter Sinalbin (4-Hydroxybenzyl-Glukosinolat). Die Kombination dieser beiden Fraktionen wirkte signifikant stimulierender als die Einzelfraktionen, sodass auf eine additive Wirkung mindestens zweier Komponenten jeder Fraktion geschlossen werden kann. Da die Aktivität dieser kombinierten Fraktionen jedoch geringer war als die der Ausgangsextrakte, könnten weitere Komponenten in das komplexe Zusammenwirken von Blattinhaltsstoffen zur Fraßstimulantion von *P. cochleariae* involviert sein. Extrakte unterschiedlich UV exponierter Pflanzen wurden getestet, um festzustellen, ob die Stärke der Fraßreaktion durch das Verhältnis von Glukosinolaten und Flavonoiden bestimmt wird. Das Verhalten von *P. cochleariae*

wurde jedoch nicht beeinflusst, sodass die Quantität der involvierten Komponenten von geringer Bedeutung sein könnte. Zur Initiierung des Fraßverhaltens könnte allein die Anwesenheit von Stimulantien genügen.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und dabei keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet habe.

Ferner erkläre ich, dass ich diese Arbeit weder einer anderen Prüfungsbehörde vorgelegt, noch anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Ich erkläre, dass ich bisher keine akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, den 19.3.2007

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