Transgene products in honeydew:

Estimating risks for non-target insects

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Summary

Insect-resistant genetically modified (GM) crops could harm organisms other than the pests targeted by the toxin. These so-called non-target organisms include parasitoids and predators that are important for natural pest regulation, which could be exposed to the expressed insecticidal proteins through feeding on transgenic plant tissue or through feeding on target or non-target herbivores containing the transgene product. In this thesis, honeydew excreted by phloem-feeding insects is addressed as an additional route of exposure to insecticidal proteins. Since honeydew is commonly used by insects as a sugar source, a broad range of non-target organisms could potentially be exposed to transgene products. The importance of this exposure route was studied for GM wheat expressing snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) to control phloem-feeding insects, such as aphids. Since risk is defined by both hazard and exposure, these aspects were studied for GNA-containing honeydew.

Three predator species (*Adalia bipunctata*, *Coccinella septempunctata* and *Chrysoperla carnea*) were tested for their sensitivity towards GNA. Longevity of the species studied was directly affected by GNA dissolved in a sucrose solution. This was confirmed by the fact that digestive enzymes in the guts of the predator larvae were unable to break down GNA. Whilst *A. bipunctata* and *C. septempunctata* excreted most of the GNA, *C. carnea* accumulated the toxin in the gut since they are not able to excrete faeces. Moreover, GNA was found to bind to glycoproteins in the gut, being a prerequisite for its toxicity, and transferred into the insects' haemolymph.

As a first step in studying honeydew as an exposure route, the nutritional suitability of honeydew from three aphid species feeding on potato or wheat plants was investigated for the aphid parasitoid *Aphidius ervi*. The different honeydews were all relatively suitable food sources for adult parasitoids, but not always as suitable as a sucrose solution. The sugar composition of the honeydews differed significantly among the three aphid species on both potato and wheat. Multivariate analysis showed that small differences in parasitoid longevity could to some extent be explained by honeydew sugar composition, indicating that sugar composition is an important factor in determining the nutritional quality of honeydew.

When sensitivity towards GNA was tested for adult *A. ervi*, a direct dose-dependent effect on longevity was found. To study GNA-exposure through honeydew, the aphid *Rhopalosiphum padi* was fed on a GNA-containing artificial diet. Honeydew collected from these aphids contained 10-40% of the GNA concentration that was present in the aphid diet. When feeding on this honeydew, *A. ervi* suffered a

reduction in longevity that was more pronounced than was to be expected based on the detected GNA concentration in the honeydew. Analysis of carbohydrate and amino acid composition revealed that a change in honeydew composition caused by a GNA-effect on the aphids could be a possible explanation for the additional reduction in parasitoid longevity. Honeydew from aphids feeding on GNA-expressing wheat did not affect the longevity of *A. ervi* compared to honeydew from non-transformed wheat plants, which was probably due to low GNA expression levels in the plants.

In order to establish the importance of honeydew feeding for adult aphidophagous insects, aphid parasitoids, hoverflies and green lacewings were collected in wheat fields. Analyses of the insects' sugar profiles revealed that the majority of the insects had recently consumed sugars. Especially the two green lacewing species (*Chrysoperla lucasina* and *C. carnea*) were found to be at a high nutritional state. Recent honeydew feeding was detected in 35% of the hoverflies (*Episyrphus balteatus*) and 63% of the parasitoids (*Aphidius* spp.) that had been classified as having consumed sugars. These results demonstrate that aphid honeydew is an important food source for aphid parasitoids, and to a lesser extent for hoverflies, in wheat fields.

To assess if honeydew consumption could be important for omnivorous predators, it was studied whether *C. carnea* larvae use honeydew as a food source in the presence of aphids, their preferred prey. Both in a choice and a no-choice situation, it was found that honeydew was utilized by the larvae, but to a lesser extent than aphids. Furthermore, previous feeding on aphids or honeydew reduced the subsequent consumption of honeydew.

Having shown that GNA in honeydew poses a hazard for a range of non-target insects and that predators and parasitoids will be exposed to GNA in honeydew, this thesis demonstrates that transgene products in honeydew pose a risk for honeydew-feeding non-target insects. This route of exposure should thus be considered in future risk assessment of transgenic plants expressing phloem-transported insecticidal compounds.

Chapter 1

General introduction

The subject of this thesis combines research on ecological risks of transgenic plants with insect food ecology. After an introduction to these two research fields, the study system and research questions are described.

Background insect-resistant transgenic plants

Insect-resistant transgenic plants

Since the first commercial release of genetically modified (GM) or transgenic crops in 1996 the global area of approved biotech crops has increased steadily, covering an estimated area of more than 90 million ha in 2005 (James 2005). These 90 million hectares were grown in 21 countries, including five EU countries (James 2005). The GM crop varieties that are grown today are altered for agronomic (input) traits, such as herbicide-, virus-, or insect resistance. Thus far, herbicide tolerance has consistently been the dominant trait with 71% in 2005, followed by insect-resistance (18%) and stacked genes to have a combination of both (11%) (James 2005). The use of input traits will be further expanded in the near future (Nickson 2005) and might also include disease resistant plants. Furthermore, plants are being developed with resistance to abiotic stresses such as drought, salt and water, and tolerance to stress. Also output traits such as elevated or reduced levels of certain nutrients, as well as plants producing pharmaceutical products are receiving research interest. If and when any of these plants will be commercialized is currently unknown.

The only commercially available insect-resistant transgenic crops are the so called *Bt* crops. *Bacillus thuringiensis* Berliner (*Bt*) is a naturally occurring soil bacterium, which produces crystallized proteins (Cry) that are toxic to insects. A large number of *Bt*-subspecies are known, each producing a specific *Bt*-protein. These Cry toxins are classified depending on their insecticidal properties and molecular relationships (Höfte and Whiteley 1989, Crickmore et al. 1998). The toxins are known to have a narrow spectrum of insecticidal activity and are quickly degraded (Glare and O'Callaghan 2000). In general, Cry1, Cry2 and Cry9 toxins are active against Lepidoptera, Cry3, Cry7 and Cry8 toxins affect Coleoptera, and Cry4, Cry10 and Cry11 toxins affect Diptera. *Bt*-genes have been introduced in over 35 plant species

(Ely 1993), but only *Bt*-transgenic maize, cotton, rice and potato varieties have been commercially released so far (AGBIOS 2006).

However, to enhance insect resistance in crops, attention has also been given to other insecticidal proteins to broaden the application of insect-resistant crops as well as to include pests that are not sensitive to the currently used *Bt* toxins (Jouanin et al. 1998, Christou et al. 2006, Ferry et al. 2006). The insect gut is the obvious target for orally acting insecticidal compounds to be expressed in plants. Until recently, most research on insect resistance genes has focused on single insecticidal genes, following two approaches: (1) Disruption of the membranes lining the gut by binding of an insecticidal protein to specific receptors; for example *Bt* Cry-toxins and lectins. (2) Disruption of the insect's digestive system by inhibition of the insect's digestion; for example proteinase inhibitors (inhibitors of protein digestion) and α-amylase inhibitors (inhibitors of carbohydrate digestion) (Jouanin et al. 1998, Schuler et al. 1998). Currently, interest is devoted to the development of a novel generation of insect-resistant plants that would ensure durability and sustainability of pest resistance. An already widely applied technique is the pyramiding (stacking) of genes encoding different (*Bt*) toxins to obtain a higher level of pest control and to render the development of resistance within the insect pest even more difficult (Maqbool et al. 2001, Zhao et al. 2003). Furthermore, hydrid or fusion proteins form alternative strategies that can enhance and extend the activity of *Bt* toxins (Mehlo et al. 2005, Ferry et al. 2006). Besides research on the afore mentioned plant-derived lectins and digestion inhibitors, the use of toxins from insect pathogens other than *B. thuringiensis*, and from plants or animals is being explored (Ferry et al. 2004a, 2006, Christou et al. 2006).

Ecological risks and risk assessment of insect-resistant transgenic plants

GM plants are developed by engineering DNA from any source into the plants genome. This revolutionary technology allows plant breeders to develop new cultivars much more quickly than with conventional plant breeding methods and has opened possibilities to develop cultivars expressing novel traits. However, the use of this technology has also raised concerns about the impact of GM crops on the environment. The primary issues in the ecological assessment of GM crops are (Conner et al. 2003, Snow et al. 2005): (1) Possible invasiveness in ecosystems: transgenic plants could become agricultural weeds or could invade natural habitats and form self-sustaining populations there. (2) Out-crossing (or vertical gene transfer): The process of hybridization of transgenic plants with cultivated or wild relatives, and the possibility of introgression of transgenes in these populations. (3) Horizontal gene transfer: the gene transfer from plants to other organisms through asexual mechanisms. (4) Development of resistance: the continuous expression of a toxin in pest- and disease-resistant crops can allow pests or pathogens to develop resistance against this toxin, which would make their control even more difficult. (5) Effects on non-target arthropods, soil organisms, birds and other animals. (6) Effects on biodiversity: disruption of biotic communities or changes in species diversity or genetic diversity.

Prior to commercial release, a detailed risk assessment of potential adverse effects on the environment has to be conducted for any novel transgenic plant (Conner et al. 2003, Nap et al. 2003). Compared to the use of pesticides, the adoption of insect-resistant plants, can bear additional risks and benefits to the environment (NAS 2002). One of the most widely discussed environmental effects of insect-resistant crops is their potential effect on non-target organisms including biological control agents (Dale et al. 2002). This group of organisms is of major concern as they often play an important role in natural pest regulation, and are considered to be of economic value. They may at the same time be a good indicator of potential ecological impacts of transgenic plants since they belong to the third trophic level in the food chain (Groot and Dicke 2002).

In the case of risk assessment of insect-resistant transgenic plants on non-target entomophagous arthropods (predators and parasitoids), a step-wise (tiered) approach has been proposed, where the assessment increases in complexity and realism based on the knowledge gained during previous tests (Cowgill and Atkinson 2003, Dutton et al. 2003, EuropaBio 2004, Poppy and Sutherland 2004). This tiered approach for the assessment of insect-resistant transgenic plants has been adapted from assessment of risks of chemicals (Hill and Sendashonga 2003), in particular the tiered system presently used for pesticide testing (Rose 2006).

Effects of transgenic plants on predators and parasitoids may be either direct, caused by the action of the insecticidal protein on the non-target organism itself, or indirect (Schuler et al. 1999). Indirect effects on entomophagous arthropods can be caused by a reduction of quantity and/or quality of the susceptible host or prey. Furthermore, the insertion of a gene into the plant genome may inactivate or alter the expression of endogenous genes, leading to unintended effects which are not related to the altered trait (Cellini et al. 2004). However, such effects are generally detected and eliminated during the variety development process and agronomic evaluation before commercialization (Bradford et al. 2005). As a consequence, environmental risk assessment can focus on potential effects caused by the introduced trait (stressor) (Romeis et al. 2006).

As a first step in the risk assessment procedure, an evaluation of which species need to be tested should be conducted. These arthropod species should be ecologically and economically important in a given crop system and should be likely to be exposed to the insecticidal protein (Dutton et al. 2003). Furthermore, attention should be given to systematic diversity among the tested species, their amenability and availability, as well as knowledge on the toxic specificity of the insecticidal protein should be taken into account (Dutton et al. 2003). For practical reasons, however, surrogates for ecologically and economically important non-target arthropods often need to be selected. The benefit of the application of this surrogate species concept is that it enhances the transferability of risk assessment data.

In the first tier, laboratory tests should be performed to test the potential toxicity on the selected organisms. In these 'worst-case' bio-assays the arthropods should be exposed to high dose of pure insecticidal protein and/or to transgenic plant material containing maximal amounts of toxin. These studies could be complemented with additional laboratory behavioural or physiological studies. If effects are observed in the 'worst-case' laboratory studies, second tier tests need to be conducted. These should be conducted under semi-field conditions with more realistic exposure levels and should allow observations at the population level. The third tier consists of field studies. Such studies generally require expensive and time-consuming procedures and results are often difficult to interpret. They should therefore only be performed if risk assessment in lower tier tests does not give a definite answer.

Exposure of non-target arthropods to transgene products

The risk that a GM crop poses for a non-target organism depends on both the sensitivity of the organism to the transgene product as well as on the likelihood and the level at which the organism is exposed to the compound (Hickson et al. 2000, Dutton et al. 2003, Poppy and Sutherland 2004).

Studies on the potential impact of transgene products on non-target entomophagous insects have mainly focused on the three exposure routes presented in Figure 1.1 (Schuler et al. 1999, Romeis et al. 2006): (1) direct exposure as a result of herbivory, for example when a predator feeds on pollen from a transgenic plant, or (2) indirect exposure through predation or parasitism of the target herbivores containing the transgene product, or (3) indirect exposure through predation or parasitism on a non-target herbivore containing the transgene product. Field studies have shown that exposure to *Bt* toxins can vary among different predators depending on the varying amounts of toxin present in the consumed prey or plant tissue as well as on the feeding biology of the predators (Harwood et al. 2005, Obrist et al. 2006).

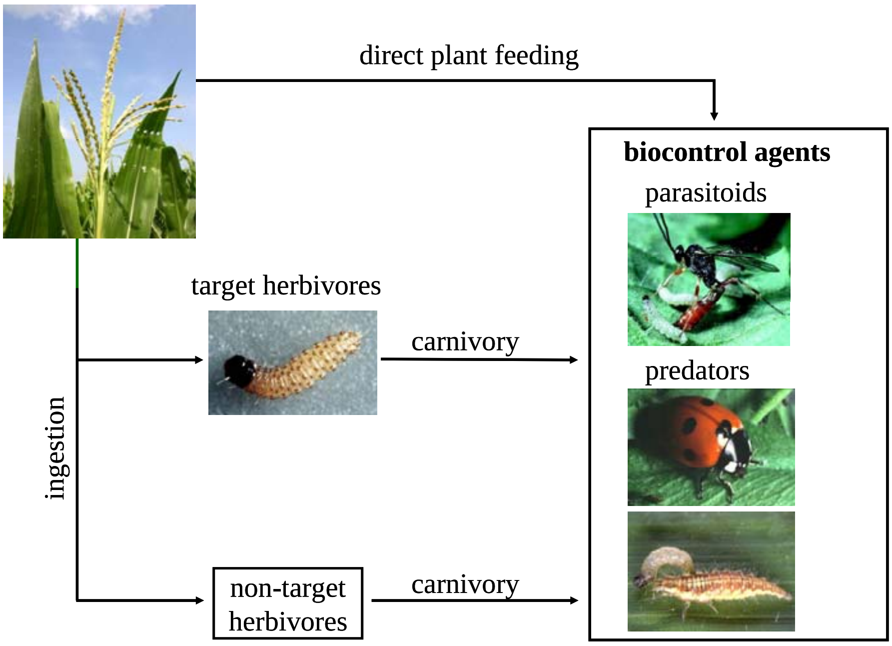

Figure 1.1 Commonly studied routes of exposure to transgene products for non-target entomophagous arthropods

Lectin-expressing transgenic plants

Lectins are a very heterologous class of carbohydrate binding (glyco)proteins, which are capable of reversible binding to at least one specific class of mono- or oligosaccharides. There are four major subgroups: the legume lectins, the monocot mannose-binding lectins, the chitin-binding lectins, and the type 2 ribosome-inactivating proteins (Van Damme et al. 1998). There are some more lectins, not belonging to either one of these groups.

Lectins are thought to play a defensive role in plants in response to attack by herbivores or pathogens (Peumans and Vandamme 1995) and naturally occur in hundreds of plant species. Artificial diet studies have shown that a number of lectins affect phloem-sucking Sternorrhynchae, including aphids (Aphididae), planthoppers (Delphacidae) and leafhoppers (Cicadellidae) (Habibi et al. 1993, Powell et al. 1993, Cole 1994, Rahbé et al. 1995, Sauvion et al. 1996, Powell 2001). Sternorrhynchan pests are not affected by the currently known *Bt* Cry toxins. Genes encoding specific lectins are therefore of particular interest as candidates for expression in transgenic crops. Lectin genes that have been engineered into food crops for protection against insects include the snowdrop lectin (GNA), garlic leaf lectin (ASAL), pea lectin (p-lec), wheat germ agglutinin (WGA), jack fruit jacalin, and rice lectin (Schuler et al. 1998, Legaspi et al. 2004, Saha et al. 2006).

Probably the most widely studied lectin is snowdrop lectin (*Galanthus nivalis* agglutinin; GNA). A gene encoding GNA has successfully been engineered into a number of crop plants (Gatehouse 1999, Legaspi et al. 2004). These plants have been found to be partially resistant to phloem-sucking pests indicating that GNA is transported in the phloem sap. Examples include tobacco (Hilder et al. 1995, Yuan et al. 2001), potato (Down et al. 1996, Gatehouse et al. 1996), wheat (Stoger et al. 1999), and rice (Rao et al. 1998, Foissac et al. 2000, Sun et al. 2002, Wu et al. 2002). This partial resistance to aphids by GNA-transgenic plants has primarily resulted in a reduced growth, development, and fecundity rather than an increased mortality. In contrast, GNA-expressing rice has been found to cause a significant increase in nymphal mortality of planthoppers and leafhoppers (Rao et al. 1998, Foissac et al. 2000, Sun et al. 2002). In order to better target transgene expression to the phloem sap and thus enhance resistance towards sternorrhynchan pests, the use of phloem-specific promoters is being studied (Shi et al. 1994, Rao et al. 1998, Foissac et al. 2000). In addition to effects on sap-sucking pests, insecticidal effects of GNA were also found to extend to lepidopteran pests (Fitches et al. 1997, Gatehouse et al. 1997, Fitches and Gatehouse 1998, Sétamou et al. 2002b, 2003), a coleopteran herbivore (Nutt et al. 1999), and root-knot nematodes (Ripoll et al. 2003). This reveals that in contrast to *Bt* Cry toxins, GNA has a broad range of activity.

Apart from the evidence that GNA has a rather unspecific mode of action, the exact functional mechanism is complex and not yet fully elucidated. Binding to glycoproteins in the midgut of the insect is considered a prerequisite for toxicity (Czapla 1997), but binding is not an absolute predictor of subsequent toxicity (Harper et al. 1995). GNA binds specifically to D-mannose (Van Damme et al. 1995). Furthermore, it is resistant to gut proteolysis in lepidopteran larvae (Gatehouse et al. 1995). In target insects binding to midgut epithelial cells causes morphological changes that are thought to affect nutrient absorption (Powell et al. 1998). For the leafhopper *Nilaparvata lugens* (Stal.) and the lepidopteran larva *Lacanobia oleracea* L., GNA has been reported to be transported across the midgut epithelial barrier into the haemolymph of orally exposed insects (Powell et al. 1998, Fitches et al. 2001). Du et al. (2000) demonstrated that one of the major receptors for GNA in *N. lugens* was a subunit of ferritin, suggesting that this particular lectin may interfere with the insects' iron homeostasis.

Effects of GNA-expressing plants on non-target arthropods

As studies on the target-effects of GNA have only shown partial resistance against the different pests, the additional impact of biological control organisms is required to further regulate the pest (Van Emden 1999). It is therefore crucial to investigate possible effects of GNA on entomophagous insects.

Studies investigating the development of larvae of hymenopteran aphid parasitoids, where GNA was delivered to the target aphids via transgenic plants or artificial diet, reported sublethal effects of GNA on *Aphidius ervi* Haliday (Couty et al. 2001b), and *Aphelinus abdominalis* (Dalman) (Couty et al. 2001a, c). It could not be distinguished, however, whether these were indirect host-quality mediated effects, or direct effects. GNA was found to bind to the gut tissue of *A. abdominalis* (Couty et al. 2001a). When adult *A. abdominalis* were allowed to host-feed and oviposition on GNA-containing aphid hosts, no negative effects of GNA were detected, neither was GNA detected in host-fed females (Couty and Poppy 2001). In studies on parasitoids of lepidopteran larvae, Bell et al. (1999, 2001) found no adverse effects on *Eulophus pennicornis* (Nees) parasitizing larvae that had fed on GNA-expressing tomato or potato plants, whereas Sétamou et al. (2002a) detected small to marginal negative effects of larvae fed with an artificial diet containing GNA-sugarcane tissue on *Cotesia flavipes* (Cameron). However, when direct effects of GNA on the adult hymenopteran parasitoids *Aphidius colemani* Viereck, *Cotesia glomerata* (L.), *Trichogramma brassicae* Bezdenko (Romeis et al. 2003), and *E. pennicornis* (Bell et al. 2004) were investigated, by feeding them a sugar solution containing GNA, a significant reduction in longevity was found for all parasitoid species. Effects on fecundity were found for *T. brassicae* and *E. pennicornis*, but not for *A. colemani*. These results suggest that the lack of evidence for any direct toxic effects of GNA in the tritrophic studies may either have been due to exposure to low protein concentrations or due to the fact that direct toxic effects were masked or outweighed by host/prey-quality mediated effects.

For predators, studies have been limited to the ladybird *Adalia bipunctata* (L.). Birch et al. (1999) showed reduced fecundity, egg viability and a reduction in female longevity of *A. bipunctata* preying on the aphid *Myzus persicae* (Sulzer), which was reared on GNA-expressing potatoes. Conversely, Down et al. (2000, 2003) found no acute toxicity of *A. bipunctata* larvae feeding on *M. persicae* containing GNA (delivered through artificial diet), although some sub-lethal effects of GNA-fed aphids as well as binding of GNA to gut tissue were found (Down et al. 2000). For both studies, however, it is not clear whether these sublethal effects are direct (caused by GNA toxicity) or indirect (caused by GNA-effects on the aphids).

Background honeydew feeding

Honeydew as food source for insects

Arthropod predators and parasitoids often require carbohydrate rich foods, such as (extra-) floral nectar and honeydew, at least during part of their life cycle. There is strong theoretical as well as empirical evidence that the availability of sugar sources can be a key factor determining the population dynamics of entomophagous insects (Křivan and Sirot 1997, Wäckers 2005). However, the level at which predators and parasitoids depend on these carbohydrate sources varies. A distinction can be made between 'facultative' consumers, for which plant-derived foods are complementary to their prey, and 'obligatory' consumers of plant-provided foods during certain stages of their life cycle (Wäckers and Van Rijn 2005). This second group is entirely dependent on non-prey food for their survival and metabolic upkeep, usually during the adult stage, and includes for example many parasitoids (Jervis et al. 1996), aphidophagous hoverflies (Gilbert 1981, 1985), and some lacewings (Canard 2001).

It is therefore not surprising that carbohydrate feeding has been found to increase reproductive fitness of parasitoids by increasing their longevity and fecundity (Jervis et al. 1996, Fadamiro and Heimpel 2001, Siekmann et al. 2001, Wäckers 2001) as well as their parasitism rate (Winkler et al. 2006). It has further been suggested that carbohydrates are key to fuelling parasitoid flight, thereby increasing search area and host encounter rate (Wäckers 1994, Siekmann 2002, Tenhumberg et al. 2006). Also the survival of adult hoverflies (Diptera: Syrphidae) and green lacewings (Neuroptera: Chrysopidae) is enhanced when access to sugar sources is available (Principi and Canard 1984, Van Rijn et al. 2006). Even host-feeding parasitoids commonly depend on sugar feeding to cover their energetic needs (Jervis and Kidd 1986). Moreover, larvae of the common green lacewing, *Chrysoperla carnea* (Stephens), that are predators of soft-bodied arthropods (Principi and Canard 1984), can benefit from feeding on sugar-rich non-prey foods when prey is scarce or of low quality (Sunby 1966, McEwen et al. 1993, Limburg and Rosenheim 2001, Patt et al. 2003). This has also been found for adult ladybirds (Coleoptera: Coccinellidae) (Mensah and Madden 1994, Evans 2000).

Honeydew is a generic term for sugar-rich excretions produced by sap-feeding Sternorrhynchae (Wäckers 2005). It is regarded as an important food source for insects including pollinators, predators and parasitoids (Zoebelein 1956, Wäckers 2005). In early spring and late autumn, when flowering plants are scarce, honeydew is often the primary source of sugar (Leius 1960). In agricultural ecosystems, honeydew can be particularly important as a carbohydrate source due to the fact that these systems often lack floral nectar or other sugar sources (Wäckers 2005). Moreover, the foraging risk for honeydew-consuming insects is relatively low in such systems (Wäckers 2005).

Although honeydew is regarded as an important food source for insects, there is very little quantitative information on the use of honeydew under field conditions. A study of parasitoids collected in a cabbage field showed that 80% of *Cotesia glomerata* (L.) and 55% of *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae) contained sugars that indicated honeydew feeding (Wäckers and Steppuhn 2003). A recapturing study of released *Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae) demonstrated that the parasitoids were able to maintain a nearly constant level of energy over 48 h in a flower-poor habitat, where honeydew may have been the primary sugar source available (Casas et al. 2003). Laboratory studies further showed that one feeding bout on a suitable sugar solution can already prolong parasitoid longevity significantly (Azzouz et al. 2004, Hausmann et al. 2005), but parasitoids still need to locate food regularly (at least every 1-2 days) to avoid starvation (Fadamiro and Heimpel 2001, Siekmann et al. 2001, Azzouz et al. 2004).

Nutritional quality of honeydew

Since honeydew is a waste product of phloem-feeding insects, rather than a sugar source to attract pollinators or natural enemies of pests (as is the case with nectar), honeydew feeding is not always beneficial to entomophagous insects. A number of studies have shown that honeydew can vary considerably with respect to its nutritional quality. There are several examples of studies where honeydew feeding provided no benefit to parasitoids or even reduced their longevity (Leius 1961a, b, Avidov et al. 1970, Elliott et al. 1987). In other studies honeydew did increase parasitoid survival, but not as much as nectar, honey or sugar solutions (Idoine and Ferro 1988, Hagley and Barber 1992, Lee et al. 2004, Fadamiro and Chen 2005, Faria 2006), whereas other studies found honeydew to be equally suitable as nectar, honey or sugar solutions to parasitoids (Hocking 1967, England and Evans 1997, Singh et al. 2000, Burger et al. 2004).

It has not yet been completely elucidated which honeydew components are responsible for these varying effects, but several factors have been opted to play a role. As almost all honeydew producers feed directly on the phloem sap of the host plant, secondary plant metabolites that are transported in the phloem sap will be ingested as well. For many of these secondary plant compounds, such as alkaloids (Wink and Römer 1986) and cardenolides (Malcolm 1990), unaltered passage through the sap feeder's digestive tract has been reported. However, little is known about the effects of these compounds on honeydew-feeding insects (Mendel et al. 1992).

Low suitability of honeydew may also be based on an unfavourable sugar composition (Wäckers 2005). Honeydew contains various sugars, up to 90-95% of the dry weight (Maurizio 1985). Besides plant-derived phloem sugars such as sucrose, fructose, glucose or maltose, it often also contains more complex sugars synthesized by the sap feeder, like melezitose, erlose (fructomaltose), raffinose and trehalose (Wäckers 2000, 2005). Studies addressing the effects of sap feeder synthesized sugars all roughly indicate that the plant-derived sugars are relatively suitable sugars, whereas the aphid-synthesized oligosaccharides can lower the nutritional value of honeydew (Wäckers 2000, 2001, Hausmann et al. 2005, Winkler et al. 2005). However, the spectrum of honeydew sugars that can be utilized may vary between parasitoids (Wäckers 2001, Beach et al. 2003, Jacob and Evans 2004, Winkler et al. 2005) and other insects (Romeis and Wäckers 2002, Winkler et al. 2005).

A further factor influencing the nutritional quality of honeydew is the amino acid composition (Wäckers 2005). Even though honeydew usually contains some amino acids, these are often non-essential and can be strongly dominated by a few compounds (Wäckers 2005). Honeydew sugar and amino acid composition show great variation depending on the sap feeder itself and the plant species (Němec and Starý 1990, Hendrix et al. 1992, Völkl et al. 1999, Fischer et al. 2002), as well as on environmental factors.

This thesis

Research aim

Apart from the exposure routes to transgene products for non-target arthropods that were presented in Figure 1.1, alternative routes of exposure have received little or no attention. However, with the development of new insect-resistant crops expressing other insecticidal proteins, fusion proteins or stacked genes, other routes of exposure might become important. In transgenic crops targeting sap-sucking pests, the insecticidal protein needs to be transported in the phloem sap in order to reach the target insect. Due to the fact that several compounds are not degraded during the passage through the digestive system of sap-feeding insects (Douglas 2003), the composition of the phloem sap has a direct impact on honeydew composition. This applies in particular to proteins, as phloem-sucking insects typically possess low proteolytic activity in the gut (Srivastava and Auclair 1963, Rahbé et al. 1995), even though cysteine protease activity in the aphid gut has recently been demonstrated (Cristofoletti et al. 2003, Rahbé et al. 2003). These phloem-transported insecticidal proteins may therefore be excreted by phloem feeders via the honeydew. This way, honeydew-feeding insects would potentially be exposed to these transgenic compounds (Figure 1.2). Since honeydew is consumed by a broad range of insects, the potential impact of this course of exposure exceeds previously considered routes.

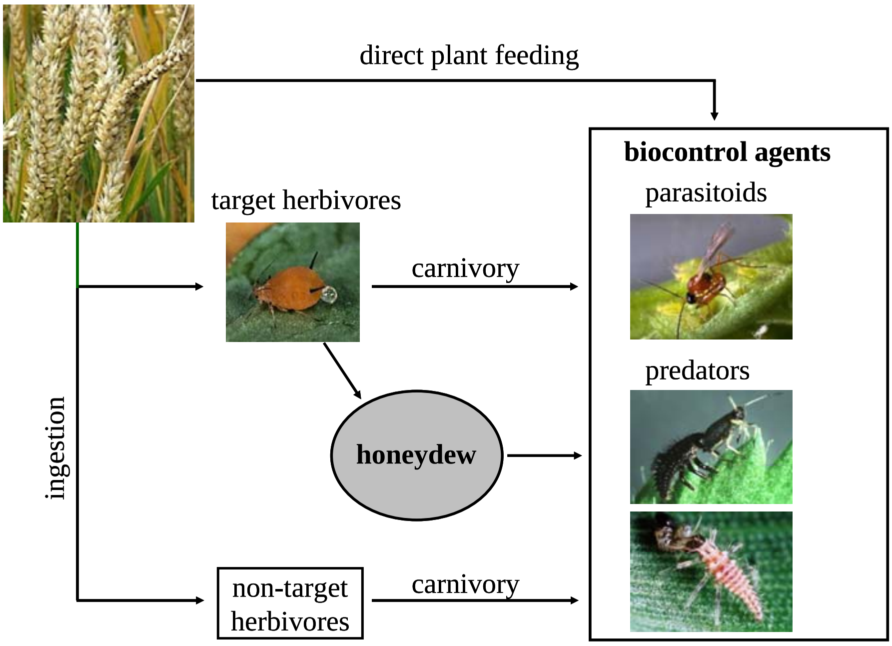

Figure 1.2 Commonly studied routes of exposure to transgene products for non-target entomophagous arthropods, including the exposure route through honeydew that is the topic of this thesis.

Unaltered passage through the digestive tract has been reported for plant lectins and protease inhibitors when provided via artificial diets (Rahbé et al. 1995, Powell et al. 1998, Cowgill et al. 1999, Down et al. 2000, Sauvion et al. 2004). Examples where insecticidal proteins have been detected in the honeydew of sternorrhynchan insects feeding on GM plants are given in Table 1.1. Similar observations have also been reported for secondary plant compounds (Wink and Römer 1986, Malcolm 1990). It appears that in current *Bt* crops Cry toxins are barely expressed in the phloem sap (Raps et al. 2001) as aphids feeding on *Bt*-transgenic maize (Head et al. 2001, Dutton et al. 2002, Obrist et al. 2006), oilseed rape (Schuler et al. 2005) and cotton (Zhang et al. 2006) were found to contain no or very low amounts of the toxins (at ng level), making excretion of Cry toxins in honeydew unlikely. The latter has been confirmed for sap feeders on *Bt* maize (Raps et al. 2001) and rice (Bernal et al. 2002).

Table 1.1 Examples where the transgene product was detected in sternorrhynchan honeydew

Toxin	Sternorrhynchae	Plant	Reference
Lectins			
Snowdrop lectin	*Myzus persicae*	tobacco	(Shi et al. 1994)
Wheat germ agglutinin	*Lipaphis erysimi*	Indian mustard	(Kanrar et al. 2002)
Protease-Inhibitor			
Oryzacystatin I	*Myzus persicae*	oilseed rape	(Rahbé et al. 2003)

The concentration at which the transgene products may appear in sternorrhynchan honeydew depends on two factors. In the first place, it depends on the level at which the compound is present in the phloem sap. This will vary with the plant species and the environmental conditions. Levels of GNA in a transgenic potato have been found to vary within a line of clonal replicates between different plant parts, with age of the plant, and can also be affected by environmental factors (Down et al. 2001). In the second place it depends on the sternorrhynchan species, as the amount of protein that is retained in the insect will vary between species (Foissac et al. 2000). Levels of transgene products in honeydew are therefore likely to vary considerably. Nevertheless, data from the literature can give an estimation in what order of magnitude one would expect GNA contamination in honeydew to be. Artificial diet studies using snowdrop lectin (GNA) have revealed that GNA-concentrations of 0.1% (w/v) are required to achieve a detectable impact on the target aphids (Down et al. 1996, Sauvion et al. 1996, Couty et al. 2001c). Transgenic plants expressing GNA at these levels, only conferred partial resistance to aphids, implicating that honeydew-producing aphids would still be present on these plants. Powell et al. (1998) found that GNA concentration in the honeydew of the planthopper *Nilaparvata lugens* (Stål) can reach the GNA levels ingested by the sap feeder, however, results were very variable. Couty and Romeis (2004) estimated that aphids may excrete more than 90% of the ingested GNA in their honeydew. This shows that on GNA-expressing plants that suppress aphid population growth, a concentration of 0.1% GNA (or higher) in honeydew could be realistic.

The aim of this PhD thesis is to assess whether the exposure to transgene products in honeydew would pose a risk for non-target honeydew-consuming insects.

The study system

To study honeydew as a route of exposure, snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) was used as a model compound. The study system consisted of wheat (*Triticum aestivum* L.), variety Bobwhite, genetically engineered with a gene from snowdrop that encodes for GNA (Stoger et al. 1999), several important aphid pests and some of their most prevalent natural enemies. GNA-expressing wheat plants had earlier been reported to have negative effects on the grain aphid, *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae) (Stoger et al. 1999). Besides this aphid species, the bird-cherry aphid *Rhopalosiphum padi* (L.), also known to be a common pest in cereals, were mainly used for honeydew collection. Studies were performed with several major aphid antagonists, such as the aphid parasitoid *Aphidus ervi* Haliday (Hymenoptera, Braconidae), the common green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), the ladybirds *Adalia bipunctata* (L.) and *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), and the hoverfly *Episyrphus balteatus* (DeGeer) (Diptera: Syrphidae).

This study system was used as a model system. Data collected in this study can to some extent and with caution be extrapolated to other transgenic compounds and natural systems in which insecticidal compounds (such as secondary plant compounds) appear in sternorrhynchan honeydew.

Thesis outline

As a first step in non-target risk assessment of transgenic crops, the susceptibility of non-target arthropods to the insecticidal compound has to be determined (Romeis et al. 2006). In **Chapter 2** it was investigated whether larvae of *C. carnea*, *A. bipunctata* and *C. septempunctata* are sensitive towards GNA. Direct effects of GNA on food uptake and longevity of the predators were studied, as well as the stability of GNA to digestive proteolysis in the gut, binding to the gut proteins, and presence in the haemolymph of the predators.

Since GNA-exposure of non-target entomophagous insects through honeydew is investigated, the nutritional suitability of honeydew itself should also be taken into account. The nutritional quality of honeydew can be variable and may be negatively affected by an unfavourable sugar composition (Wäckers 2005). Sugar composition has been found to vary considerably between different aphid species and different host plants (Hendrix et al. 1992). The aim of **Chapter 3** was therefore to study how natural changes in sugar composition of honeydew influence the nutritional suitability for *A. ervi*. Parasitoid survival was tested when fed with honeydew collected from three different aphid species feeding on potato or wheat plants or artificial diet. This was related to the sugar composition of the different honeydews.

In **Chapter 4** direct effects of GNA on food uptake and longevity of *A. ervi* were assessed. It was furthermore addressed if the insecticidal effect of GNA was affected by the passage through the aphid gut or by the nutritional quality of the honeydew. This was done by studying the longevity of the adult parasitoid on honeydew from *R. padi* kept on artificial diet with or without GNA, or on honeydew from *S. avenae* and *R. padi* kept on GNA-expressing or non-transformed wheat plants. With the results of this chapter it can be determined whether honeydew is a realistic route through which honeydew-feeding parasitoids would be exposed to GNA.

Since a risk posed by a transgene product for a non-target organism not only depends on the toxicity of the compound, but also on the frequency and intensity of exposure to this compound, the question addressed in **Chapter 5** is whether honeydew feeding in wheat fields forms an important nutritional contribution to the diet of aphidophagous insects. Adult aphid parasitoids, lacewings and hoverflies were collected in wheat fields near Zurich (Switzerland) and analysed for their sugar profile to reveal their nutritional state and recent honeydew feeding.

The adult beneficial insects studied in chapter 5 are vegetarian during that stage in their life cycle and are therefore obligatory consumers of plant-derived foods and honeydew. Predacious stages, such as larvae of *C. carnea*, mainly consume small, soft-bodied arthropods such as aphids. However, honeydew consumption in addition to prey has also been observed (Downes 1974). When honeydew is available in the field, the sternorrhynchan producers are usually there too. **Chapter 6** focuses on the importance of honeydew for aphid predators. In a choice and a no-choice feeding behaviour experiment it was investigated if honeydew contributes to the diet of *C. carnea* larvae when aphid prey is available.

In **Chapter 7** the most important results of this thesis are summarized, the importance of honeydew as a route of exposure to GNA and the potential risks for non-target insects are discussed based on the obtained results and knowledge gaps are pointed out.

Chapter 2

Direct effects of GNA on larvae of three aphid predators and fate of GNA after ingestion[1]

Abstract

Plants genetically modified to express *Galanthus nivalis* agglutinin (GNA) have been found to confer partial resistance to homopteran pests. Laboratory experiments were conducted to investigate direct effects of GNA on larvae of three species of aphid predators that differ in their feeding and digestive physiology, i.e. *Chrysoperla carnea*, *Adalia bipunctata* and *Coccinella septempunctata*. Longevity of all three predator species was directly affected by GNA, when they were fed a sucrose solution containing 1% GNA. However, a difference in sensitivity towards GNA was observed when comparing the first and last larval stage of the three species. *In vitro* studies revealed that gut enzymes from none of the three species were able to break down GNA. *In vivo* feed-chase studies demonstrated accumulation of GNA in the larvae. After the larvae had been transferred to a diet devoid of GNA, the protein stayed present in the body of *C. carnea*, but decreased over time in both ladybirds. Binding studies showed that GNA binds to glycoproteins that can be found in the guts of larvae of all three predator species. Immunoassay by Western blotting of haemolymph samples only occasionally showed the presence of GNA. Fluorescence microscopy confirmed GNA accumulation in the midgut of *C. carnea* larvae. Implications of these findings for non-target risk assessment of GNA-transgenic crops are discussed.

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[1] Based on: Hogervorst P.A.M., Ferry N., Gatehouse A.M.R., Wäckers F.L. and Romeis J., 2006. Direct effects of snowdrop lectin (GNA) on larvae of three aphid predators and fate of GNA after ingestion. Journal of Insect Physiology 52: 614-624

Introduction

Insect-resistant transgenic crops expressing *cry* genes from the soil bacterium *Bacillus thuringiensis* (Bt) have been grown commercially since 1996 (Shelton et al. 2002). However, many other insecticidal proteins, including endogenous plant defence proteins have been studied for expression in transgenic plants. One of the most widely studied is snowdrop lectin (*Galanthus nivalis* agglutinin; GNA). The particular interest in this protein is based on the fact that it acts on sap-sucking insects; pest species that are not targeted by the known Bt-toxins. GNA delivered in artificial diet has been shown to result in reduced performance of homopteran pests belonging to the Aphididae (Rahbé et al. 1995, Sauvion et al. 1996), Cicadellidae and Delphacidae (Powell et al. 1993, 1995, 1998, Powell 2001). Partial resistance to homopteran pests has been achieved in several crop plants as a result of having been genetically modified to express GNA. These include tobacco (Hilder et al. 1995, Yuan et al. 2001), potato (Down et al. 1996, Gatehouse et al. 1996), rice (Rao et al. 1998, Foissac et al. 2000, Sun et al. 2002, Wu et al. 2002), and wheat (Stoger et al. 1999). In addition to effects on sap-sucking pests, insecticidal effects of GNA were also found to extend to lepidopteran pests (Fitches et al. 1997, Gatehouse et al. 1997, Fitches and Gatehouse 1998, Sétamou et al. 2002b, 2003) and a coleopteran herbivore (Nutt et al. 1999). These studies have revealed the broad activity of GNA, but the exact mechanism of action is complex and not yet fully elucidated. Binding to glycoproteins in the midgut of the insect is considered a prerequisite for toxicity of lectins (Czapla 1997), but binding is not an absolute predictor of subsequent toxicity (Harper et al. 1995). GNA binds specifically to D-mannose (Van Damme et al. 1995). Furthermore, it is resistant to gut proteolysis in lepidopteran larvae (Gatehouse et al. 1995). For the homopteran *Nilaparvata lugens* (Stål.) and lepidopteran *Lacanobia oleracea* L., GNA has been reported to be transported across the midgut epithelial barrier into the circulatory system (Powell et al. 1998, Fitches et al. 2001). Du et al. (2000) demonstrated that one of the major receptors for GNA in *N. lugens* was a subunit of ferritin, suggesting that this particular lectin may interfere with the insects' iron homeostasis.

As studies on GNA-transgenic plants have only revealed partial resistance against different pests, the additional impact of biological control organisms is required to further regulate pest populations (Van Emden 1999). Thus possible effects of GNA on entomophagous insects have been widely studied. Studies on larvae of hymenopteran aphid parasitoids, where GNA was delivered to the target pests via transgenic plants or artificial diet, reported sublethal effects on *Aphelinus abdominalis* (Dalman) (Couty et al. 2001a, c), and *Aphidius ervi* Haliday (Couty et al. 2001b). However, the studies did not allow to distinguish whether these effects were indirect host-quality mediated or direct. Bell et al. (1999, 2001) found no adverse effects on *Eulophus pennicornis* (Nees) parasitizing *L. oleracea* that had been feeding on GNA-expressing tomato/potato plants. However, investigating direct effects, Romeis et al. (2003) and Bell et al. (2004) fed adult parasitoids, including *Aphidius colomani* Viereck and *E. pennicornis,* a sugar solution containing GNA and found a significant reduction in longevity and effects on parasitoid fecundity. For predators, studies investigating possible effects of GNA have been limited to the ladybird *Adalia bipunctata* (L.). Birch et al. (1999) showed reduced fecundity, egg viability and a reduction in female longevity of *A. bipunctata* preying on the aphid *Myzus persicae* (Sulzer), which was reared on GNA-expressing potatoes. Conversely, Down et al. (2000, 2003) found no acute toxicity on *A. bipunctata* larvae, when feeding on *M. persicae* containing GNA (delivered through artificial diet), although some sub-lethal effects of GNA-fed aphids were found (Down et al. 2000). For both studies, however, it is not clear whether these sublethal effects were direct (caused by GNA toxicity) or indirect (caused by GNA-effects on the aphids).

The present study investigates direct effects of GNA on larvae of three different aphid predator species that differ in their feeding and digestive physiology to obtain more information for non-target risk assessment of GNA-expressing plants. We offered larvae of three predator species GNA dissolved in a sucrose solution and studied the following parameters: a) GNA acceptance, to determine the insects' gustatory response when provided with GNA in a sugar solution; b) longevity, to investigate the sensitivity of the larvae to GNA; stability of GNA to digestive proteolysis in the predators' gut through c) *in vitro* studies and d) *in vivo* studies; e) GNA binding to gut proteins; and f) movement of GNA across the gut membrane, by assessing presence of GNA in the haemolymph. Furthermore, we compared the three predatory species with respect to the relative level of GNA present in various body tissues using fluorescence microscopy. The green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), and the ladybirds *A. bipunctata* and *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) were chosen as study objects as they are very common aphid predators and important for biological control.

Materials and methods

Reagents

Lyophilized *Galanthus nivalis* agglutinin (GNA) was obtained from Prof. E. van Damme (Ghent University, Belgium). GNA primary antibodies were supplied by Prof. J.A. Gatehouse (University of Durham, UK). Bradford reagent and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories GmbH, München, Germany. The ECL (enhanced chemiluminescence) kit and ECL protein biotinylation reagents were obtained from Amersham International, Bucks, UK. Tissue-Tek® O.C.T.™ compound was purchased from Sakura Finetek Europe B.V. (Zoeterwoude, The Netherlands). Sucrose was purchased from Merck (Darmstadt, Germany). All other chemicals were from Sigma Chemical Company and were of analytical grade unless otherwise stated.

Insect material

Eggs of *C. carnea* were collected from our permanent laboratory colony (Romeis et al. 2004), *A. bipunctata* eggs and *C. septempunctata* eggs were received from Andermatt Biocontrol (Switzerland) and Katz Biotech AG (Germany), respectively. Eggs were kept separately in a climatic chamber (23 ± 1 °C, 85 ± 5% RH, 16:8 L:D) until they hatched. When last instar larvae (L3 for chrysopids and L4 for coccinellids) were used, the predators were reared on an *ad libitum* supply of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs, which were provided by INRA (France).

Effects of GNA on predators

GNA acceptance

Immediately after egg hatch or ecdysis into the last larval stadium, larvae were placed individually in plexiglass containers (2 x 2 x 1.5 cm) devoid of food. After 24-36 h individual weights of the food deprived larvae were measured on a microbalance (Mettler Toledo, MX5, d = 1 µg; ± 2 µg). The larvae were subsequently placed back into their container and provided with a pellet of cotton wool soaked with a 2 M sucrose solution, with or without GNA dissolved at 1% (w/v). This concentration is considered to represent a worst case scenario, since effects on the target pest (aphids) were already detectable at a ten times lower concentration (Down et al. 1996, Sauvion et al. 1996, Couty et al. 2001c). The containers were kept in a climate chamber at 23 ± 1 °C, 85 ± 5% RH, 16:8 L:D. After 1 h the larvae were reweighed. A no food treatment was used as a control to verify balance variation and weight decrease of unfed larvae during this period. The larvae that had not found the food solution, according to the no food treatment, were deleted from further analysis [0-10 larvae per treatment; weight increase ≤ 5 µg (3-10% of body weight) for L1 and ≤ 30 µg (< 1% of body weight) for the last instar larvae]. In no case was the number of deleted larvae significantly higher for the GNA treatment (χ^2 test, $P > 0.05$). This resulted in a total of 19-36 replications for the L1 and 38-43 for the last instar larvae. Data on the relative weight increase (%) of the larvae that had fed on the corresponding sucrose solution were statistically analysed using Student's *t*-test. When necessary, data were log or square root transformed to fulfil normality and equal variance assumptions. All statistical analyses were computed in Statistica (version 6, Statsoft Inc., Tulsa, USA).

Predator longevity

Freshly hatched first instar larvae and larvae that had just moulted into the last larval stadium were used for this experiment. The last instar larvae that were used, had been removed from the food and placed individually in plexiglass containers (2 x 2 x 1.5 cm) a day before ecdysis into the last instar. The larvae were individually placed in plexiglass Petri dishes (5 cm Ø, 1 cm high, containing a gauze window in the lid for ventilation) in a climate chamber at 23 ± 1 °C, 85 ± 5% RH, 16:8 L:D. Larvae were supplied with a pellet of cotton wool soaked with water, 2 M sucrose solution or 2 M sucrose solution with GNA dissolved at 1% (w/v). Sugar solutions were renewed every 2-3 days. Water was changed twice a day. Survival of the larvae was scored daily between 10 AM and noon. Since assumptions for parametric statistics could not be fulfilled, the longevity data were analysed using Kruskal-Wallis ANOVA. Post hoc comparisons of mean ranks were made using Mann-Whitney *U*-test with Bonferroni correction. Two pairwise comparisons (water *vs.* sucrose and sucrose *vs.* sucrose + GNA) lead to an adjusted $\alpha = 0.025$.

Stability of GNA in the predator's gut

In vitro studies

Studies were carried out with guts of predators in the last larval stadium that had either fed on *E. kuehniella* eggs or a 2 M sucrose solution during the 48 h before dissection. These two treatments were used because previous studies have shown that proteolytic gut enzymes are induced in some cases in response to ingestion of protein, as has been seen in ladybirds (N. Ferry, pers. com.), predatory bugs (Bell et al. 2005), and mosquitos (Okuda et al. 2005). Gut samples (20 guts for egg treatment, 60 guts for sucrose treatment) were homogenized in 50 µl PBS (Phosphate Buffered Saline), shaken at 4 °C for 5-10 min, centrifuged at 13,000 *g* for 5 min and the supernatant was collected. Total soluble protein was determined by Bradford assay according to the manufacturer's instructions. To investigate the stability of the lectin, 1 µg GNA (dissolved in PBS) was incubated with 100 µg total soluble protein of gut digestive enzymes with gentle agitation at room temperature. Samples were taken after 0, 1, 6 and 20 h and the reaction was terminated by flash freezing the samples in liquid nitrogen. Sample proteins (containing approximately 1 µg of GNA or its digestion products) were separated by SDS-PAGE (12.5%) and subsequently electrophoretically transferred to 0.2 µm nitrocellulose membranes (Schleicher & Schuell, BA83). The membranes were developed for immunoassay by Western blotting, using poly-clonal antibodies raised against GNA as the primary antibody, and HRP-conjugated goat antirabbit IgG as the secondary antibody as described by Gatehouse et al. (1996). Remaining GNA was detected by enhanced chemiluminescence (ECL) according to the manufacturer's instructions.

In vivo studies

Last instar larvae were fed a 2 M sucrose solution containing GNA dissolved at 1% (w/v) for 48 h, after which they were provided with a sucrose solution devoid of GNA. Samples were taken after 1 and 2 days of feeding on the GNA-sucrose solution, and subsequently after 1, 2, 3, 5 and 8 days of feeding on the sucrose solution by freezing and storing larvae at -20 °C. 14-26 pooled larvae were used for total insect protein extraction to reduce individual feeding variation. Larvae were surface washed in PBS to remove GNA that was potentially stuck to the cuticle, and subsequently homogenized in 10 µl PBS per insect. Protein extraction was performed as described for the gut samples above and the presence of GNA in insect tissues was analysed by SDS-PAGE and immunoassay using Western blotting as described above. For *C. carnea*, 10 µg total soluble protein was loaded and for the ladybirds 250 µg total soluble protein was used as these were found to be the optimal amounts for detection by Western blotting.

GNA binding to gut proteins

Total gut protein extraction from predator larvae was used, prepared as described in the *in vitro* studies. Freeze dried GNA equilibrated in 40 mM sodium bicarbonate buffer, pH 8.6 was biotinylated according to the manufacturer's instructions. Gut proteins (20 µg protein) from the predator larvae were electrophoretically separated on SDS-PAGE (10%), transferred to a nitrocellulose membrane (0.2 µm), and rinsed in PBS with 0.1% Tween 20 (PBST) prior to probing with the ligand (0.5 µg/ml of biotinylated GNA) for 1 h. Unbound toxin was removed with three washes of PBST and the membrane was then incubated with streptavidin-HRP (Amersham, UK) (1 in 1500 in PBST) for a further 1 h. After three additional washes the ligand blots were developed using ECL detection reagents (see manufacturer's protocol).

Presence of GNA in the haemolymph

Haemolymph samples from last instar predator larvae were collected using two different methods:

Method 1: *C. carnea* and *C. septempunctata* larvae were offered a sucrose solution with 0.1% GNA (w/v) for 48 h. Haemolymph was obtained by piercing the cuticle with a needle and collecting the droplet with a pipette. Haemolymph samples from 10 larvae were pooled and added to a 10 µl PBS solution in an eppendorf; 10 µl sample was analysed. This was replicated three times for *C. carnea* and twice for *C. septempunctata*. Control haemolymph was collected from larvae that were fed a sucrose solution.

Method 2: *C. carnea* and *A. bipunctata* larvae were offered a sucrose solution with 0.1% GNA (w/v) for 48 h. A leg was pulled out with small forceps and the haemolymph droplet that had formed on the wound was collected with a 5 µl micropipette (Blaubrand® intramark, Brand GmbH + Co kG, Wertheim, Germany). Samples were pooled (from 3-7 insects) until 1 µl of haemolymph was collected in the micropipette. This was replicated four times per species. Control haemolymph was collected from larvae that were fed a sucrose solution. In addition, 1 µl of the *C. carnea* larvae's defence fluid that is excreted through the anus was also collected by touching the anus of the larvae with a micropipette. The presence of GNA in the haemolymph and defence fluid was analysed by SDS-PAGE and immunoassay using Western blotting as described above.

Immunohistochemical studies

For labelling, GNA and casein were dissolved in 50 mM Borate buffer, pH 8.5. 45 µl of rhodamine B isothiocyanate in dimethylformamide (20 mg/ml) was added to 955 µl of GNA or casein solution. The reaction was incubated in the dark for 1 h. Unlabelled excess rhodamine was dialysed out against water. Labelling was confirmed by running an aliquot of the dialysed protein on SDS-PAGE and visually evaluating fluorescence.

One day old last instar larvae of each predator species were fed a 5 µl drop of rhodamine labelled GNA or casein at 0.1% (w/v) in a 2 M sucrose solution. Control insects were fed a 2 M sucrose solution. After 24 h the insects were transferred to a new container with a 5 µl drop of sucrose solution. After 2 days (ladybirds) or 5 days (lacewings) the insects were placed onto filter paper and embedded in Tissue-Tek O.C.T. compound. Intact insects were subsequently flash frozen in isopentane (2-methyl-butane). Samples were stored at -80 °C until sectioning. Frozen whole insects were cryo-sectioned at 10 µm with a Leica cryostat cm1900. Persistence in the midgut of the predators and transportation into the haemolymph was studied for rhodamine labelled GNA and casein using a Leica DMRB research microscope, equipped with differential interference contrast (DIC) optics, and a Leica Rhodamine Filter Block (N2.1: 515-560/590). Images of predator cross sections were recorded digitally using an Olympus 5050 digital camera (at 2776 x 2074 pixels).

Results

Effects of GNA on predators

GNA acceptance

For all of the predator species tested, there was no significant difference between relative weight increase of larvae on a 2 M sucrose solution and a sucrose solution containing 1% GNA, although *P*-values obtained for both instars of *C. septempunctata* were close to the significance level (Table 2.1).

Table 2.1 Acceptance of GNA by first and last instar larvae of three aphidophagous predators. Larvae were fed a 2 M sucrose solution with or without 1% GNA (w/v) for a period of 1 h. The relative weight increase in this hour was measured.

Predator species	Larval instar	Relative weight gain (%) ± SE		Student's *t*-test		
		sucrose	sucrose + GNA	df	*t*-value	*P*-value
C. carnea	L1	21.56 ± 1.35	19.72 ± 1.18	50	1.03	0.309
	L3	53.58 ± 3.58	56.82 ± 3.90	77	-0.61	0.541
A. bipunctata	L1	9.19 ± 0.55	8.45 ± 0.48	41	0.99	0.328
	L4	11.15 ± 1.03	9.89 ± 0.83	75	0.90	0.371
C. septempunctata	L1	7.42 ± 0.36	6.43 ± 0.43	67	1.78	0.079
	L4	8.28 ± 0.49	7.00 ± 0.58	84	1.92	0.058

Longevity

Longevity of larvae of the three predator species was significantly prolonged by a factor of 3 or more when they were fed a sucrose solution, compared to water (Figure 2.1). None of the larvae developed to the next stage, since the offered food solution does not provide the necessary proteins and essential amino acids for development.

Survival of first instar larvae of *A. bipunctata* and *C. septempunctata* was significantly decreased by 33% and 15%, respectively, after GNA ingestion, whereas for *C. carnea* no significant longevity reduction could be detected (Figure 2.1). However, the presence of GNA was shown to significantly reduce longevity of all three species in the last larval stage by 47%, 28% and 21% for *C. carnea*, *A. bipunctata* and *C. septempunctata*, respectively (Figure 2.1).

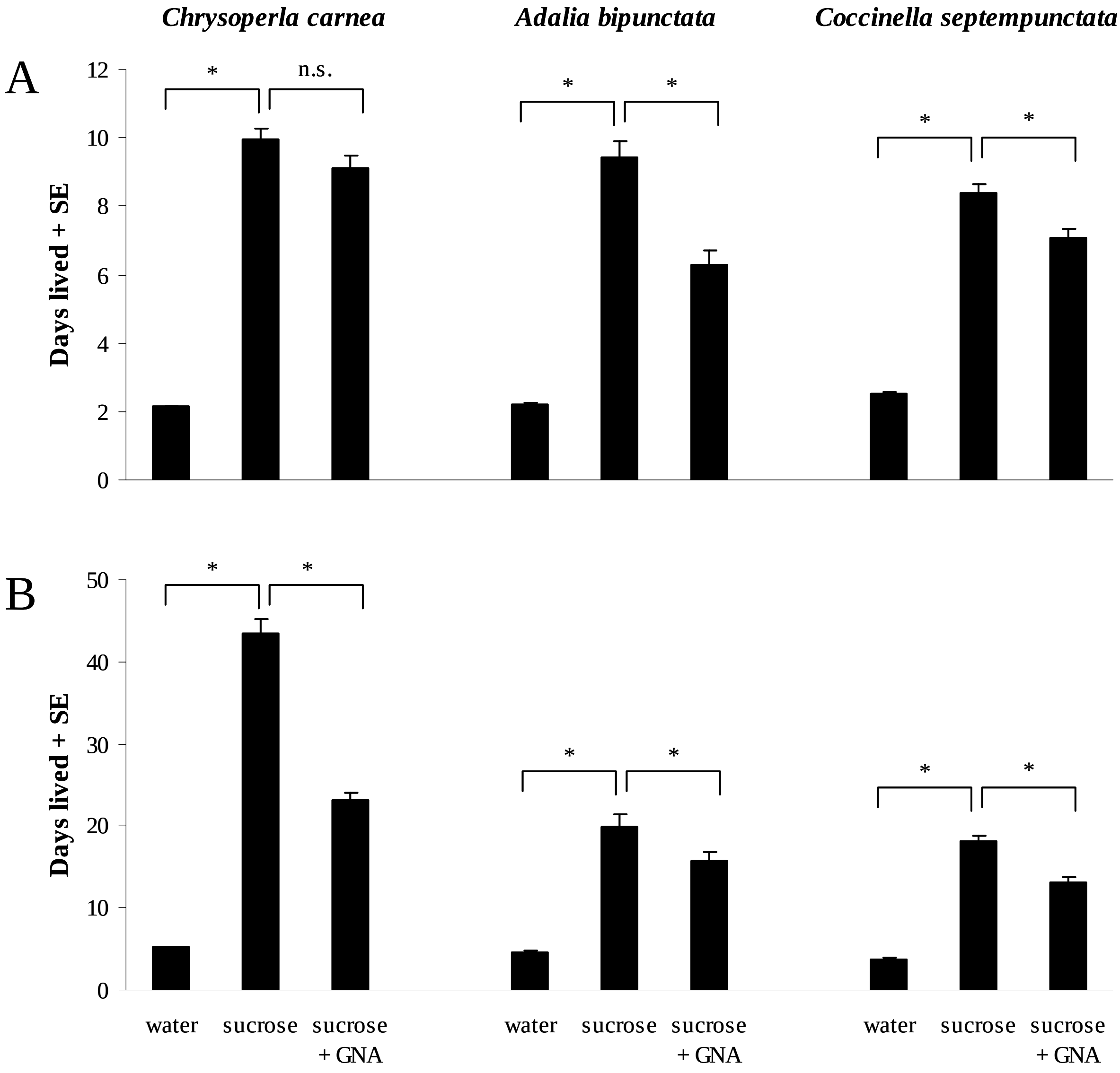

Figure 2.1 Mean longevity (+ Standard Error bars) of predators in the first (A) or last (B) larval stage when fed water, a 2 M sucrose solution, or a 2 M sucrose solution with 1% GNA (w/v) (n = 65-111 for first instar and n = 17-45 for last instar). Non-parametric statistics with two pairwise comparisons: water *vs.* sucrose; sucrose *vs.* sucrose + GNA (Mann-Whitney *U*-test with Bonferroni-adjusted $\alpha = 0.025$). *: $P < 0.025$, n.s.: not significant ($P = 0.036$).

Stability of GNA in the predator's gut

In vitro studies

To determine whether GNA can be broken down by proteolytic enzymes from the gut, GNA solution was incubated with protein extracts from the gut and analysed by immunoassay. The Western blots (Figure 2.2) showed no detectable sign of breakdown of GNA by digestive proteins from larvae of the three predator species studied, even after 22 h of incubation. There was no difference in breakdown of GNA between gut extract from larvae previously fed sucrose or eggs.

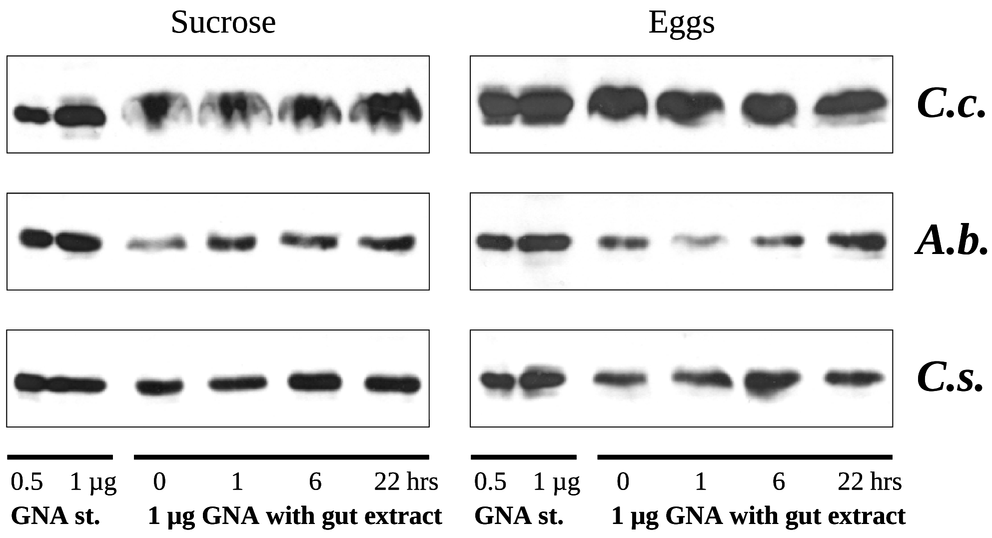

Figure 2.2 Western blots of breakdown time series of GNA with predator larvae gut extract. Freshly ecdysed last instar larvae of *Chrysoperla carnea* (*C.c.*), *Adalia bipunctata* (*A.b.*), and *Coccinella septempunctata* (*C.s.*) were either provided a 2 M sucrose solution (Sucrose) or eggs of *Ephestia kuehniella* (Eggs) for 48 h before dissection. 100µg of larval gut extract total soluble protein was incubated with 1 µg of GNA for 0, 1, 6, or 22 h. The first two lanes (GNA st.) contain GNA standards of 0.5 and 1 µg, respectively. The GNA bands appeared at a size of approximately 12.5 kDa.

In vivo studies

Protein extracts from GNA-fed larvae were analysed at different time intervals following ingestion of the lectin to investigate endogenous stability of GNA in the predators. As shown in Figure 2.3, GNA could be detected within the larvae after 24 h of feeding on a 1% GNA-sucrose solution, and was found to accumulate over the next 24 h. After the predator larvae had been transferred to a sucrose solution devoid of GNA, the protein could be detected in the body of *C. carnea*, even 8 days after GNA uptake. For both *A. bipunctata* and *C. septempunctata* the amount of GNA present in their body tissues decreased over time, with only trace amounts detected 8 days after uptake.

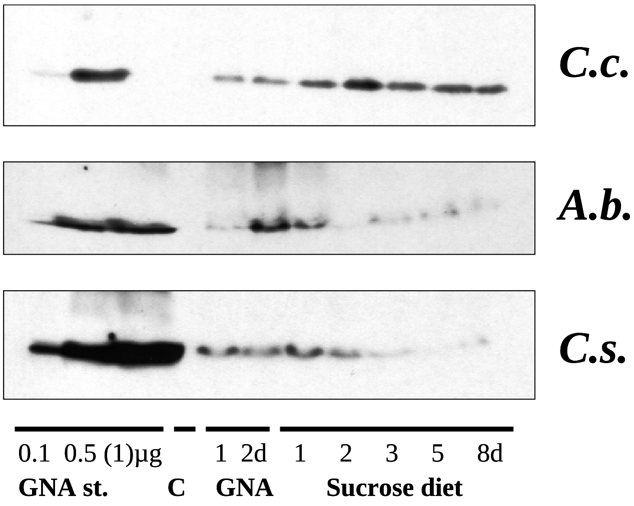

Figure 2.3 Western blots of feed-chase studies with *Chrysoperla carnea* (*C.c.*), *Adalia bipunctata* (*A.b.*), and *Coccinella septempunctata* (*C.s.*) larvae. Freshly ecdysed last instar larvae were provided a 2 M sucrose solution with 1% GNA (w/v) for 48 h, and subsequently offered a pure 2 M sucrose solution. Samples were taken after 1, 2, 3, 4, 5, 7, 10 days. Protein extract from several pooled larvae was resolved by SDS-PAGE, electroblotted onto nitrocellulose and probed with anti-GNA antibodies. The first three lanes (GNA st.) contain known amounts of GNA (first two lanes for *C. carnea*). The fourth lane (C) is a control treatment containing a protein extract from last instar larvae fed on eggs of *E. kuehniella*. The GNA bands appeared at a size of approximately 12.5 kDa.

GNA binding to gut proteins

The ligand blot of gut protein, probed with recombinant GNA, showed at least 6 different glycoproteins capable of binding GNA, within the *C. carnea* gut extract (Figure 2.4). For both ladybird species at least 9 GNA-binding glycoproteins were detected.

Presence of GNA in the haemolymph

Haemolymph was collected from GNA-fed larvae after an exposure of 48 h. Western blotting of haemolymph samples from *C. carnea* or *C. septempunctata*, collected by piercing the cuticle (method 1), showed low, but detectable levels of GNA in the haemolymph from *C. carnea* in one out of three samples. GNA could not be detected in either of the two haemolymph samples from *C. septempunctata*. Western blots of haemolymph samples from *C. carnea* or *A. bipunctata*, collected by removing a leg (method 2), gave similar results. For both species one out of four samples showed the presence of GNA. As an example a Western blot is included on which two haemolymph samples from GNA fed *C. carnea* larvae as well as two control haemolymph samples are shown (Figure 2.5). GNA could not be detected in the defence fluid from *C. carnea*.

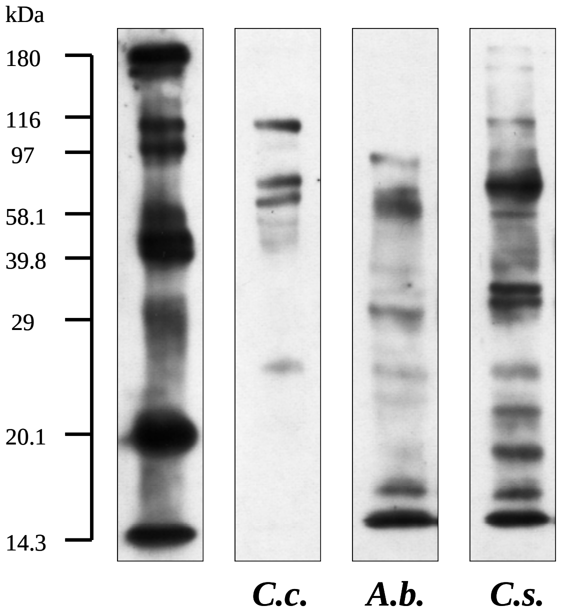

Figure 2.4 Ligand blot of predator larvae gut extract, incubated with biotinylated GNA. Bands represent glycoproteins within the gut extract to which GNA will bind. The first lane contains a biotinylated marker, the following lanes contain gut extract of sugar-fed *Chrysoperla carnea* (*C.c.*), *Adalia bipunctata* (*A.b.*), and *Coccinella septempunctata* (*C.s.*).

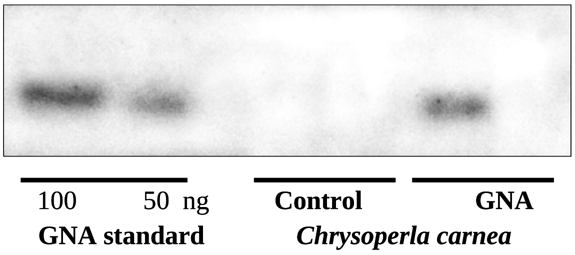

Figure 2.5 Western blot of four haemolymph samples from *Chrysoperla carnea* larvae. Freshly ecdysed L3 larvae were provided with a 2 M sucrose solution with 0.1% GNA (w/v) or a plain 2 M sucrose solution (Control) for 48 h. Haemolymph was collected by removing a leg and collecting the droplet of haemolymph with a micropipette (method 2). In each lane a haemolymph sample of 1 µl was loaded. Two GNA standards were added. The GNA bands appeared at a size of approximately 12.5 kDa.

Immunohistochemical studies

Fluorescence microscopy images (Figure 2.6) confirm GNA accumulation in the midgut of *C. carnea* larvae. After 2 days GNA was still detectable in the midguts of both ladybird species, but at lower concentrations than for the lacewing 5 days after GNA ingestion. Unfortunately the level of magnification of fluorescence microscopy was not sufficient to show binding to the midgut epithelial cells. No fluorescent GNA or casein could be detected in the body cavity of any of the insects.

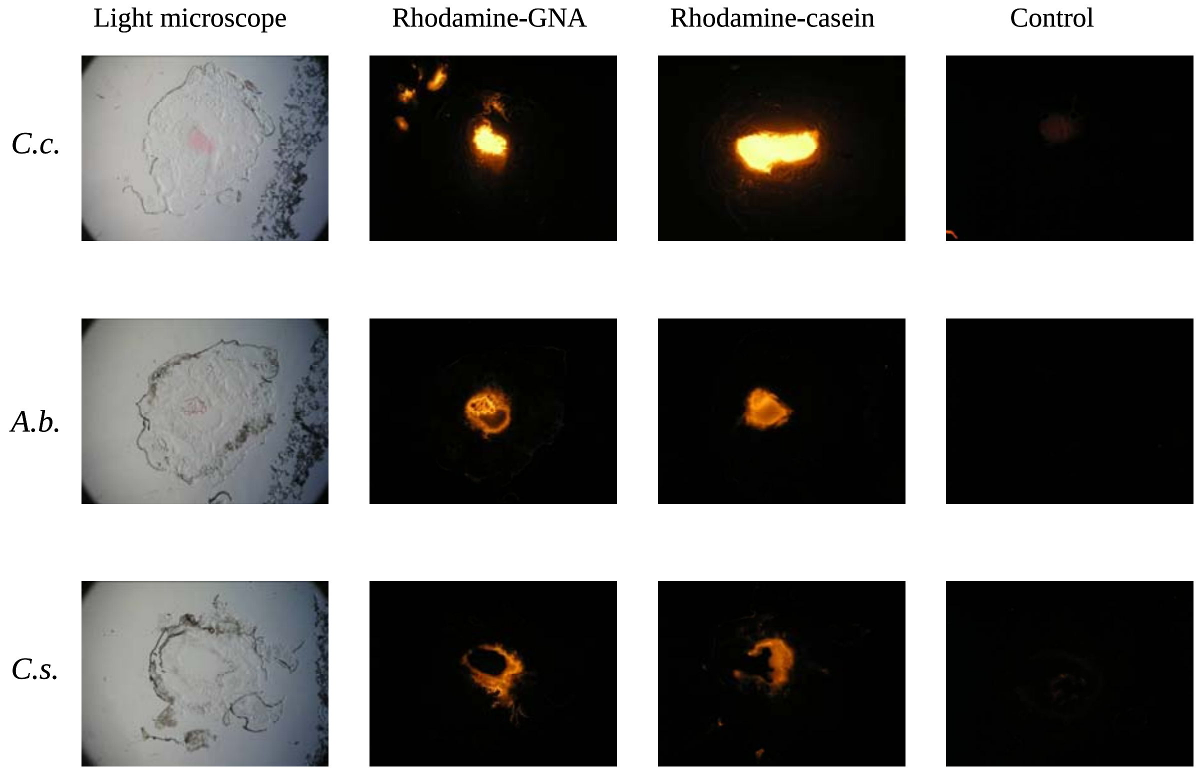

Figure 2.6 Light microscope images of cross-sections of the midgut region of *Chrysoperla carnea* (*C.c.*), *Adalia bipunctata* (*A.b.*), and *Coccinella septempunctata* (*C.s.*) larvae, at a magnification of 100. Larvae were fed on a sucrose solution (control), or a sucrose solution containing 0.1% w/v rhodamine-labelled casein or GNA for 24 h, and subsequently fed a sucrose solution for 2 (ladybirds) or 5 days (lacewings), before cryo-sectioning at 10 μm.

Discussion

Effects of GNA on predators

Since no significant difference between relative weight increase of the predator larvae on a sucrose solution with or without GNA was detected, the results demonstrate that none of the tested predator species was strongly deterred from feeding by GNA. It can therefore be excluded that effects of GNA on longevity are due to a lack of energy uptake, but are caused by the protein itself (direct effect). For both first and fourth instar larvae of *C. septempunctata* a lower uptake of sucrose solution was seen when GNA was incorporated. Despite the fact that the reduced weight increase was not significant, data suggest there might have been a gustatory response to GNA. An antifeedant effect of GNA has previously been observed for the rice brown planthopper (*N. lugens*) (Powell et al. 1995) and to a lesser extent for the egg parasitoid *Trichogramma brassicae* (Romeis et al. 2003). Powell et al. (1995) suggested that the observed antifeedant effects of this lectin in *N. lugens* may be caused by binding to glycoproteins in chemoreceptor sites involved in diet recognition. However, despite feeding deterrent effects, GNA was still ingested by both *N. lugens* and *T. brassicae* (Powell et al. 1998, Romeis et al. 2003). Another possible reason for the reduced weight increase could be an increase in viscosity when GNA is added to the sucrose solution. The larvae might have greater difficulty ingesting this more viscose (sticky) solution. However, a feeding deterrent effect of garlic leaf lectin (ASAL), offered in a less viscous 0.5 M sucrose solution, on L1 *C. septempunctata* has also been observed (Romeis et al., unpublished results). Thus uptake problems due to increased viscosity seem unlikely.

A sucrose solution alone does not provide the nutrients essential for the predator to develop to the next larval stage or pupa. The provision of GNA dissolved in a sucrose solution does therefore only allow assessment of the impact on a specific larval stage. The experiments on predator longevity showed that *A. bipunctata* and *C. septempunctata* in L1 and L4 were directly affected by ingestion of GNA at a 1% concentration. This is in contrast to Down et al. (2000), who exposed *A. bipunctata* larvae to GNA-containing aphids, and found no effects on survival and only marginal effects on development of the larvae, which were hypothesized to be indirect, i.e. caused by the fact that the aphids were affected by the GNA. The fact that they didn't find any strong direct effects is probably a matter of toxin concentration. The aphid diet contained 0.1% (w/v) GNA, so GNA concentrations in the aphids were likely even lower. The GNA concentrations used in the present study were at least ten fold higher and did show a direct effect of GNA on ladybird larvae.

GNA expressing potatoes adversely affected the longevity and reproductive biology of *A. bipunctata* females via aphids (Birch et al. 1999), although there was no direct evidence to show that this was due to expression of the GNA. It could not be distinguished whether these effects were direct or indirect. Down et al. (2003) exposed adult *A. bipunctata* to aphids fed a GNA diet (0.1% w/v), and did not find any negative effects on ladybird longevity and fecundity. Down et al. (2003) argued that the effects found in the study by Birch et al. (1999) were most likely to be indirect and due to suboptimal quality of the aphids. However, since the present study has shown sensitivity in larval stages, direct effect on adult *A. bipunctata* cannot be excluded.

In contrast to the two ladybird species, no significant effect of GNA on larval longevity could be detected for L1 of *C. carnea*. However, L3 *C. carnea* appeared to be more sensitive to GNA than the L4 of the two ladybird species.

Stability of GNA in the predator's gut

GNA is known to be highly resistant to proteolytic digestion (Van Damme et al. 1987) and even passes unaltered through the gut system of Lepidoptera larvae (Gatehouse et al. 1995, Fitches and Gatehouse 1998). The present *in vitro* studies confirm this by showing that GNA remained undigested when it was incubated with the larval gut digestive enzymes of the three predator species. There was no difference in breakdown of GNA between eggs and sucrose-fed larvae suggesting that either digestive proteases are not induced in response to dietary protein in any of these larvae, or, more likely, the enzymes are unable to hydrolyse GNA.

Differences in biology between the insect predators can explain differences observed in the *in vivo* experiment. The three predator species are likely to apply different protein detoxification processes. *C. carnea* larvae lack a connection between the mid- and hindguts and do therefore not excrete faeces at all (Yazlovetsky 2001). Since they are unable to break down GNA, it accumulates together with other undigested remnants in the distal part of the midgut as meconium. This accumulation of (rhodamine-labelled) GNA in the midgut is clearly visible on the microscope images (Figure 2.6). The filling of the midgut with GNA might cause part of the observed adverse effect on the larvae as this can result in hypertrophy and a premature cease of larval food uptake (Yazlovetsky 2001). This effect was not observed in first instar larvae, as their lifespan was too short to show accumulative effects of GNA. Ladybird larvae, on the other hand, are able to excrete faeces (Baumgartner et al. 1987), and are therefore able to excrete most of the GNA.

GNA binding to gut proteins

Binding studies showed that glycoproteins that bind to GNA can be found in the larval guts of all three predator species. For both ladybirds at least 9 GNA-binding glycoproteins with very similar banding patterns were found, suggesting that the two species have similar glycoprotein profiles in their guts. For *A. bipunctata* the region between 30 and 95 kDa appeared to be very similar to the banding pattern reported by Down et al. (2000). Several more bands were found in the present study, perhaps due to a different method of protein extraction. For *C. carnea* at least 6 different glycoproteins interacting with GNA were detected. The bands partly appear at different sizes compared with the ladybirds. This indicates that part of the binding of GNA in the midgut of *C. carnea* larvae involves glycoproteins that differ from those in the two ladybird species.

Presence of GNA in the haemolymph

Passage of GNA into the haemolymph, organs and glands can make an important contribution to its toxicity, since the lectin may cause general systemic effects as has been suggested by Powell et al. (1998). It could also provide an alternative way of toxin excretion as *C. carnea* has been found to excrete pesticides in its defence fluids (Medina et al. 2003). Independent of the method of haemolymph collection, immunoassay by Western blotting of haemolymph samples of the predator larvae only occasionally showed the presence of GNA in the haemolymph of *C. carnea* and *A. bipunctata*, and this at levels close to the limit of detection. This leads us to assume that GNA is indeed transferred into the haemolymph, but in low and variable amounts. These findings would agree with earlier work showing transportation of GNA into the haemolymph in *N. lugens* (Powell et al. 1998) and *L. oleracea* (Fitches et al. 2001), and the dependency of delivery to the circulatory system upon lectin binding to gut glycoproteins (Fitches et al. 2001). To date very little is known about the mechanisms of protein movement across the gut into the body-cavity of insects (Jeffers et al. 2005). Unfortunately, the microscopy images failed to visualize the presence of rhodamine-labelled GNA in the predators' body-cavity, but again this might have been due to the detection limits as rhodamine-labelled casein was not detected either. Other studies have found that intact casein is transported into the insect haemolymph, but that it is extensively degraded in gut and haemolymph (Habibi et al. 2002). However, caution must be taken in interpretation of these results as we cannot completely eliminate the possibility of contamination of haemolymph samples with GNA-containing sucrose solution stuck to the insect cuticle.

Implications for ecological risk assessment

In worst case laboratory studies larvae of all three predator species have been found to be directly affected by ingestion of a sucrose solution containing 1% GNA. This sensitivity was expressed in a significant reduction in longevity. This worst case scenario might not be unrealistic as currently technologies are being developed to better target transgene expression to the phloem sap, for example by using phloem-specific promoters (Shi et al. 1994, Rao et al. 1998, Foissac et al. 2000). Furthermore, a minimum concentration of 0.1% GNA is required to achieve a detectable impact on target aphids (Down et al. 1996, Sauvion et al. 1996, Couty et al. 2001c). Exposure to lower GNA-levels showed no or only marginal effects on *A. bipunctata* (Birch et al. 1999, 2000, Down et al. 2003). Increased expression levels in the phloem sap will cause stronger effects on sap feeders, but might result in stronger effects on predators as well. It would be interesting to see how the sensitivity to GNA of these predators compares with the sensitivity of the different sap-sucking prey at different lectin concentrations, considering that different species show different dose-dependent responses to GNA (e.g. Rahbé et al. 1995, Sauvion et al. 1996, Romeis et al. 2003). To assess the risk one should not only consider the hazard (here: toxicity), but also the level at which a non-target organism (here: the aphid predator) is exposed to the toxin (Dutton et al. 2003, Poppy and Sutherland 2004). Therefore, the amount of GNA that would be transferred to the predator when feeding on sap-sucking prey should be considered. The levels are likely to be different from phloem sap expression levels. In addition, other prey insects should be investigated. The three predators studied here are known to feed preferentially on aphids, but have been reported to also attack other soft-bodied prey. Furthermore, many predaceous insects, including ladybirds and lacewing larvae have been observed to consume honeydew (Zoebelein 1956, Downes 1974). Honeydew as well as nectar and pollen have been reported to enhance development of *C. carnea* larvae (McEwen et al. 1993, Patt et al. 2003). As GNA has been detected in the honeydew from aphids feeding on transgenic tobacco plants (Shi et al. 1994), and lectin concentrations in freshly excreted honeydew can reach the lectin levels ingested by the sap feeder (Powell et al. 1998, Couty and Romeis 2004), this might be an additional route of exposure for predator larvae.

As long as the mechanisms that cause the insecticidal properties of GNA remain unclear, one should be careful with generalizations. This study revealed that the feeding and digestive physiology of the predator should be taken into account, as first instar lacewings seem less susceptible than ladybirds. This observation is reversed in final instar larvae, possibly caused by GNA-accumulation in *C. carnea* larvae due to their lack of ability to excrete faeces during their larval development.

Because a direct effect of GNA has been found in these worst case laboratory studies, a next step in the risk assessment process would be to study the effects of GNA on the predator larvae under more realistic conditions (Dutton et al. 2003, Poppy and Sutherland 2004). Studies performed by Down et al. (2000, 2003), where GNA has been delivered to aphids through artificial diet and transgenic plants, can be seen as such. The fact that they found only marginal effects indicate that the ecological risks for predator larvae would be limited under the GNA concentrations and expression levels used in these studies. However, when plants with higher GNA-expression levels become available, further studies should be conducted to assess the risk for aphid predators, as the present study demonstrated that they are sensitive to the toxin. Studies should take into account the GNA expression levels in the phloem sap, routes of exposure, and environmental conditions which may affect the predators' performance as a biological control agent (Dutton et al. 2003, Poppy and Sutherland 2004). Should these studies show an impact of GNA-expressing transgenic plants on aphid predators, one would need to evaluate the ecological relevance of such an impact. As with the evaluation of any novel pest control strategy, it is important to place the work in context by comparing its potential impact on non-target organisms with that caused by conventional methods of pest control.

Acknowledgements

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Chapter 3

Effects of honeydew sugar composition on the longevity of *Aphidius ervi*[2]

Abstract

Feeding on sugar-rich foods such as nectar and honeydew is important for survival of many adult parasitoids. Especially in agricultural systems, honeydew is often the most prevalent carbohydrate source. However, relative to plant nectar, honeydew may be relatively unsuitable, due to an unfavourable sugar composition or the presence of secondary plant compounds. We studied survival of the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) on honeydew collected from different aphid species feeding on potato (*Solanum tuberosum* L., cv Desiree), wheat (*Triticum aestivum* L., cv Bobwhite), or artificial diet, as well as the sugar composition of the different honeydews. Honeydews from the tested aphid species on potato, wheat or artificial diet were found to be relatively suitable food sources for adult *A. ervi*, although not always as suitable as a 2 M sucrose solution. There were differences in honeydew sugar composition among the different aphid species on the different host plants. Multivariate statistics showed that the factor 'aphid species' had a significant influence on the sugar composition of the honeydew explaining 27% of the variation in the potato system and 89% in the wheat system. When exploring the relationship between carbohydrate composition of the honeydews from aphids on potato and wheat plants, and their nutritional value for *A. ervi*, data revealed that differences in parasitoid longevity can to some extent be explained by carbohydrate composition. Furthermore, our results confirm that sucrose and its hexose components glucose and fructose are very suitable carbohydrate sources for hymenopteran parasitoids and show that parasitoid survival on an equimolar solution of the two monosaccharides glucose and fructose does not exceed performance on the disaccharide sucrose.

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[2] Based on: Hogervorst P.A.M., Wäckers F.L. and Romeis J., Effects of honeydew sugar composition on the longevity of *Aphidius ervi*. Entomologia Experimentalis et Applicata, in press

Introduction

Carbohydrate feeding is important for survival of many adult parasitoids. It can increase reproductive fitness of parasitoids by increasing their longevity, fecundity and/or parasitism rate (Jervis et al. 1996, Fadamiro and Heimpel 2001, Siekmann et al. 2001, Wäckers 2001, Winkler et al. 2006). Even host-feeding parasitoids commonly depend on sugar feeding to cover their energetic needs (Jervis and Kidd 1986). It has been suggested that carbohydrates are key to fuelling parasitoid flight, thereby increasing search area and host encounter rate (Wäckers 1994, Siekmann 2002, Tenhumberg et al. 2006). Even though one feeding bout on a suitable sugar solution can already prolong parasitoid longevity significantly (Azzouz et al. 2004, Hausmann et al. 2005), parasitoids still need to locate food regularly (at least every 1-2 days) to avoid starvation (Fadamiro and Heimpel 2001, Siekmann et al. 2001, Azzouz et al. 2004).

Nectar (floral and extrafloral) and honeydew produced by sap-feeding Sternorrhynchae (Hemiptera) appear to be the carbohydrate sources most commonly exploited by hymenopteran parasitoids (Wäckers 2005). Honeydew is often the most prevalent source of carbohydrates available, especially in agricultural systems where flowering plants are frequently scarce (Wäckers 2005). Moreover, the foraging risk for honeydew-consuming insects is relatively low in such systems (Wäckers 2005). A study of parasitoids collected in a cabbage field showed that 80% of *Cotesia glomerata* (L.) and 55% of *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae) had recently fed on honeydew (Wäckers and Steppuhn 2003). A recapturing study of released *Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae) demonstrated that the parasitoids were able to maintain a nearly constant level of energy over 48 h in a flower-poor habitat, where honeydew may have been the primary sugar source available (Casas et al. 2003).

The most abundant and important aphid parasitoids belong to the Aphidiidae, a family of solitary endoparasitoids. Host feeding is not common among Aphidiidae and adults feed mostly on honeydew from their aphid hosts (Starý 1970). Honeydew also has a second function for Aphidiids, as it is used as a kairomone to locate hosts (reviewed by Hågvar and Hofsvang 1991). Since parasitoids of Sternorrhynchae encounter honeydew deposits during the normal course of their host-searching activities (Ayal 1987), exploitation of this food is likely most efficient in terms of both time budget and energy budget (Jervis et al. 1996).

Even though honeydew might be the primary food source for parasitoids, it can be far inferior to nectar or sucrose. Low suitability of honeydew may be based on an unfavourable sugar composition. However, amino acid composition as well as the presence of plant secondary chemicals (Wäckers 2000) or transgene products (Chapter 4) may also play a role. Honeydew contains various sugars, up to 90-95% of the dry weight (Maurizio 1985). Besides plant-derived phloem sugars such as sucrose, fructose, glucose or maltose, it often also contains more complex sugars synthesized by the sap feeder, like melezitose, erlose (fructomaltose), raffinose and trehalose (Wäckers 2000, 2005). Honeydew composition shows great variation both in the type of sugars present and the overall sugar concentration depending on the sap feeder and plant species (Němec and Starý 1990, Hendrix et al. 1992). Moreover, parasitoid species may differ in their utilization of honeydew sugars (Wäckers 2001, Beach et al. 2003, Jacob and Evans 2004, Winkler et al. 2005).

The aim of the current study was to investigate the nutritional suitability of honeydew from different aphid species (Hemiptera: Aphididae) on different crop plants for the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae). We assessed parasitoid survival on honeydew collected from aphid species feeding on potato, wheat plants or artificial diet, and analysed the sugar composition of the different honeydews. For wheat and potato, two aphid species were chosen that are commonly associated with these crops, i.e. the fox glove aphid *Aulacorthum solani* (Kaltenbach) and the potato aphid *Macrosiphum euphorbiae* (Thomas) for potato, and the English grain aphid *Sitobion avenae* (Fabricius), and the bird cherry-oat aphid *Rhopalosiphum padi* (L.) for wheat. Additionally, a generalist species the green peach aphid *Myzus persicae* (Sulzer) was tested on both plant species. For comparison honeydew was collected from *R. padi* feeding on artificial diet. Differences in sugar composition among the honeydews from the different aphid species were compared and linked to the nutritional quality of the honeydews for *A. ervi*.

Materials and Methods

Plant material

Plants (*Solanum tuberosum* L., cv Desiree) were grown in plastic pots in compost soil and cultivated in the glasshouse at 24 ± 4 °C. Each pot was fertilized with 3 g Osmocote Exact (N15:P9:K9:Mg3 and trace elements) slow release granule (Scotts Italia SRL, Treviso, Italy). All pots were watered as needed. Potato plants (*Solanum tuberosum* L., cv Desiree) were grown individually and were used when four to six weeks old.

Wheat plants (*Triticum aestivum* L., cv Bobwhite) were grown with three plants per pot. Pots received additional 0.3 g Arbosol (N10:P6:K15:Mg3:Bor0.2) (AGROline AG, Basel, Switzerland) at sowing. Plants then received Superwux 0.2%

(N10:P10:K8) (Samen Mauser, Winterthur, Switzerland) once a week. Four weeks after sowing an additional application of ammonium nitrate (N27.5) and Foskal (P13:K26:Mg2.5:Ca13:$CaCO_3$34) (AGROline) was given. All pots were watered as needed. Seven weeks old plants were used for the experiment.

Insect material

Colonies of the different aphid species were kept as a continuous culture in the glasshouse (24 ± 2 °C) on four to six weeks old plants of the species they would be kept on during the experiment, i.e. *A. solani* and *M. euphoribiae* on potato, *R. padi* and *S. avenae* on wheat, and separate *M. persicae* colonies on both potato and wheat.

Pupae of *Aphidius ervi* (Hymenoptera: Braconidae) were purchased from Andermatt Biocontrol AG (Switzerland) and kept in a climatic chamber (23 ± 1 °C, 85 ± 5% RH, L16:D8). After emergence, parasitoids were provided with water and kept in a cage for 6-12 h to allow mating, before the experiment was started.

Parasitoid longevity on aphid honeydew

Three separate experiments were performed to test the nutritional quality of honeydew from aphids fed on either plants or artificial diet for female *A. ervi*. The first experiment included honeydew from *A. solani*, *M. euphoribiae*, and *M. persicae* on potato (*n* = 20 parasitoids per honeydew treatment), the second included *R. padi*, *S. avenae*, and *M. persicae* on wheat (*n* = 30), and the third involved *R. padi* on artificial diet (*n* = 30-31). This third experiment was included to study honeydew sugar composition from aphids on an unnatural food source that has a known carbohydrate composition, i.e. 20% sucrose w/v.

For the experiments with plants, colonies of three different aphid species were setup on plants in separate cages in one glasshouse compartment (24 ± 2 °C). Honeydew was collected on glass plates (18 x 18 mm) that had been placed under aphid infested leaves. The glass plates were renewed every 24 h and kept at -20 °C until further use. After two weeks of honeydew collection, plants were replaced.

For the third feeding experiment artificial aphid diet was prepared according to the A5 diet described by Febvay et al. (1988), but with a sucrose content of 20% (w/v). The diet was provided in parafilm sachets on Plexiglas cylindrical cages (Ø 3.4 cm, height 3.0 cm) as described by Powell et al. (1993). Approximately 30 apterous, parthenogenetic *R. padi* of different ages were put in each cage from the stock culture. Cages were then kept in a climatic chamber at 23 ± 1 °C, 85 ± 5% RH, L16:D8. The artificial diet was renewed every two days to avoid contamination and deterioration of the diet. One day after aphid settling, glass plates (Ø 3.0 cm) were placed at the bottom of the cages to collect the honeydew. Glass plates were replaced every 24 h and honeydew was collected for a total of five consecutive days. Honeydew sprinkled glass plates were stored at –80 °C until further use.

For each experiment, individual female parasitoids were placed in plexiglas containers (12 x 10 x 5 cm) and provided with water-soaked cotton wool, which was renewed every 2-3 days. In addition to the different honeydew treatments mentioned above, each experiment included a 2 M sucrose solution and a no food treatment as controls. Honeydew was provided on the glass plates on which it had been collected and was renewed daily. Glass plates with honeydew from aphids on artificial diet were offered in the consecutive order they had been collected, starting with honeydew collected from aphids that had been on the diet for 24-48 h on the first day of the experiment. After five days the feeding cycle started over. This was done to give the parasitoids honeydew of similar quality, in case the honeydew composition would change with the time the aphids were on the diet. Sucrose droplets were also provided on glass plates and renewed every 2-3 days. For both the sucrose and honeydew treatments food was provided *ad libitum*. Survival of the parasitoids was scored daily between 10 AM and noon. The no food (water only) treatment served as a control to confirm that the parasitoids were 'unfed' and was not included in the subsequent data analysis. Longevity data were analysed by Student's *t*-test or one-way ANOVA. Means were subsequently separated using Tukey's HSD-Test.

Parasitoid longevity on sugar solution

As our experiments indicated a positive correlation between the glucose and fructose concentration in honeydew with parasitoid longevity, and a negative correlation in the case of sucrose concentration, we conducted an additional experiment to test parasitoid survival on sucrose relative to its two hexose components. In this experiment, individual *A. ervi* females were placed in plexiglas containers with wet cotton wool, and were provided a 5 µl drop of 1 M sucrose [D-(+)-Sucrose, Fluka] or a solution of 1 M glucose [D-(+)-Glucose anhydrous, Fluka] and 1 M fructose [D-(-)-Fructose, Merck] (the hexose components of sucrose). A third treatment consisted of a solution of 0.5 M sucrose, 0.5 M glucose and 0.5 M fructose. A no food treatment served as control. Thirty females were tested per treatment. The concentrations of the sugar solutions in this experiment were chosen to be equivalent to the 1 M sucrose solution and all solutions were provided *ad libitum*. Cotton wool and food solutions were renewed every 2-3 days. Survival of the parasitoids was again scored daily. Longevity data (excluding the no food control) were analysed by one-way ANOVA.

Carbohydrate analysis of honeydew

For analysis of carbohydrate composition, additional honeydew samples were collected by placing a Petri dish lid under aphid infested leaves or under aphids on artificial diet for 24 h. Subsequently, a water-satiated piece of cotton wool was placed in the bottom part of the Petri dish, before the dishes were closed by the honeydew-sprinkled lid. The closed dishes were then placed in a climatic chamber for one hour (23 ± 1 °C, 85 ± 5% RH) reducing the viscosity of the honeydew through hygroscopy, and thus facilitating its collection with micropipettes.

For carbohydrate analysis, samples of approximately 1 μl of honeydew from aphids on plants and 0.5 μl from aphids on artificial diet were collected and diluted in 50 μl 70% ethanol and subsequently stored at room temperature. For analysis, samples were diluted 400-fold with Milli-Q water (Millipore, Amsterdam, The Netherlands). Samples were analysed using a Dionex DX 500 HPLC-system (Dionex Corp., Sunnyvale, CA, USA) and concentrations of the individual sugars were calculated using the program PEAKNET Software Release 5.1 (DX-LAN module) (Dionex, Breda, The Netherlands). For further details of the equipment and methodology used see Steppuhn and Wäckers (2004). For source and purity of sugar standards see Wäckers (1999). The samples were analysed for common nectar and honeydew sugars and sugar alcohols (sucrose, fructose, glucose, erlose, trehalose, maltose, mannitol, sorbitol, melezitose, melibiose and raffinose).

Multivariate statistics on honeydew sugar composition

To calculate and visualize the association between sugar composition and honeydews from the different aphid species on potato and wheat, we used an unconstrained linear ordination (indirect gradient analysis) using Principal Components Analysis (PCA) on sugar percentages (log-transformed). Regression of the ordination axes for the two treatment factors was then depicted by projecting the factors into the ordination diagram. The eigenvalue of an axis in the ordination diagram represents the proportion of the variance in the dataset that can be explained by this axis. In addition, the distribution of the sugar concentrations was investigated by constrained linear ordination (direct gradient analysis) using Redundancy Analysis (RDA), that uses both the sugar composition data and the treatment information in a single, integrated analysis. This was done to examine the variability in sugar composition that can be explained by the different honeydew types. Following RDA, a Monte Carlo permutation test with unrestricted permutations enabled significance testing. The software package CANOCO 4.5 was used to conduct the multivariate analyses (Ter Braak and Šmilauer 2002).

To explore the relationship between mean parasitoid longevity and sugar composition of the honeydews from the different aphid species (*A. solani*, *M. euphoribiae*, *R. padi*, *S. avenae*, and *M. persicae*) on the two plant species (potato and wheat), an indirect gradient analysis was performed. Mean parasitoid longevity was standardized prior to analysis, using mean parasitoid longevity on the 2 M sucrose solution as a reference value. This was done to account for differences between the parasitoid batches and experimental conditions. The log-transformed sugar composition data of the six different honeydews were first subjected to a PCA. Subsequently, mean parasitoid longevity was added to the ordination by regressing its data on to the existing ordination axes (Ter Braak and Šmilauer 2002).

Results

Parasitoid longevity on aphid honeydew

The availability of a sugar source increased the life span of female *A. ervi* when compared to water-fed parasitoids (Figure 3.1). Honeydew from the three aphid species feeding on potato and the 2 M sucrose control (Figure 3.1A) showed no significant differences among the treatments (ANOVA, $F_{3,76} = 2.55$, $P = 0.062$). In the other two feeding experiments there was a significant treatment effect [wheat (Figure 3.1B): $F_{3,115} = 5.89$, $P = 0.0009$; artificial diet (Figure 3.1C): Student's *t*-test, $t = 3.97$, df = 59, $P = 0.0002$]. In general, parasitoids fed with a 2 M sucrose solution had the longest mean longevity when compared with parasitoids fed with any of the honeydews tested. The 2 M sucrose solution was a significantly better food source than honeydew from *M. persicae* and *S. avenae* on wheat ($P < 0.005$) (Figure 3.1B), and *R. padi* on artificial diet ($P < 0.001$) (Figure 3.1C). There was no significant difference between parasitoid longevity on any of the honeydews within an experiment.

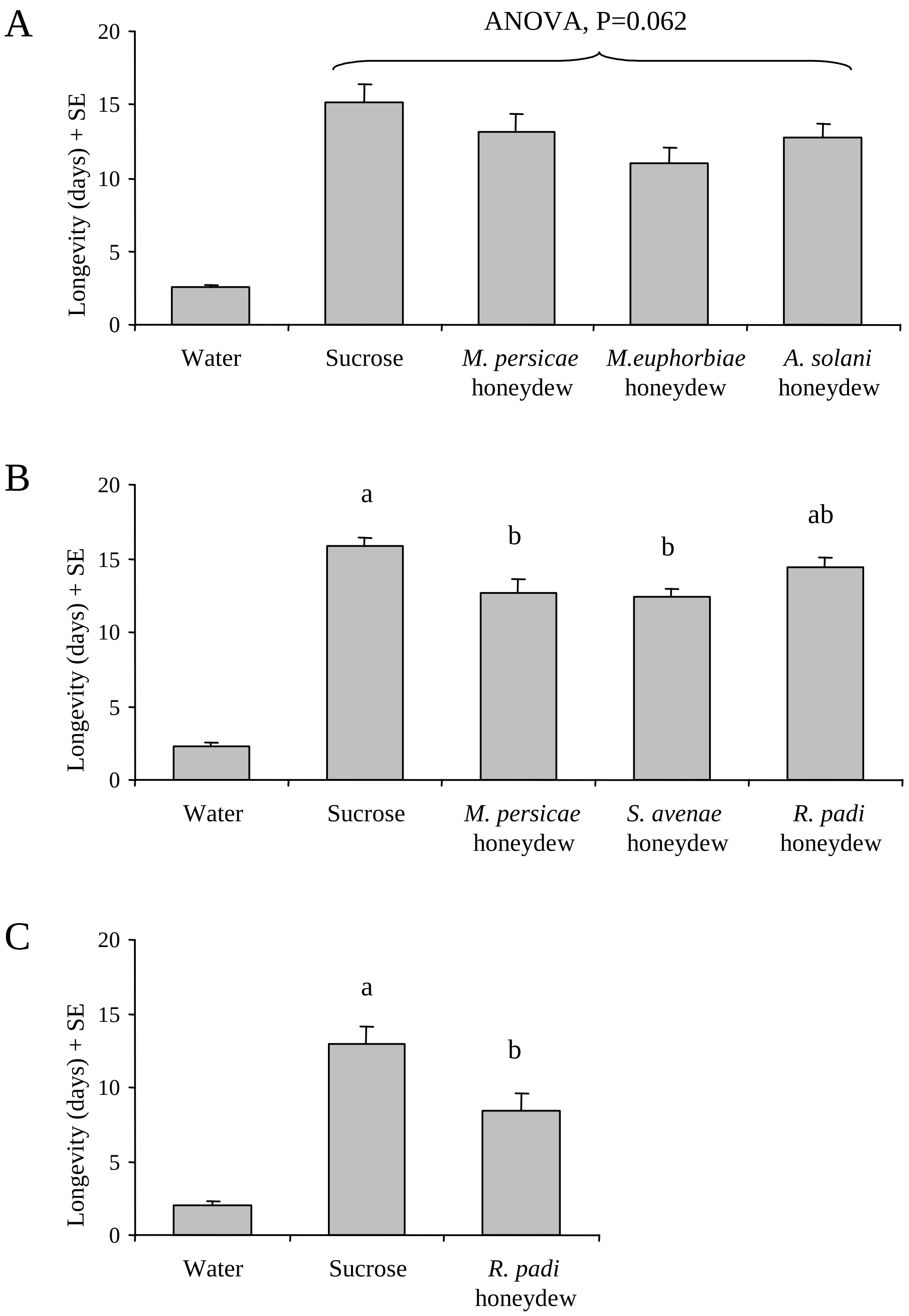

Figure 3.1 Mean longevity (+ SE) of *Aphidius ervi* females when fed with honeydew from different aphid species kept on (A) potato ($n = 20$), (B) wheat ($n = 29$-30), and (C) artificial diet ($n = 30$-31). Two control treatments were added to each of the three experiments: water and a 2 M sucrose solution. The water treatment was not included in the analysis. Different letters indicate statistically significant differences ($P < 0.05$).

Parasitoid longevity on sugar solution

Longevity of female *A. ervi* fed on the different sugar solutions is shown in Figure 3.2. All three sugar solutions tested were found to be equally suitable as a food source (ANOVA, $F_{2,86} = 1.73$, $P = 0.18$).

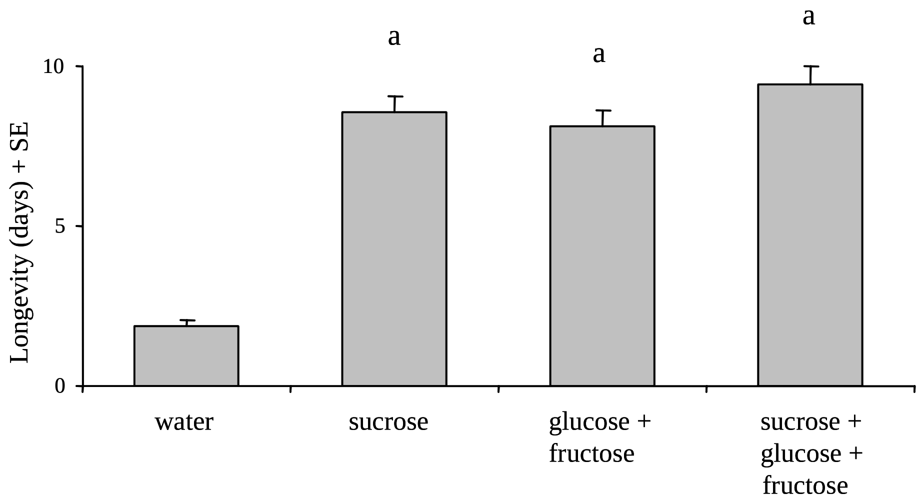

Figure 3.2 Mean longevity (+ SE) of *Aphidius ervi* females when fed with a sugar solution containing 1 M sucrose or 1 M glucose + 1 M fructose or 0.5 M sucrose + 0.5 M glucose + 0.5 M fructose (n = 29-30). A 'water only' treatment was added as a control, but not included in the analysis. No statistically significant differences were found (one-way ANOVA; $P = 0.18$).

Carbohydrate analysis of honeydew

The average total sugar concentration (calculated as the sum of the measured sugars; Table 3.1) was extremely variable between the different samples, as the consequence of differences in evaporation of the honeydew samples and the collection method used. For this reason, the sugar composition of the honeydew is presented as percentage of total sugar (Table 3.1). There were differences in honeydew sugar composition among the different aphid species on the different plants. The most common plant-derived phloem sugars (sucrose, glucose and fructose) made up the majority of the carbohydrates for all aphid species. For aphids on potato this group of sugars constituted 74% (*A. solani*), 78% (*M. euphoribiae*), and 79% (*M. persicae*) of the sugars in the honeydew. For aphids on wheat, this figure was 79% (*S. avenae*), 89% (*R. padi*), and 74% (*M. persicae*). For *R. padi* on artificial diet, however, they only made up 67% of the total honeydew sugars. The trisaccharide erlose was the most important aphid-synthesized sugar in the honeydew of all aphid-plant combinations. The disaccharide maltose constituted 17% of the sugars in honeydew from *R. padi* on artificial diet, even though the aphid diet only contained sucrose as sugar source.

Table 3.1 The sugar composition of honeydew samples from five aphid species feeding on potato or wheat plants, or on artificial diet. Mean total sugar is given in µg/µl (*n* = 8-12). The sugar composition of the individual sugars is expressed as mean percentage of total sugar. Standard errors (SE) are given below the mean value in *italics*. The sugar raffinose was not found in any of the samples.

	Potato			**Wheat**			**Artificial diet**
Sugar	*A. solani*	*M. euphorbiae*	*M. persicae*	*S. avenae*	*R. padi*	*M. persicae*	*R. padi*
Mean total (µg/µl)	**273**	**328**	**311**	**304**	**202**	**211**	**210**
	21.8	*26.5*	*42.5*	*33.4*	*9.8*	*33.0*	*27.3*
Composition (%)							
sucrose	**44.5**	**60.0**	**30.9**	**45.3**	**5.9**	**26.0**	**4.0**
	3.14	*3.00*	*2.14*	*3.09*	*0.72*	*1.80*	*0.22*
fructose	**20.0**	**15.7**	**41.2**	**28.3**	**68.4**	**43.2**	**32.3**
	1.95	*2.41*	*1.70*	*1.76*	*2.04*	*0.59*	*0.35*
glucose	**9.2**	**2.0**	**7.1**	**5.9**	**14.8**	**5.1**	**30.3**
	2.47	*0.36*	*1.85*	*0.69*	*1.79*	*0.48*	*1.75*
erlose	**18.3**	**18.0**	**12.1**	**16.7**	**5.6**	**15.6**	**7.9**
	3.49	*0.62*	*1.82*	*1.08*	*0.53*	*0.56*	*1.06*
trehalose	**4.8**	**2.8**	**6.2**	**1.8**	**1.7**	**7.1**	**0.1**
	2.08	*0.74*	*1.35*	*0.41*	*0.95*	*1.53*	*0.01*
maltose	**1.5**	**0.9**	**2.1**	**1.5**	**2.7**	**2.3**	**17.1**
	0.98	*0.53*	*0.44*	*0.10*	*0.39*	*0.48*	*0.44*
mannitol	**1.6**	**0.6**	**0.4**	**0.4**	**0.7**	**0.6**	**3.2**
	0.72	*0.11*	*0.08*	*0.05*	*0.11*	*0.06*	*0.19*
sorbitol	**0.1**	**0.0**	**0.0**	**0.1**	**0.1**	**0.1**	**0.5**
	0.02	*0.00*	*0.01*	*0.08*	*0.03*	*0.03*	*0.07*
melezitose	**0.0**	**0.0**	**0.0**	**0.0**	**0.1**	**0.0**	**4.7**
	0.00	*0.00*	*0.00*	*0.02*	*0.04*	*0.00*	*0.45*
melibiose	**0.0**	**0.0**	**0.0**	**0.0**	**0.1**	**0.0**	**0.0**
	0.02	*0.00*	*0.00*	*0.04*	*0.05*	*0.01*	*0.00*

Multivariate statistics on honeydew carbohydrate composition

Multivariate statistics showed that aphid species had a significant influence on the sugar composition of the honeydew from aphids on both potato and wheat plants (Figure 3.3). The ordination diagrams of the PCAs of carbohydrate composition of honeydew from aphids on potato and wheat are shown in Figure 3.3A and B, respectively. The ellipses grouping the different aphid species are partly overlapping for the samples from aphids on potato, whereas the ellipses grouping the honeydew from aphids on wheat are completely segregated. The honeydew sample from *A. solani* on potato that can be found at the bottom right of Figure 3.3A, was considered an outlier and therefore ignored when the ellipse was drawn.

Monte Carlo permutation test on sugar composition of honeydew from potato revealed that aphid species could explain 27% of the variation in the data ($F = 2.86$, $P = 0.030$, $n = 27$). For honeydew from aphids on wheat plants the differences in sugar composition among the aphid species was much higher. Monte Carlo permutation test showed that here aphid species could explain 89% of the variance ($F = 28.51$, $P = 0.001$, $n = 24$).

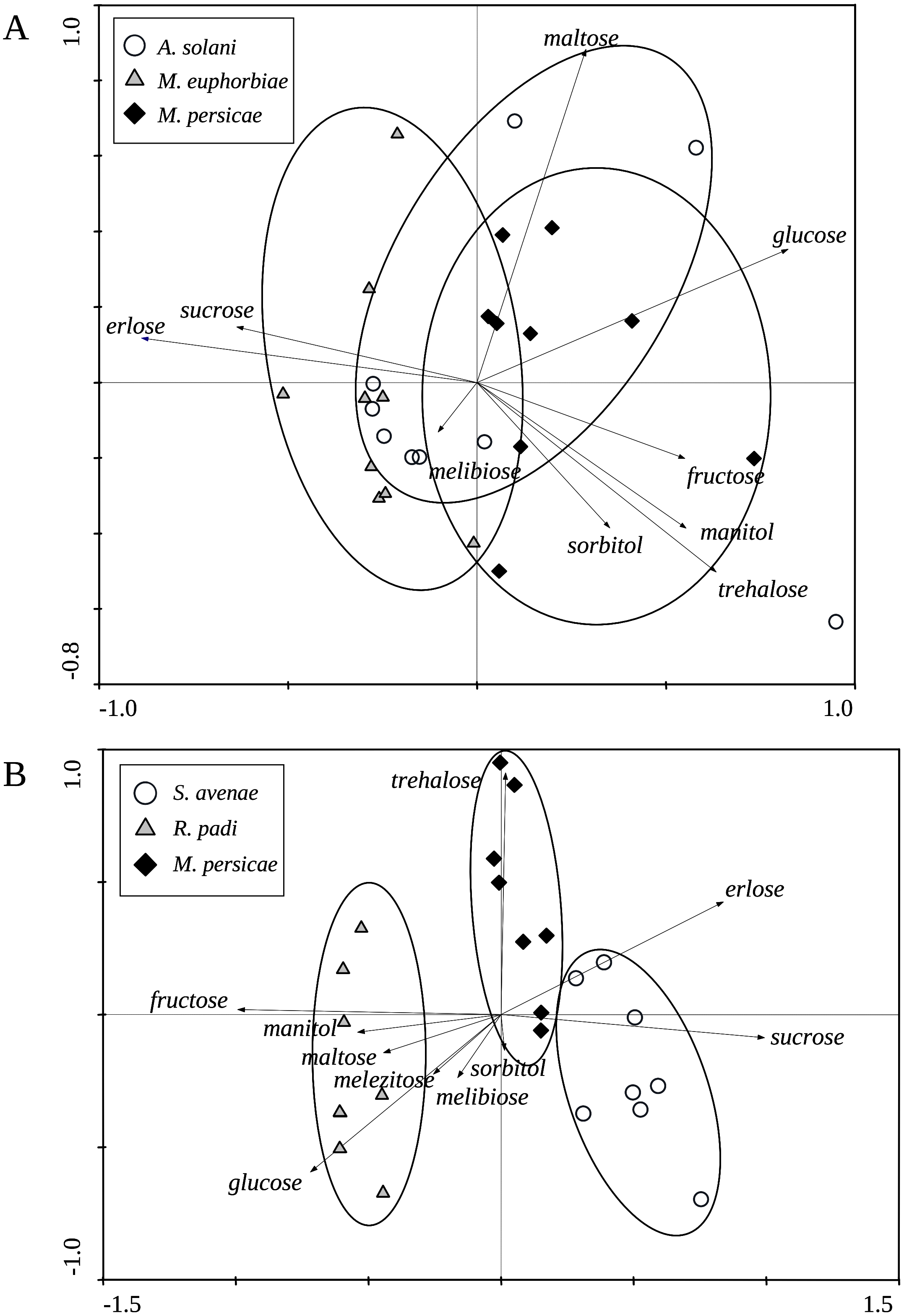

Figure 3.3 Distribution of sugar composition of honeydew samples from three different aphids feeding on (A) potato or (B) wheat plants (see Table 3.1 for details of honeydew sugar composition), in the ordination biplot of a PCA. (A) Eigenvalues: axis 1: 0.473, axis 2: 0.235; (B) Eigenvalues: axis 1: 0.903, axis 2: 0.065. Sugar composition data (plotted as vectors) were expressed as percentage of total sugar and the data were log-transformed. The ellipses group the samples from one aphid species. One sample of *Aulacorthum solani* (Figure 2A) was considered an outlier and was not included in the ellipse.

In addition, honeydew sugar composition of *M. persicae* on potato and wheat plants was compared by PCA and RDA followed by Monte Carlo permutation test. For this polyphagous aphid species Monte Carlo permutation test revealed that no significant difference between the sugar composition of the honeydew from the two plant species existed ($F = 0.82$, $P = 0.548$, $n = 17$).

The ordination diagram of the indirect gradient analysis shows the distribution of the different honeydew samples from the five aphid species on potato and wheat in relation to the mean longevity of female *A. ervi* on the six treatments (Figure 3.4). The first two axes of the PCA (Figure 3.4) explained 69% of the variance in the data. The length of the vector for mean parasitoid longevity that is projected into the ordination is an indicator for the correlation of the variable (mean parasitoid longevity) with the displayed ordination axes (Ter Braak and Šmilauer 2002). The strong correlation of the vector with the first ordination axis is a further indication for a relation between honeydew sugar composition and parasitoid longevity.

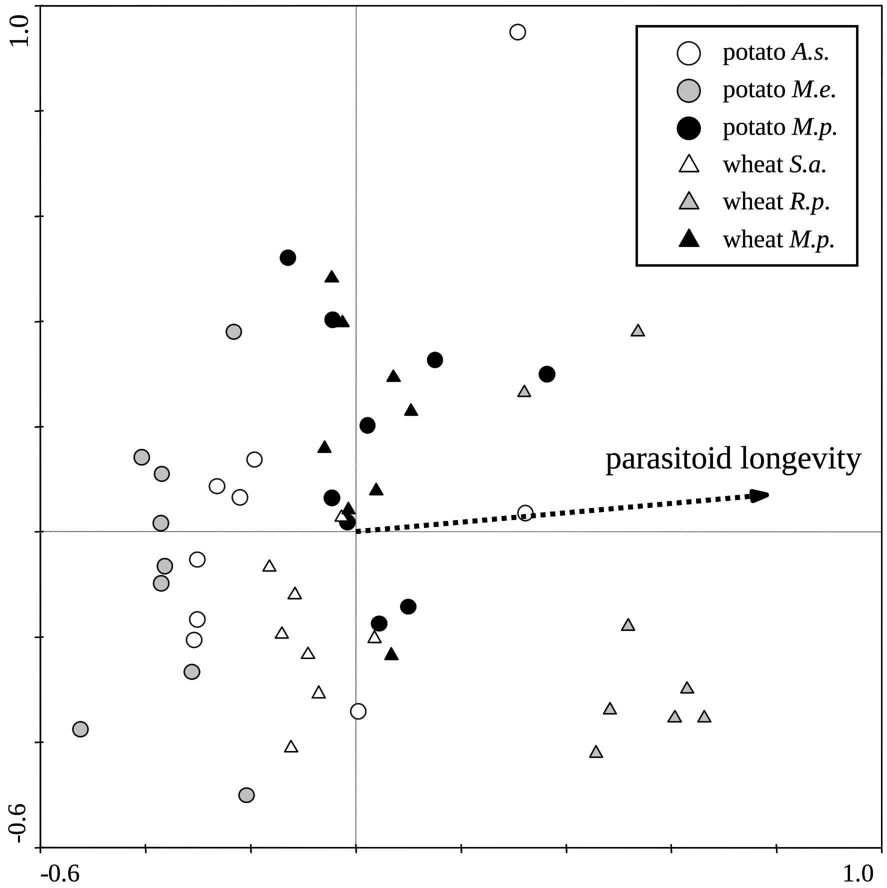

Figure 3.4 Distribution of sugar composition of honeydew samples from *Aulacorthum solani* (*A.s.*) and *Macrosiphum euphoribiae* (*M.e.*) on potato, *Rhopalosiphum padi* (*R.p.*) and *Sitobion avenae* (*S.a.*) on wheat and *Myzus persicae* (*M.p.*) on potato and wheat (see Table 3.1 for details of honeydew sugar composition), in the ordination biplot of a PCA. Eigenvalues: axis 1: 0.467, axis 2: 0.223. Sugar composition data were expressed as percentage of total sugar and the data were log-transformed. Mean parasitoid longevity (standardized to the 2 M sucrose treatment) was added to the ordination diagram by indirect gradient analysis.

Discussion

Compared to females of *A. ervi* that only had access to water, the additional access to honeydew increased the mean parasitoid lifespan by a factor 4.4 to 6.3 depending on the aphid and plant species. No significant differences were found among honeydews from different aphid species feeding on the same plant species, indicating that these honeydews are equally suitable for female *A. ervi*. However, in a similar experiment described in Chaper 4 *A. ervi* longevity was found to be significantly prolonged on honeydew from *R. padi* as compared to honeydew from *S. avenae* on wheat. Whereas honeydews from the tested aphids on potato and wheat are thus relatively suitable carbohydrate sources for *A. ervi*, they were nevertheless less suitable than a 2 M sucrose solution. The suitability of honeydew as a food source has been shown to vary substantially. There are several studies in which honeydew feeding provided no benefit to parasitoids or even reduced their longevity (Leius 1961a, b, Avidov et al. 1970, Elliott et al. 1987). Other papers, like this study, report that honeydew did increase parasitoid survival, but not as much as nectar, honey or sugar solutions (Idoine and Ferro 1988, Hagley and Barber 1992, Lee et al. 2004, Fadamiro and Chen 2005, Faria 2006). Others still, found honeydew to be equally suitable as nectar, honey or sugar solutions (Hocking 1967, England and Evans 1997, Singh et al. 2000, Burger et al. 2004).

It is difficult to compare results among studies given the variability in honeydew chemistry. Honeydew composition has been found to vary not only with aphid and plant species (Byrne and Miller 1990, Hendrix et al. 1992, Völkl et al. 1999), but also with aphid stage (Arakaki and Hattori 1998, Costa et al. 1999) and age (Fischer et al. 2002), the presence of bacterial symbionts in the digestive tract (Wilkinson et al. 1997), ant attendance (Fischer and Shingleton 2001, Yao and Akimoto 2001), and rate and duration of aphid infestation (Faria 2006).

Sugar composition is one of the key factors determining the nutritional value of honeydew (Wäckers 2000). Further factors affecting the nutritional value of honeydew include amino acid composition (Chapter 4, Wäckers 2005), as well as secondary plant metabolites (Wink and Römer 1986, Malcolm 1990) and transgene products (Chapter 4, Romeis et al. 2003).

When using multivariate statistics to compare the different honeydews in our study with regard to their carbohydrate composition, significant differences in composition were detected among the three aphid species on both potato and wheat. However, these differences were much more pronounced among the three aphid species on wheat plants. The differences in sugar composition did not impact the nutritional quality of these honeydews for *A. ervi* in the longevity experiment. The relation between carbohydrate composition of the honeydews from aphids on potato and wheat plants, and its nutritional value for *A. ervi* was further explored by adding mean parasitoid longevity to the PCA of honeydew carbohydrate composition. We showed that the small and non-significant differences in parasitoid longevity can to some extent be explained by differences in honeydew carbohydrate composition. This finding is a further indication that sugar composition can play an important role in determining the nutritional quality of honeydew for parasitoids. However, in this particular case, it is unlikely to have a high ecological relevance as all honeydews tested were relatively suitable for *A. ervi*. It will be interesting to study the effect of honeydew sugar composition for a group of honeydews that show a larger variation in their sugar composition as well as their nutritional suitability.

The plant-derived sugars sucrose, glucose and fructose generally have a high nutritional quality, whereas the aphid-synthesized oligosaccharides such as melezitose, erlose, raffinose, and trehalose can lower the nutritional value of honeydew (Wäckers 2000, 2001). We found that all honeydews analysed here contained a relatively high percentage of sucrose and its breakdown products glucose and fructose (sum of 74-89% of the three sugars). When combining the experiments on honeydew suitability for *A. ervi* with the sugar composition of the honeydews, it is noticeable that for both the potato and the wheat system, the honeydews on which the parasitoids had the longest average longevity (honeydew from *M. persicae* on potato and from *R. padi* on wheat) contained the highest percentage of the two monosaccharides glucose and fructose and the lowest percentage of the disaccharide sucrose, whereas the honeydews on which the parasitoids had the shortest average longevity (honeydew from *M. euphorbiae* on potato and from *S. avenae* on wheat) contained the lowest percentage of glucose and fructose and the highest percentage of sucrose. The same can be seen when observing the sugar vectors in the two ordination diagrams of Figure 3.3. Even though the differences in average parasitoid longevity were only marginal, this trend suggested that the monosaccharides fructose and glucose might be as a better carbohydrate source for *A. ervi* than the disaccharide sucrose (Ashford et al. 2000, Karley et al. 2005). Therefore, we investigated the longevity of female *A. ervi* on a sucrose solution, on a solution containing glucose and fructose, and on a solution containing all three sugars. We found no differences among the three treatments (Figure 3.2), showing that a solution of the two monosaccharides glucose and fructose does not provide an additional nutritional benefit compared with a solution of the disaccharide sucrose. Our results further confirm that sucrose and its hexose components glucose and fructose are all suitable carbohydrates for hymenopteran parasitoids as has previously been demonstrated for other species by Wäckers (2001), Hausmann et al. (2005), and Winkler et al. (2005).

Whilst honeydew from *R. padi* on wheat plants was found to contain the highest percentage of the phloem sugar, sucrose, and its hexose units glucose and fructose, honeydew from *R. padi* on artificial diet, was found to have the lowest percentage of these sugars (67%). Furthermore, longevity of *A. ervi* feeding on honeydew from *R. padi* feeding on artificial diet was reduced by 35% compared to a 2 M sucrose solution, whereas honeydew from *R. padi* feeding on wheat only caused a 9% longevity reduction compared to a 2 M sucrose solution. The reduction in parasitoid survival may be explained solely by the sugar composition, but may also have been affected by other aphid-diet mediated factors.

The enzymatic hydrolysis and polymerization of sugars is common in phloem-feeding insects (Karley et al. 2005). The excretion of oligosaccharides in the honeydew has often been described and is generally viewed as having an osmoregulatory function (Fisher et al. 1984, Wilkinson et al. 1997). Phloem sap composition of the host plant can play an important role in determining the sugar composition of the honeydew excreted by the sap feeder. However, the sugar composition of the honeydew from aphids may be similar on different host plants as we saw for honeydew from *M. persicae* fed on potato and wheat. For the polyphagous *M. persicae* wheat is not a common host plant. However, apart from a higher percentage of trehalose in some of the samples, the honeydew from *M. persicae* did not have an altered sugar composition compared to the honeydew from any of the other tested aphids on wheat. This indicates that the polyphagous *M. persicae* may have a similar sugar metabolism as the more specialized wheat aphids, *R. padi* and *S. avenae*.

We have shown that honeydew from several aphid species on potato and wheat can be considered suitable as sugar source for *A. ervi*. In addition, minor differences in parasitoid longevity could to some extent be explained by differences in honeydew sugar composition. The fact that this aphid parasitoid benefits from feeding on its hosts honeydew, represents a double benefit as it not only provides the carbohydrates essential for survival, it also avoids having to divide its time between searching for hosts and carbohydrate sources (Křivan and Sirot 1997, Lewis et al. 1998, Tenhumberg et al. 2006). The parasitoid seems to be able to make optimal use of its hosts 'waste product' honeydew. Honeydew quality is not likely to be a limiting factor in the aphid-plant systems studied here. However, the availability and accessibility of honeydew might be the limiting factor in the field (Wäckers 2005). Intake of honeydew may be more difficult due to higher viscosity or crystallization of the honeydew (Faria 2006), but see Starý (1970). Further studies should be done to investigate the utilization of this obvious and suitable sugar source in the field and the possible effects of honeydew on population dynamics of parasitoids.

Acknowledgements

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Chapter 4

Effects of GNA-containing honeydew on the aphid parasitoid *Aphidius ervi*[3]

Abstract

Insecticidal proteins can be excreted in the honeydew when sap-sucking insects feed on insect-resistant transgenic plants. Given that honeydew can be an important source of carbohydrates, this could potentially expose a broad range of honeydew-feeding insects such as parasitoids to transgene products. Our studies show that snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) dissolved in a sucrose solution has a direct negative effect on adult longevity of the aphid parasitoid *Aphidius ervi*. Honeydew from *Rhopalosiphum padi* feeding on a GNA-containing artificial diet contained 10-40% of the GNA concentration in the diet. When feeding on this honeydew, *A. ervi* suffered a reduction in longevity that was more pronounced than was to be expected based on the detected GNA concentration in the honeydew. Analysis of carbohydrate and amino acid composition revealed that a change in honeydew composition caused by a GNA-effect on the aphids could be a possible explanation for the additional reduction in parasitoid longevity. When comparing the effect of honeydew from *Sitobion avenae* and *R. padi* feeding on GNA-expressing or non-transformed wheat plants on *A. ervi* longevity, aphid species was found to have a significant effect, whereas wheat variety had no effect. The latter result was probably due to low GNA expression levels in the plants. Differences in the nutritional suitability between honeydew from *R. padi* and *S. avenae* could be explained by differences in carbohydrate and amino acid composition. This is the first study showing that GNA in honeydew negatively affects honeydew-consuming parasitoids. We recommend that honeydew as a route of exposure to transgene products should be considered in future risk assessment studies.

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[3] Based on: Hogervorst P.A.M., Wäckers F.L., Woodring J. and Romeis J., Effects of GNA-containing honeydew on the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae). Submitted

Introduction

Honeydew is a generic term for sugar-rich excretions produced by sap-feeding Sternorrhynchae (Wäckers 2005). It is regarded as an important food source for insects including pollinators, predators and parasitoids (Zoebelein 1956, Wäckers 2005). In agricultural systems, where nectar sources are often scarce, honeydew might be the primary carbohydrate source for insects (Wäckers 2005). Wäckers and Steppuhn (2003) showed that 80% of *Cotesia glomerata* (L.) and 55% of *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae) collected in a cabbage field contained honeydew specific sugars, indicating widespread honeydew feeding. Honeydew feeding can increase parasitoid longevity and fecundity (Chapter 3, Singh et al. 2000, Burger et al. 2004) and the presence of carbohydrate supplements can increase parasitism rates in the field (Winkler et al. 2006) with clear impacts on parasitoid-host dynamics (Wäckers 2003).

Given that almost all honeydew-producers feed directly on the phloem sap of the host plant and due to the fact that several compounds are not degraded during the passage through the digestive system of sap-feeding insects (Douglas 2003), the composition of the phloem sap has a direct impact on honeydew composition. This applies in particular to proteins, as phloem-sucking insects typically possess low proteolytic activity in the gut (Srivastava and Auclair 1963, Rahbé et al. 1995), even though cysteine protease activity in the aphid gut has recently been demonstrated (Cristofoletti et al. 2003, Rahbé et al. 2003). Unaltered passage through the sap feeder's digestive tract has also been reported for secondary plant compounds, such as alkaloids (Wink and Römer 1986) and cardenolides (Malcolm 1990).

One would expect a similar unaltered passing of insecticidal proteins expressed by genetically modified (transgenic) plants into honeydew. The current generation of insect-resistant transgenic crops all express *cry* genes from the soil bacterium *Bacillus thuringiensis* (*Bt*). It appears that the Cry toxins are barely expressed in the phloem sap (Raps et al. 2001) as aphids feeding on *Bt*-transgenic maize (Dutton et al. 2002), oilseed rape (Schuler et al. 2005) and cotton (Zhang et al. 2006) were found to contain no or very low amounts of the toxins (at ng level), making excretion of Cry toxins in honeydew unlikely. The latter has been confirmed for sap feeders on *Bt* maize (Raps et al. 2001) and rice (Bernal et al. 2002).

However, other insecticidal proteins have also been expressed in crops to render them resistant to insects. Among these, the toxins that are directed at sap feeders, such as lectins, that need to be transported in the phloem to reach the target pest.

Unaltered passage through the sap feeder's digestive tract and excretion in the honeydew has been reported for several of these insecticidal proteins, including lectins (Shi et al. 1994, Kanrar et al. 2002) and protease inhibitors (Rahbé et al. 2003). This way, honeydew-feeding insects would potentially be exposed to these transgenic compounds. This exposure route has received little attention until now as most studies on the potential non-target impact of insecticidal proteins expressed in transgenic plants have exclusively focused on direct exposure as a result of herbivory, and indirect exposure in the case of predators or parasitoids attacking herbivores containing the transgene product (Romeis et al. 2006). However, the potential for exposure to transgene products through honeydew may exceed the previously considered routes, as honeydew is consumed by a broad range of organisms including both vertebrates and invertebrates (Wäckers, 2005).

Depending on the sap feeder and the plant species, honeydew in itself can be relatively unsuitable as a food source when compared to nectar or a sucrose solution (for references see Chapter 3). Low suitability of honeydew can in part be attributed to the presence of certain aphid synthesized oligosaccharides or secondary plant compounds (Wäckers 2000). The impact of a transgene product in honeydew on a honeydew consumer may be altered by this variation in honeydew composition.

To study honeydew as a route of exposure, we used snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) as a model compound. This insecticidal protein is of particular interest because it is transported in the phloem sap of the transgenic plant and acts on sap-feeding Sternorrhynchae, which are typically not targeted by the known *Bt* Cry-toxins. GNA binds specifically to D-mannose (Van Damme et al. 1995), and is resistant to gut proteolysis (Chapter 2, Gatehouse et al. 1995). In several cases GNA has been reported to be transported across the midgut epithelial barrier into the circulatory system (Powell et al. 1998, Fitches et al. 2001), but the exact mechanism of toxicity is complex and not yet fully elucidated. A gene encoding GNA has been successfully engineered into a number of crops, causing partial resistance to sap-sucking insects including aphids (Aphididae) (Down et al. 1996, Gatehouse et al. 1996, Stoger et al. 1999), planthoppers (Delphacidae) (Rao et al. 1998) and leafhoppers (Cicadellidae) (Foissac et al. 2000). In addition, it has proven to be also active against tissue feeding insects, including lepidopteran (Fitches et al. 1997) and coleopteran pests (Nutt et al. 1999). Among beneficial arthropods, direct effects of GNA dissolved in a sugar solution have been found for a range of adult hymenopteran parasitoids (Romeis et al. 2003, Bell et al. 2004) as well as for larvae of predatory insects (Chapter 2).

We used the aphid parasitoid *Aphidus ervi* Haliday (Hymenoptera, Braconidae) to address whether GNA contained in honeydew poses a risk for adult parasitoids. We first performed behavioural observations to investigate whether GNA affects food uptake by *A. ervi*. Subsequently, we studied whether longevity of *A. ervi* was affected when feeding on sucrose solution containing GNA. To investigate how the passage through the aphid gut affects GNA toxicity to honeydew consumers and whether the impact of GNA interacts with the nutritional quality of honeydew, we tested *A. ervi* longevity when fed with honeydew from the bird-cherry aphid, *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) kept on artificial diet with or without GNA, or honeydew from the grain aphid, *Sitobion avenae* (Fabricius) and *R. padi* kept on GNA-expressing or non-transformed wheat plants.

Materials and Methods

Plant material

The wheat variety Bobwhite (*Triticum aestivum* L.) has been genetically engineered with a gene from snowdrops (*Galanthus nivalis*) that encodes for GNA (Stoger et al. 1999). Plants were transformed with three genes, i.e. the *gna* gene *pLECGNA2* and the selectable BASTA resistance marker gene (*bar*), both controlled by the corn ubiquitin promoter (Stoger et al. 1999). These plants had earlier been reported to have some effect on *S. avenae*. Seeds of GNA-transgenic wheat of line BW150 (T3 seeds) (Stoger et al. 1999) and the corresponding non-transformed near isoline were provided by A.M.R. Gatehouse (University of Newcastle, Newcastle upon Tyne, UK).

Plants were grown in plastic pots (three per pot) in compost soil and cultivated in the glasshouse at 24 ± 4 °C. Each pot was fertilized with 3 g Osmocote Exact (N15:P9:K9:Mg3 and trace elements) slow release granule (Scotts Italia SRL, Treviso, Italy) and approximately 0.3 g Arbosol (N10:P6:K15:Mg3:Bor0.2) (AGROline AG, Basel, Switzerland) at sowing. They then received the fertilizer Superwux 0.2% (N10:P10:K8) (Samen Mauser, Winterthur, Switzerland) once a week. Four weeks after sowing an additional application of ammonium nitrate (N27.5) and Foskal (P13:K26:Mg2.5:Ca13:$CaCO_3$34) (AGROline) was given. All pots were watered as needed. Seven week old plants were used for the experiment.

The presence of GNA was determined for each plant that was used for honeydew collection. For extraction, leaves (without tips and bases) used to collect honeydew (see below) were taken at the end of honeydew collection and put into Eppendorf tubes, which were directly flash frozen in liquid nitrogen and stored at -80 °C. Quantification of transgene expression was determined by immuno-assay using dot-blotting based on the methods described by Gatehouse et al. (1996). Frozen leaf samples were ground in liquid nitrogen and homogenized in PBST (phosphate buffered saline + 1% Tween 20). Samples were incubated for 1 h at 4 °C under shaking, and subsequently centrifuged at 13,000 *g* for 10 min. The supernatant was collected and total soluble protein in the extraction was estimated by Bradford assay according to the manufacturer's instructions, using bovine serum albumin (BSA) as a protein standard. For the dot blots, aliquots containing 25 μg total soluble protein per well were loaded in duplicate unto a 0.2 μm nitrocellulose membrane (Schleicher & Schuell, BA83) in a Bio-Rad dot-blot apparatus. Eight GNA standards, containing 0, 1, 2.5, 5, 7.5, 10, 25 and 50 ng GNA, were also added in duplicate. The membrane was processed as described by Gatehouse et al. (1996), using poly-clonal antibodies raised against GNA as primary antibody, and HRP-conjugated goat antirabbit IgG (Bio-Rad) as secondary antibody and detected by enhanced chemo-luminescence (ECL). GNA was detected in all plants but at variable levels, ranging from levels below 0.001% to 0.02% of total soluble protein. The maximum expression levels found, were about 10-fold below expression levels for wheat line BW150 reported by Stoger et al. (1999).

Insect material

Pupae of *Aphidius ervi* (Hymenoptera: Braconidae) were purchased from Andermatt Biocontrol AG (Switzerland) and kept in a climatic chamber (23 ± 1 °C, 85 ± 5% RH, 16:8 L:D). After emergence, parasitoids were provided with water and kept in a cage for 6-12 h to allow mating, before the experiment was started.

Colonies of the two aphid species, *R. padi* and *S. avenae*, were kept as a continuous culture on non-transgenic wheat plants in the glasshouse (24 ± 2 °C).

Snowdrop lectin (GNA)

Lyophilized GNA was obtained from E. van Damme (Ghent University, Belgium). The lectin was isolated from snowdrop bulbs as described by Van Damme et al. (1987) (methods adjusted to production of larger quantities), and has a purity of at least 99%. The activity of the lectin from our batch has been confirmed by agglutination assays using rabbit erythrocytes (method according to Van Damme et al. 1987).

Gustatory response to GNA

We assessed whether GNA affects the feeding behaviour of *A. ervi*. Food deprived, water satiated, one day old female parasitoids were provided with a 1 μl droplet of 1% GNA (w/v) dissolved in a 2 M sucrose solution. Thirty individuals were observed for each treatment through a binocular microscope. Feeding time was measured as the time between first mouth parts contact with the food solution until first retreat from the droplet. The data were log transformed and analysed using Student's *t*-test for independent variables.

Parasitoid longevity on GNA

Individual female *A. ervi* were provided with a 5 µl droplet consisting of different GNA concentrations (0, 0.01%, 0.1% and 1%, w/v) dissolved in a 2 M sucrose solution as described for *Aphidius colemani* Viereck by Romeis et al. (2003). Thirty females were tested per treatment. Data were analysed using one-way ANOVA. Means were separated using Tukey's HSD test.

Artificial diet experiment

For this bioassay *R. padi* was kept on an artificial aphid diet as described in Chapter 3, with or without 0.1% GNA (w/v) dissolved in the diet. Honeydew was collected for a total of 5 consecutive days on glass plates that were replaced daily and stored as described in Chapter 3.

Individual female parasitoids were placed in Plexiglas containers (12 x 10 x 5 cm) and provided with cotton wool soaked with water that was renewed every 2-3 days. In addition, parasitoids were provided with one of the following: 2 M sucrose solution with or without 0.1% (w/v) GNA, honeydew from *R. padi* that had fed on artificial diet with or without 0.1% (w/v) GNA, or no food ($n = 30$). Honeydew was provided on the glass plates on which it had been collected and was renewed daily. The glass plates were offered in the order they had been collected, starting with honeydew collected from aphids that had been on the diet for 24-48 h on the first day of the experiment. After 5 days the feeding cycle started over. This was done to give the parasitoids honeydew of similar quality, in case the honeydew composition would change with the time the aphids were on the diet. Sucrose droplets were also provided on glass plates and renewed every 2-3 days. For both sucrose- and honeydew-fed parasitoids food was in ample supply. Survival of the parasitoids was scored daily between 10 AM and noon. The no food (water only) treatment was used as a control to ensure that the wasps were truly starved. This treatment was not included in the subsequent data analysis. Longevity data were square root transformed prior to statistical analysis by two-way ANOVA, with food type (sugar solution or honeydew) and presence of GNA as parameters. Means were subsequently separated using Tukey's HSD-Test.

For analysis of GNA content and carbohydrate and amino acid composition, honeydew sprinkled glass plates were put in a box with water-satiated pieces of cotton wool that was then placed in a climatic chamber (23 ± 1 °C, 85 ± 5% RH), reducing the viscosity of the honeydew through hygroscopy, and thus facilitating its collection with 0.5 µl micropipettes (end to end minicaps, Hirschmann® Laborgeräte, Germany). Honeydew samples (of approximately 0.5 µl) were analysed for GNA content using the immunodot-blot assay detection system as described above. For carbohydrate analysis, samples of approximately 0.5 µl were collected and analysed as described in Chapter 3. For amino acid analysis, samples of approximately 0.5 µl were collected and diluted in 10 µl 0.1 N HCl. Subsequently derivitized by adding 20 µl Stock Dansyl chloride, neutralized by 20 µl $NaHCO_3$ (buffer) and incubated for 3 h at 23 ± 1 °C. Finally another 50 µl $NaHCO_3$ buffer was added. The samples were analysed for amino acid composition as described by Woodring et al. (2004) for a total of 19 amino acids, including the ten amino acids that are generally considered essential for insects: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Dadd 1985).

The software package CANOCO 4.5 was used to conduct multivariate analyses (Ter Braak and Šmilauer 2002, Lepš and Šmilauer 2003) in order to study the association between sugar / amino acid composition and diet type (control and GNA diet), as well as the time the aphids spent on the diet. The lengths of gradient were low, justifying the use of linear models for the relationships among samples. Therefore, we used Principal Components Analysis (PCA), an unconstrained linear ordination (indirect gradient analysis), on log-transformed sugar and amino acid percentages to visualize the important patterns in the data. Regression of the ordination axes for the two treatment factors was depicted by projecting the factors into the ordination diagram. Additionally, the distribution of the sugar / amino acid concentrations was investigated by Redundancy Analysis (RDA), a constrained linear ordination (direct gradient analysis), to examine the variability in sugar / amino acid composition that can be explained by the two treatment factors. The significance was tested by a Monte Carlo permutation test with unrestricted permutations ($N = 999$).

Aphid performance on GNA wheat

Performance of *R. padi* and *S. avenae* on GNA-transgenic wheat and non-transformed control plants (seven plants per treatment) was analysed in the glasshouse at 24 ± 4 °C. Three reproductive adults from the rearing colony were caged on a fully expanded leaf of seven week old plants in a clip cage (3.4 cm diameter). Two to three cages were attached per plant. The next morning, all but three newly laid nymphs were removed from the cages. The development of the nymphs was checked daily until the start of reproduction. At four days (*R. padi*) and six days (*S. avenae*) aphids were weighted individually on a microbalance (Mettler Toledo, MX5, d = 1 µg; ± 2 µg). Data for the aphids per cage were pooled to avoid pseudo-replications, resulting in 19 and 18 replications for *R. padi*, and 18 and 16 replications for *S. avenae*, for control and GNA plants, respectively.

Once the first aphid in a cage started producing nymphs, only this adult was kept in the cage and over the next days the number of offspring produced by this aphid was counted. Aphid reproductive performance on the different plant treatments was assessed by calculating the intrinsic rate of natural increase [$r_m = 0.74$ (ln F_D/D), where F_D = number of aphids produced over a period of time equal to that of the pre-reproductive period (D)] (Wyatt and White 1977). The r_m values and average weights per cage were compared among the GNA-transgenic wheat and its control line using Student's *t*-test. In one case (r_m of *R. padi*) data were log transformed to fulfill the assumptions for a normal distribution. All statistical analyses were computed in Statistica (version 6, Statsoft Inc., Tulsa, USA).

Parasitoid longevity on aphid honeydew from GNA wheat

Colonies of *R. padi* and *S. avenae* were setup in separate cages in one glasshouse compartment (24 ± 2 °C) on either GNA-transgenic wheat plants or control plants. Honeydew from the two aphid species was collected on glass plates (18 x 18 mm) that had been placed under aphid infested leaves. The glass plates were renewed every 24 h and kept at -20 °C until further use. After two weeks of honeydew collection, plants were replaced with new (seven weeks old) plants.

Individual female parasitoids were placed in Plexiglas cages (12 x 10 x 5 cm) and provided with cotton wool soaked with water which was renewed every 2-3 days. In addition, parasitoids were provided with one of the following: honeydew from one of the two aphid species from either GNA or control wheat plants, 2 M sucrose solution or no food ($n = 30$). Honeydew was provided on the glass plates on which it had been collected and was renewed daily. Sucrose droplets were also provided on glass plates and renewed every 2-3 days. For both the sucrose and honeydew treatments food was provided *ad libitum*. Survival of the parasitoids was scored daily between 10 AM and noon. The no food (water only) treatment served as a control and was not included in the data analysis. Longevity data were analysed by one-way ANOVA. Means were subsequently separated using Tukey's HSD-Test.

Honeydew samples were analysed for GNA content (samples of 1-5 µl), and carbohydrate and amino acid composition (samples of 2 µl), as described above with dilutions adjusted to higher amounts of honeydew per sample.

The association between sugar / amino acid concentrations and the two treatment factors (aphid species: *R. padi* and *S. avenae*, and plant type: control and GNA-transgenic), were calculated and visualized by PCA, RDA and Monte Carlo permutation test as described above.

Results

Gustatory response to GNA

GNA did not have a significant effect on feeding time ($t = 0.99$, df = 58, $P = 0.33$). Female parasitoids provided with a 2 M sucrose solution fed an average (± SE) of 78.1 ± 9.82 s, whereas this figure was 75.1 ± 13.21 s on a 2 M sucrose solution with 1% GNA (w/v).

Parasitoid longevity on GNA

GNA dissolved in a sucrose solution had a dose-dependent negative effect on longevity of female *A. ervi* ($F_{3,116} = 84.9$, $P < 0.0001$). Whilst females fed with a 2 M sucrose solution with 0 and 0.01% GNA (w/v) lived in average (± SE) for 21.80 ± 0.82 and 21.90 ± 0.44 days, respectively, longevity was already significantly compromised at 0.1% GNA to 14.87 ± 0.57 days (Tukey's HSD test, $P = 0.0001$). At 1% GNA, parasitoid longevity was again significantly reduced to 14.07 ± 0.56 days ($P = 0.0001$), which is a reduction of over 50% when compared to control females fed a sucrose solution devoid of GNA.

Artificial diet experiment

Dot blot of honeydew samples showed the presence of GNA in the honeydew at concentrations ranging from 0.01% to 0.04% (w/v), i.e. 10-40% of the concentrations consumed by the aphids (Figure 4.1). The strongest signal in the honeydew was seen after 24-48 h, but GNA was present in the honeydew over the five days of collection.

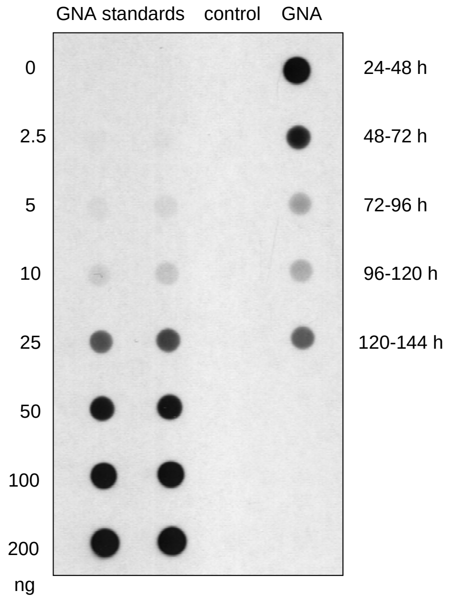

Figure 4.1 Immuno-blot of *Rhopalosiphum padi* honeydew from artificial diet. The first two columns contain standards with known amounts of GNA (indicated on the left). Wells in column three and four were loaded with 0.25 μl honeydew from aphids fed on control and GNA-containing diet respectively. The honeydew samples were collected over different periods of time since the aphids were put on the diet (indicated on the right).

Statistical analysis of female parasitoid longevity revealed that there was a significant difference between sucrose and *R. padi* honeydew ($F_{1,117} = 45.8$, $P < 0.00001$) as well as a GNA effect ($F_{1,117} = 30.9$, $P < 0.00001$) (Figure 4.2). The ANOVA showed no significant interactions between the two parameters ($F_{1,117} = 0.76$, $P = 0.38$). The addition of 0.1% (w/v) GNA to a 2 M sucrose solution reduced parasitoid longevity by 30%. Honeydew from *R. padi* on artificial diet without GNA reduced *A. ervi* longevity by 35% compared to a 2 M sucrose solution. When the aphid diet contained 0.1% (w/v) GNA, mean parasitoid longevity was reduced by another 47% compared to honeydew from aphids on a control diet. Even though the measured GNA content of the honeydew was only 10-40% of the original 0.1% (w/v) GNA in the aphid diet, the effect of the honeydew from *R. padi* on GNA diet on the longevity of *A. ervi* was considerably more than expected based on the reduction due to the addition of 0.1% GNA to a sucrose solution (30% reduction).

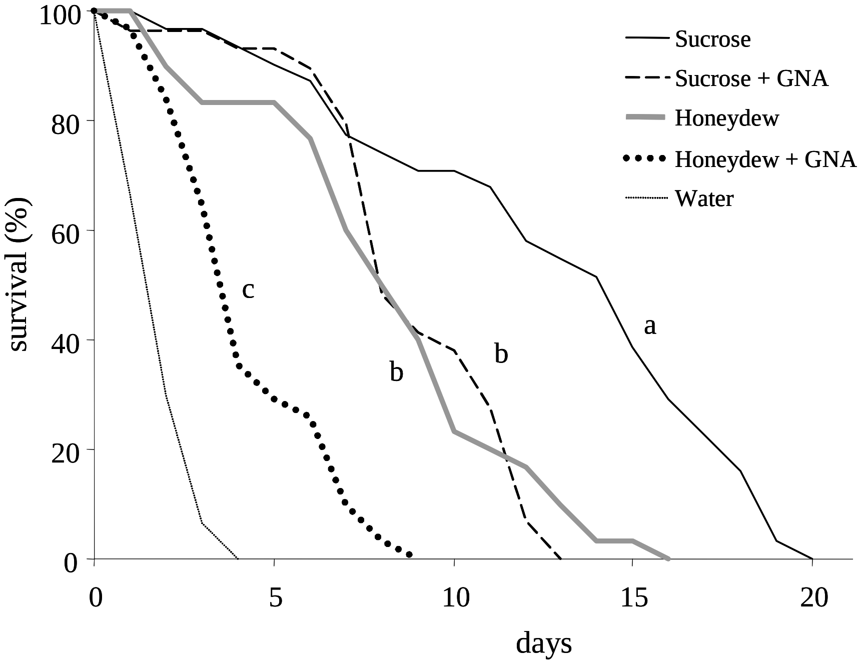

Figure 4.2 Survival percentage of female *Aphidius ervi* when fed a 2 M sucrose solution, a 2 M sucrose solution with 0.1% GNA (w/v), or honeydew from *Rhopalosiphum padi* feeding on an artificial diet without or with 0.1% GNA (w/v) (n = 29-31). Different letters indicate significant differences among the square root transformed data (Tukey's HSD-Test; $P < 0.05$). The water treatment served as a control and was not included in the analysis.

Since it was difficult to estimate the exact amount of honeydew in the honeydew samples, the sugar and amino acid composition of the honeydew was measured as percentage of total sugar / amino acids measured. There were slight differences in honeydew composition between honeydew from *R. padi* feeding on control diet or diet containing 0.1% GNA (Figure 4.3). The plant-derived phloem sugars, sucrose, glucose and fructose, were the most prevalent constituting 67% of the sugars in honeydew from control diet and 69% in honeydew from GNA-containing diet (Figure 4.3A).

A total of 17 amino acids were detected in the honeydew (Figure 4.3B). The average content of essential amino acids in the honeydew was 12% and 8% for aphids from the control diet and GNA diet respectively. This is substantially lower than the fraction essential amino acids (53%) in the aphid diets. The essential amino acid methionine could not be detected in any of the samples, even though this amino acid was present in the aphid diet. The non-essential amino acid glutamic acid (Glu) was the dominant amino acid in all treatments, accounting for 80% of the amino acid percentage in *R. padi* honeydew from control diet and 87% from GNA-diet (Figure 4.3B). In the aphid diet, glutamic acid only constituted about 4% of the total amino acid percentage.

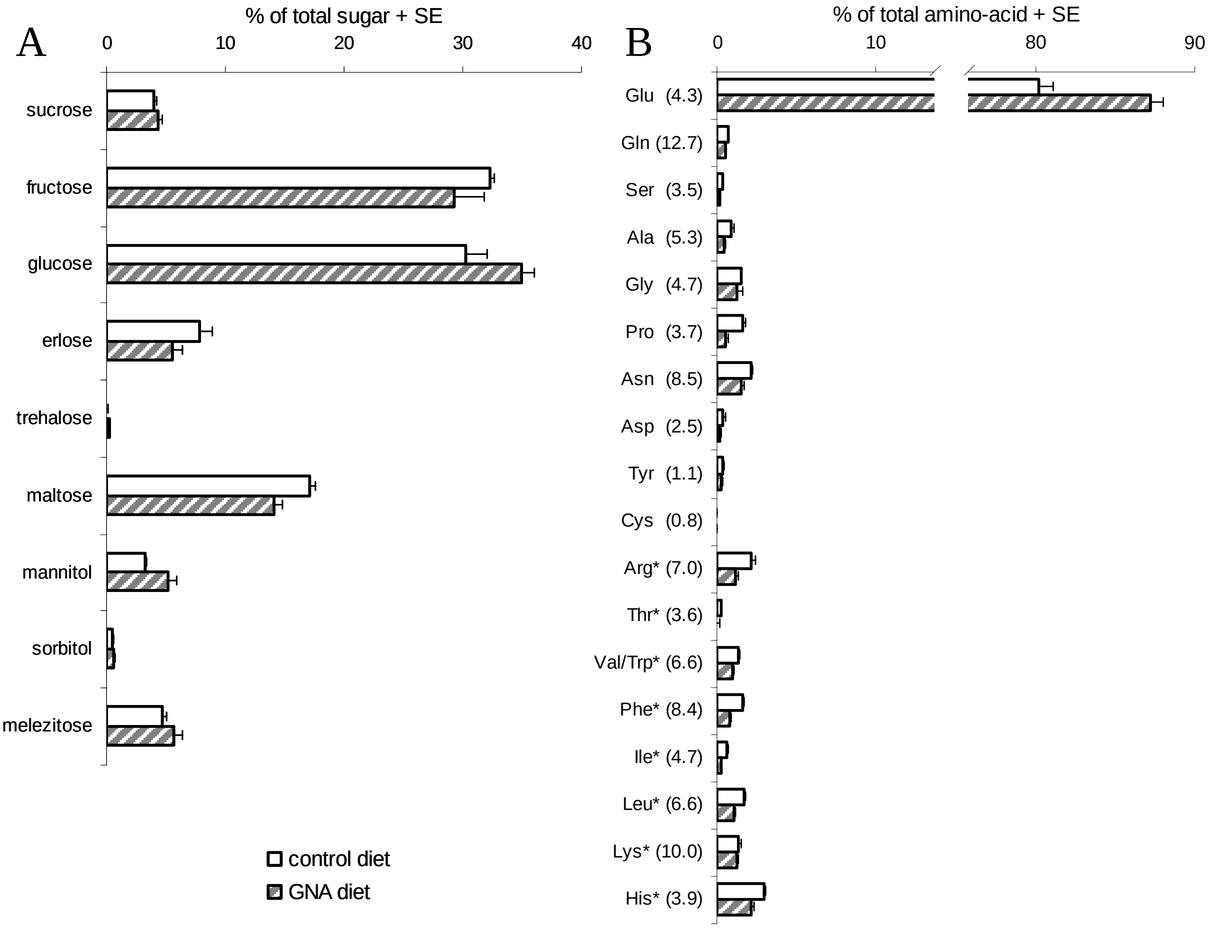

Figure 4.3 Relative amounts of (A) honeydew sugars (+SE) (*n* = 9-12), and of (B) honeydew amino acids (+SE) (*n* = 10-12) from *Rhopalosiphum padi* either feeding on a control artificial diet or on an artificial diet with 0.1% GNA (w/v). The essential amino acids are marked with an asterisk. The relative amounts of the individual amino acids in the aphid diet (%) are given in parentheses behind amino acids on the y-axis.

Multivariate statistics showed that for both sugar composition and amino acid composition the addition of GNA to the artificial diet had a significant influence on the sugar / amino acid composition (Figure 4.4). The time that the aphids had spent on the diet also had a significant influence on honeydew composition. The results of the ordination diagrams for the PCA of sugar and amino acid composition are shown in Figure 4.4A and B respectively. RDA on sugar composition revealed that 34% of the variation in the data could be explained by the presence of GNA in the diet (Monte Carlo permutation test; $F = 9.84$, $P = 0.001$, $n = 21$). When the factor time on the aphid diet was added to the model 49% of the variance was explained ($F = 5.43$, $P = 0.003$, $n = 21$), indicating that the honeydew sugar composition changes over time. For the amino acid distribution the presence of GNA in the diet explained 33% of the variance

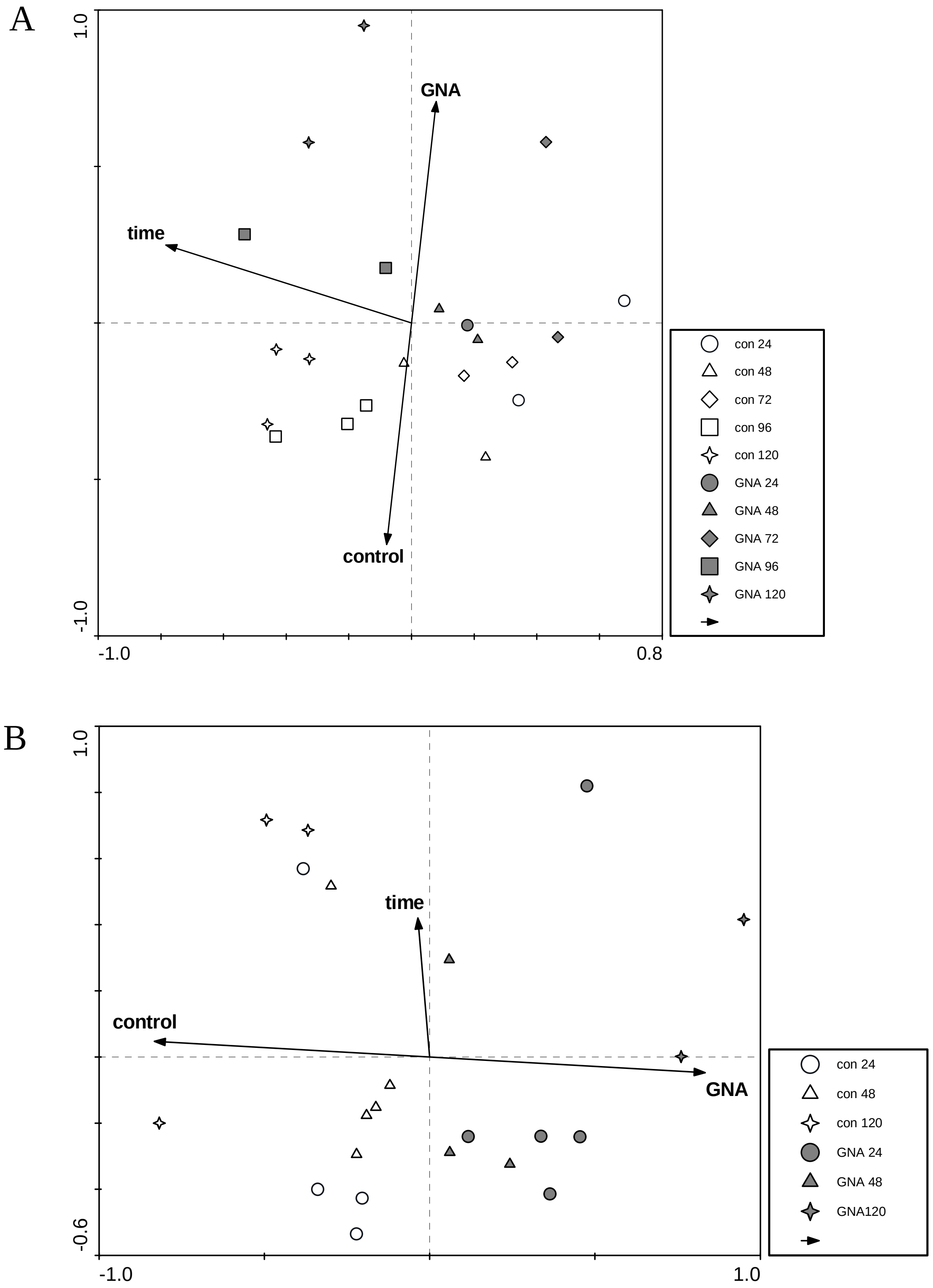

Figure 4.4 Distribution of (A) sugar composition and (B) amino acid composition of honeydew samples from *Rhopalosiphum padi* feeding on an artificial diet without (con) or with 0.1% GNA (w/v) (GNA), in the ordination biplot of a PCA. In addition, samples were labelled to the time the aphids had spent on the diet when honeydew was collected (24-48 h = 24; 48-72 h = 48, etc.). (A) Eigenvalues: axis 1: 0.516, axis 2: 0.282; (B) Eigenvalues: axis 1: 0.468, axis 2: 0.167. The vectors represent the treatment factors. Both sugar and amino acid data were expressed as percentage of total sugar / amino acid and the data were log-transformed.

($F = 9.91$, $P = 0.001$, $n = 22$), and when the factor time was added to the model 40% of the variance was explained ($F = 2.27$, $P = 0.025$, $n = 22$).

Aphid performance on GNA wheat

The average weight (± SE) of *R. padi* nymphs after four days was 352 ± 29 µg on control wheat and 352 ± 31 µg on GNA-transgenic wheat. For *S. avenae* the average weight (± SE) after six days was 621 ± 31 µg on control wheat and 637 ± 38 µg on GNA-transgenic wheat. In both cases there was no difference in average aphid weight between GNA-transgenic and control wheat (*R. padi*: $t = 0.0077$, df = 35, $P = 0.99$; *S. avenae*: $t = -0.33$, df = 32, $P = 0.75$). The GNA wheat also failed to show an effect on average (± SE) intrinsic rate of increase (r_m) of *R. padi* (control: 0.43 ± 0.010; GNA: 0.43 ± 0.009; $t = 0.25$, df = 36, $P = 0.81$) and *S. avenae* (control: 0.35 ± 0.007; GNA: 0.37 ± 0.008; $t = -1.61$, df = 34, $P = 0.12$). This indicates that levels of GNA expression were insufficient to cause any detectable effects on the target organism.

Parasitoid longevity on aphid honeydew from GNA wheat

Parasitoid longevity was affected by feeding treatment ($F_{4,149} = 10.3$, $P < 0.0001$) (Figure 4.5). *A. ervi* lived significantly longer on a 2 M sucrose solution than on honeydew from *S. avenae* (Tukey HSD test, $P < 0.0001$). Honeydew from *R. padi* was of intermediate quality, but significantly more suitable than honeydew from *S. avenae* ($P = 0.0497$). No effect of the genetic transformation on honeydew suitability could be detected for both *R. padi* ($P = 1.00$) and *S. avenae* ($P = 0.99$) (Figure 4.5).

GNA could not be detected in the honeydew collected from the aphids fed on GNA wheat. The detection limit of the dot blot was 0.0001% GNA (w/v). This confirms the low levels of GNA expression.

Again, there were only slight differences in honeydew composition between the two aphid species, but no differences were found between honeydew from GNA expressing and non-transformed plants (Figure 4.6). The plant-derived phloem sugars, sucrose, glucose and fructose, were once more the most prevalent. For *S. avenae* and for *R. padi* they constituted around 80% and 86% of the honeydew sugars, respectively (Figure 4.6A). It is noticeable that these percentages were much higher than the percentages found for honeydew from *R. padi* feeding on artificial diet. Honeydew from *S. avenae* had higher concentrations of the aphid-synthesized trisaccharide erlose and the disaccharide sucrose, whereas *R. padi* had higher concentrations of the two hexose components of sucrose, namely the monosaccharides fructose and glucose.

A total of 18 and 17 amino acids could be separated and identified in the honeydew of *R. padi* and *S. avenae* on wheat, respectively (Figure 4.6B). The honeydew from the four treatments showed high similarities in their amino acid composition. The average content of essential amino acids in the honeydew ranged from 7-21%. The essential amino acid methionine was present in the standards, but could not be detected in any of the samples. The non-essential amino acid glutamic acid (Glu) was once again the dominant amino acid in all treatments, accounting for around 30% and 50% of the amino acid percentage in *R. padi* and *S. avenae* honeydew, respectively (Figure 4.6).

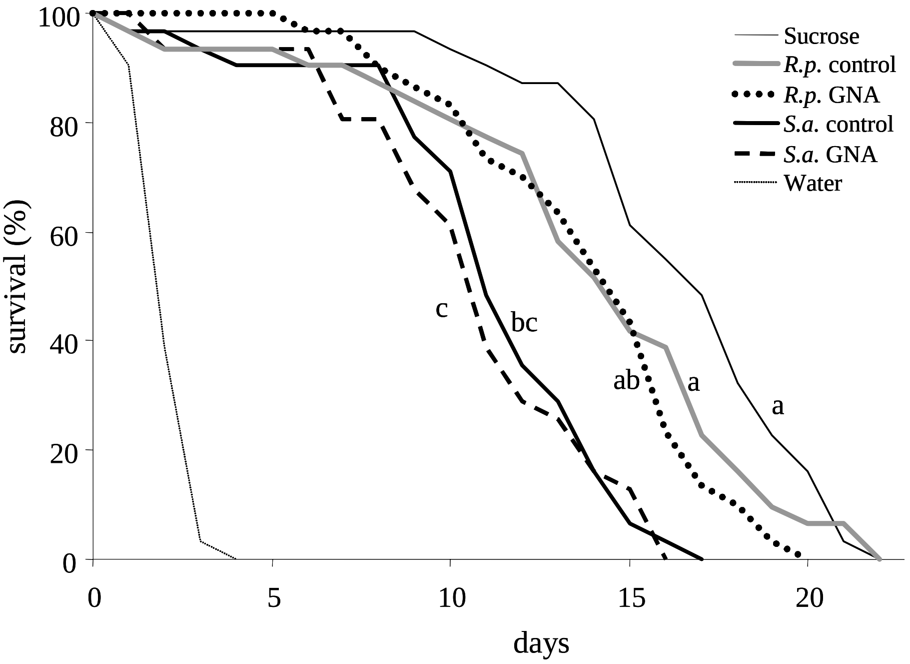

Figure 4.5 Survival percentage of female *Aphidius ervi* when fed a 2 M sucrose solution or honeydew from *Rhopalosiphum padi* (R.p.) or *Sitobion avenae* (S.a.) either feeding on control wheat plants or on GNA-transgenic wheat plants ($n = 30$). Different letters indicate significant differences (Tukey's HSD-Test; $P < 0.05$). The water treatment served as a control and was not included in the analysis.

In the PCA of sugar composition two clusters were evident: one comprising the honeydew from *S. avenae*, and a second one comprising the honeydew from *R. padi* (Figure 4.7A). RDA revealed that 50% of the variation in the data could be explained by the aphid species (Monte Carlo permutation test; $F = 40.79$, $P = 0.001$, $n = 42$). The addition of the treatment factor 'plant type' (control and GNA) did not make a significant contribution to the model ($F = 0.14$, $P = 0.99$, $n = 42$), and could only explain the variance by an additional 1%. A similar pattern was observed by the multivariate analysis of the amino acid distribution (Figure 4.7B).

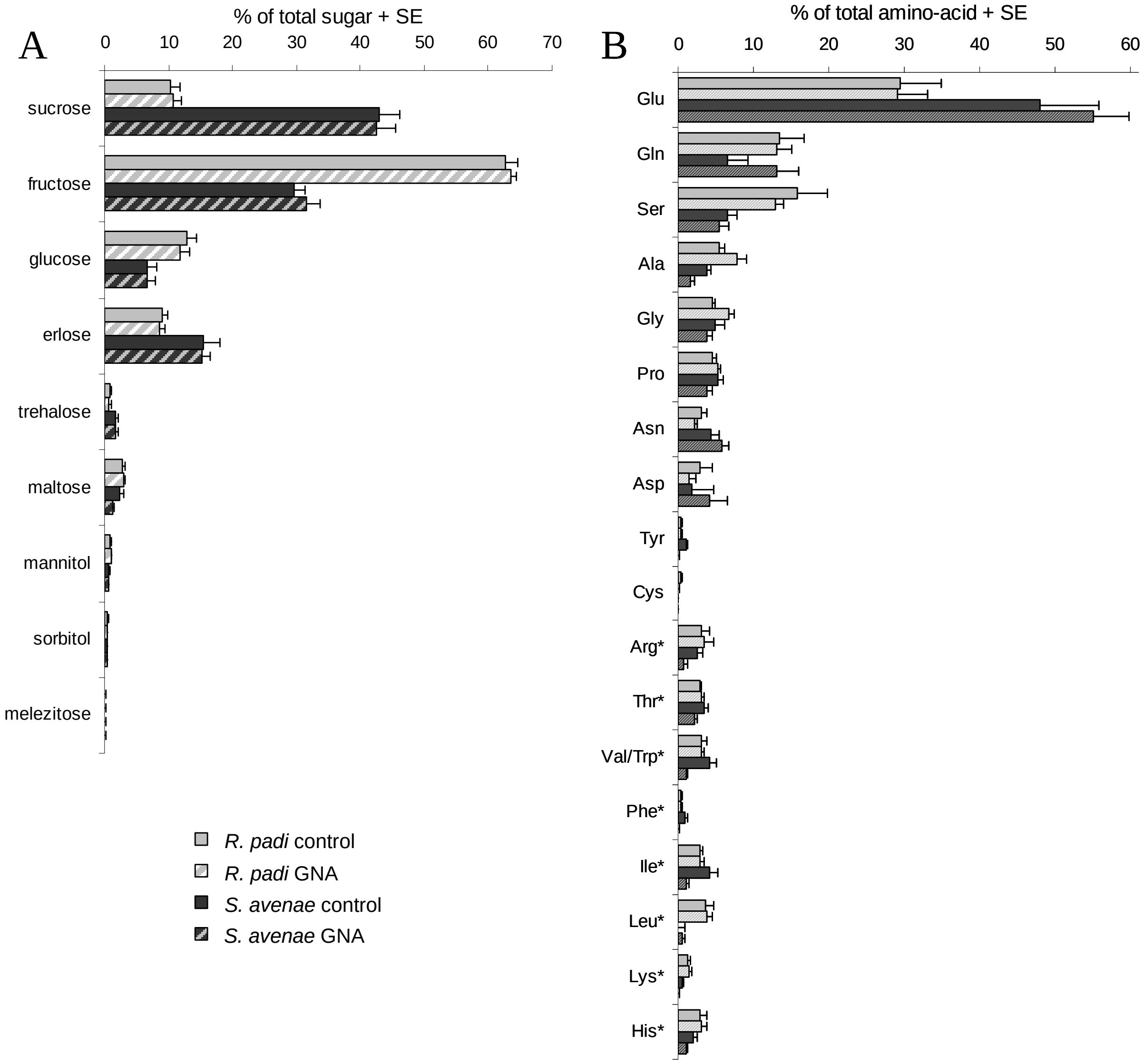

Figure 4.6 Relative amounts of (A) honeydew sugars (+SE) (*n* = 10-11), and of (B) honeydew amino acids (+SE) (*n* = 8-10) from *Sitobion avenae* or *Rhopalosiphum padi* either feeding on a control wheat plant or on a GNA-transgenic wheat plants. The essential amino acids are marked with an asterisk.

However, the separation of the two clusters formed by the factor 'aphid species' was not as clear for the amino acid composition. This was confirmed by RDA, where only 19% of the variation in the data could be explained by the aphid species ($F = 8.00$, $P = 0.001$, $n = 36$). The addition of the treatment factor 'plant type' explained the variance by a mere further 2%, which again was not significant ($F = 0.64$, $P = 0.76$, $n = 36$).

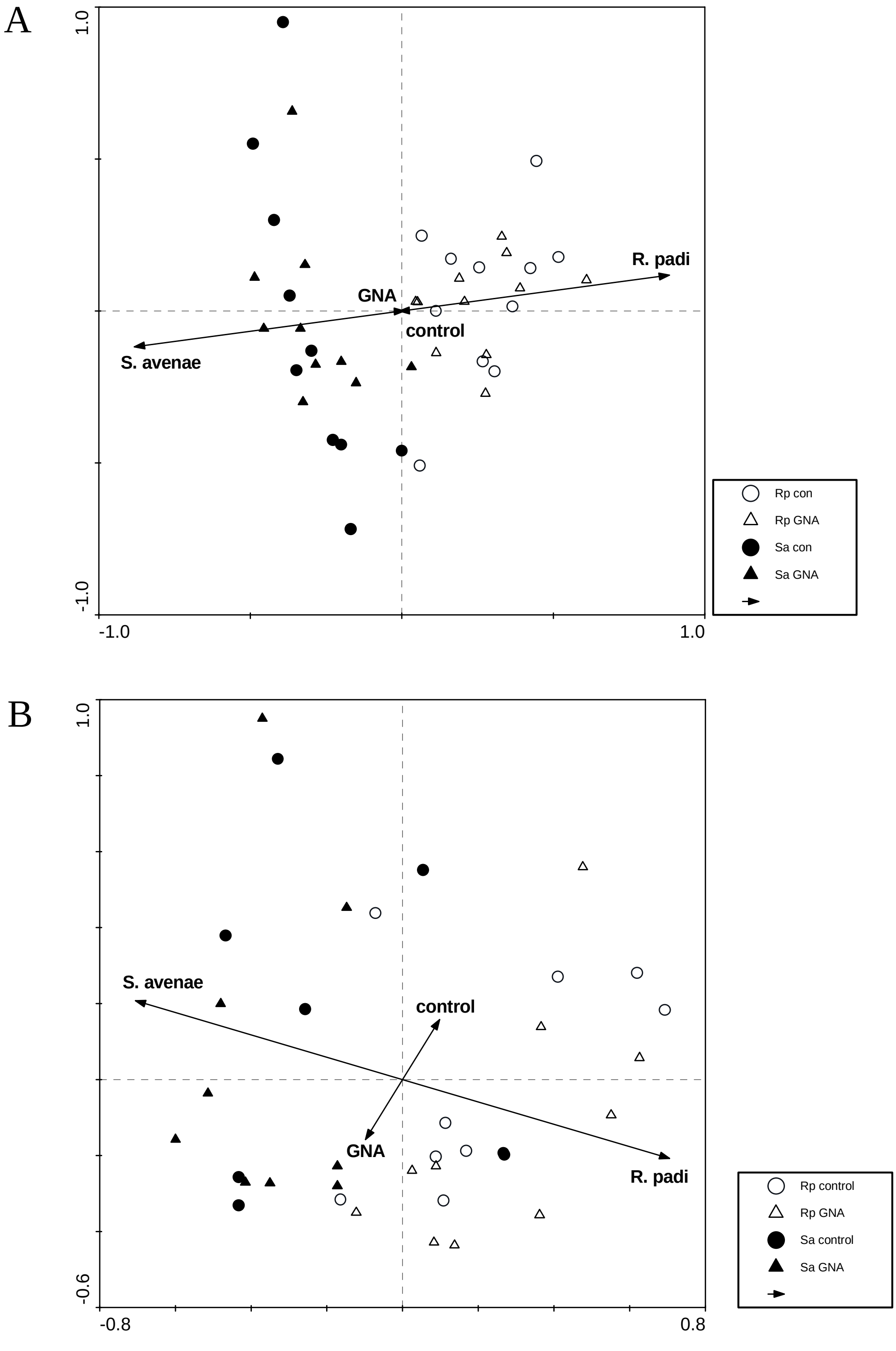

Figure 4.7 Distribution of (A) sugar composition and (B) amino acid composition of honeydew samples from *Rhopalosiphum padi* (Rp) or *Sitobion avenae* (Sa), feeding on GNA-transgenic (GNA) or control (con) wheat, in the ordination biplot of a PCA. (A) Eigenvalues: axis 1: 0.628, axis 2: 0.180; (B) Eigenvalues: axis 1: 0.328, axis 2: 0.203. The vectors represent the treatment factors. Both sugar and amino acid data were expressed as percentage of total sugar / amino acid and the data were log-transformed.

Discussion

Impact of GNA on *A. ervi* longevity

The gustatory experiment demonstrates that female *A. ervi* are not deterred from feeding by GNA. We can therefore exclude that effects of GNA on parasitoid longevity are due to reduced food uptake, indicating that such effects are caused by the protein itself (direct effect).

When effects of GNA dissolved in a sucrose solution were tested, it was found that GNA has a dose-dependent negative effect on female *A. ervi* longevity. A GNA concentration of 0.1% (w/v) in a sucrose solution is sufficient to cause a significant reduction in parasitoid longevity. Interestingly, this concentration is also the minimum concentration required to achieve a detectable impact on target aphids (Down et al. 1996, Couty et al. 2001c). At the highest GNA concentration tested (1% w/v) lifespan was reduced by over 50%. A similar dose-dependent effect on adult parasitoid longevity has been found when GNA was fed directly to *A. colemani*, *Cotesia glomerata* (L.), *Trichogramma brassicae* Bezdenko, and *Eulophus pennicornis* (Nees) (Romeis et al. 2003, Bell et al. 2004). These studies also show that GNA reduces fecundity in *T. brassicae* and *E. pennicornis*, but not in *A. colemani*.

Parasitization as a route of exposure to GNA, appears to have a less pronounced effect on hymenopteran parasitoids. Bell et al. (1999, 2001) found no adverse effects on *E. pennicornis* when developing in *Lacanobia oleracea* (L.) (Lepidoptera: Noctuidae) that had been feeding on GNA-expressing tomato or potato plants. Tomov and Bernal (2003), on the other hand, report that several life history parameters of the parasitoid *Parallorhogas pyralophagus* (Marsch) were affected when its host *Eoreuma loftini* (Dyar) (Lepidoptera: Pyralidae) was fed a diet containing GNA-transgenic sugarcane. When GNA was delivered to larvae of *Aphelinus abdominalis* (Dalman) (Couty et al. 2001a, c) and *A. ervi* (Couty et al. 2001b) via aphids fed on GNA-containing artificial diet, sublethal effects on some life history parameters were found. However, those studies reporting effects could not distinguish between the direct impact of GNA and indirect host-quality mediated effects. The present study suggests that a direct effect of GNA could have played a role, as adult *A. ervi* were found to be sensitive to the lectin.

Impact of GNA-containing honeydew on parasitoid longevity

When GNA was delivered to *R. padi* through an artificial diet, the honeydew excreted by the aphids did contain GNA at 10-40% of the concentration in the diet.

Passing of lectins and protease inhibitors through the digestive tract of phloem-sucking insects has been reported before for both when kept on artificial diets (Rahbé et al. 1995, Powell et al. 1998, Sauvion et al. 2004) or transgenic plants (Shi et al. 1994, Kanrar et al. 2002, Rahbé et al. 2003). The estimated GNA concentration in honeydew relative to the diet is not very precise, since the honeydew had been exposed to evaporation and re-absorption of water before collection. Powell et al. (1998) found that GNA concentration in the honeydew of the planthopper *Nilaparvata lugens* (Stål) can reach the GNA levels ingested by the sap feeder, however, results were very variable. The data by Powell et al. (1998) cannot be directly compared with our results, as the method of honeydew collection was not specified. Couty and Romeis (2004) estimated that aphids may excrete more than 90% of the ingested GNA in their honeydew.

GNA in aphid diet can have a twofold effect on the nutritional quality of the honeydew for honeydew-consuming arthropods. There can be a direct toxic effect of GNA as demonstrated by the 30% reduction in longevity of female *A. ervi* when fed 0.1% GNA in a 2 M sucrose diet. Alternatively, GNA appears to cause changes in the metabolic processes in the aphids resulting in changes in honeydew composition (see below). This second mechanism may explain the fact that honeydew from *R. padi* kept on artificial diet (containing 0.1% GNA) reduced *A. ervi* longevity by 47% compared to honeydew from control diet, even though the GNA concentrations in honeydew were only 0.01-0.04%. Another explanation could be that the honeydew from *R. padi* on artificial diet by itself already represents a suboptimal food source for the parasitoids. When GNA is added to the diet there might not only be an additive negative effect of GNA, but also a negative synergistic effect.

Impact of honeydew from GNA-transgenic wheat on parasitoid longevity

The results from the present study show that honeydew from both *R. padi* and *S. avenae* prolongs survival of *A. ervi*, however not as much as a 2 M sucrose solution. Moreover, there was an aphid species effect, whereby *A. ervi* performed better on honeydew from *R. padi* as compared to *S. avenae* honeydew.

Despite the fact that *A. ervi* was found to be sensitive to GNA, there was no effect on parasitoid longevity when feeding on honeydew from aphids on GNA-transgenic wheat. This was probably due to the fact that the transgenic plants were expressing the toxin at very low levels. We assume that if GNA was transported in the phloem, this was only at very low concentrations. Inefficient directing of GNA to the phloem sap and resulting limited effects on the targeted phloem feeder have been observed previously for GNA-transgenic rice and potatoes (Foissac et al. 2000, Couty et al. 2001b). This assumption was corroborated by the fact that GNA could not be detected in the honeydew of the aphids, and the fact that neither aphid species showed a reduction in weight or in intrinsic rate of increase on the GNA expressing plants.

Crop breeding by both conventional means and by genetic modification has the potential to generate 'unintended effects' (Cellini et al. 2004). This has for example been reported by Wakefield et al. (2006) who unexpectedly found positive effects of GNA-transgenic tomato on the tomato moth *L. oleracea*. In the present study with GNA-transgenic wheat, there was no indication of any unintended transformation-related effects on aphids or parasitoids.

Impact of honeydew composition on parasitoid longevity

A number of studies have shown that honeydew can vary considerably with respect to its nutritional quality. Whilst certain types of honeydew are equally suitable to parasitoids as nectar, honey or sugar solutions, others are clearly inferior (for references see Chapter 3). Both sugar and amino acid composition are key factors determining the nutritional value of honeydew (Wäckers 2000, 2005). When honeydew from the two aphid species *S. avenae* and *R. padi* on GNA-transgenic wheat or control wheat was analysed, it was found that there were some differences in honeydew sugar and amino acid composition. Compared to honeydew from *S. avenae*, honeydew from *R. padi* contained a slightly higher percentage of the most common phloem sugars: sucrose, glucose and fructose, with relatively higher levels of the two monosaccharides fructose and glucose. Longevity of female *A. ervi* was significantly higher on honeydew from *R. padi*, compared to honeydew from *S. avenae*, both feeding on wheat. *A. ervi* longevity on honeydew from *R. padi* feeding on artificial diet, however, was much shorter. This honeydew contained the lowest percentage of sucrose, glucose and fructose. These findings concur with earlier work by Wäckers (2000, 2001), showing that these three plant-derived sugars are more effective in enhancing parasitoid longevity than aphid-synthesized sugars. A similar pattern was observed when the amino acid composition of the honeydew was related to parasitoid longevity. The average percentage of essential amino acids was slightly higher for honeydew from *R. padi* (feeding on wheat), with a broader distribution over the different amino acids, compared to honeydew from *S. avenae*, where there was a much stronger dominance by glutamic acid. Honeydew from *R. padi* feeding on artificial diet contained the lowest average percentage of essential amino acids, as well as the highest dominance of glutamic acid. RDA and Monte Carlo permutation test showed significant correlation between aphid species and sugar and amino acid composition. PCA showed that sugar composition of *R. padi* and *S. avenae* are separated into two clusters. For GNA-transgenic and control plants no differences in sugar or amino acid distribution of the honeydew was discovered. This reflects the low expression levels of the GNA-wheat, but also indicates that the transformation event has not altered the sugar and amino acid composition of the phloem sap. When GNA was dissolved in the diet of *R. padi* it did have an influence on the honeydew composition. Not only did PCA, RDA and Monte Carlo permutation test reveal that the sugar and amino acid distribution were significantly altered, also the time the aphids had spent on the artificial diet had a significant influence on the honeydew composition. Honeydew production by aphids feeding on diet containing GNA was reduced relative to aphids on the control diet (data not shown). This indicates that the aphids consumed less of the GNA-containing diet. Similar effects have been described for other sap-feeding insects (Shi et al. 1994, Rao et al. 1998). This reduction in food uptake possibly resulted in a more complete assimilation of the available amino acids and other nutrients from the diet, explaining the lower concentration of essential amino acids in the excreted honeydew, as well as the changed sugar composition.

Ecological implications

The reduced nutritional quality of aphid honeydew produced on a GNA-containing diet, combined with a possible decrease in honeydew production as a result of an antifeedant action of GNA, could affect parasitoid survival under field conditions. In agricultural systems, where alternative food sources are often lacking, this can have distinct implications for parasitoid-host dynamics and biological control. The effect on parasitoids might exceed the effect on the phloem feeders, as the direct toxic effects of GNA on the parasitoids appear to be compounded by unfavourable changes in sugar and amino acid composition of the honeydew.

We only studied effects on longevity of female *A. ervi*, an endoparasitoid of aphids that doesn’t host-feed with a relatively high ovigeny index (a large proportion of mature eggs at emergence) (Jervis et al. 2001). We can argue that the reduced lifetime of the parasitoid on GNA-containing honeydew will translate in an overall reduction in total reproductive success, since the oviposition period of most of the Aphidiinae starts on the first day after emergence and lasts for 7-23 days depending on the species (Hågvar and Hofsvang 1991). For parasitoids with a more synovigenic mode of egg maturation (low ovigeny index) the effect of GNA-containing honeydew might be even more pronounced, since the fecundity of these parasitoids largely depends on the food uptake during the adult stage. Host-feeding parasitoid species might be affected twofold by GNA expressing plants, as they would both ingest GNA through honeydew, and by feeding on host haemolymph. However, haemolymph of GNA-fed aphid has been found to contain relatively low amounts of GNA (Powell et al. 1998, Fitches et al. 2001).

This is the first study showing that GNA in honeydew negatively affects honeydew-consuming parasitoids, not only directly due to the action of the insecticidal compound, but also indirectly through an altered honeydew composition. Whilst the present study clearly demonstrates that transgene products in honeydew may pose a hazard for biocontrol agents, further studies under (semi-)field conditions with plants expressing GNA at realistic levels are required to better estimate the actual risk of exposure to transgene products through honeydew (Dutton et al. 2003, Poppy and Sutherland 2004). Since honeydew can be an important route of exposure to transgene products for a wide range of non-target organisms, it should be considered in future risk assessment studies.

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Chapter 5

Detecting nutritional state and food source use in nectar/honeydew feeding insects that synthesize oligosaccharides[4]

Abstract

During the adult stage many arthropod species, including aphid predators and parasitoids, depend on sugar-rich foods such as nectar and honeydew for survival and reproduction. Despite the importance of sugar feeding for these organisms, we often know little about how they cover their nutritional needs under field conditions. Here we assessed the nutritional state of adult parasitoids, hoverflies and lacewings in a winter wheat (WW) and a spring wheat (SW) field in Switzerland and investigated the contribution of honeydew to the diet of these aphidophagous insects. The total sugar level and the glucose-fructose ratio were used as indicators for nutritional state and sugar feeding. More than 50% of the insects in all four insect groups collected and in both fields had recently consumed carbohydrates. The mean nutritional state was significantly higher in the SW field for *Chrysoperla carnea*, *Chrysoperla lucasina* and *Aphidius* spp. Honeydew consumption by insects is commonly investigated by analysing target insects for the presence honeydew 'signature' sugars, such as melezitose and erlose. However, our laboratory studies show that adults of the three insect orders investigated show synthesis of these 'honeydew specific' sugars after sucrose feeding. As the erlose-melezitose ratio of sucrose-fed insects differed clearly from the sugar profiles of honeydew from wheat infesting aphids, this ratio could be used as indicator for honeydew feeding by aphid parasitoids (*Aphidius* spp.) and the hoverfly *Episyrphus balteatus*, but not for the lacewings *C. carnea* and *C. lucasina*. Our data show that 63% (WW) and 64% (SW) of field-collected *Aphidius* spp. which showed evidence of sugar feeding could be classified as having consumed honeydew within the 24-48 h before collection. Evidence of honeydew feeding by hoverflies, on the other hand, was found to be much more variable, ranging from 0% in the WW field to 44% in the SW field.

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[4] Based on: Hogervorst P.A.M., Wäckers F.L. and Romeis J., Detecting nutritional state and food source use in nectar/honeydew feeding insects that synthesize oligosaccharides. In preparation

Introduction

Arthropod predators and parasitoids often require carbohydrate-rich foods, such as (extra-)floral nectar and honeydew, at least during part of their life cycle. One can distinguish ‘facultative’ consumers, that use plant-derived foods complementary to their prey, and ‘obligatory’ consumers which are entirely dependent on plant-provided foods for their survival and metabolic upkeep, usually during the adult stage (Wäckers and Van Rijn 2005). This second group includes many parasitoids (Jervis et al. 1996), aphidophagous hoverflies (Gilbert 1981, 1985), and some lacewings (Canard 2001).

Nectar and honeydew appear to be the most commonly exploited non-host foods for hymenopteran parasitoids (Heimpel and Jervis 2005, Wäckers 2005). Adult green lacewings have been reported to feed on honeydew, nectar and pollen (Hagen et al. 1970, Principi and Canard 1984, Canard 2001). Adult hoverflies on the other hand are thought to feed almost exclusively on nectar and pollen, in varying proportions according to species (Gilbert 1981, 1985), whereas honeydew feeding has only rarely been reported (Lyon 1965, Ssymank and Gilbert 1993, Hickman and Wratten 1996). However, Van Rijn et al. (2006) showed that honeydew of cabbage aphids enhanced the survival of adult *Episyrphus balteatus* (DeGeer) (Diptera: Syrphidae) in cage experiments.

In addition to the vital importance of honeydew as a source of energy (Haslett 1989, Wäckers 1994, Tenhumberg et al. 2006, Winkler et al. 2006), aphid predators and parasitoids can use honeydew as a host-location kairomone (Hågvar and Hofsvang 1991), and an oviposition stimulus (Hagen 1986, Budenberg and Powell 1992, Bargen et al. 1998). Given that pollen and nectar sources are often scarce in areas of intensive agriculture, honeydew is likely to be the primary exogenous carbohydrate source available (Wäckers 2005). However, honeydew can be very variable in its nutritional quality for honeydew consumers (see Chapter 3 for references), and can be far inferior to nectar and sucrose (Leius 1961a, b, Avidov et al. 1970, Elliott et al. 1987, Wäckers 2000).

Although honeydew is regarded as an important food source for aphidophagous predators and parasitoids, there is very little quantitative information on the use of honeydew in the field. A study by Wäckers and Steppuhn (2003) demonstrated that 80% of *Cotesia glomerata* (L.) and 55% of *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae) collected in a cabbage field had recently fed on honeydew. A recapturing study of released *Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae) demonstrated that the parasitoids were able to maintain a nearly constant level of energy over 48 h in a habitat, where honeydew appeared to be the only food source available (Casas et al. 2003).

Insight in the contribution of honeydew feeding to the diet of beneficial insect is also of importance to the risk assessment of genetically modified (GM) plants as honeydew has been found to be a potential route of exposure to insecticidal proteins expressed by GM plants (Chapter 4). In cases where the transgene product appears in honeydew, this information is important to assess the risks that this may pose to honeydew-feeding non-target organisms (Dutton et al. 2003).

The aim of the current study was to determine whether adult aphid predators and parasitoids feed on honeydew under field conditions. One method to detect recent honeydew feeding is by analysing the insect's sugar profile using high performance liquid chromatography (HPLC) as has been described for parasitoids (Wäckers and Steppuhn 2003, Steppuhn and Wäckers 2004) and sandflies (Macvicker et al. 1990). This methodology is based on the detection of honeydew specific 'signature sugars' (Macvicker et al. 1990, Byrne et al. 2003, Heimpel et al. 2004) in the study organism. These signature sugars are di- and oligosaccharides that are synthesized by phloem-feeding Sternorrhynchae and excreted in the honeydew. Until recently these sugars were believed to be 'honeydew-specific'. However, Wäckers et al. (2006) showed that the synthesis of melezitose and erlose is not restricted to honeydew producers, but that *Diadegma* spp. (Hymenoptera: Ichneumonidae) also produce these oligosaccarides following sucrose-consumption. Before analysing the field collected insects, laboratory studies were performed with the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae), the hoverfly *E. balteatus* and the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) to test for synthesis of 'honeydew specific' sugars. It was further investigated how long the sugars melezitose and erlose stay present in these insects after one feeding bout on a honeydew sugar mimic.

Materials and methods

Laboratory experiments

Experimental conditions

Laboratory experiments were conducted in a climatic chamber at 23 ± 1 °C, 85 ± 5% RH, with a 16:8 L:D photoperiod.

Insect material

Aphid mummies containing *A. ervi* and pupae of *E. balteatus* were received from Andermatt Biocontrol (Switzerland) and Katz Biotech AG (Germany), respectively. Eggs of *C. carnea* were collected from our permanent laboratory colony as described by Romeis et al. (2004), and freshly emerged larvae were placed in individual glass tubes (with a cotton wool plug) and provided with *ad libitum* eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), which were provided by Biotop (Valbonne, France). The glass tubes were kept at 22 ± 2 °C until larvae had pupated. Mummies and pupae of all three insect species were subsequently kept in a climate chamber until they hatched. Adults of *A. ervi* and *E. balteatus* were kept in cohorts in a cage, and were presumed to be mated, whereas *C. carnea* were kept in individual tubes, so were unable to mate.

Sugar spectrum upon emergence

Within one day after hatching, 10 unfed females of *C. carnea*, *A. ervi* and *E. balteatus* were individually put in an Eppendorf tube containing 250 µl (*C. carnea* and *E. balteatus*) or 100 µl (*A. ervi*) of 70% ethanol and subsequently stored at room temperature until analysis of the sugar spectrum by HPLC.

Sugar synthesis capacities

Within one day after hatching, 10 females of *C. carnea*, *A. ervi* and *E. balteatus* were individually placed in plexiglass Petri dishes (5 cm Ø, 1 cm high, containing a gauze window in the lid for ventilation) and provided with a 10 µl drop of a 2 M sucrose solution and kept in a climatic chamber for 48 h before being put in ethanol, as described above.

Sugar retention time

To investigate how long the ingested sugars in honeydew stay present in adult *C. carnea*, *A. ervi* and *E. balteatus*, insects were fed well-defined sugar solutions and subsequently analysed at 0, 12, 24 and 48 h following feeding.

Upon adult emergence the insects were kept in a cage in a climatic chamber for 16-40 h. Twenty females were provided with a 1 M sugar solution containing 60% fructose, 20% glucose, 10% sucrose and 10% erlose (molar concentrations), simulating the honeydew sugar spectrum of the aphid *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) on wheat (Table 5.3). A separate group of 20 females was provided an identical sugar solution but with 10% melezitose instead of erlose. For source and purity of sugars see Wäckers (1999).

Lacewings and hoverflies were picked up by their wings using soft forceps and offered a 3 µl drop of sugar solution until the whole drop was consumed. The parasitoids were placed in individual plexiglass containers (2 x 2 x 1.5 cm) containing a 2 µl drop of sugar solution for 1 h. All parasitoids were recorded feeding at least once. Subsequently, females were individually placed in plexiglass Petri dishes (5 cm Ø, 1 cm high, containing a gauze window in the lid for ventilation) and provided with water only. At 0, 12, 24 and 48 h following feeding five females of each species and each treatment were individually placed in an Eppendorf tube containing 250 µl (*C. carnea* and *E. balteatus*) or 100 µl (*A. ervi*) 70% ethanol. The hoverflies died of starvation within 48 h. Therefore the 48 h treatment was omitted for this group. Samples were stored at room temperature until HPLC analysis of the sugar spectrum.

Field experiment

Field site

The study was conducted in two wheat (*Triticum aestivum* L.) fields close to Zurich, Switzerland. Both fields were extensively managed without use of fungicides, insecticides and growth regulators. Field collections of parasitoids, hoverflies and green lacewings were done on dry and sunny days from 15 May - 3 July 2003 in a winter wheat field (WW), and from 29 May - 16 July 2003 in a spring wheat field (SW).

The two fields were situated approximately 2 km apart. Both fields were mainly surrounded by other agricultural fields. There were few flowering weeds in either field.

The first field (indicated as WW) contained winter wheat (cv Zinal) that was sown on 29 October 2002, and had a size of 1.8 ha. It was directly surrounded by potato and oilseed rape fields, an apple orchard and a road on one side. The oilseed rape was already past its flowering stage, when our field collection started. A mixed forest started a few hundred meters from the field.

The second field (indicated as SW) contained spring wheat (cv Greina) that was sown on 18 March 2003, and had a size of 2.0 ha. The field was directly surrounded by a winter and spring wheat field, a mixed forest and an area of fallow land with naturally emerged grasses and weeds.

Aphid counts

The aphid densities in both sampling fields were monitored weekly throughout the whole sampling period by counting the aphids on groups of five tillers in 20 randomly picked spots per field, located at least 20 m from the field border.

Honeydew collection

Honeydew samples were collected in the field from the three occurring aphid species: *Metopolophium dirhodum* Walker, *Sitobion avenae* (F.) and *R. padi*. Honeydew was collected during 24 h by placing a 3.4 cm Ø clip cage around a group of aphids on a leaf. Within the clip cage a small glass plate (3.0 cm Ø) was placed to collect the honeydew. In the laboratory, the honeydew was dissolved in 10 µl distilled water and collected with microcapillaries and subsequently diluted in 50 µl 70% ethanol to be stored at room temperature until HPLC analysis of the sugar spectrum.

Insect sampling

Adult hoverflies, hymenopteran parasitoids and lacewings were collected in the two fields by sweep net sampling. Insects were collected throughout the centre of the fields (20 m from the field borders). When caught, insects were directly transferred into individual Eppendorf tubes containing 250 µl (hoverflies and lacewings) or 100 µl (parasitoids) of 70% ethanol. Collected specimens were examined under a binocular for identification and to establish sex. Of the collected insects, the aphid parasitoids *Aphidius* spp., the aphidophagous hoverfly *E. balteatus*, and the green lacewings *C. carnea* and *C. lucasina* (Lacroix) (Neuroptera: Chrysopidae) (following the classification by Henry et al. 2002) were selected for HPLC analysis.

HPLC sugar analysis

To prepare the insect samples for HPLC analysis, the parasitoids were homogenized in the ethanol solution using a pestle, whereas the hoverflies and lacewings were mechanically homogenized using a mixer mill (Retsch, MM 301). Samples were first diluted by adding 500 µl Milli-Q water (Millipore, Amsterdam). Then 200 mg glass beads (Ø 3 mm) were added. Samples were vibrated for 15 min at 30 s^{-1}. All homogenized insect samples were diluted 10-fold before being analysed and honeydew samples were diluted 400-fold. Diluted samples were filtered (0.2 mu PTFE Alltech filter) prior to analysis. Of each sample 10 µl was injected into a Dionex DX 500 HPLC-system (Dionex Corp., Sunnyvale, CA). The system was equipped with a GP 40 gradient pump, a Carbopac PA1 guard (4 x 50 mm^2), and analytical column (4 x 250 mm), as well as an ED 40 electrochemical detector for pulsed amperimetric detection (PAD) (Dionex, Breda, The Netherlands). The column was eluted with 1 M NaOH and Milli-Q water (10:90%, 1 ml min^{-1}) and kept at 20 °C during analysis. Daily reference curves were obtained for sorbitol, mannitol, trehalose, glucose, fructose, melibiose, sucrose, maltose, raffinose, melezitose, and erlose by injecting calibration standards with concentrations of 2.5, 5, 7.5. and 10 p.p.m. of each of these sugars. After 10 samples an additional standard was run to check for deviation from the calibrated values. The concentrations of the individual sugars were analysed using the program CHROMELEON Version 6.40.

Interpretation of HPLC data

Insect nutritional state and recent sugar feeding

To establish whether the field collected insects were 'fed' or 'unfed', the nutritional state and recent sugar feeding were used as classification parameters, measured by total sugar content (μg/insect) and the glucose-fructose ratio respectively. The overall sugar content of an insect was obtained from the sum of the concentrations of the individual sugars. The glucose-fructose ratio was calculated as the glucose fraction of the sum of both monosaccharides. The average total sugar level and the average glucose-fructose ratio of the different insect groups were compared between the two fields using Student's *t*-test (data were transformed when necessary to fulfil normal distribution and equal variance assumptions). For insect groups where a significant difference between the fields was found, the relation between aphid density and average total sugar level or average glucose-fructose ratio was investigated using linear regression analysis.

The threshold level to classify the insect as either 'fed' or 'unfed' was set as the maximum total sugar level and the minimum glucose-fructose ratio of the unfed control insects from the laboratory experiment The threshold levels for the total sugar content and glucose-fructose ratio were 4.32 μg/wasp and 0.71 for *A. ervi*, 251 μg/fly and 0.94 for *E. balteatus*, and 59.2 μg/lacewing and 0.85 for *C. carnea* and *C. lucasina*. Insects were classified as 'unfed' when the total sugar level was below the established threshold and glucose-fructose ratio was above the threshold. The total sugar level and glucose-fructose ratio of insects that were starved for 48 h (24 h for *E. balteatus*) after previous feeding could not be distinguished from individuals that had never fed before (Table 5.1; Figure 5.1).

Honeydew feeding

As sucrose-fed insects in our study were able to synthesize erlose and melezitose, the mere presence of these sugars could not be used as indicator for honeydew feeding. However, as the honeydew of the three aphid species in our system is highly erlose dominated, whereas *A. ervi* and *E. balteatus* synthesize more melezitose than erlose (Table 5.3), the erlose-melezitose ratio could be used as an indicator for honeydew feeding in these two species. The erlose-melezitose ratio was calculated as the erlose fraction of the sum of both sugars. When the erlose-melezitose ratio in the insect exceeded a defined threshold level, the insect was classified as 'honeydew-fed'. This threshold was set as the maximum erlose-melezitose ratio that was found in the insects that were sucrose-fed for 48 h, and was 0.64 for *Aphidius* spp. and 0.48 for *E. balteatus*. This method could not be applied for the lacewings, since these insects generally synthesized erlose and melezitose in a similar ratio to that found in the different honeydews. The classification 'honeydew-fed' does not exclude the possibility that individuals may have also consumed nectar. Furthermore, the group 'honeydew-fed' only includes insects that have recently fed honeydew as honeydew feeding is not detectable anymore after 24-48 h due to hydrolysis of the sugars.

Results

Laboratory experiments

Sugar spectrum upon emergence

The HPLC sugar spectrum of newly emerged individuals of all three species was dominated by glucose, resulting in a high glucose-fructose ratio (Table 5.1). In addition, low levels of fructose and sucrose were present in all three species. The sugar alcohols mannitol (not in *A. ervi*) and sorbitol occurred as well. Maltose was sometimes detected in *A. ervi* and *E. balteatus*, but not in *C. carnea*, whereas trehalose and raffinose were only detected in *E. balteatus*. Melezitose, erlose and melibiose were not present in unfed individuals.

Table 5.1 Average total sugar level and average glucose-fructose ratio [glucose / (glucose + fructose)] upon eclosion, calculated from the HPLC sugar spectrum.

Insect species	Sex	Total sugar (µg/insect)			Glucose-fructose ratio		
		n	mean	SE	*n*	mean	SE
Aphidius ervi	♀	7	1.49	0.239	13	0.87	0.041
	♂	6	1.81	0.435			
Episyrphus balteatus	♀	10	96.5	13.59	20	0.98	0.003
	♂	10	143.8	15.78			
Chrysoperla carnea	♀	10	36.4	3.94	15	0.97	0.004
	♂	5	20.2	2.37			

Sugar synthesis

After *ad libitum* feeding on a 2 M sucrose solution for 48 h, total sugar level had dramatically increased in all three species, accompanied by a decrease in glucose-fructose ratio (Table 5.2) compared to unfed newly emerged adults (Table 5.1). All three insect species contained levels of melezitose and erlose after feeding on the sucrose solution. All *E. balteatus* produced higher levels of melezitose than erlose. This was also the case for 7 out of 10 *A. ervi*. *C. carnea*, on the other hand, produced erlose at twice the level of melezitose.

Table 5.2 Average total sugar level and average glucose-fructose ratio after 48 h of *ad libitum* feeding on a 2 M sucrose solution, calculated from the HPLC sugar spectrum.

Insect species	Sex	Total sugar (µg/insect)			Glucose-fructose ratio		
		n	mean	SE	*n*	mean	SE
Aphidius ervi	♀	10	24.73	5.614	10	0.62	0.054
Episyrphus balteatus	♀	5	2723	451.8	12	0.52	0.011
	♂	7	1838	390.0			
Chrysoperla carnea	♀	10	371.9	30.36	15	0.50	0.032
	♂	5	432.2	61.05			

Sugar retention time

After a single feeding bout of a 1 M honeydew sugar mimic average total sugar content increased 8- to 14-fold in *A. ervi*, 5-fold in *E. balteatus*, and 12- to 26-fold in *C. carnea*, compared to freshly emerged unfed specimens (Table 5.1). During a consecutive period of starvation, the sugar content of the insects dropped again to levels of 'unfed insects' after 24 h for *E. balteatus* and after 48 h for *A. ervi* and *C. carnea* (Figure 5.1A). Males of *A. ervi* tended to have a higher average total sugar level than the females, whereas for *C. carnea* males tended to have a lower average total sugar level than females after a couple of hours of starvation. For this reason the total sugar level was calculated separately for the two sexes of these two species.

The glucose-fructose ratio of all three species is below 0.5 shortly after feeding, but increases again over time (Figure 5.1B). After 24 h (*E. balteatus*) or 48 h (*A. ervi* and *C. carnea*) the glucose-fructose ratio is mostly back to that of unfed insects (see Table 5.1).

When fed with the sugar mixture containing erlose, *E. balteatus* digested this sugar completely (below detection limits) within 12 h. In the case of *A. ervi* and *C. carnea* this figure was 24 h. When fed with the melezitose-containing sugar mixture, this oligosaccharide was completely digested by the majority of *E. balteatus* and *C. carnea* within 24 h and 48 h respectively. However melezitose was still detectable in 50% of *A. ervi* after 48 h.

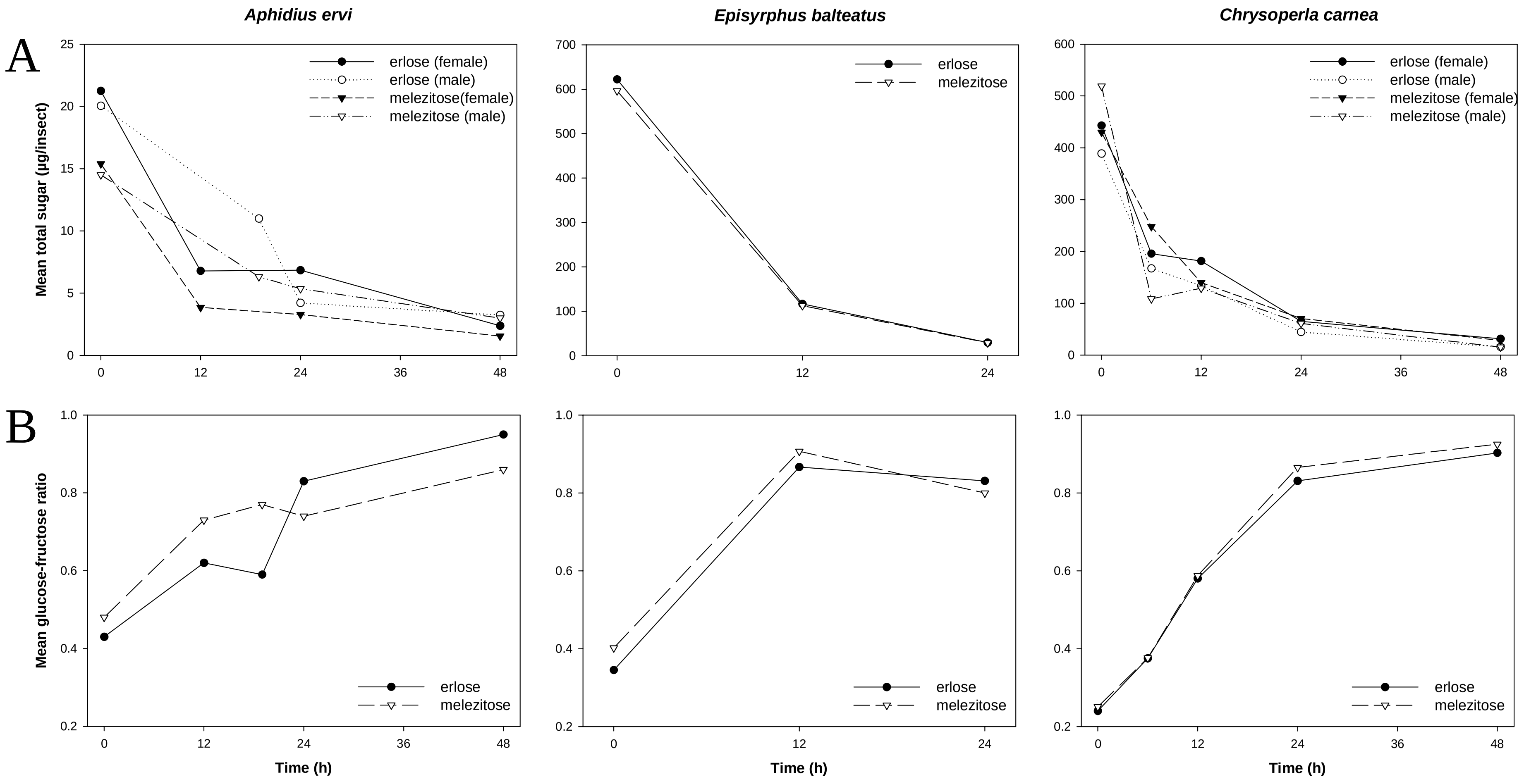

Figure 5.1 (A) Mean total sugar level and (B) mean glucose-fructose ratio of *Aphidius ervi*, *Episyrphus balteatus* and *Chrysoperla carnea* after one feeding bout of a 1 M sugar solution containing 60% fructose, 20% glucose, 10% sucrose and 10% melezitose or erlose (indicated as ‘melezitose’ and ‘erlose’). Insects were subsequently starved for 24 h (*E. balteatus*) or 48 h (*A. ervi* and *C. carnea*), and HPLC sugar spectrum was measured at several time points during this period. For *A. ervi* and *C. carnea* a distinction was made between mean total sugar levels of males and females.

Field experiment

Aphid counts

During the period of field collections the aphid densities ranged from 0.1 ± 0.10 (mean ± SE) to 10.2 ± 5.28 aphids / 5 tillers in field WW, and from 0.2 ± 0.13 to 5.7 ± 1.35 aphids / 5 tillers in field SW.

Three aphid species were present in our sampling plots: *M. dirhodum*, *S. avenae* and *R. padi*. *Metopolophium dirhodum* was the dominant species in the beginning of the sampling period (mid Mai – mid June), and *S. avenae* was the dominant species afterwards (mid June – mid July).

Honeydew composition

HPLC analysis of honeydew from the three aphid species infesting the wheat fields used for insect collection revealed that sucrose was the most prevalent sugar occurring in honeydew of *M. dirhodum* and *S. avenae*, whereas honeydew from *R. padi* is dominated by fructose (Table 5.3). Honeydew from all three aphid species only contains small amounts of melezitose (not in all samples), but a relatively high concentration of erlose. Honeydew from *S. avenae* contained the highest concentration of erlose at an average of 21% of the overall sugar content.

Insect nutritional state and sugar feeding

The average total sugar level and the average glucose-fructose ratio of insect groups that were analysed are shown in Table 5.4. These mean values allow comparison of the average nutritional state and sugar feeding of a group of insects between the two wheat fields. Student's *t*-test revealed significant differences between the total sugar and glucose-fructose ratio in the two fields for *C. carnea* (total sugar: $t = -4.43$, df = 98, $P < 0.0001$; glucose-fructose ratio: $t = 2.41$, df = 98, $P = 0.018$) and *C. lucasina* (total sugar: $t = -2.90$, df = 106, $P = 0.0046$; glucose-fructose ratio: $t = 3.64$, df = 106, $P = 0.0004$). For *Aphidius* spp. only a significant difference in the glucose-fructose ratio ($t = 5.14$, df = 116, $P < 0.0001$) was found. In all comparisons where a significant difference was found, insects were better fed in the SW field.

Table 5.3 Average HPLC sugar spectrum of (A) the honeydew from the three aphid species infesting the experimental fields, and of (B) adult insects after 48 h of *ad libitum* feeding on a 2 M sucrose solution. Average sugar composition of honeydew is given as percentage of overall sugars.

	Species	n	sorbitol	mannitol	trehalose	glucose	fructose	melibiose	sucrose	melezitose	raffinose	maltose	erlose
A	Aphid honeydew							(%)					
	Metopolophium dirhodum	5	0.99	0.19	11.43	3.87	21.68	0.00	58.81	0.52	0.00	0.28	2.23
	Rhopalosiphum padi	5	2.85	0.23	0.60	13.80	64.41	0.00	6.55	0.19	0.00	2.29	9.07
	Sitobion avenae	6	0.54	0.44	1.11	7.64	25.61	0.00	40.16	0.27	0.00	3.18	21.06
B	Sucrose fed insects							(µg/insect)					
	Aphidius ervi	10	0.47	0.00	0.01	7.16	4.12	0.00	12.40	0.25	0.01	0.16	0.15
	Episyrphus balteatus	12	13.40	0.46	3.29	858.54	832.16	0.00	243.91	79.24	0.59	142.67	32.32
	Chysoperla carnea	15	4.22	2.61	0.31	140.38	151.32	0.00	36.82	10.64	2.15	18.93	24.62

By comparing the total sugar level and the glucose-fructose ratio values of the field collected individuals with the threshold values for ‘unfed’ and ‘fed’ insects, the percentage of insects that had fed was calculated (Figure 5.2). The threshold levels were set as the highest total sugar level and lowest glucose-fructose ratio of the unfed controls, and only the individuals that scored ‘negative’ for both parameters were classified as ‘unfed’. For *C. lucasina* only unfed controls from field collected pupae were available and these data were highly variable, showing three clear outliers for the glucose-fructose ratio (Figure 5.2). Therefore, threshold levels from the closely related *C. carnea* were used for this species. For *E. balteatus* the separation between ‘fed’ and ‘unfed’ individuals was very clear, showing only a few individuals that scored differently for the two parameters. *C. carnea* and *C. lucasina*, on the other hand, both contained a rather large group that was classified as ‘fed’ based on the raised total sugar levels, but that did not have a reduced glucose-fructose ratio. For *Aphidius* spp. it was most difficult to distinguish ‘fed’ and ‘unfed’ individuals. There is great variability in total sugar level and glucose-fructose ratio within the two control groups, and the unfed and fed controls are not separated very strongly, especially for the glucose-fructose ratio. Moreover, most field collected parasitoids tended to have relatively low total sugar levels. The percentages of insects that had were classified as ‘fed’ were 51.2% and 82.4% for *Aphidius* spp., 85.7% and 89.3% for *E. balteatus*, 97.7% and 98.2% for *C. carnea*, and 93.3% and 97.9% for *C. lucasina* in field WW and field SW, respectively.

Honeydew feeding

For *Aphidius* spp. and *E. balteatus* we could further attribute food source to the ‘fed’ insects by looking at the erlose-melezitose ratio. The results presented in Figure 5.3 show that of the individuals classified as ‘fed’ 62.8% (field WW) and 64.3% (field SW) of *Aphidius* spp. and 0% (field WW) and 44.0% of *E. balteatus* had recently consumed honeydew.

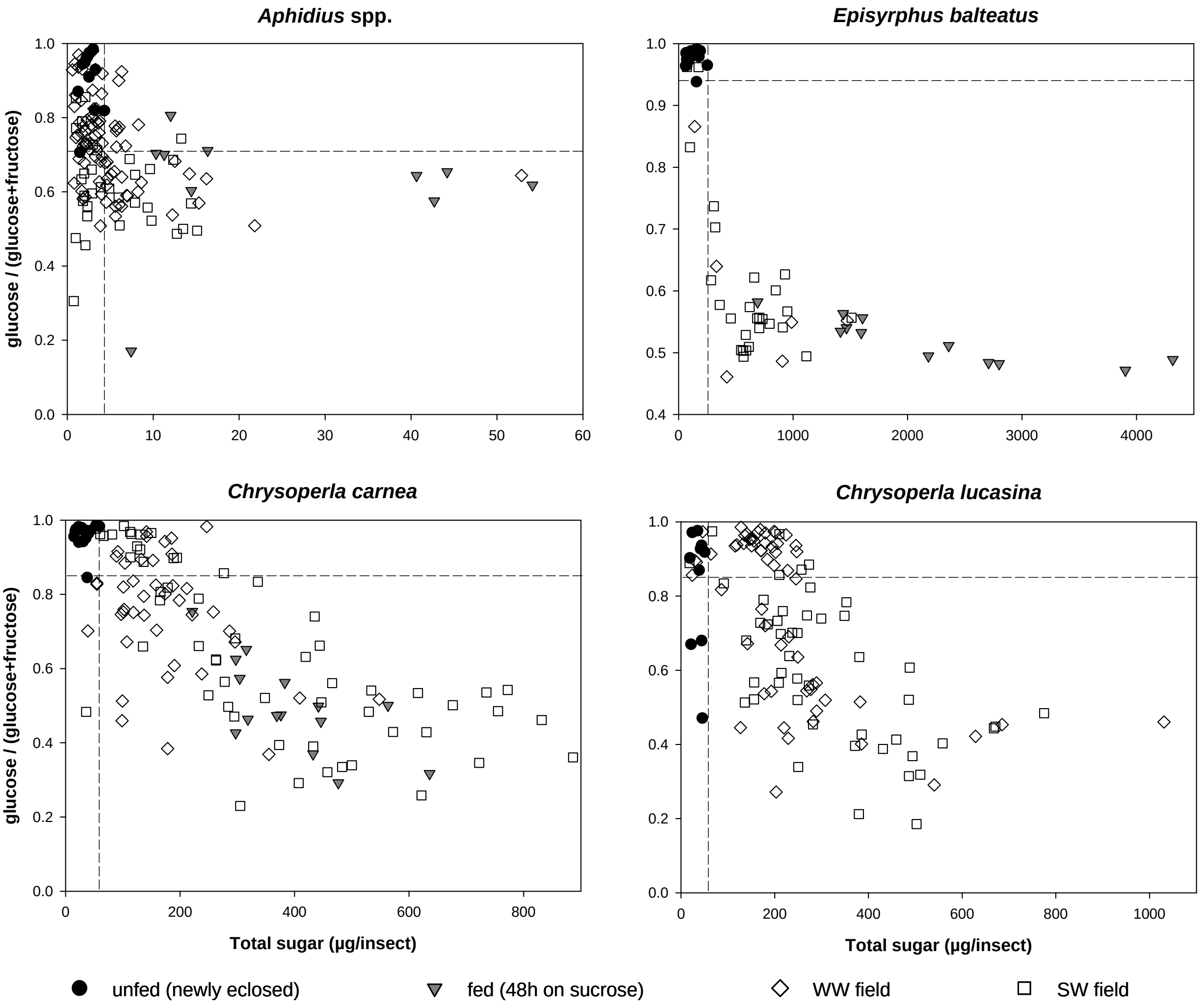

Figure 5.2 Plots of the total sugar content (µg/insect) *vs* the glucose-fructose ratio of adults from field collections of *Aphidius* spp., *Episyrphus balteatus*, *Chrysoperla carnea* and *Chrysoperla lucasina* in a winter wheat (WW) and a spring wheat field (SW) in Switzerland. Unfed controls were freshly emerged adults from the laboratory experiments (*Aphidius* spp., *E. balteatus*, *C. carnea*) and from pupae collected in the field (*E. balteatus*, *C. carnea*, *C. lucasina*). Well fed positive controls had *ad libitum* access to a 2 M sucrose solution during 48 h. Thresholds (indicated by dashed lines) were estimated from the highest total sugar level and lowest glucose-fructose ratio of the unfed controls, and separate the unfed individuals (box top left). For *C. lucasina* thresholds from the closely related *C. carnea* were used, since no laboratory data were available and field collected controls showed very variable results.

Table 5.4 Average total sugar level and average glucose-fructose ratio as detected by HPLC analysis of four groups of insects caught in a winter wheat (WW) or a spring wheat (SW) field. Significant differences between the two fields are marked with an asterisk (Student's *t*-test, $P < 0.05$).

Insect group	Field	Number of insects	Total sugars (µg/insect)		glucose-fructose ratio	
			Mean	SE	Mean	SE
Aphidius spp.	WW	84	5.00	0.704	0.74 *	0.013
	SW	34	5.69	0.778	0.61	0.020
Episyrhus balteatus	WW	7	627	191.5	0.65	0.076
	SW	28	602	61.4	0.62	0.094
Chrysoperla carnea	WW	43	170 *	15.1	0.76 *	0.024
	SW	57	348	29.9	0.64	0.030
Chrysoperla lucasina	WW	60	223 *	20.8	0.76 *	0.028
	SW	48	311	23.6	0.61	0.029

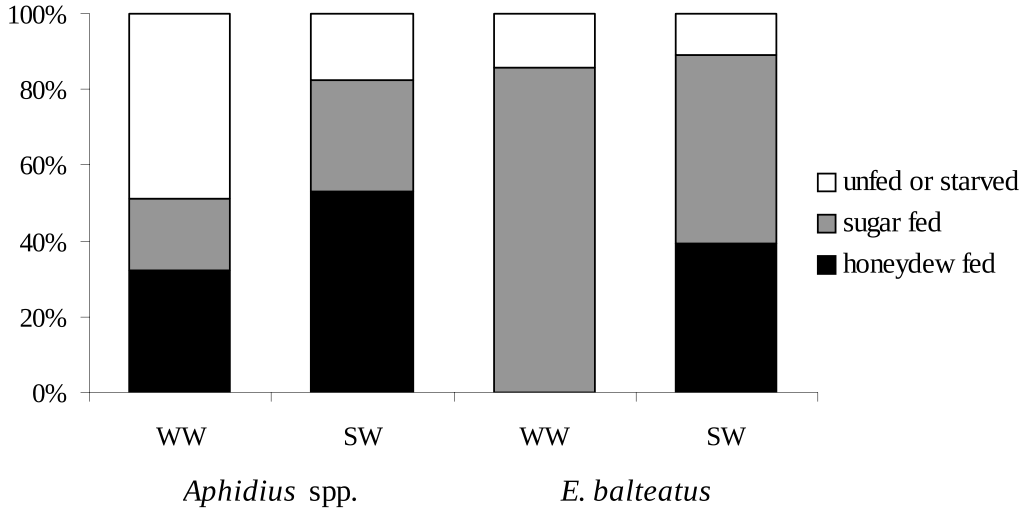

Figure 5.3 Feeding history and estimated food source of *Aphidius* spp. and *Episyrphus balteatus* collected in a winter wheat (WW) and a spring wheat field (SW) in Switzerland (see Table 4 for number of insects analysed). Insects classified as 'unfed or starved' had no elevated sugar level and can include insects that have never fed or that have fed previously but were starved for at least 1-2 days (Figure 2). Of the adults that were classified as 'fed', individuals with an erlose-melezitose ratio [erlose / (erlose + melezitose)] above the threshold (0.64 for *Aphidius* spp. and 0.48 for *E. balteatus*) were classified as 'honeydew-fed', although this group does not exclude additional nectar feeding. For insects classified as 'fed' with a low erlose-melezitose ratio the sugar source could not be identified (referred to as 'sugar-fed').

Discussion

Synthesis of oligosaccharides

Wäckers et al. (2006) were the first to report the synthesis of melezitose and erlose by arthropods other than honeydew-producing Sternorrhynchae. The findings of the present study show that the synthesis of what has been commonly considered to be 'honeydew-specific' oligosaccharides is a more general phenomenon among sugar feeding insects. We were able to show synthesis of melezitose and erlose after feeding on a sucrose solution in three different insect orders. Although the examples of two parasitoids (*C. glomerata* and *M. mediator*) that have been established not to synthesize these sugars using the same sensitive methodology exist (Steppuhn and Wäckers 2004), our findings emphasize that the use of signature sugars to detect recent honeydew feeding is not as widely applicable as previously believed.

Methods to detect feeding status and honeydew feeding

Both the total sugar level and the glucose-fructose ratio were used as indicators for the feeding status of the three insect species studied. According to expectation, the total sugar level was found to increase upon feeding and to decrease during a period of starvation. The glucose-fructose ratio showed an opposite response. To distinguish fed from unfed insects, these two parameters have been used before to study sugar feeding in the parasitic wasps *C. glomerata* and *M. mediator* (Steppuhn and Wäckers 2004). However, these parasitoids differ from our insect species as their glucose-fructose ratio stays balanced, even after three days of starvation. Whereas the glucose-fructose ratio could thus be used to separate individuals that had fed earlier from those that had never fed in this earlier study, the ratio always returned to a strong glucose dominance within two days of starvation in the three insects tested in the current study. The total sugar level and glucose-fructose ratio in freshly emerged insects was variable (Table 5.1, Figure 5.2). Under these conditions the joint use of both parameters to establish feeding reduces the number of insects falsely classified as 'unfed'.

Even though the use of melezitose and erlose as signature sugars to detect honeydew feeding was precluded by the fact that our insects proved capable of synthesizing these sugars, in our system we were able to use the erlose-melezitose ratio as an indicator for recent honeydew consumption by *Aphidus* spp. and *E. balteatus*. Based on this method we could conclude that 63% of *Aphidius* spp. and 35% of *E. balteatus* had recently consumed honeydew. This is likely to be a conservative estimate for several reasons. Honeydew composition can vary among different aphid and plant species (Hendrix et al. 1992), but also within species

(Costa et al. 1999). It can therefore not be excluded that other honeydews might have been present in or near our collection fields which could not be detected due to a low erlose-melezitose ratio. Furthermore, honeydew feeding is no longer detectable after 24-48 h due to rapid hydrolysis of the sugars. The group of individuals classified as 'honeydew-fed' does therefore only include the insects that have recently consumed honeydew and is therefore likely to underestimate the importance of honeydew as a food source.

Whether or not the erlose-melezitose ratio can be used as an indicator of honeydew feeding depends on the honeydew sugar profile as well as the insects' sugar profile. In order to use this method, the honeydew sugar profile needs to be compared with the insects' sugar profile case-by-case, which means that laboratory testing of sugar synthesis capabilities is essential, as well as the collection of reference parameters to determine threshold values.

Sugar feeding and nutritional state of field collected aphidophagous insects

We compared average total sugar content and glucose-fructose ratio of field collected insects with the laboratory data of the freshly emerged and sucrose-fed individuals (Figure 5.2, and Table 5.1, 5.2 and 5.4 for average values). Values from the freshly emerged individuals show the baseline sugar levels in unfed individuals and these low initial levels will drop further with age in absence of feeding (Steppuhn and Wäckers 2004). Values from the sucrose-fed individuals can be seen as maximum values as the combination of *ad libitum* provision of a 2 M sucrose solution and captivity in a small Petri dish results in a high food satiation of the insects. The comparison with these reference values shows that the adult aphid predators and parasitoids collected in the field had on average consumed sugars. Adults of *Aphidius* spp. from the two fields contained sugar levels that were on average 3.1 and 3.5 times higher than the initial total sugar levels, but 4.3 and 5.0 times lower than well fed laboratory adults, indicating that aphid parasitoids on average had a relatively low nutritional state. Total sugar level of *E. balteatus* had increased 5.2 and 5.0 times compared to newly eclosed hoverflies, but were still 3.5 and 3.7 times lower than sucrose-fed controls. However, this might also be due to the active nature of these insects in the field, causing a higher energy consumption compared to control insects kept in a small Petri dish. For *C. carnea* there was a large difference between the two fields, with an increase in total sugar level of 5.5 times for WW and 11.2 times for SW. Average total sugar levels of *C. carnea* in SW where close to those of the sucrose-fed controls (Figure 5.2), indicating that the adult lacewings in this field were on average very well fed. The comparison of glucose-fructose ratios between field collected insects and laboratory reared controls shows roughly the same trend as the comparison of total sugar levels (Figure 5.2).

Significant differences between the total sugar levels and the glucose-fructose ratio in the two fields were found for *C. carnea* and *C. lucasina*, and for *Aphidius* spp. between the glucose-fructose ratio in the two fields. The insects were better fed in the SW field. Reasons for this difference in nutritional state between the two fields remain speculative. As honeydew availability is dependent on aphid density, difference in aphid densities could possibly have played a role. There were very few flowering weeds in and next to the fields, so other sugar sources were scarce. Possibly, the fact that the SW crop was grown later in the season than the WW crop could have had an influence. Furthermore, hymenopteran parasitoids have been found to have of considerable foraging range (Lee and Heimpel 2003, Dorn et al. 2006). Even though a sampling margin of 20 m from the field border was kept, foraging beyond the field border cannot be excluded for the aphid parasitoids. The mobility of *Chrysoperla* spp. is even larger, including migration flights (Chapman et al. 2006) and continued nomadism throughout the reproductive period (Duelli 2001). It is therefore also possible that differences between the surrounding habitats of the two fields are responsible for the increased nutritional state in SW.

Attributing food source of field collected *Aphidius* spp. and *E. balteatus*

Our data show that 62.8% (field WW) and 64.3% (field SW) of *Aphidius* spp. that had been classified as 'fed' had recently fed on honeydew. It cannot be excluded that these parasitoids did feed on nectar sources in addition to honeydew. Nevertheless, these results demonstrate that aphid honeydew is an important food source for aphid parasitoids in a wheat field. Honeydew from the main aphid species in wheat has previously been demonstrated to be a suitable food source for these parasitoids (Chapter 3). The percentages found in our study were in the same range as was previously found in a cabbage field for the non-aphid parasitoids *C. glomerata* (80%) and *M. mediator* (55%) (Wäckers and Steppuhn 2003).

Verifiable honeydew feeding by hoverflies was found to be much more variable, ranging from 0% in the WW field to 44.0% in the SW field. However, only a relatively small number of samples (Table 5.4) could be collected for this species. Since hoverflies are known to have a high flight activity and migration rate (Salveter and Nentwig 1993), it is not possible to give an explanation for this variability as we did not perform a detailed analysis of other possible food sources in the surrounding landscape. Adult hoverflies require pollen feeding for reproduction (Schneider 1948, Van Rijn et al. 2006) and they are often described as flower feeders (Hickman and Wratten 1996). However, since *E. balteatus* uses honeydew as a contact kairomone to initiate oviposition (Sutherland et al. 2001), honeydew might be a good energy source within a crop lacking nectar and pollen sources (Van Rijn et al. 2006). No experimental data could be collected for adult green lacewings. However, literature suggests that honeydew is the main food for adult lacewings (Hagen et al. 1970, Principi and Canard 1984, Canard 2001).

In conclusion, the majority of the field collected adult aphid antagonists had recently fed carbohydrates, as was determined by a raised total sugar levels and a decreased glucose-fructose ratio. The erlose-melezitose ratio could be used in this aphid-wheat system to detect recent honeydew feeding by *Aphidius* spp. and *E. balteatus*. Honeydew feeding in a wheat system was found to be most important for the aphid parasitoids. For *E. balteatus*, honeydew feeding was found to be much more variable. These hoverflies, however, are likely to make facultative use of honeydew when searching for oviposition sites.

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Chapter 6

The importance of honeydew for larvae of *Chrysoperla carnea* when aphids are available[5]

Abstract

Larvae of the common green lacewing *Chryoperla carnea* are predacious and feed on a wide range of small, soft-bodied arthropods. Despite their dependence on prey arthropods for growth and development, the consumption of non-prey foods such as honeydew has been reported for lacewing larvae. It is commonly believed that these food supplements are primarily exploited by the larvae when prey is scarce or of low nutritional quality. Here we assess whether *C. carnea* larvae use honeydew, in the presence of high-quality aphid prey. In a choice experiment the feeding behaviour of *C. carnea* larvae was observed in the presence of both aphids and honeydew. The larvae were starved, aphid-fed, or honeydew-fed prior to the experiment. The time spent feeding on honeydew compared to feeding on aphids was highest with starved larvae and lowest with honeydew-fed larvae. The aphid-fed larvae spent the most time resting and the least time searching of the three treatments. In an additional experiment weight change was assessed when larvae were provided with an *ad libitum* supply of either aphids or honeydew. Larvae yielded a significant lower relative weight increase on honeydew compared to aphids. The reduced weight increase on honeydew was compensated when larvae were subsequently provided with aphids, but not when honeydew was provided again. This study indicates the importance of alternative foods by demonstrating that larvae of *C. carnea* still use honeydew as a food source in the presence of suitable prey.

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[5] Based on: Hogervorst P.A.M., Wäckers F.L. and Romeis J., The importance of honeydew for larvae of *Chrysoperla carnea* when aphids are available. In preparation

Introduction

Larvae of the common green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) are active, efficient predators with a high prey consumption and excellent searching capacity (Sunby 1966, Bond 1980). They are therefore valued as effective biological control agents (Senior and McEwen 2001). In contrast to the larval stage, the adults are not predaceous, feeding instead on nectar, pollen and honeydew (Hagen et al. 1970, Principi and Canard 1984).

Chrysoperla carnea larvae feed on a wide range of small, soft-bodied arthropods including aphids (Principi and Canard 1984), but the consumption of non-prey foods such as sternorrhynchan honeydew, (extra-)floral nectar and pollen in addition to prey has also been reported (Downes 1974, Limburg and Rosenheim 2001). Despite their dependence on prey arthropods for growth and development, the omnivorous lacewing larvae may benefit from feeding on sugar-rich non-prey foods, because they can provide nutrients that increase longevity and prolong foraging activity (Sunby 1966, McEwen et al. 1993, Limburg and Rosenheim 2001, Patt et al. 2003).

A study by Patt et al. (2003) demonstrated that the inclusion of pollen and sucrose in a diet of low-quality prey (larvae of *Drosophila melanogaster* Meigen), decreased development time and generated larger adults of *C. carnea*. Limburg and Rosenheim (2001) showed that extrafloral nectar consumption was important for neonate lacewing larvae on cotton and increased strongly as the availability of aphid prey declined. Furthermore, McEwen et al. (1993) showed that the applications of artificial honeydew in the field would allow *C. carnea* to complete its development at lower prey densities. These examples show that *C. carnea* utilizes non-prey foods at times when high-quality prey is scarce. However, the question remains whether larvae of *C. carnea* would still consume non-prey food in the presence of high-quality prey.

The aim of the work reported here was to tackle this question by investigating whether *C. carnea* larvae still feed on honeydew, when aphids are available. Honeydew is an excretion product of sap-feeding Sternorrhynchae, which is dominated by sugars. In addition to plant-derived fructose, glucose and sucrose it commonly also contains aphid-synthesized sugars (Chapter 3, Wäckers 2000, 2005). Lower levels of amino acids (Chapter 4, Sandstrom and Moran 2001, Fischer et al. 2002) and other compounds, including some vitamins occur in honeydew as well (Hagen 1986). However, when honeydew is available in the field, the aphids that produced this honeydew are usually not far away. Aphids are generally considered a relatively high-quality (Principi and Canard 1984, Obrycki et al. 1989, Dutton et al. 2002) and common (Canard 2001) prey for larvae of *C. carnea*. Moreover, they also provide proteins and other nutrients essential for growth and developments (McEwen et al. 1993, Patt et al. 2003).

In a first (no-choice) experiment it was studied how previous honeydew or aphid consumption affects larval weight change with subsequent provision of one of these food sources. In a second (choice) experiment the feeding behaviour of *C. carnea* larvae was observed in the presence of both aphids and honeydew.

Materials and methods

Insects and honeydew

Eggs of *C. carnea* were collected from our permanent laboratory colony as described by Romeis et al. (2004), and were kept separately in a climatic chamber (23 ± 1 °C, 85 ± 5% RH, 16:8 L:D) until they hatched. Larvae were individually reared in plexiglass containers (2 x 2 x 1.5 cm) on an *ad libitum* supply of eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), which were provided by Biotop (Valbonne, France). Larvae that had moulted into the second instar (L2) overnight were removed from the food in the morning and placed individually into new plexiglass containers. In addition, larvae that had moulted into L2 in the morning were used for a second batch of experiments in the afternoon. Larvae were subsequently starved for 24 h before the experiment started.

A colony of the bird cherry-oat aphid *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) was kept as a continuous culture on 4-6 week old wheat plants in the glasshouse (24 ± 2 °C). In preparation of the bio-assays, aphids were collected form the rearing and placed in an empty Petri dish (5 cm diameter, 1 cm high) in a climatic chamber (23 ± 1 °C, 85 ± 2% RH) for 3 h to prevent honeydew production during the bio-assays.

Honeydew from *R. padi* was collected in the aphid culture by placing Petri dish lids under infested leaves during 24 h. Subsequently, a water-satiated piece of cotton wool was placed in the bottom part of the Petri dish for two hours, with the honeydew-sprinkled lid on top. Honeydew droplets were scraped together using a plastic spatula and then slightly diluted with 2-5 µl water (depending on the amount of honeydew). Diluted honeydew was collection with a 5 µl micropipette (Blaubrand® intramark, Brand GmbH + Co kG, Wertheim, Germany) and stored at –20 °C.

No-choice experiment

In this experiment the weight increase of larvae with access to aphids or honeydew during one hour was determined, as well as the weight increase with access to these food sources for a second hour. Second instar larvae were weighed individually on a microbalance (Mettler Toledo MX5, $d = 1$ µg; ± 2 µg). Subsequently, larvae were placed back into their plexiglass containers in a climatic chamber (23 ± 1 °C, 85 ± 5% RH) and provided with either 10 aphids of different developmental stages (*ad libitum*) or a 2 µl drop of honeydew (*ad libitum*). After one hour larvae were re-weighed and again provided either aphids or honeydew for another hour. After this second hour, larvae were weighed again. The four treatments were (i) aphids in first and second hour, (ii) aphids in first hour, honeydew in second hour, (iii) honeydew in first hour, aphids in second hour, and (iv) honeydew in first and second hour. In addition, a control treatment consisting of no food during the first and second hour was added. A total of 37-38 replications were collected for each treatment. The relative weight increase after one or two hours of feeding was calculated as percentage of the initial weight ($t = 0$ h). This was done to be able to show the weight increase in the second hour independently of differences in weight increase in the first hour. Data on the relative weight increase were separately analysed for the two hours using one-way ANOVA. Means were subsequently separated using Tukey's HSD Test. All statistical analyses were computed in Statistica (version 6, Statsoft Inc., Tulsa, USA).

Choice experiment

The plexiglass containers with second instar larvae were randomly divided into three groups. During one hour before the start of the observations, the first group of larvae was provided with wet cotton wool whereas the second group was provided with three aphids (*ad libitum*) in addition to wet cotton wool, and the third group had access to a 3 µl drop of honeydew (*ad libitum*) and wet cotton wool.

A wet filter paper (5 cm ∅) with a wheat leaf disc (1 cm ∅) placed in the middle of the filter paper formed the experimental arena. The leaf disc contained four food sources distributed in the corners of a square. These food sources consisted of two diagonally opposite droplets of 1 µl honeydew and two diagonally opposite fourth instar *R. padi*. Aphids had had 15 min of settling time and the observations were aborted when an aphid would walk around the leaf disc. A *C. carnea* larva was placed in the arena on the filter paper in close vicinity of the leaf disc. The observation was started once the larvae had contacted the leaf disc. Larvae that did not find the leaf disc within 15 min were replaced. Using the software equipment 'The Observer', Base Package for Windows, Version 3.0 (Noldus Information Technology, Wageningen, The Netherlands) the feeding behaviour of the larvae was recorded. The position (on leaf disc or on filter paper) of the larva was monitored as well as its behaviour. Five activity parameters were distinguished: resting, searching, feeding on aphids, feeding on honeydew, and drinking from the wet filter paper. In addition, the number of aphids that were fed on was recorded. Aphids were not always completely consumed.

The observation was terminated when (i) the larvae left the experimental arena, (ii) one of the two food sources was finished completely, or (iii) after one hour. An observation period of one hour was chosen since feeding to repletion can occur within the first 30 min of exposure to food (Bond 1978). In total 16 replications were conducted for each of the three treatments.

The number of larvae that had fed on aphids, on honeydew, and that had fed in general were compared between the three treatments using χ^2 contingency tables (3 x 2 tables). Furthermore, it was investigated using χ^2 test whether the pre-treatment (starved, honeydew or aphid-fed) affected the choice of the first food source (honeydew or aphids). Differences in the number of aphids fed on by the larvae in the three treatments were analysed using Kruskall-Wallis ANOVA.

Results

No-choice experiment

The relative weight change as percentage of the initial weight of the larvae after the first and second hour are summarized for the five treatments in Figure 6.1. The no food treatment (control) showed a small but steady weight decrease during the two hours. For the other four treatments, one-way ANOVA of relative weight increase during the first hour showed significant differences among the treatments ($F_{3,147} = 8.46$, $P < 0.0001$). Subsequent mean comparison demonstrated a significant lower relative weight increase (Tukey's HSD-Test, $P < 0.01$) after an hour of honeydew feeding compared to aphid feeding (except for one out of four comparisons that showed a P-value of 0.053). ANOVA results of the second hour again showed significant differences between the treatments ($F_{3,147} = 22.35$, $P < 0.0001$). Tukey's HSD-Test revealed that the relative weight increase during the second hour of feeding was highest when feeding on aphids following an hour of honeydew feeding ($P < 0.0001$). After having fed on aphids during the first hour, there was no significant difference in relative weight increase between honeydew and aphid feeding in the second hour ($P = 0.17$). There was also no significant difference between the larval relative weight increase when feeding on honeydew during the second hour ($P = 0.83$), independent of the food source consumed during the first hour (Figure 6.1).

The total relative weight increase in two hours was 74% for larvae that had fed aphids during both hours, 42% for those that had only fed honeydew, 63% when aphids were consumed during the first hour and honeydew during the second hour, and 75% when honeydew was consumed during the first hour and aphids during the second hour.

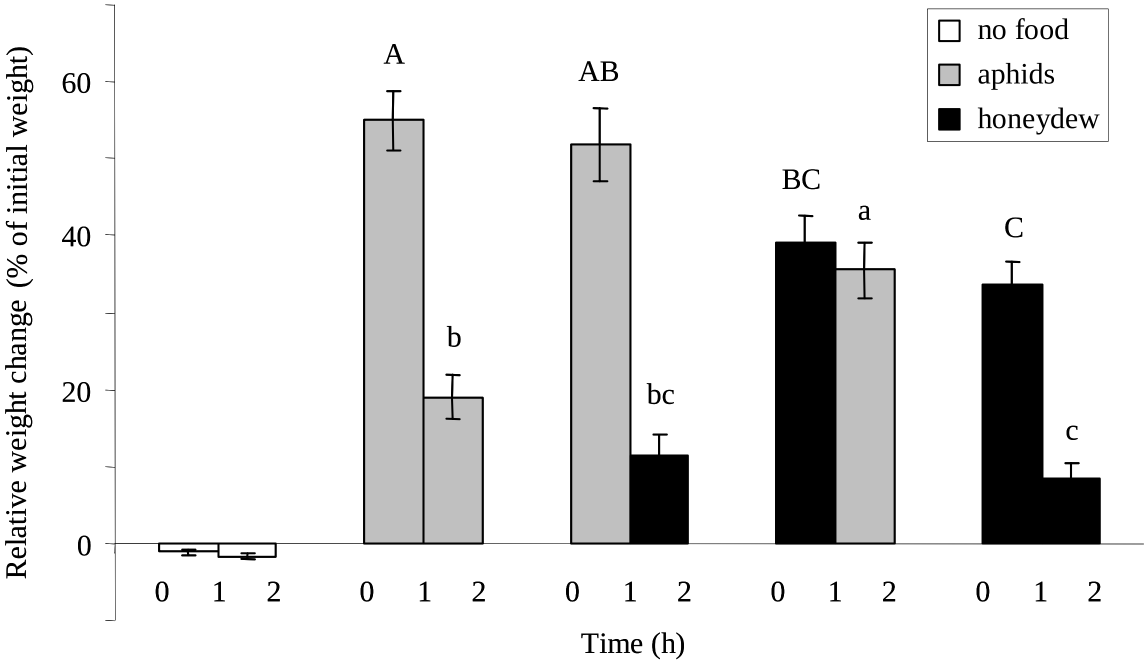

Figure 6.1 Relative weight change (to initial weight, t = 0 h) of second instar *Chrysoperla carnea* larvae, when fed on either aphids or honeydew for 1 h and subsequently on one of the food sources for a second hour ($n = 37\text{-}38$). Different letters above the bars indicate significant differences (One-way ANOVA, followed by Tukey's HSD-Test, $P < 0.05$; capital letters: feeding from 0-1 h; small letters: feeding from 1-2 h).

Choice experiment

When the *C. carnea* larvae were starved (but water satiated) prior to the experiment, 15 out of 16 larvae managed to find and feed on either honeydew or aphids during the experiment. This number was lower (however, not significantly; χ^2 test, $\chi^2 = 4.48$, $P > 0.1$) for larvae that had *ad libitum* access to aphids or honeydew in the last hour before the experiment (Table 6.1). Both starved and aphid-fed larvae showed no increased tendency to accept honeydew or aphids. For the honeydew-fed larvae, on the other hand, more larvae fed on aphids whilst only a few continued feeding on honeydew during the experiment (Table 6.1). This resulted in no significant difference in the number of larvae that fed on aphids among the three treatments ($\chi^2 = 5.25$, $P > 0.05$), however for honeydew feeding a significant difference among the treatments was found ($\chi^2 = 12.76$, $P < 0.05$). It was further investigated whether the larvae had a preference for aphids or honeydew as first food source. Although starved larvae fed more frequently on honeydew as a first food source, whereas honeydew-fed larvae more frequently started feeding on an aphid (Table 6.1), χ^2 tests revealed no significant differences ($P > 0.05$). Aphid-fed larvae showed the lowest tendency to attack aphids during the experiment, whereas starved larvae showed the highest aphid consumption (Table 6.1), however, these differences were not statistically significant (Kruskall-Wallis ANOVA, $H_{2,\,n=48} = 5.14$, $P = 0.077$).

Table 6.1 Summary of the feeding behaviour observations in the choice experiment of second instar *Chrysoperla carnea* larvae that had been provided either with wet cotton wool (starved), or three aphids, or with a 3 µl drop of honeydew, both in addition to wet cotton wool (*n* = 16). In the first four columns the number of individuals that showed the listed behaviour are recorded. The last column shows the average amount of aphids fed from the two aphids available.

Pre-treatment	Feeding	Feeding on aphids	Feeding on honeydew	First food source		Mean number of aphids fed on
				aphids	honeydew	
starved	15	14	14	5	10	1.38
aphid fed	12	8	8	7	5	0.75
honeydew fed	10	10	4	7	3	0.88

The larval behaviour during the time spent on the leaf disc is shown in Figure 6.2. Overall, *C. carnea* larvae spent more time on the leaf disc when they had been starved for 24 h prior to the experiment. This additional time is mostly spent feeding. The time of honeydew consumption, compared to aphid consumption (ratio time spent honeydew feeding / time spent aphid feeding), is highest for starved lacewing larvae (0.31), a bit lower for aphid-fed larvae (0.13), and lowest for honeydew-fed larvae (0.04). The aphid-fed larvae spent the most time resting and the least time searching of the three treatments.

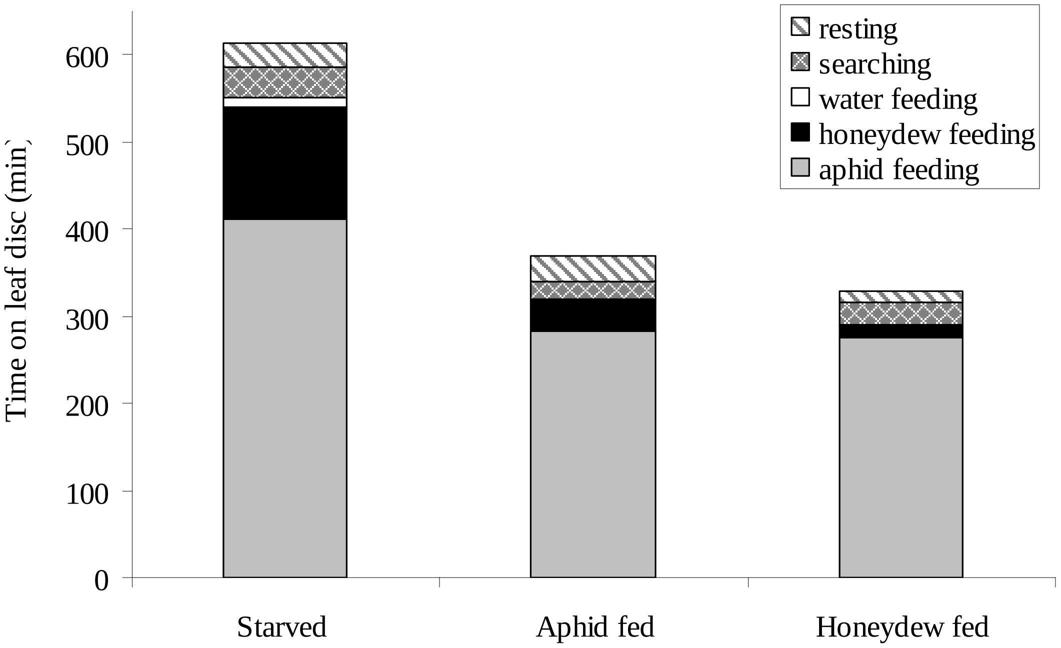

Figure 6.2 Behaviour during presence on the leaf disc of *Chrysoperla carnea* larvae that had been provided either with wet cotton wool (starved), with three aphids, or with a 3 μl drop of honeydew, both in addition to wet cotton wool, prior to the choice experiment (*n* = 16). The time was recorded that larvae were feeding on aphids, feeding on honeydew, drinking water from the wet filter paper, searching or resting.

Discussion

Our results clearly demonstrate that larvae of *C. carnea* still use honeydew as a food source in the presence of high-quality prey. It can therefore be noted that carbohydrate sources are not only used to extend longevity in the absence of prey, but form an integral part of the diet of this omnivorous predator. However, these non-prey foods remain alternative food and the omnivorous predator is still dependent on prey to complete larval development. Limburg and Rosenheim (2001) showed that lacewing larvae were able to utilize nutrients in extrafloral nectar, but were unable to moult to the next instar. Both (extra-)floral nectar and honeydew mainly contain carbohydrates. Even though honeydew often contains higher levels of amino acids compared to (extra-)floral nectar, these are often non-essential and can be strongly dominated by a few compounds (Wäckers 2005). The nutritional contribution of honeydew to growth and development of predator larvae is therefore likely to be very low.

In both experiments described here it was seen that previous feeding reduced absolute and relative consumption of honeydew. The reduced likelihood of further honeydew feeding is most pronounced after previous honeydew feeding, but also clearly present when aphids were consumed. This could be due to satiation or, in the case of aphid feeding, caused by the fact that aphids also contain sugars and the larvae may therefore not be sugar limited. With one exception, the total relative weight increase in two hours is for all treatments of the no-choice experiment more or less equal (Figure 6.1). Only when honeydew is consumed in both hours, total relative weight increase is much lower. This implies that larvae are not completely satiated after honeydew consumption alone. Moreover, when encountering honeydew, a trade-off seems to take place in lacewing larvae between consumption of this carbohydrate source and leaving enough space for digestion of a more nutritionally balanced aphid prey that could be encountered next. Limburg and Rosenheim (2001) suggested that lacewing larvae do not reject opportunities to feed on extrafloral nectar, but reduce their foraging time in abundance of prey. This reduced foraging time would lead to a lower encounter rate and therefore lower consumption of extrafloral nectar. However, our laboratory studies have shown that at least when food is available in excess, regulation of food uptake does take place, because both aphids and honeydew droplets were abandoned on several occasions before they were completely consumed.

In the no-choice experiment it was found that relative weight increase of lacewing larvae during the first hour was about one third (32%) lower on honeydew than on aphids. This shows that the alternative food source, honeydew, is still consumed in rather high amounts when larvae have been starved for a long period (24 h). In the second hour, after one hour of aphid feeding, a similar difference in relative weight gain between aphid and honeydew feeding was observed (39% lower on honeydew). Although the total amounts consumed in the second hour are much lower than in the first hour, the ratio between aphid and honeydew consumption is very similar. A multidimensional geometric approach to insect feeding and nutrition, described by Raubenheimer and Simpson (1993, 1999) and Simpson et al. (1995), could have a further explanation for this reduced relative weight increase on honeydew and the stable intake ratio between aphids and honeydew. In this approach, the intake of nutrients optimal for the insect's fitness is defined as 'intake target' and when this cannot be reached due to nutritionally imbalanced food, the 'point of best compromise' (Simpson et al. 1995). This point of best compromise for honeydew feeding is reached at a lower relative weight increase compared to aphid feeding. Lacewing larvae require both carbohydrates and proteins at a certain ratio. Whilst honeydew provides almost exclusively carbohydrates, it is considered as a nutritionally imbalanced food. Animals given two or more imbalanced but complementary foods, have been found to be able to reach the intake target by 'compensatory feeding' (Raubenheimer and Simpson 1993, Simpson et al. 1995). Compensatory feeding can explain the high relative weight increase of lacewing larvae when given access to aphids (a source of both proteins and carbohydrates) after having access to honeydew during the previous hour (Figure 6.1).

The choice experiment showed that larvae spent much less time feeding on honeydew than on aphids. This can of course be explained by a preference of the larvae for essential prey to alternative food. However, another part of the explanation could be that the time spent feeding is not equal to food uptake. Feeding on honeydew is possibly more efficient than feeding on aphids as honeydew is easily imbibed (Downes 1974), and does not require energy-consuming acts of prey identification, prey catching and possibly requires a strongly reduced predigestion (Canard 2001). The hypothesis that honeydew is a quick and easy source of energy (and water) may also explain the observation in the choice experiment that most starved lacewing larvae fed on honeydew as a first food source, before attacking an aphid (Table 6.1).

Honeydew-fed larvae spent 7.7% of the time on the leaf disc actively searching, whereas this number was 5.6% for starved larvae and 5.5% for aphid-fed larvae (Figure 6.2). Due to increased energy levels, intense foraging activity of lacewing larvae is also prolonged, as has been shown for extrafloral nectar feeding (Limburg and Rosenheim 2001). In addition, honeydew has been found to serve another purpose for aphidophagous lacewing larvae. The honeydew of aphids may also serve as a kairomone, increasing the intensity of searching behaviour (Canard and Duelli 1984). Downes (1974) suggested that honeydew guides lacewing larvae towards aphids. Kawecki (1932) assumed that *C. carnea* larvae can orient towards honeydew of sternorrhynchan insects with the help of chemical attractants emitted by the honeydew. Adult *C. carnea* use the amino acid tryptophan, that is present in honeydew, as kairomone (Hagen et al. 1976). An increased exploitation of prey patches in response to honeydew has also been observed for larvae of *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) (Carter and Dixon 1984).

The results presented here give a further indication of the importance of alternative foods, such as honeydew, for omnivorous green lacewing larvae. These non-prey foods are not only important during a period of prey scarcity (Limburg and Rosenheim 2001) or in the absence of high-quality prey (Patt et al. 2003), but also form part of the diet when suitable prey is present.

Acknowledgements

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Chapter 7

Synthesis and outlook

This thesis addresses an exposure route to transgene products for non-target arthropods that previously had received little attention. Honeydew-feeding predators and parasitoids can be exposed to phloem-transported transgene products that are excreted in the honeydew of sternorrhynchan insects. This exposure route is likely to be particularly important in transgenic crops targeting sap-sucking pests, due to the fact that the insecticidal protein needs to be transported in the phloem sap in order to reach the target pest. Therefore, transgenic wheat expressing snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) to target aphid pests, was used as model plant. Several important aphid antagonists were chosen as non-target species. The specific research objectives were twofold: on the one hand to estimate the insect sensitivity to GNA in honeydew, and on the other hand to determine the contribution of honeydew feeding to the diet of these predators and parasitoids. Together this allows assessing if transgene products in honeydew pose a risk for non-target honeydew-consuming insects.

Sensitivity of predators and parasitoids to GNA

All predator and parasitoid species studied in this thesis were found to be sensitive to GNA. When provided with a sucrose solution containing 1% GNA (w/v) a substantial direct effect on longevity was observed for the predator larvae of *Chrysoperla carnea*, *Adalia bipunctata* and *Coccinella septempunctata* (Chapter 2) as well as the aphid parasitoid *Aphidius ervi* (Chapter 4). Differences in sensitivity were found among the three predator species and between the first and last larval stage of *C. carnea*. It was further shown that GNA is not broken down by the digestive enzymes in the predators' gut. Several glycoproteins that bind to GNA, being a prerequisite for toxicity of lectins, were found in the larval guts of all three predator species. Most of the GNA that had been ingested, was excreted by the ladybird larvae, whereas it was retained in the case of lacewing larvae because of their inability to excrete faeces during the larval stages. Passage of GNA into the haemolymph of the larvae likely occurred, but in low and variable amounts (Chapter 2). These results found for GNA are in contrast to Cry toxins from *Bacillus thuringiensis* (*Bt*), that are broken down in the gut (Romeis et al. 2004) of *C. carnea* larvae. Moreover, Cry toxins require binding to the midgut epithelium as an obligatory step in the toxic action, but were found to lack this capability in *C. carnea* larvae (Rodrigo-Simón et al. 2006).

Earlier studies investigating direct effects of GNA on adult parasitoids have found similar longevity reduction for *Aphidius colemani*, *Cotesia glomerata*, *Trichogramma brassicae* (Romeis et al. 2003) and *Eulophus pennicornis* (Bell et al. 2004). For *A. ervi* different GNA concentrations were tested and a clear dose-dependent effect on longevity was found (Chapter 4). A sucrose solution with 0.1% (w/v) GNA was needed to cause a significant reduction in parasitoid longevity. Interestingly, this concentration is also the minimum concentration required to achieve a detectable impact on target aphids (Down et al. 1996, Sauvion et al. 1996, Couty et al. 2001c). Since effects of GNA on both target and non-target insects have been found to be dose-dependent, provoking more severe effects at higher intake, the degree of exposure to GNA is crucial for the eventual detrimental effect on the organism.

A number of tritrophic studies investigated effects of GNA on predators when preying on GNA-fed aphids or parasitoid larvae developing in GNA-fed hosts (Table 7.1). The effects on non-target insects found in these studies are quite variable. However, when negative effects have been found in these tritrophic studies, they were only marginal and generally much smaller than the effects found in the direct feeding studies mentioned above. Taking into account that effects of GNA are dose- dependent, the smaller effects in tritrophic studies compared to direct feeding studies can largely be explained by the limited GNA ingestion by the predator or parasitoid larva. In the artificial diet studies, where the GNA concentration in both the artificial diet and the aphid pest were determined, the GNA concentration in the aphid was at least 40% lower than in its diet. When studying tritrophic interactions with GNA-expressing plants, establishing GNA concentrations in the target insect is even more important, as expression levels can be variable (Down et al. 2001). Moreover, sternorrhynchan pests feed directly on the phloem sap, where GNA concentrations can be an order of magnitude lower than the expression levels measured in a leaf extract (Foissac et al. 2000, Chapter 4).

Table 7.1 Studies investigating effects of GNA-containing GM plants or artificial diets on predators and parasitoids via tritophic interactions

Non-target insect	Developmental stage exposed	Host/prey insect	Ingestion through[a]	GNA source[a]	GNA in target insect[a]	Non-target effects	Ref.[b]
Adalia bipunctata	larvae	*Myzus persicae*	predation	art. diet 0.1% w/v	0.08 µg = 0.06% (w/w)	marginal negative effects on the developmental biology	1
				potato 0.07-0.2% tsp	below detection limit (< 5 ng)	no effects	2
	adults	*Myzus persicae*	predation	art. diet 0.1% w/v	0.08 µg = 0.06% (w/w)[c]	positive effects on fertility and fecundity (older GNA-fed aphids used to ensure similar size as controls)	2
				potato 0.02-0.2% tsp	below detection limit (< 5 ng)	marginal negative effects, but only small effect on fertility when older, but similar size aphids were used	2, 3
Aphelinus abdominalis	larvae	*Macrosiphum euphorbiae*	parasit.	art. diet 0.1% w/v	230-300 ng/aphid = 0.05% (w/w)	negative effect on adult weight (not when older, but similar size aphids were used) and adult fecundity	4, 5
	adults	*Macrosiphum euphorbiae*	host-feeding	art. diet 0.1% w/v	below detection limit in haemolymph	no effects	6
Aphidius ervi	larvae	*Myzus persicae*	parasit.	art. diet 0.045% w/v	60 ng/aphid = 0.025% (w/w)[d]	dose-dependent negative effects on several parameters including development time, hatch rate and adult weight	7
				potato 0.01-0.12% tsp	below detection limit	no effects	7
Cotesia flavipes	larvae	*Diatraea saccharalis*	parasit.	sugarcane diet 0.49% tsp	n.a.	small to marginal negative effects on several parameters including hatch rate and adult longevity	8
Eulophus pennicornis	larvae	*Lacanobia oleracea*	parasit.	leaf-based diet 2% tsp	n.a.	no effects	9
				potato 0.08-1.0% tsp	n.a.	small negative effect on adult mean size and longevity, no effect on fecundity	9, 10
Meteorus gyrator	larvae	*Lacanobia oleracea*	parasit.	tomato 2.0% tsp	n.a.	no effects	11
Parallorhogas pyralophagus	larvae	*Eoreuma loftini*	parasit.	sugarcane diet 0.47% tsp	n.a.	variable marginal effects on development time, adult size, longevity and fecundity in 1st and 2nd generation	12

[a] Abbreviations: parasit. = parasitisation; w/v = weight/volume; w/w = weight/weight; tsp = total soluble protein in leaf; n.a. = not assessed; ref. = reference

[b] References: 1: Down et al. 2000; 2: Down et al. 2003; 3: Birch et al. 1999; 4: Couty et al. 2001a; 5: Couty et al. 2001c; 6: Couty and Poppy 2001; 7: Couty et al. 2001b; 8: Sétamou et al. 2002; 9: Bell et al. 1999; 10: Bell et al. 2001; 11: Wakefield et al. 2006; 12: Tomov and Bernal 2003

[c] Data from Down et al. 2000; [d] Percentage calculated based on aphid weight from Sauvion et al. 1996

Even when the GNA concentration in the target pest has been established, one also needs to consider whether the predator or parasitoid consumes the pest insect completely or only partly. The host-feeding parasitoid *Aphelinus abdominalis*, for example, is known to feed exclusively on its host's haemolymph. This is important since the highest concentration of GNA is generally found in the gut, whilst the haemolymph has been found to contain no or only small amounts of GNA (Powell et al. 1998, Down et al. 2000, Foissac et al. 2000, Fitches et al. 2001). Furthermore, effects on the entomophagous insect can be either direct, caused by the insecticidal protein, or indirect, caused by a reduced quality of the affected host/prey. In none of the studies listed in Table 7.1 can these different effects be separated, since all host/prey insects were also directly affected by the GNA.

Importance of honeydew as food source

The importance of honeydew as a food source for parasitoids and several adult predators that are 'obligatory' consumers of plant-derived foods has often been recognized. Also for omnivorous predators and host-feeding parasitoids this carbohydrate source can be important, especially when prey/hosts are scarce or of low nutritional quality. However, quantitative information on honeydew exploitation in the agricultural ecosystems, where honeydew is believed to be the most important carbohydrate source available (Wäckers 2005), is largely lacking. The only exception being one study by Wäckers and Steppuhn (2003) demonstrating that 80% of the hymenopteran parasitoid *C. glomerata* and 55% of *Microplitis mediator* collected in a cabbage field had recently fed on honeydew. More detailed information on the contribution of honeydew consumption to the diet of entomophagous insects is important for the assessment of exposure to transgene products in honeydew.

To investigate the prevalence of honeydew feeding in our system, adult aphid antagonists, i.e. parasitoids, lacewings and hoverflies were collected in winter wheat and spring wheat fields near Zurich (Switzerland) and their sugar profile was subsequently analysed by high performance liquid chromatography (HPLC). This method can reveal the overall nutritional state as well as recent honeydew feeding (Chapter 5). Raised total sugar levels or a decreased glucose-fructose ratio in the field collected adult aphid antagonists was used to classify them as having recently fed on carbohydrates. It was found that 60% of *Aphidius* spp., 89% of *Episyrphus balteatus*, 98% of *C. carnea*, and 95% of *Chrysoperla lucasina* collected in wheat fields, had recently fed. The erlose-melezitose ratio was used to detect recent honeydew feeding by *Aphidius* spp. and *E. balteatus* in this aphid-wheat system. It was concluded that at least 63% of *Aphidius* spp. and 35% of *E. balteatus*, which were classified as 'fed', had consumed honeydew during the two days before collection. These results demonstrate that aphid honeydew is an important food source for aphid parasitoids, and to a lesser extent for hoverflies in wheat fields.

Aphid parasitoids are known to use honeydew as a contact kairomone to locate hosts (Hågvar and Hofsvang 1991), and are thus more likely to encounter honeydew during their normal course of activity than parasitoids of other insects such as lepidopteran pests. I would therefore expect that honeydew utilization would be higher for this first group. This hypothesis could be tested in semi-field studies (for example field tents described by Winkler et al. 2006) with different aphid densities. The same would apply for aphid predators that use honeydew to locate aphid prey (Hagen 1986, Budenberg and Powell 1992, Bargen et al. 1998, Sutherland et al. 2001).

Detection of recent honeydew consumption based on the sugar profile of the predator or parasitoid could only be done for non-aphidophagous stages, since honeydew feeding could otherwise not be distinguished from aphid feeding. However, when honeydew is available in the field, the sternorrhynchan honeydew producers are generally present as well. In Chapter 6 it was therefore investigated whether honeydew would be consumed by the omnivorous larvae of *C. carnea* in the presence of aphids, the preferred prey of this predator. When larvae were supplied with an excess of either aphids or honeydew during one hour, the relative weight increase on honeydew was about one third lower than on aphids. When aphid-fed larvae were supplied with honeydew during a second hour, a similar difference in relative weight gain between aphid and honeydew feeding was observed. However, when honeydew-fed larvae were supplied with honeydew during a second hour, their relative weight increase was two thirds lower as compared to larvae allowed to switch to aphids, indicating that the larvae compensate for the reduced nutrient intake on honeydew when aphids are available again. When *C. carnea* larvae that had been either starved, aphid-fed, or honeydew-fed prior to the experiment were allowed to choose between aphids and honeydew, the honeydew/aphid consumption ratio (measured in time spent feeding) was highest for starved lacewing larvae (0.31), lower for aphid-fed larvae (0.13), and lowest for honeydew-fed larvae (0.04). In both experiments it was seen that previous feeding reduced the consumption of honeydew, especially after honeydew feeding. Nevertheless, these studies show that larvae of *C. carnea* still use honeydew as a food source in the presence of suitable prey.

In a comparative study of three aphid predators, longevity of newly emerged lacewing (*C. carnea*), hoverfly (*Syrphus ribesii*), and ladybird (*C. septempunctata*) larvae was determined when kept either on water or on water and honey (Sunby 1966). The provision of honey resulted in a 6.4-fold longevity increase for *C. carnea*, but only increased lifespan 3.2-fold for *C. septempunctata* and 1.4-fold for

S. ribesii. These remarkably large differences in the utilization of a carbohydrate source could also implicate differences among omnivorous predators in honeydew utilization in the field and hence require further investigation.

GNA in honeydew

Honeydew nutritional quality

Honeydew can vary in its nutritional quality for honeydew-feeding entomophagous insects. In Chapter 3 honeydews from different aphid species feeding on potato, wheat or artificial diet were found to be a suitable food source for adult *A. ervi*, although not always as suitable as a 2 M sucrose solution. I showed that the impact of honeydew on parasitoid longevity can to some extent be explained by its carbohydrate composition. This indicates that the carbohydrate composition plays a role in determining the nutritional quality of honeydew for parasitoids. There were differences in honeydew sugar composition among the different aphid species on the different plants. Multivariate statistics showed that aphid species had a significant influence on the sugar composition of the honeydew in both the potato and wheat system. The fact that this aphid parasitoid benefits from feeding on its host's honeydew represents a double advantage. Firstly, honeydew provides the carbohydrates essential for survival; secondly the parasitoid avoids the trade off between having to divide its time between searching for hosts and carbohydrate sources (Křivan and Sirot 1997, Lewis et al. 1998, Tenhumberg et al. 2006).

Honeydew quality is not likely to be a limiting factor for parasitoid nutrition in the conventional aphid-plant systems studied here. However, in the case of GNA-expressing wheat this may be different. I therefore investigated at which concentrations GNA would appear in the honeydew and whether honeydew quality would interfere with GNA toxicity (Chapter 4). When the aphid *Rhopalosiphum padi* fed on an artificial diet containing 0.1% (w/v) GNA, its honeydew contained 0.01-0.04% GNA. Being supplied with this honeydew as a food source, the mean longevity of adult *A. ervi* was reduced by 47% compared to parasitoids fed with honeydew from aphids on a control diet. Even though the measured GNA content of the honeydew was only 10-40% of the GNA concentration in the aphid diet, the effect of the honeydew from *R. padi* on GNA diet on the longevity of *A. ervi* was more pronounced than expected based on the reduction due to the addition of 0.1% GNA to a sucrose solution (30% reduction). Analysis of carbohydrate and amino acid composition revealed that a change in honeydew composition caused by a GNA-effect on the aphids could be a possible explanation for the additional reduction in parasitoid longevity. Transgene products in honeydew have thus been shown to have a two-fold effect on the nutritional quality of the honeydew for honeydew-consuming arthropods. Apart from causing a direct toxic effect, GNA appears to cause changes in the metabolic processes in the aphids resulting in changes in honeydew composition. Alternatively, a negative synergistic effect between GNA and suboptimal honeydew quality could be an explanation for the additional longevity reduction of *A. ervi*.

Additional studies comparing the effects of GNA in honeydew with a high nutritional quality with those of GNA in honeydew with a low nutritional quality for non-target insects could determine the underlying mechanism explaining the more pronounced effect of GNA in honeydew on *A. ervi*. In connection with this research, it would also be interesting to study the effect of honeydew sugar / amino acid composition for a group of honeydews that show a larger variation in their composition as well as their nutritional suitability for honeydew-feeding insects.

GNA concentrations in honeydew

In order to quantify the amount of GNA excreted in the honeydew of *R. padi* (Chapter 2), the aphids were kept on an artificial diet containing 0.1% (w/v) GNA for five days. Dot blot analysis of the honeydew samples collected on different days showed the presence of GNA in the honeydew at concentrations ranging from 0.01% to 0.04% (w/v). The strongest signal in the honeydew was seen on the second day of feeding, but GNA was present in the honeydew over the whole five day collection period. However, since the honeydew samples had been exposed to evaporation and re-absorption of water before analysis, the estimated GNA concentration in honeydew relative to the diet is not very precise. Powell et al. (1998) have determined the GNA concentration in honeydew of the planthopper *Nilaparvata lugens* fed on artificial diet containing 0.02 and 0.05% (w/v) by dot blot analysis. Unfortunately, the method of honeydew collection was not specified. Honeydew from *N. lugens* collected during the first day contained approximately 60% of ingested GNA, and up to an average of 130% of ingested GNA concentration over the next two days. These results suggest that the honeydew samples were probably condensed. Couty and Romeis (2004) estimated possible GNA-excretion levels in honeydew of more than 90% of ingested GNA based on the GNA amounts of 200-300 ng detected in the aphid *Macrosiphum euphorbiae* (assuming a diet ingestion rate of 25 nl h^{-1} (Wright et al. 1985) and no proteolysis of GNA by the aphid). However, these estimations might be a bit too high since the authors did not account for a reduced food uptake on the GNA-containing diet due to an antifeedant effect of GNA (Powell et al. 1995). Even though the results of the different studies are variable, it can be concluded that high GNA concentrations in honeydew are likely to occur, especially when taking into account that GNA concentrations can increase even further under field conditions due to water evaporation from the honeydew.

Do transgene products in honeydew pose a risk for non-target insects?

In this thesis I have shown that all four non-target insect species that were tested for their sensitivity towards GNA, suffer a significant reduction in their longevity when directly exposed to the insecticidal protein in a sucrose solution (Chapter 2 and 4). I further demonstrated that GNA excreted in aphid honeydew negatively affects the lifespan of an aphid parasitoid, and that this longevity reduction is more pronounced than the impact that could be expected from the GNA-toxicity alone (Chapter 4). This indicates that GNA in honeydew poses a hazard for a range of aphidophagous insects.

Based on the fact that GNA in honeydew can reach levels close to those ingested by the sap feeder, it can be argued that aphid predators and parasitoids will be exposed to GNA in honeydew. The field study described in Chapter 5 demonstrated that both the adult aphid parasitoid *Aphidius* spp. and the adult aphid predator *E. balteatus* would be exposed to transgene products in honeydew, since honeydew was found to be used as food source in wheat fields. The results from Chapter 6 show that in a choice experiment a quarter of hungry *C. carnea* larvae's feeding time was spent feeding on honeydew, and in a no-choice situation weight increase on honeydew was two-third of the weight increase on aphids. Omnivorous aphid predators can therefore be exposed to GNA, not only through aphid prey (Birch et al. 1999, Down et al. 2000, Down et al. 2003), but also through honeydew. Since the adult aphid parasitoid *A. ervi* refrains from host feeding, honeydew is probably their primary route of exposure to GNA. As larvae they develop in the aphid host which exposes them to GNA as well. However, the concentration of GNA would be at least 40% lower than GNA concentrations in the aphid diet (Table 7.1). Tritrophic effects may further be direct and indirect. Exposure through honeydew does of course extend to other honeydew-consuming insects as well.

The exposure to GNA through honeydew, combined with a reduced nutritional quality of the aphid honeydew and a possible decrease in honeydew production as a result of an antifeedant effect of GNA, could affect the survival of non-target organisms under field conditions. In agricultural systems, where alternative food sources are often lacking, this can have distinct implications for parasitoid-host and predator-prey dynamics and biological control. However, more research is required to investigate this. Especially cumulative effects of the different routes of exposure should be considered. Since honeydew can be an important route of exposure to transgene products for a wide range of non-target organisms, it should be considered in future risk assessment of phloem-transported insecticidal compounds. The possible risk of GNA-containing honeydew depends very much on the expression levels of the insecticidal protein in the phloem sap of the transgenic plant. The ecological impact can only be determined with transgenic plants expressing the insecticidal protein at levels sufficient to control the target pest and under more realistic conditions. It is important in future studies to address the relative sensitivity and exposure to the insecticidal protein for both the target and the non-target organisms.

Honeydew could also be a route of exposure to other transgenic compounds, systemic pesticides and secondary plant compounds. Tests on systemic pesticides have revealed that certain pesticides can be found in sap-sucking insects shortly after spraying, and that this level increases over the next days (Michaela Gandolfi, pers. com.). Pesticides could be excreted in honeydew, as has been shown for alkaloids (Wink and Römer 1986), cardenolides (Malcolm 1990), plant lectins (Rahbé et al. 1995, Powell et al. 1998, Down et al. 2000, Sauvion et al. 2004) and protease inhibitors (Cowgill et al. 1999), and then expose non-target insects. This could be an interesting topic requiring further investigation.

Methodological aspects

GNA as model compound

GNA has proven to be a good model compound to study routes of exposure, for several reasons: (1) It is a very stable protein; several studies have shown that it is very resistant to proteolysis (Gatehouse et al. 1995, Chapter 2). (2) GNA has been found to have a broad range of activity, affecting several pest and non-target arthropods (Chapter 2 and 4). (3) The insecticidal protein has been found to cause fitness consequences for the target and non-target insects. The sublethal effects of GNA are dose-dependent (Romeis et al. 2003, Bell et al. 2004, Chapter 4). The compound can provide a good reference (positive control) for toxicity tests that aim to measure sublethal effects of novel insecticidal compounds. (4) The pure toxin is commercially available. (5) Antibodies binding to GNA are available allowing the detection of the protein in plant and insect tissues.

GNA-expressing wheat

In Chapter 4 the performance of the aphids *R. padi* and *Sitobion avenae* on GNA-transgenic wheat and non-transformed control plants was analysed in the glasshouse. No differences were found between GNA-transgenic and control wheat for the average weight and intrinsic rate of increase of both aphid species. GNA could not be detected in the honeydew of the aphids and no effect on *A. ervi* longevity when feeding on honeydew from aphids on GNA-transgenic wheat was found. GNA was detected in all plants, but levels were variable, ranging from below 0.001% to 0.02% of total soluble protein in leaf extract. The maximum expression levels found, were about 10-fold below expression levels for the same wheat line (BW150) reported by Stoger et al. (1999). I assume that if GNA was transported in the phloem, this was only at very low concentrations. These results consistently indicate that the transgenic plants would not be functional in suppressing phloem-feeding pests.

Variation in protein accumulation within a line of clonal replicates of GNA-expressing potato plants has been found by Down et al. (2001). Since the wheat plants were propagated by seeds, the variation in GNA accumulation between different plants of the same line is likely to be even higher. Levels of GNA in a transgenic plant also vary between different plant parts, age of the plant and can also be affected by environmental factors (Down et al. 2001). Inefficient directing of GNA to the phloem sap and resulting limited effects on the targeted phloem feeder have been observed previously for GNA-transgenic rice and potatoes (Foissac et al. 2000, Couty et al. 2001b).

Signature sugars to detect honeydew feeding

Detecting honeydew feeding by analysing the insect's sugar profile using HPLC was first described for sandflies (Macvicker et al. 1990) and has been applied only recently for parasitoids (Wäckers and Steppuhn 2003, Steppuhn and Wäckers 2004). This methodology is based on the detection of 'honeydew specific signature sugars' (Macvicker et al. 1990, Byrne et al. 2003, Heimpel et al. 2004) in the tested insect. These signature sugars are di- and oligosaccharides that are synthesized by phloem-feeding Sternorrhynchae and excreted in the honeydew, but cannot be synthesized by the study organism itself. The 'honeydew specificity' of these sugars has been put into question by Wäckers et al. (2006), who were the first to report the synthesis of both melezitose and erlose by arthropods other than honeydew-producing Sternorrhynchae. In Chapter 5 I showed synthesis of melezitose and erlose in three further insect species from different taxonomical orders following consumption of a sucrose solution. Although there are insects that have been shown not to synthesize these sugars, using the same methodology (Steppuhn and Wäckers 2004), these findings emphasise that the use of signature sugars to detect recent honeydew feeding is not as widely applicable as previously believed.

Constricted by the fact that the study insects were capable of synthesising the sugars melezitose and erlose, the method to detect honeydew feeding was adapted (Chapter 5). Laboratory studies demonstrated that *A. ervi* and *E. balteatus* synthesized more melezitose than erlose, whereas the honeydew of the three aphid species present in the wheat fields was highly erlose dominated. For this reason, the erlose-melezitose ratio could be used as an indicator for recent honeydew consumption by *Aphidus* spp. and *E. balteatus*. However, the use of this method bears several limitations: (1) It cannot be used for all insects, since species show clear variation in the ratio at which they synthesize these sugars. This is exemplified by *C. carnea* that contained an erlose-melezitose ratio after sucrose feeding that could not be distinguished from that of the honeydew. (2) Honeydew composition as well can vary depending on aphid and plant species (Hendrix et al. 1992), but also within species (Costa et al. 1999). This will limit the applicability of this method to systems where honeydews of various sugar compositions are present simultaneously. Whether or not the erlose-melezitose ratio (or a ratio of other oligosaccharides) can be used as an indicator for honeydew feeding depends on the honeydew sugar profile as well as the insects' sugar profile. In order to use this method, the honeydew sugar profile needs to be compared with the insects' sugar profile case-by-case, which means that laboratory testing of sugar synthesis capabilities is essential, as well as the collection of reference parameters to determine threshold values. (3) The threshold level to classify an insect as having fed on honeydew depends on the variation among the unfed individuals from the laboratory controls. The use of too few controls can lead to wrong threshold levels. (4) No information was collected about the synthesis of oligosaccharides by insects feeding on food sources other than a sucrose solution. However, as the synthesis of melezitose and erlose is believed to be caused by a single enzyme acting specifically on sucrose molecules (Ashford et al. 2000), other sugars in the insect's diet are unlikely to affect the erlose-melezitose ratio (Wäckers et al. 2006). (5) Results from the laboratory experiments have shown that honeydew sugars were no longer detectable after 24-48 h due to rapid digestion. Honeydew feeding can therefore only be detected in individuals that have consumed this food source in the last 1-2 days. (6) The amount of honeydew consumption (by individual insects) can only be approximately assessed.

Future of GNA-expressing plants

Over the last decade, since genetically modified (GM) crops were first commercialized in 1996, the global area of GM crops has consistently increased with double-digit growth rates every single year (James 2005), and is likely to increase even further in the future. In order to confer insect-resistance in plants, expanded use of the *Bt* crops will be a focus area in the immediate future (Nickson 2005). Developments regarding stacking of several *Bt* genes or genes with different modes of action (pyramiding) will also continue (Moar 2003, Nickson 2005). Another approach that will be followed is the use of hybrid/fusion toxins. Their potential has been demonstrated by a fusion protein combining the Cry1Ac-toxin from *Bt* with the galactose-binding domain of the ricin B lectin that extended the range and level of toxicity compared to the original Cry toxin (Mehlo et al. 2005). Also GNA has been proven to fulfil a possible role as carrier protein. Due to its ability to bind to the gut epithelium and to pass into the haemolymph, GNA can be used to transport other toxic proteins to the insects' circulatory system. So far, the *gna* gene has been fused to a gene encoding a neuropeptide (*Manduca sexta* allatostatin) (Fitches et al. 2002) and to a gene encoding spider venom neurotoxin (*Segestria florentina* toxin 1) (Fitches et al. 2004). The resulting fusion proteins were found to be significantly more effective than the component proteins themselves. Based on data obtained in this thesis one would expect that fusion proteins containing GNA may also pose a risk for a range of non-target insects. To what extent they will be affected needs further investigation.

Whether plants expressing lectins, in particular GNA, will ever be commercialized is uncertain for several reasons. The pest reduction efficacy of current plants expressing GNA as a single gene is generally not high enough to be considered commercially viable (Ferry et al. 2004b). Furthermore, its broad spectrum of activity might raise environmental concerns, especially regarding potential effects on non-target organisms. Moreover, some lectins are known to be toxic to mammals. Even though short-term nutritional experiments have shown that GNA is non-toxic for rats (Pusztai et al. 1996, Ewen and Pusztai 1999), effects on brush border membrane enzyme activity were observed (Ewen and Pusztai 1999). GNA-effects on mammals by longer exposure are unknown, hence impact of GNA on human health is still debated. On the other hand, the absence of other genes with high-insecticidal activity against sternorrhynchan pests may mean that GNA-crops could find acceptance in agriculture. Irrespective of the commercialization of GNA, the concepts and mechanisms addressed in this thesis will extend to any insecticidal compound targeting phloem-feeding pests or being transported in the phloem sap.

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Résumé français

Les cultures génétiquement modifiées (GM) résistantes aux insectes ravageurs sont susceptibles d'affecter des organismes autres que ceux ciblés par les toxines. Ces organismes non-cibles incluent des parasitoïdes et des prédateurs importants pour le contrôle naturel des ravageurs et pourraient être exposés aux protéines insecticides en se nourrissant de tissu végétal transgénique ou d'herbivores cibles ou non-cibles contenant le produit transgénique. Dans cette thèse, le miellat sécrété par les insectes opophages (qui se nourrissent de sève) est considéré comme une nouvelle voie possible d'exposition aux protéines insecticides. Le miellat étant couramment utilisé comme une source de sucre, de nombreux organismes non-cibles sont susceptibles d'être exposés aux produits transgéniques. L'importance de cette voie d'exposition a été évaluée en considérant le blé GM exprimant la lectine perce-neige (*Galanthus nivalis* agglutinine, GNA) afin de contrôler les insectes opophages tels que les pucerons. Le risque étant défini à la fois par le danger potentiel et par l'exposition, ces deux aspects ont été étudiés pour le miellat contenant la GNA.

La sensibilité de trois espèces de prédateurs (*Adalia bipunctata*, *Coccinella septempunctata* et *Chrysoperla carnea*) à la GNA a été testée. Les résultats montrent que la longévité des espèces étudiées est directement affectée par la GNA dissoute dans la solution de sucrose. Cela est confirmé par le fait que les enzymes digestives présentes dans le système digestif des larves de prédateurs n'ont pu dégrader la GNA. Alors que *A. bipunctata* et *C. septempunctata* parviennent à excréter la majeure partie de la GNA, *C. carnea* accumule la toxine dans son système digestif puisqu'elle ne peut excréter les fèces. En outre, il a été observé que la GNA se lie aux glycoprotéines dans l'estomac - condition nécessaire à sa toxicité - et qu'elle est transférée dans l'hémolymphe des insectes.

Dans une première étape de l'étude du miellat comme voie possible d'exposition, la qualité nutritionnelle du miellat de trois espèces de pucerons se nourrissant de pomme de terre ou de blé a été étudiée pour le parasitoïde de puceron *Aphidius ervi*. Les résultats montrent que les différents miellats constituent une source de nourriture relativement bonne pour les adultes parasitoïdes, mais pas toujours autant que la solution de sucrose. La composition en sucre des miellats diffère significativement entre les trois espèces de pucerons, qu'ils soient nourris avec des pommes de terre ou des plants de blé. Les analyses multivariées révèlent que les petites différences de longévité des parasitoïdes sont expliquées dans une certaine mesure par la composition en sucre du miellat. Cela indique que la composition en sucre est un facteur important de la qualité nutritionnelle du miellat.

Le test de la sensibilité des adultes de *A. ervi* à la GNA révèle un effet direct qui dépend de la dose administrée. L'exposition du parasitoïde à la GNA dans le miellat a été étudiée en soumettant le puceron *Rhopalosiphum padi* à une nourriture artificielle contenant la protéine. Le miellat issu de ces pucerons contenait entre 10 et 40% de la concentration en GNA initialement présente dans la nourriture. Les *A. ervi* nourris avec ce miellat présentent une réduction de leur longévité plus prononcée que celle attendue au regard de la concentration de GNA détectée dans le miellat. L'analyse de la composition en glucides et en acides aminés montre que la réduction de la longévité des parasitoïdes pourrait être liée à un changement de la composition du miellat causé par la GNA. Le miellat de pucerons nourris avec du blé exprimant la GNA n'a pas plus affecté la longévité de *A. ervi* que du miellat issu de blé non transgénique, ce qui est probablement lié au faible niveau d'expression de la GNA dans les plantes.

Afin d'établir l'importance du miellat comme ressource alimentaire pour les insectes adultes prédateur de pucerons, des parasitoïdes de pucerons, des syrphes et des chrysopes ont été collectés dans des champs de blé. Des analyses des teneurs et composition en sucre chez ces insectes ont révélé que la majorité des insectes ont récemment consommé des sucres. En particulier, un état nutritionnel très élevé a été observé chez deux espèces de chrysopes (*Chrysoperla lucasina* and *C. carnea*). Des preuves d'une ingestion récente de miellat ont été détectées pour 35% des syrphes (*Episyrphus balteatus*) et 63% des parasitoïdes (*Aphidius* spp.) ayant consommé des sucres. Ces résultats montrent que le miellat de puceron est une ressource alimentaire importante pour les parasitoïdes de pucerons, et pour les syrphes dans une moindre mesure, dans les champs de blé.

Afin d'évaluer l'importance de la consommation de miellat pour les prédateurs omnivores, une étude a été conduite afin de déterminer si les larves de *C. carnea* utilisent le miellat comme ressource alimentaire en présence des pucerons, ou si elles préfèrent des proies. Les résultats révèlent une consommation de miellat par les larves, que ce soit en présence de miellat uniquement, ou en présence de miellat et de proies. La consommation de miellat reste toutefois moindre en comparaison de la consommation de pucerons. Par ailleurs, une ingestion préalable de pucerons ou de miellat réduit les consommations suivantes de miellat.

Cette thèse a montré que la GNA présente dans le miellat représente un danger pour divers insectes non-cibles, et que les prédateurs et parasitoïdes seront exposés à la GNA contenue dans le miellat. Les produits transgéniques présents dans le miellat représentent donc un risque pour les insectes non-cibles qui se nourrissent de miellat. Cette voie possible d'exposition devrait être prise en compte dans les futures évaluations des risques liés aux plantes transgénique exprimant des composés insecticides véhiculés par la sève.

Deutsche Zusammenfassung

Insektenresistente, gentechnisch veränderte (GV) Nutzpflanzen können neben den Schadorganismen potentiell auch Nicht-Zielorganismen schädigen wie zum Beispiel Schlupfwespen und räuberische Arthropoden, die für die natürliche Schädlingsregulierung von Bedeutung sind. Diese Nützlinge können die exprimierten insektiziden Proteine aufnehmen indem sie transgenes Pflanzenmaterial fressen oder sich von Herbivoren ernähren, die das Toxin aufgenommen haben. Im Rahmen dieser Dissertation habe ich untersucht, inwiefern Nützlinge transgenen Proteinen ausgesetzt sind, wenn sie Honigtau aufnehmen, der von Insekten, die Phloemsaft saugen, ausgeschieden wird. Da Honigtau eine bedeutende Nahrungsquelle für viele Nützlinge darstellt, besteht ein potentielles Risiko für ein breites Spektrum von Nicht-Zielorganismen. Als Modellsystem dienten GV Weizenpflanzen, die Lektin aus dem Schneeglöckchen (*Galanthus nivalis* agglutinin, GNA) exprimieren und so eine erhöhte Resistenz gegenüber Phloemsaft saugenden Insekten, wie zum Beispiel Blattläusen zeigen. Das Risiko für Honigtau fressende Nützlinge wird einerseits bestimmt durch deren Empfindlichkeit gegenüber dem Lektin und anderseits durch das Mass, in dem sie dem Toxin ausgesetzt sind. Beide Aspekte wurden in dieser Dissertation untersucht.

Die Larvenstadien dreier räuberischer Insektenarten (*Adalia bipunctata*, *Coccinella septempunctata* und *Chrysoperla carnea*) wurden auf ihre Empfindlichkeit gegenüber GNA geprüft. GNA (aufgelöst in einer Saccharoselösung) wirkte sich direkt negativ auf die Lebensdauer der untersuchten Arten aus. Untersuchungen zu den Verdauungsenzymen im Magen der räuberischen Larven haben ergeben, dass das aufgenommene GNA nicht abgebaut werden konnte. Während Larven von *A. bipunctata* und *C. septempunctata* den grössten Teil des GNA ausscheiden konnten, akkumulierte *C. carnea* das Toxin im Darm, da sie im Larvenstadium keinen Kot ausscheidet. Außerdem wurde die Bindung von GNA an verschiedene Glycoproteine im Magen nachgewiesen, was eine Voraussetzung für die Toxizität des Proteins ist. Geringe Mengen des Lektins schienen auch in die Hämolymphe der Insekten zu gelangen.

Bevor die Auswirkungen von GNA im Honigtau untersucht wurden, wurde getestet, wie gut sich Honigtau von drei Blattlausarten auf Kartoffel- oder Weizenpflanzen als Nahrung für die Schlupfwespe *Aphidius ervi* eignet. Honigtau aller Blattlausarten war für die Schlupfwespen eine geeignete Nahrungsquelle, aber nicht immer gleich gut geeignet wie eine Saccharoselösung. Die Zuckerzusammensetzung des Honigtaus der drei Blattlausarten auf Kartoffeln und Weizen war signifikant unterschiedlich. Eine multivariate Analyse zeigte, dass kleine Unterschiede in der Lebensdauer der Schlupfwespen zum Teil durch die Zuckerzusammensetzung des

Honigtaus erklärt werden können. Dies bedeutet, dass die Zuckerzusammensetzung ein wichtiger Faktor für die Nahrungsqualität des Honigtaus ist.

Für adulte *A. ervi* wurde eine direkte, dosisabhängige Wirkung des GNA auf die Lebensdauer gefunden. Um die Auswirkungen von GNA-haltigem Honigtau zu untersuchen, wurden *Rhopalosiphum padi* Blattläuse mit einer GNA-haltigen Kunstdiät ernährt und der gesammelte Honigtau den Schlupfwespen verfüttert. Dieser Honigtau enthielt 10-40% der GNA Konzentration, die in der Blattlausdiät gelöst war. Die Auswirkung auf die Lebensdauer von *A. ervi* war stärker als aufgrund der ermittelten GNA Konzentration im Honigtau zu erwarten war. Analysen der Kohlenhydrat- und der Aminosäure-Zusammensetzunge zeigten, dass GNA eine Veränderung der Honigtau-Zusammensetzung verursachte. Dies könnte die unerwartete verkürzte Lebensdauer der Schlupfwespen erklären. Honigtau von Blattläusen auf transgenen Weizenpflanzen, die GNA exprimieren, hatte dagegen keine Auswirkungen auf die Lebensdauer von *A. ervi*, im Vergleich zu Honigtau von nicht-transgenen Weizenpflanzen. Das ist vermutlich dadurch zu erklären, dass die transgenen Pflanzen nur auf niedrigem Niveau GNA exprimierten.

Um die Bedeutung von Honigtau als Nahrungsquelle für adulte Blattlausantagonisten zu erfassen, wurden Schlupfwespen, Schwebfliegen und Florfliegen in Weizenfeldern gesammelt. Analysen der Zuckerprofile der Insekten zeigten, dass die meisten Tiere in den letzten Tagen an einer zuckerreichen Nahrungsquelle gefressen hatten. Besonders für die zwei Florfliegenarten (*Chrysoperla lucasina* und *C. carnea*) wurde ein guter Ernährungszustand festgestellt. Bei 35% der Schwebfliegen (*Episyrphus balteatus*) mit hohem Zuckerprofil konnte nachgewiesen werden, dass sie kürzlich Honigtau gefressen hatten. Bei den Schlupfwespen (*Aphidius* spp.) lag dieser Anteil bei 63%. Diese Resultate zeigen, dass Honigtau von Blattläusen in Weizenfeldern eine wichtige Nahrungsquelle für Schwebfliegen und vor allem auch für Schlupfwespen darstellt.

Um festzustellen, ob Honigtau eine wichtige Nahrungsquelle für omnivore räuberische Insekten ist, wurde untersucht, ob *C. carnea* Larven auch in Anwesenheit von Blattläusen, ihrer bevorzugten Beute, Honigtau fressen. Sowohl in Versuchen mit als auch ohne Wahlmöglichkeit wurde festgestellt, dass die Larven Honigtau aufnahmen, wenn auch Blattläuse bevorzugt wurden. Vorheriges Fressen von Blattläusen oder Honigtau verringerte zusätzlich die weitere Honigtauaufnahme.

Diese Dissertation konnte zeigen, dass GNA im Honigtau einerseits eine potentielle Gefahr für Nicht-Zielinsekten darstellt und dass Nützlinge anderseits dem Toxin ausgesetzt sind. Das bedeutet ein potentielles Risiko von insektiziden Proteinen, die in Honigtau vorkommen, für Honigtau fressende Nicht-Zielinsekten und sollte bei der zukünftigen Risikobeurteilung von transgenen Pflanzen, die Insektizide exprimieren und im Phloemsaft transportieren, beachtet werden.

Nederlandse samenvatting

Insectenresistente genetisch gemodificeerde gewassen kunnen, naast de beoogde effecten op plaaginsecten, ook een negatieve invloed hebben op niet-doelorganismen. Wanneer deze laatsten een belangrijke ecologische functie hebben zoals bijvoorbeeld sluipwespen en predatoren die belangrijk zijn voor de natuurlijke plaagbestrijding, dan kunnen deze neveneffecten negatief zijn voor het gewas. Predatoren en sluipwespen kunnen aan het transgene product blootgesteld worden wanneer zij aan transgeen plantenmateriaal vreten, of aan insecten, die het toxine bevatten. In dit proefschrift wordt een nieuwe wijze van blootstelling aan transgene stoffen beschreven, namelijk via honingdauwconsumptie, een suikerrijke uitscheiding van sapzuigende insecten zoals bijvoorbeeld bladluizen. Omdat honingdauw voor vele insecten een belangrijke voedselbron is, bestaat er hierdoor een potentieel risico voor een breed spectrum aan niet-doelinsecten. Als modelsysteem zijn transgene tarweplanten gekozen die een lectine uit het sneeuwklokje (*Galanthus nivalis* agglutinin, GNA) tot expressie brengen met als doel de plant tegen sapzuigende insecten te beschermen. Aangezien een risico wordt gedefinieerd door zowel het gevaarpotentieel als de blootstelling, zijn hier beide apecten onderzocht voor GNA-bevattende honingdauw.

Larven van drie predatorsoorten (*Adalia bipunctata*, *Coccinella septempunctata* en *Chrysoperla carnea*) werden op hun gevoeligheid voor GNA getest. De levensduur van alledrie de soorten bleek gereduceerd door directe consumptie van GNA (opgelost in een sacharoseoplossing). Dit effect werd bevestigd doordat aangetoond kon worden dat de verteringsenzymen in de maag van de predatorlarven GNA niet konden afbreken. Terwijl *A. bipunctata* en *C. septempunctata* GNA grotendeels uitscheidden, hoopte het toxine zich in *C. Carnea* op, omdat zij geen uitwerpselen uitscheiden. Bovendien bond GNA zich aan glycoproteinen in de maag en werd het in het hemolymf van de insecten overgebracht.

Voordat de effecten van GNA in honingdauw onderzocht werden, is de geschiktheid van honingdauw van drie bladluissoorten, die aan aardappel- of tarweplanten vraten, onderzocht voor de bladluisparasitoïd *Aphidius ervi*. Hoewel de honingdauw van de geteste combinaties over het algemeen redelijk geschikt was als voedselbron voor de sluipwespen, bleek het soms toch minder geschikt dan een sacharoseoplossing. De suikersamenstelling van de honingdauw verschilde significant tussen de drie bladluissoorten op zowel aardappel- als tarweplanten. Multivariate analyse liet zien dat kleine verschillen in de levensduur van de sluipwespen in zekere mate door de suikersamenstelling van de honingdauw konden worden verklaard. Dat betekent dat suikersamenstelling een belangrijke factor kan zijn voor de voedingskwaliteit van honingdauw.

Bij het testen van de gevoeligheid van adulte *A. ervi* voor GNA, werd een dosis-gerelateerd effect gevonden op de levensduur. Om de effecten van GNA-houdende honingdauw te onderzoeken werden *Rhopalosiphum padi* bladluizen met een dieet gevoed dat GNA bevatte. De van deze bladluizen verzamelde honingdauw bevatte 10-40% van de GNA concentraties in het bladluis dieet. Door het vreten van deze honingdauw, werd de levensduur van *A. ervi* in sterkere mate verkort dan op basis van de gemeten GNA concentraties te verwachten was. Analyse van de koolhydraat- en aminozuursamenstelling liet zien dat er een verandering in honingdauw samenstelling had plaatsgevonden, die waarschijnlijk veroorzaakt was door het effect van GNA op de bladluizen. Dit zou een mogelijke verklaring kunnen zijn voor de versterkte levensduurvermindering van de sluipwespen. Honingdauw van bladluizen die aan GNA-transgene tarweplanten hadden gevreten had geen effect op de levensduur van *A. ervi* in vergelijking met honingdauw van niet-transgene tarweplanten. Dit was waarschijnlijk veroorzaakt door een zeer lage expressie van GNA in de planten.

Om vast te stellen of honingdauw een belangrijke voedselbron is voor adulte bladluisantagonisten, werden bladluis parasitoïden, zweefvliegen en gaasvliegen in tarwevelden gevangen. De analyse van de suikerprofielen van de insecten toonde aan dat de meerderheid van de insecten recentelijk honingdauw gevreten had. Vooral de twee gaasvliegsoorten (*Chrysoperla lucasina* en *C. carnea*) waren zeer goed gevoed. Recente honingdauw consumptie kon worden vastgesteld in 35% van de zweefvliegen (*Episyrphus balteatus*) en 63% van de sluipwespen (*Aphidius* spp.), die als suikergevoed geclassificeerd waren. Deze resultaten tonen aan dat honingdauw van bladluizen in tarwevelden een belangrijke voedselbron is voor bladluis parasitoïden en in mindere mate ook voor zweefvliegen.

Om vast te stellen of honingdauw een belangrijke voedselbron zou kunnen zijn voor omnivore predatoren, werd onderzocht of de larven van *C. carnea* honingdauw als voedselbron gebruikten in de aanwezigheid van bladluizen, hun voorkeursprooi. Zowel in een keuzesituatie als in situatie waarin slechts een enkele voedselbron werd aangeboden vraten de larven van honingdauw, maar in mindere mate dan van de bladluizen. Bovendien veroorzaakte het vreten van bladluizen of honingdauw een reductie in de daaropvolgende consumptie van honingdauw.

Door te hebben aangetoond dat enerzijds GNA in honingdauw een gevaar vormt voor vele niet-doelinsecten en dat anderzijds predatoren en parasitoïden blootgesteld kunnen worden aan GNA in honingdauw, laat dit proefschrift zien dat insecticide eiwitten in honingdauw een risico vormen voor honingdauwvretende insecten. Deze manier van blootstelling moet daarom worden meegewogen bij toekomstige risicobeoordeling van transgene planten, die in het floëem getransporteerde insecticiden tot expressie brengen.

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Curriculum vitae

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Education and Work Experience

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2002 – 2003	**Research fellowship** from European Science Foundation, Scientific Programme on the Assessment of Impacts of Genetically Modified Plants (AIGM), to conduct research at Agroscope Reckenholz-Tänikon Research Station ART, Zurich **Title** Transgenic snowdrop lectin in honeydew: estimating risks for non-target insects
1996 - 2001	**MSc in Biology**, Specialization: ecosystem biology, Wageningen University (NL) Degree awarded 'with distinction' **First MSc thesis** West Africa Rice Development Association (WARDA), Senegal and Crop and Weed Ecology group, Wageningen University (NL), supervized by Prof. Martin J. Kropff and Dr. Marco C.S. Wopereis **Title** Understanding of weed competition in irrigated rice in the Senegal river valley – Parameterization of the INTERCOM model

Second MSc thesis Soil Science and Geology group, Wageningen University (NL), supervized by Dr. Harry Booltink

Title Creation of maps of nitrogen content in the soil using crop growth simulations and remote sensing observations of the vegetation at field scale

Third MSc thesis Agroscope RAC Changins, Centre des Fougères, Conthey (CH) and Crop and Weed Ecology group, Wageningen University (NL), supervized by Dr. Wopke van der Werf and Werner Pfammatter

Title The influence of biological vs. integrated production and surfaces of ecological compensation on biodiversity in an apple orchard in Valais (Switzerland)

1990 – 1996 Preuniversity degree (secondary education)
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List of publications

Hogervorst P.A.M., Romeis J. and Wäckers F.L., 2003. Suitability of honeydew from potato infesting aphids as food fource for *Aphidius ervi*. Proceedings of the Section Experimental and Applied Entomology of the Netherlands Entomological Society 14: 87-90

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Submitted

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In preparation

Hogervorst P.A.M., Wäckers F.L. and Romeis J., Detecting nutritional state and food source use in nectar/honeydew feeding insects that synthesize oligosaccharides

Hogervorst P.A.M., Wäckers F.L. and Romeis J., The importance of honeydew for larvae of *Chrysoperla carnea* when aphids are available

Romeis J., Waldburger M., Streckeisen Ph., Hogervorst P.A.M., Keller B., Winzeler M. and Bigler F., Performance of transgenic spring wheat plants and effects on insect herbivores under glasshouse and semi-field conditions

List of presentations

Oral presentations

Hogervorst P.A.M., Romeis J. and Wäckers F.L., Estimating risks of exposure to transgene products through honeydew feeding: the role of honeydew quality.
14e Nederlandse Entomologendag, 20 December 2002, Amsterdam, The Netherlands

Hogervorst P.A.M., Romeis J. and Wäckers F.L., Importance of honeydew as food source for beneficials in agricultural fields.
15e Nederlandse Entomologendag, 19 December 2003, Groningen, The Netherlands

Hogervorst P.A.M., Wäckers F.L. and Romeis J., Transgene products in honeydew: estimating risks for non-target insects.
Royal Entomology Society (SIG) Meeting on GMOs and insects, 19 May 2004, Rothamsted, United Kingdom

Hogervorst P.A.M., Wäckers F.L. and Romeis J., Do transgene products in honeydew pose a risk for aphidophagous insects?
Ecology of Aphidophaga 9, 6-10 September 2004, Ceske Budejovice, Czech Republic

Hogervorst P.A.M., Wäckers F.L. and Romeis J., Transgene products in honeydew: estimating risks for non-target insects.
NCCR Plant Survival International Conference 2005, 31 March – 3 April 2005, Leysin, Switzerland

Hogervorst P.A.M., Ferry N., Gatehouse A.M.R., Wäckers F.L. and Romeis J., Impact of snowdrop lectin (GNA) on aphidophagous insects.
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VIIIth European Congress of Entomology, 17-22 September 2006, Izmir, Turkey.

Poster presentations

Hogervorst P.A.M., Romeis J. and Wäckers F.L., Impact of honeydew quality on parasitoid survival.
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XXII International Congress of Entomology, 15-21 August 2004, Brisbane, Australia

Hogervorst P.A.M., Wäckers F.L. and Romeis J., Do transgene products in honeydew pose a risk for an aphid parasitoid.
8th International Symposium on the Biosafety of Genetically Modified Organisms, 26-30 September 2004, Montpellier, France

16e Nederlandse Entomologendag, 17 December 2004, Haren, The Netherlands.

2nd International Symposium on Biological Control of Arthropods, 12-16 September 2005, Davos, Switzerland

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From January 2003 to September 2006 Mrs Petra Hogervorst obtained 9 credit points within the Graduate School (GS, minimum 8 required) and 7.5 credit points outside the Graduate School (EX) with the following activities:

Communication activities: 6.5 credit points (5.0 within the Graduate School)

EX	ZOeK PhD student conference 2003, Ascona (TI), Switzerland (poster and oral presentation)	October 2003	1.5
GS	Peer review and writing manuscripts	February 2004	2.0
GS	Effective public speaking	April - June 2004	1.0
GS	Planning a career strategy - Part 1 - Job finding methodology and networking	November 2005	1.0
GS	Preparing for the next step - Improving your CV and practical training to meet your future employer's expectations	January 2006	1.0

Research tools activities: 2.5 credit points (0 within the Graduate School)

EX	Datananalyse, Bundesamt für Statistik, Bern	August 2004	2.5

Scientific activities: 7.5 credit points (4.0 within the Graduate School)

GS	Risk assessment of genetically modified (GM) crops	January 2003	1.5
EX	15th Nederlandse entomologendag, Groningen, The Netherlands (oral presentation)	December 2003	1.0
GS	NCCR Plant Survival international conference, Leysin, Switzerland (oral presentation)	April 2005	1.0
GS	Integrated management of pests and diseases	February 2005	1.5
Ex	International conference 2004, Ecology of aphidophaga 9, Czech. Republic (oral presentation)	September 2004	1.0
EX	2nd meeting of ecological impact of GMOs, Lleida, Spain (oral presentation)	June 2005	1.0
EX	International symposium on biological control of arthropods, Davos, Switzerland (poster)	September 2005	0.5

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