The nutritional value of aphid honeydew for parasitoids of lepidopteran pests

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Contents

Summary

Keywords: nutritional ecology, aphids, honeydew, parasitoids, learning, herbivore induced volatiles, risk assessment, transgenic plants

Feeding on carbohydrate food sources is critical for survival and reproductive success of adult parasitoids. This means that females of many parasitoids will have to periodically interrupt host foraging to find food. If host and food are located in different patches, food searching can be time and energy consuming. This is especially true for most agricultural monocultures were nectar is available for a short period of time or not available at all. Situations where host and food sources are in close proximity from each other are highly beneficial for parasitoid females as this allows them to concentrate in host foraging and thereby minimize time spend on food searching. In this context, aphid-produced honeydew might be regarded as an alternative food source of key importance as it usually occurs in close association with the hosts even for non-aphid parasitoids. We tested the effect that honeydew feeding has on the performance of larval parasitoids (Chapter 1). Parasitoid females lived longer and produced a higher number of offspring when feeding on honeydew when compared to unfed females. However, they lived shorter and produced a smaller number of offspring than females fed with a sucrose solution. The honeydew was composed of both plant-derived and aphid-produced sugars and its composition changed over time and with different infestation rates. The relatively poor performance on honeydew cannot be explained by the honeydew composition, but rather by the slow uptake of the very viscose honeydew as compared to the sucrose solution. Due to its wide availability and its accessibility, honeydew would still make it a very useful food source for many parasitoids. Therefore an ability to learn to distinguish between host and food associated cues should be adaptive as it would allow the parasitoid to save energy and time when switching from host to food searching and vice-versa. We assessed whether *C. marginiventris* learns honeydew associated odours and how their physiological state affects the choice between host and

food searching (Chapter 2). *C. marginiventris* was not innately attracted to aphid-infested barley. However, when the females had a food reward associated with the odour of aphid-infested plants they were subsequently attracted to this odour when hungry. When given a choice between food and host associated odours *C. marginiventris* decides between host and food foraging based on their physiological status and previous experience. Moreover, hungry females were less responsive to host-associated odours than well fed ones.

The fast increase in the commercial use of transgenic plants has raised concerns about their potential risks for non-target organisms. In this context, we investigated possible effects of Bt-transgenic plants on *C. marginiventris* through aphid-produced honeydew (Chapter 3). Surprisingly, transgenic plants were more susceptible to aphids than their corresponding untransformed counterparts. Higher amino acid concentrations in the phloem of the transgenic lines partially explained these differences in aphid performance. The differences in aphid performance also affected the performance of *C. marginiventris*. Females of this parasitoid lived longer and produced more offspring when in the presence of transgenic plants infested with aphids compared to females that had access to near isogenic lines infested with aphids. The sugar composition of the honeydew did not explain these differences in parasitoid performance. Further tests suggested that the increase in parasitoid performance was rather due to a greater availability of honeydew on the transgenic plants due to their higher susceptibility to aphids.

Résumé

Mots-clés: écologie nutritionnelle, aphides, miellat, parasitoïdes, apprentissage, volatiles induits par des herbivores, évaluation de risque, plantes transgéniques

La consommation d'hydrates de carbone est indispensable pour la survie et reproduction des parasitoïdes adultes. Ceci veut dire que les femelles de nombreux parasitoïdes devront périodiquement arrêter la recherche des hôtes afin de trouver de la nourriture. Si les hôtes et la nourriture se trouvent à différents endroits, la recherche de nourriture peut conduire à une grande perte en temps et en énergie. Ceci est le cas dans la plupart des monocultures agricoles où le nectar est disponible pour une période limitée voire complètement indisponible. Lorsque les hôtes et les sources de nourriture sont proches l'une de l'autre, les parasitoïdes femelles en bénéficient grandement car elles peuvent se concentrer sur la recherche d'hôtes et minimiser le temps consacrée à trouver la nourriture. Dans ce contexte, le miellat produit par les pucerons peut être considéré comme une source de nutrition alternative d'importance vitale car il se trouve en association proche avec les hôtes même pour les parasitoïdes autres que ceux du puceron. Les effets de l'alimentation avec du miellat sur la performance des parasitoïdes larvaires ont été testés (Chapitre 1). Les femelles parasitoïdes ont vécu plus longtemps et ont produit une descendance plus nombreuse en se nourrissant de miellat comparé à des femelles non nourries. Toutefois, elles ont vécu moins longtemps et ont produit moins de descendants que des femelles nourries avec une solution de sucrose. Le miellat était composé de sucres dérivés de la plante et des pucerons et sa composition changeait avec le temps et avec des taux d'infestations différents. La performance relativement basse avec du miellat ne peut pas être expliquée par sa composition mais plutôt par la difficulté à ingérer le miellat qui est très visqueux comparé à la solution de sucrose. Du miellat facilement accessible serait toutefois une source de nourriture très utile pour de nombreux parasitoïdes. Ainsi donc, l'aptitude à apprendre à distinguer entre hôte et les signaux associés à la nourriture devrait être adaptive

car cela permettrait aux parasitoïdes d'économiser de l'énergie et du temps lors du changement entre recherche de nourriture et recherche d'hôte et vice-versa. Nous avons évaluée si le parasitoïde apprend les odeurs associées au miellat et si leur état physiologique affecte le choix entre recherche de nourriture et recherche d'hôte (Chapitre 2). *C. marginiventris* n'est pas attiré de façon innée à de l'orge infesté par des pucerons. Toutefois, quand les femelles avaient une récompense sous forme de nourriture associée avec l'odeur des plantes infestées par des pucerons, elles étaient attirées par ces odeurs les fois suivantes. Lorsque le choix entre odour de nourriture et odeur associée à l'hôte est donné à *C. marginiventris*, celle-ci décide selon son état physiologique et ses expériences passées. De plus, les femelles affamées réagissaient moins aux odeurs associées aux hôtes que des femelles bien nourries.

La grande augumentation de l'utilisation commerciale des plantes transgéniques a provoqué de nombreuses inquiétudes sur leurs risques potentiels pour des organismes non-cibles. Dans ce contexte nous avons testé les effets potentiels des plantes transgéniques Bt sur *C. marginiventris* à travers le miellat produit par les pucerons (Chapitre 3). De façon surprenante les plantes transgéniques furent plus susceptibles aux pucerons que leurs analogues non-transformés. Des concentrations supérieures en acides aminés dans le phloème des transgéniques explique partiellement la différence de performance des pucerons. Ces différences ont aussi affectés les performances de *C. marginiventris*. Les femelles de ce parasitoïde ont vécu plus longtemps et ont produits plus de descendants quand elles étaient en présence des transgéniques infestées de pucerons comparés aux femelles qui avaient accès aux plantes isogéniques infestées. La composition en sucre du miellat n'a pas pu expliquer les différentes performances des parasitoïdes. D'autres tests ont permis de suggérer que l'amélioration des performances des parasitoïdes était due à un accès plus facile du miellat sur les plantes transgéniques à cause de leur plus grande susceptibilité aux pucerons.

General Introduction

Studies on parasitoid behaviour have focused mainly on parasitoid-host interactions. However, hosts are not the only resources needed by parasitoids. In order to optimize their reproductive success adult parasitoids also need food. Feeding not only affects adult parasitoid survival, but enhances fecundity through a positive effect on egg production and life span (Leius, 1961; Syme, 1975; Coombs, 1997; England & Evans, 1997; Baggen & Gurr, 1998, Schmale et al., 2001). Feeding can also affect the parasitoid's motivation to forage for hosts by a general increase in activity. After a meal parasitoids shift from food to host searching and show an increase in responsiveness to host-related odours (Lewis & Takasu, 1990; Forsse et al., 1992; Wäckers, 1994; Takasu & Lewis, 1995; Siekmann et al., 2004). Moreover, food availability can attract parasitoids and promotes their retention in an area (Idris & Grafius, 1995; Jacob and Evans, 1998; Baggen & Gurr, 1998).

Regarding their feeding behaviour, parasitoids can be divided in two groups: species whose females feed from their hosts and species that require and obtain carbohydrates from other food sources (Jervis et al., 1992; Jervis & Kidd, 1995; Jervis, 1998). For the species that feed on hosts or their by-products, host and food searching can be considered a single process. However, many parasitoid species do not feed on their hosts and even species that do host-feed often need carbohydrates from other food sources. This means that females will have to periodically interrupt host foraging to find food. This is especially critical when host and food are located in distinctly different habitats.

In most agricultural monocultures carbohydrate food sources for parasitoids (i.e. nectar sources) are available only during a short period of time or not available at all. This may greatly hamper the parasitoids' performance as biological control agents. For example, lack of food sources for adult parasitoids can be regarded as one of the reasons for the failure of some introduced parasitoids to establish in biological control programs (Stilling, 1993) and food sources can explain why some parasitoids are more successful on certain plants than on others (Streams et al., 1968; Shahjahan, 1974).

Plants that provide both hosts and food are more profitable for parasitoids and allow them to allocate more time and energy to the search for hosts and minimize food searching. In the absence of nectar, homopteran-produced honeydew can be an alternative food source of key importance for both homopteran and non-homopteran parasitoids if it occurs in close association with hosts. It is important to keep in mind that not all food sources are equally suitable to parasitoids. Factors such as availability, accessibility, nutritional quality, distribution and detectability can greatly influence the exploitation of a food source by parasitoids.

Studies that have assessed the suitability of homopteran-produced honeydew had very different outcomes. Some studies have shown that parasitoid females live longer when feeding on honeydew than when unfed, but not as long as females that fed on nectar or honey (Idoine & Ferro, 1988; Lee et al., 2004). Other studies have found that honeydew has the same effect on survival as nectar, honey or sucrose solution (England & Evans, 1997; Singh, 2000; Hogervost et al., 2003), but there are also examples that show that parasitoid survival on honeydew can be as low as when given no food (Avidov et al., 1970; Elliot et al., 1987).

Honeydew is an aqueous mixture of various sugars that makeup more than 98% of the dry weight, but also contains several other plant compounds that can affect nutritional quality, such as amino acids, sterols and other lipids, phenolics, and organic acids (Mittler, 1958; Auclair, 1963; Forrest & Knights, 1972; Hussain et al., 1974; Lombard et al., 1984; Douglas 1993; van Helden et al., 1994). Several of these honeydew components might explain the different outcomes of the studies that have assessed parasitoid longevity. The sugars are of special interest as honeydew is composed not only of plant-derived sugars, but also of homopteran-synthesized sugars. Wäckers (2000) has shown that the homopteran-synthesized sugars have lower nutritional value for parasitoids than the plant-derived ones.

For long it has been assumed that homopterans actively synthesize sugars in order to osmorregulate (Kennedy & Fosbrooke, 1972; Downing, 1978; Wilkinson et al., 1997). Wäckers (2000) proposed another (non-exclusive) function of the homopteran synthesized sugars. He suggests that these sugars impede gustatory perception and honeydew uptake and utilization by homopteran antagonists, supporting the hypothesis that the synthesis of carbohydrates by homopterans serves to reduce the exploitation of the honeydew by parasitoids and non-mutualistic predators. However, the composition of the honeydew is highly variable and depends strongly on the homopteran and plant species (Hendrix et al., 1992) and parasitoids vary considerably regarding the spectrum of sugars they can utilize (Eijs et al., 1998; Teraoka & Numata, 2000; Wäckers, 2001; Beach et al., 2003; Jacob & Evans, 2004).

Optimally balancing decisions on whether to forage for hosts or food is critical for the parasitoids reproductive success (Sirot & Bernstein 1996). It has been shown that the physiological state of the parasitoid female will determine whether host or food foraging is given priority (Lewis & Takasu, 1990; Takasu & Lewis, 1993; Wäckers, 1994; Jervis & Kidd, 1995; Sirot & Bernstein, 1996). The females use olfactory and visual cues to find both types of resources (Wäckers & Swaans, 1993, Wäckers, 1994; Stapel et al., 1997) and use not only innate behaviours, but are also able to learn to distinguish between cues that are associated with hosts and cues associated with food (Lewis & Takasu, 1990; Takasu & Lewis 1993; 1996). The importance of learning host or food associated cues by parasitoid females has been well documented (Lewis & Takasu, 1990; Vet & Groenewold, 1990; Turlings et al., 1993; Takasu & Lewis 1993; 1996; Vet et al., 1995) and it is highly adaptative as females have to periodically switch from host to food searching when these resources vary spatially and temporarily.

To guarantee the effectiveness of parasitoids as biological control agents in modern agriculture it is important to consider their need for food sources. This has also to be taken into account when assessing the ecological effects of introducing transgenic crops. The commercial use of genetically modified (GM) crops is rapidly expanding. In 2004, the global area of transgenic crops continued to grow for the ninetieth consecutive year, increasing more than 47 fold, form 1.7 million hectares in 1996 to 81.0 million hectares in 2004 (James, 2004). To date, the only insect resistant transgenic plants that are commercially available are those expressing genes that code for *Bacillus thuringiensis* (Bt) toxins. The primary targets of these toxins are insects belonging to Lepidoptera, Diptera and Coleoptera (Höfte & Whiteley 1989; MacIntosh et al., 2001) and they are only active when orally ingested as their mode of action is expressed in the midgut (Liang et al., 1995; Schnepf et al., 1998).

The rapid expansion of transgenic plants has raised concerns regarding their impact on non-target organisms, and how this might reflect on the food web. Organisms associated with the crop can potentially be affected by insect resistant transgenic plants through various ways (Schuler et al., 1999; Groot & Dicke, 2002; Dutton et al., 2003). These effects can be grouped in three types (Fig. 1): direct effects on non-target species that are susceptible to the toxin; indirect effects on carnivores (such as parasitoids and predators) due to changes in host or prey quantity or quality; and indirect effects due to unintended changes in the plant caused by the insertion of the new gene.

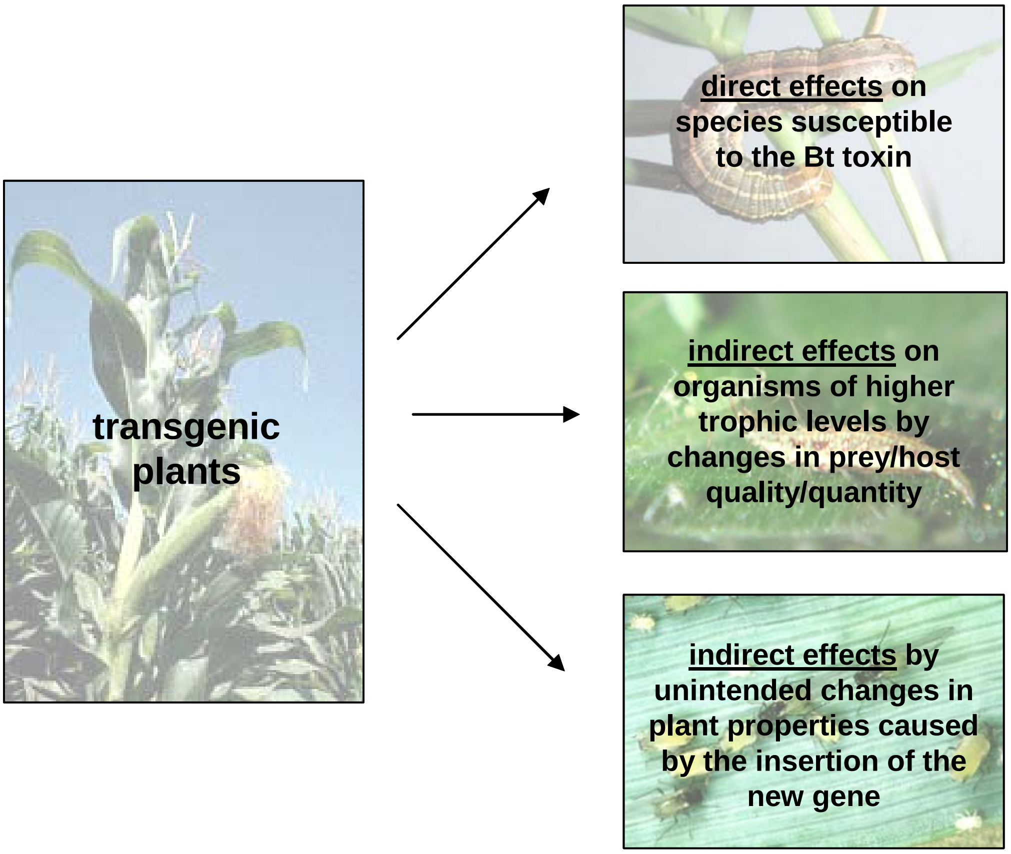

Figure 1. Types of effects through which transgenic plants can affect non-target organisms

Several studies have investigated direct and indirect effects of Bt plants on non-target organisms, finding no or various degrees of detrimental effects (reviewed by Dutton et al., 2003 and by Lövei & Arpaia, 2005). Recently some studies have reported different types of unintended effects, which show for instance: honeydew produced by planthoppers feeding on Bt rice to be more acidic (Bernal et al., 2002); better aphid performance on Bt maize (Lumbierres et al., 2004; Pons et al., 2005); poorer aphid performance on Bt potatoes (Ashouri et al., 2001); Bt maize to be higher in lignin content (Masoero et al., 1999; Saxena & Stotzky, 2001); and Bt maize to have decreased *Fusarium* infections (Munkvold et al., 1997; 1999).

The unintended (and unexpected) changes in GM and conventionally bred crops usually arise due to: pleiotropic effects that is the production of more than one genetic effectby the transferred gene; epistasic effects that is the alteration on or of the inserted gene via alteration by a non-allelic gene; tissue culturing effects via mutational, chromosomal or epigenetic changes; and direct physiological effects of the introduction of the new trait via altered metabolic pathways or physiological responses of the plant genotype to abiotic or biotic stimuli (Birch, 2003; Cellini et al., 2004). These potential changes can have important ecological implications, as for example suggested by Saxena & Stotzky (2001) for the higher lignin content of Bt maize. As lignin reduces the ability of herbivores to digest plant material, an increase in lignin might affect rates of feeding and population dynamics of herbivores affecting subsequently the upper levels of the food web. Despite recent progress in investigating how unintended changes in transgenic crops might affect non-target organisms, as yet, little is know about the causes of these changes and their effect on the food web.

Regarding the effect of Bt transgenic plants on aphids, as mentioned previously, aphids are not susceptible to the Bt toxin. Moreover, the toxin is not present in the phloem of three commercially available maize events: Mon 810, Bt11 and Event 176 (Head et al., 2001; Raps et al., 2001; Dutton, 2002). Consequently aphids do not ingest the toxin when feeding on these constructs. Experiments with aphids feeding on artificial diets have shown that even when the diet contained high concentrations of the Bt toxin, the toxin levels in the body of the aphid were 250-500 lower than the original levels (Raps et al., 2001). This means that aphid-produced honeydew is not an exposure route of Bt toxin to non-target organisms. However various plant characteristics have a significant direct influence on survival, fecundity and foraging success of parasitoids (see Bottrell et al., 1998; Cortesero et al., 2000 and references within). Any unintended change at the plant level is bound to affect higher trophic levels in the food web, either directly or indirectly.

One such indirect effect could result from possible changes in aphid susceptibility of a plant, which in turn might also change honeydew quality/availability as a food source. Such an effect could have an important impact on the wasps' reproductive success and consequently on their effectiveness in biological control of pests.

In this context, there is a substantial lack of information about the use of honeydew as a food source by non-aphid parasitoids and the factors that determine its nutritional value. The principal objective of the current thesis was to obtain such basic knowledge, thus making it possible to answer questions about how changes in transgenic plants could affect aphid performance and the suitability of their honeydew as food for non-aphid parasitoids.

Thesis outline

The system used for this study consists of the host plants maize *Zea mays* and barley *Hordeum vulgare*; the herbivores *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae); and the larval parasitoids *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) and *Microplitis rufiventris* Kokujev (Hymenoptera: Braconidae) (Fig. 2), natural enemies of important lepidopteran pests including *Spodoptera* spp. (Hoballah et al., 2004, Hegazi 1977). For each of the three parasitoids aphid honeydew could be a food source that can potentially increase their performance.

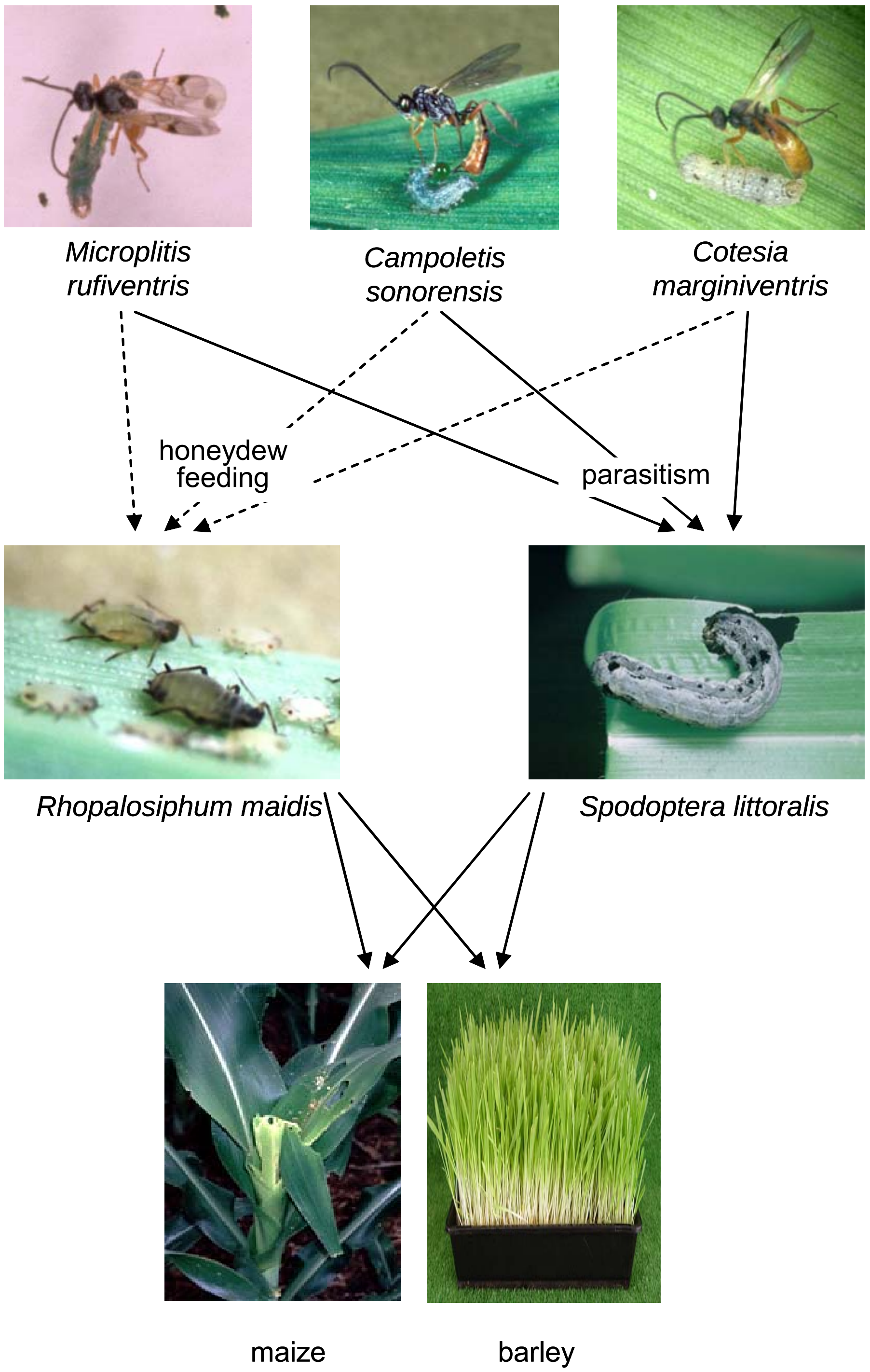

Figure 2. Main players composing the food web used in the current study: the plants maize and barley; the herbivores *Spodoptera littoralis* and *Rhopalosiphum maidis*; and the parasitoids *Cotesia marginiventris*, *Campoletis sonorensis*, and *Microplitis rufiventris*.

The present thesis addresses three main questions:

1. Are larval parasitoids able to use honeydew produced by *R. maidis* as food? As discussed above, studies on the suitability of honeydew as food for parasitoids have very different outcomes. The aim of this study was to assess the effect of *R. maidis* honeydew on the performance of the three parasitoids. It was found that honeydew does increase the parasitoids' longevity and may enhance their parasitism rate, but honeydew was found to be less suitable in comparison a sucrose solution (the positive control tested). We further investigated possible reasons for this result, especially focusing on the sugar composition of the honeydew.

2. Is *C. marginiventris* innately attracted by aphid-produced honeydew and can it learn to distinguish between honeydew-associated cues and host-associated cues? As honeydew was shown to increase *C. marginiventris* longevity (Chapter 1) and therefore an ability to learn honeydew-associated cues might be adaptive. Such an ability should decrease the time and energy spent when switching from host to food searching. *C. marginiventris* females with different experiences and feeding status were given a choice to respond to volatiles produced by plants infested by *R. maidis* (producing food) and *S. littoralis* (serving as hosts). These volatiles were also collected and analyzed to determine qualitative of quantitative differences that might be perceived and learned by the wasps.

3. Does Bt maize affect the performance of *R. maidis* and, if so, does this effect translate in the performance of *C. marginiventris* after honeydew feeding? The corn leaf aphid *R. maidis* was found to perform significantly better on Bt-transgenic maize lines than on their corresponding near-isogenic lines. Knowing that amino acids in the phloem sap are considered an indicator of nutritional quality for aphids, we compared the composition and concentration of the amino acid in the phloem of transgenic plants and their unmodified counterparts. As shown in Chapter 1, the availability of honeydew plays an important role in determining how efficiently parasitoids exploit this food source. We therefore further studied if the positive effect of Bt plants on aphid performance reflects on the performance of *C. marginiventris.* Honeydew composition and food intake were investigated as possible reasons for an increased performance of *C. marginiventris* when fed with honeydew from aphids on Bt maize.

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Chapter 1

The nutritional value of aphid honeydew for non-aphid parasitoids

Based on: Faria, CA, Wäckers, FL & Turlings, TCJ. The nutritional value of aphid honeydew for non-aphid parasitoids. To be submitted to *Entomologia Experimentalis et Applicatta*

Abstract

Intake of sugar-rich foods by adult parasitoids is crucial for their reproductive success. This is an aspect of parasitoid biology that warrants special attention in the biological control of pests. Facilitating access to suitable foods might help optimize the efficacy of parasitoids as biological control agents. In situations where nectar is not readily available, homopteran honeydew can be a key alternative food source.

We studied the impact of honeydew feeding on the longevity of the larval endoparasitoids *Cotesia marginiventris*, *Campoletis sonorensis* and *Microplitis rufiventris*, natural enemies of important lepidopteran pests. Females of these wasps lived longer when feeding on the honeydew produced by the aphid *Rhopalosiphum maidis* on barley compared to females that were provided only with water. However, they lived considerably shorter than females fed with a sucrose solution. Further investigations with *C. marginiventris* showed that honeydew also increases the number of offspring produced, but less so than the increase promoted by a sucrose solution. Moreover, females of this species need to feed several times throughout their life in order to reach optimal longevity and reproductive output. We analyzed the sugars in the honeydew produced by *R. maidis* on barley and found that it contains mainly plant-derived sugars, but also several sugars produced by the aphid. The sugar composition of the honeydew changed over time and as a function of aphid infestation. In general, the higher the aphid infestation, the smaller the percentage of aphid synthesized sugars in the honeydew. Experiments with sugar mimics of the honeydew allowed us to reject the hypothesis that the relatively poor performance of the parasitoid when fed honeydew was due to the sugar composition. Instead, the results from feeding experiments strongly suggest that poor intake of honeydew due to high viscosity limits its value as food for the wasp. The possible consequences of these findings for the biological control of pests are discussed.

Introduction

To optimize their reproductive success, adult parasitoids not only need to find hosts for their offspring, but also food to extend their longevity and thus the time they have to find hosts. The availability of suitable food should therefore be an important consideration in attempts to optimize the effectiveness of aparasitoids as biological control agents.

Adult females of some parasitoids obtain essential nutrients directly from hosts through so-called host feeding, but even these species often need non-host food sources to optimize their longevity (Jervis et al., 1996; Heimpel et al., 1997a). It has been shown that feeding by adult parasitoids not only increases longevity and fecundity (Leius 1961; England & Evans 1997; Baggen & Gurr 1998), but also affects flight activity (Forsse et al., 1992) and attraction and/or retention of the parasitoids in an area (Stapel et al., 1997; Jacob & Evans, 1998).

Parasitoid females that feed on hosts or their by-products reduce the need to shift from host to food foraging. However, parasitoids that feed on other food sources will have to forage for hosts and food separately (Sirot & Bernstein, 1996; Lewis et al., 1998). When food is located at a distance from host sites, this issue is specially important, since travelling to food sites limits the amount of time available for host foraging, costs energy and increases the risk of mortality (Jervis et al., 1996; Stapel et al., 1997). Foraging cues used to locate food may be different from cues that lead parasitoids to hosts and differences in olfactory and visual cues can be learned through association during successful food and host location (Lewis & Takasu, 1990; Wäckers, 1994),

In nature, the principal non-host food sources available for parasitoid females are floral and extra-floral nectar, and homopteran honeydew (Idoine & Ferro, 1988; Jervis et al., 1993; Jervis 1998). Nectar is often absent or very limited in its availability in large monocultures, which may greatly hamper the effectiveness of parasitoids used for biological control. In such situations, honeydew can be a key alternative food for parasitoids if honeydew producing Homoptera occur in the vicinity of hosts. Feeding on nearby honeydew instead of distant nectar sources should allow parasitoids to allocate more time to forage for hosts, resulting in higher rates of parasitism.

Several laboratory studies have shown that in the presence of honeydew parasitoid females indeed live longer and have higher fecundity than unfed females (Hocking, 1966;

England & Evans, 1997; Singh, 2000; Hogervost et al., 2003). However, nectar is usually much better food sources for parasitoids than honeydew (Avidov et al., 1970; Elliot et al., 1987; Idoine & Ferro, 1998; Wäckers, 2000; 2001; Lee et al., 2004). One possible explanation for this is that honeydew is not only composed of the main plant-derived sugars fructose, sucrose and glucose, but also contains various other compounds. These include various homopteran-synthesised sugars (Mittler, 1958; Hendrix et al., 1992) that may reduce the nutritional value of the honeydew as a food source (Wäckers, 2000; 2001). In fact, Homoptera might synthesise less nutritional sugars to reduce the suitability of the honeydew as food for insects, thus reducing the nutritional benefit to their natural enemies (Wäckers, 2000).

The composition of honeydew shows great variation both in the type of sugars present and the overall sugar concentration depending on the homopteran and plant species (Hendrix et al., 1992). Moreover, parasitoids can vary considerably with regard to the spectrum of honeydew sugars that they can utilise (Jacob & Evans, 2004; Beach et al., 2003; Wäckers, 2001). This variability both from the side of the product (honeydew) and the users (parasitoids) suggests that there is an opportunity to fine-tune and manipulate the situation in crop fields to better exploit the presence of honeydew producing insects for pest control.

With the above in mind, we investigated the effect of the honeydew produced by the aphid *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae) attacking barley (*Hordeum vulgare*), for the longevity of the solitary larval endoparasitoids *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) and *Microplitis rufiventris* Kokujev (Hymenoptera: Braconidae), natural enemies of important lepidopteran pests (Hoballah et al., 2004; Hegazi 1977). For *C. marginiventris* we also tested, in cage experiments, how the presence of aphids affects their lifetime parasitism. As it was found that *R. maidis* honeydew was considerably less suitable than a water solution with sucrose, we analysed the honeydew for the presence of aphid-produced sugars that might explain these results. Solutions mimicking the measured sugar composition of *R. maidis* honeydew were then fed to *C. marginiventris* females to determine their nutritional quality. Since sugar composition did not explain the negative effect on nutritional quality, an experiment was added to determine if differences in food uptake could explain differences in survival on honeydew and sucrose water.

Materials and Methods

Plants

All plants were individually grown from seed in a climate chamber (27±2 °C, 60% r.h., 16L:8D, and 50000 lm/m^2). Barley of the variety Lyric was used for the aphid rearing or experiments when 3-5 weeks old. Maize of the variety Delprim was used for the parasitoid performance experiments when 3-4 weeks old.

Insects

We chose the corn leaf aphid *R. maidis* for the experiments because it excretes copious amount of honeydew and it is usually considered a minor pest (Jauset et al., 2000; Kring & Gilstrap, 1986; Kröber & Carl, 1991, Waterhouse & Sands, 2001), so its presence in crop fields could indeed benefit the effectiveness of biological agents without causing additional harm to the crop. The aphids were provided by the Agroscope RAC Changins in Switzerland and reared in climate chambers (25°C, 70% r.h. and 14L:10D). *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) eggs were received weekly from Syngenta (Stein, Switzerland) and the emerging larvae were used for parasitoid rearing or experiments.

C. marginiventris, *M. rufiventris* and *C. sonorensis* colonies were maintained on *S. littoralis* larvae fed with artificial diet. Adults were kept in climate chambers (25ºC, 85% r.h. and 14L:10D) and the females used for the experiments were one day old, mated and unfed.

Effect of honeydew on parasitoid longevity

Groups of five parasitoid females of a particular species were put in a cellophane bag (30 x 15 cm) covering a barley plant that was either: sprayed with a 2M sucrose solution, infested with aphids or clean (i.e. not sprayed with sucrose nor infested by aphids). The reason for the use of a 2M concentration is that it represents the high end of sugar concentrations found in floral nectar (Baker & Baker, 1983). Aphid infestation was obtained by placing clean barley plants together with aphid-infested plants for four to six days before an experiment started. This resulted in an estimated infestation of eight aphids/cm^2 at the beginning of the experiments. To provide humidity, a plastic container with water-soaked cottonwool was provided in each cellophane bag. All plants were put in a climate chamber and survival of the wasps was recorded daily.

Effect of honeydew on C. marginiventris *performance*

Groups of three *C. marginiventris* females were placed in plastic Bugdorm-2 cages (60 x 60 x 60 cm, MegaView Science Education Services Co. Ltd., Taiwan) with two maize plants infested with around 300 *S. littoralis* larvae and one barley plant that was either: sprayed with sucrose, infested with aphids or clean. To investigate if *C. marginiventris* females need to feed several times through their life to optimally benefit from a food source, we added two other treatments, whereby barley plants sprayed with sucrose or infested with aphids were left in the tents only for the first two days of the experiments. The longevity of the females was recorded daily and the *S. littoralis*-infested maize plants were replaced every other day. The recollected larvae were reared through on artificial diet until the parasitoids formed the cocoons that were then counted.

Honeydew collection and analysis

Individual barley plants were infested with aphids in a clipcage (1.5x1.5 cm). In order to evaluate an effect of infestation rate and time since infestation on honeydew composition, three initial aphid densities were used (10, 100 and 500 aphids of mixed ages). The first honeydew collection was made three days after the infestation, and subsequently the honeydew was collected at intervals of one week from the infestation date, for three consecutive weeks.

For the first and second collections the clipcages that were used for the initial infestation were replaced with a new clipcage that was left attached to the plant for 24h. These collection cages were then placed in a 100% r.h. environment for 24h and a micro-capillary was used to collect 1µL of honeydew, which was diluted in 50µL of 70% ethanol. For the subsequent collections, the clip cages were removed, the plants were placed in new cellophane bags and Petri dishes were placed at the bottom of the plants for 24h. The honeydew was collected from the Petri dishes using the same method described for the collection from clipcages.

Just before analysing the samples they were diluted a further 1000x with Milli-Q water. Of each diluted 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 column (4 x 50 mm), an Dionex Carbopac PA1 analytical column (4 x 250 mm), as well as an ED 40 Electrochemical Detector for Pulsed Amperimetric Detection (PAD). 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, melezitose, raffinose, maltose and erlose by injecting calibration standards with concentrations of 2.5 ppm, 5 ppm, 7.5 ppm, and 10 ppm of these sugars. The concentrations of the individual sugars were analysed using the program PEAKNET Software Release 5.1 (DX-LAN module).

Longevity of C. marginiventris *on honeydew mimics*

The HPLC analysis showed that the honeydew is mainly composed of the plant sugars glucose, fructose and sucrose and the aphid synthesized sugars maltose, erlose and trehalose. To investigate if the observed reduced survival of parasitoids on the honeydew might be due to the aphid produced sugars, we measured the longevity of *C. marginiventis* when feeding on 1M sugar mixtures that mimic the honeydew with three different sugar compositions. These were: 1) glucose (33.3%), fructose (33.3%) and sucrose (33.3%); 2) glucose (25%), fructose (25%), sucrose (25%), maltose (12.5%) and erlose (12.5%); and 3) glucose (25%), fructose (25%), sucrose (25%), maltose (10%), erlose (10%) and thehalose (5%). Longevity on these solutions was compared with the longevity of wasps feeding on a 1M sucrose solution, *R. maidis* honeydew and unfed wasps.

Groups of three females that were 12-24h old and hungry were placed in plastic cups (height 2.5cm, diameter 4.5cm). Small droplets of one of the sugar solutions were distributed on the lid of the cup using a total of 8µL of solution per cup. To collect honeydew, plastic lids were placed under aphid infested barley plants for 24h, which was sufficient to cover the lids with honeydew. Humidity was kept high by placing a wet dental roll in each cup. The lids with sugar solutions were replaced twice per week and the ones with honeydew every other day. The cups were kept in a climate chamber (25ºC, 85% r.h. and 14L:10D) and the number of wasps alive recorded daily.

Correlation between honeydew intake and survival

As the aphid-produced sugars did not explain the relatively poor performance of honeydew fed parasitoids, an experiment was added to test for a possible difference in uptake of honeydew and sucrose solution during feeding. For this we measured the intake of *R. maidis* honeydew or of a 2M sucrose solution, by *C. marginiventris*, during a single feeding bout and further determined its effect on parasitoid survival.

C. marginiventris females were used when 24-30h old. To make sure that the food intake was solely motivated by sugar need, parasitoids had been provided with water *ad libidum* prior to the experiments. The food sources were presented to the wasps as a 1µL droplet on a microscope slide (7.6 x 2.6 cm) and were left in a 100% r.h. environment during the tests. Consumption was determined by weighting the individual females on a precision scale (Mettler MX5; ±1 µg) before and immediately after exposure to honeydew or a sucrose solution. The time spent feeding was recorded and each individual was placed in a glass tube (1.2 x 7.5 cm) and its survival determined. Humidity was provided by a wet strip of filter paper in the glass tubes.

Statistical Analysis

Effects of different diets on survival probability in the first experiment were compared using survival analysis. Differences between survival curves were analysed with a log-rank test using S-Plus 6.2.

Differences in parasitism rate (numbers of cocoons formed) by *C. marginiventris* females kept in cages with different food sources were determined by ANOVA and differences between means compared using the Tukey's test with SPSS 12.0.

The difference between the percentage of weight gained by *C. marginiventris* after one feeding bout on honeydew and sucrose solution were compared with the t-test and *C. marginiventris'* subsequent longevity after feeding on these two food sources were compared using the Mann-Whitney test. The degree of correlation between honeydew and sucrose solution intake and subsequent survival was determined by linear regression analysis. Longevity was analysed in a GLM with terms for food type, intake (% weight gain during one feeding bout) and their interaction. The difference between time spent feeding on honeydew and sucrose was analysed using the Mann-Whitney test. All these analysis were performed using SPSS 12.0.

Results

Effect of honeydew on parasitoid longevity

Diet significantly affected the longevity of *C. marginiventris*, *M. rufiventris* and *C. sonorensis* (n=24, χ^2=125, df=2, p<0.001 for *Cotesia*, n=20, χ^2=83.1, df=2, p<0.001 for *Microplitis* and n=24, χ^2=104, df=2, p<0.001 for *Campoletis*). For all three species, honeydew had a positive effect on survival, but survival was considerably lower in comparison to the wasps that had fed on the sucrose solution (Fig. 1).

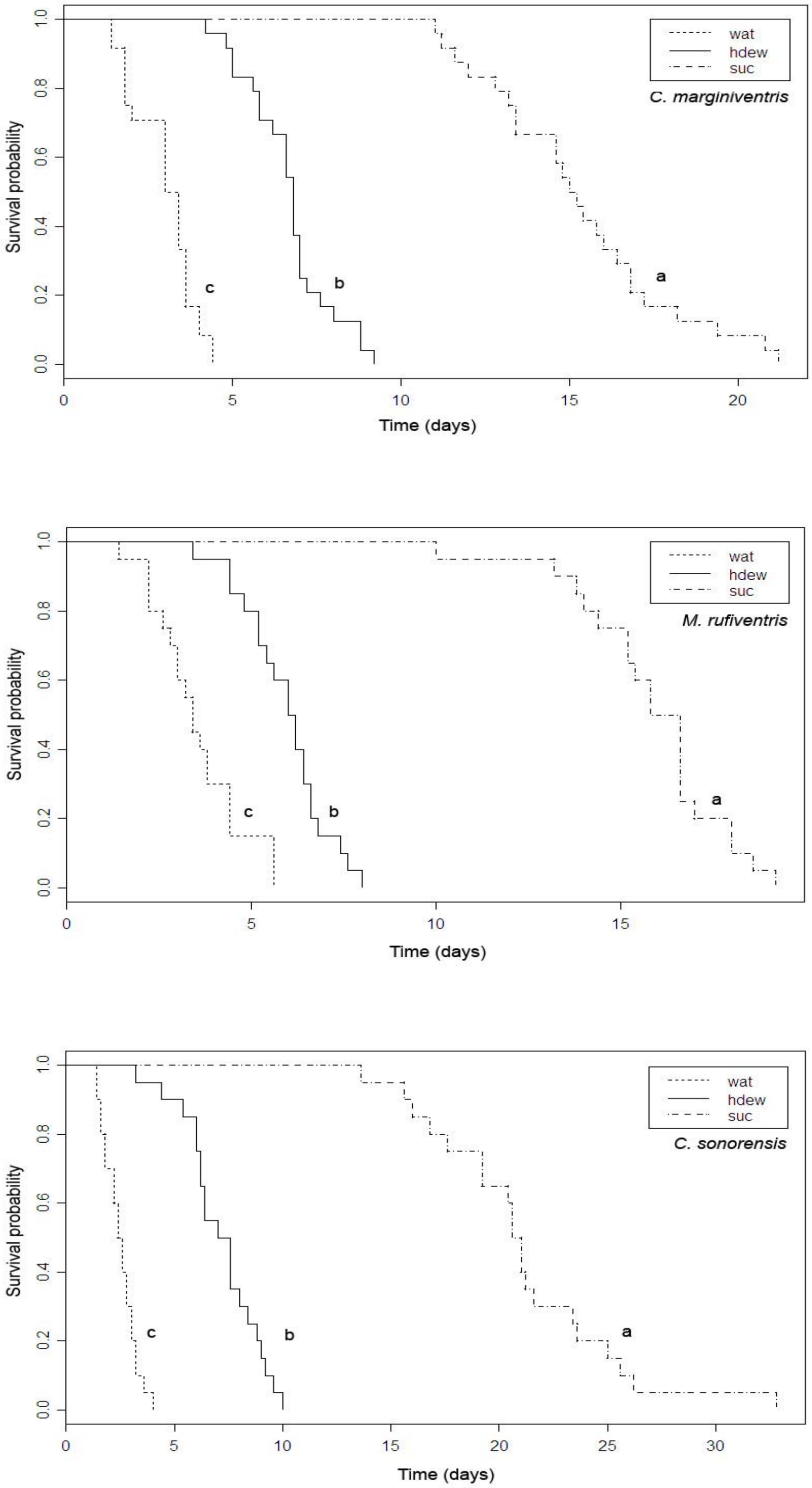

Figure 1. Survival curves showing the survival probability of *C. marginiventris*, *M. rufiventris* and *C. sonorensis* when provided water, honeydew or sucrose solution. Different letters indicate significant differences between curves ($p<0.001$)

Effect of honeydew on C. marginiventris *performance*

Results for longevity were similar to the ones obtained in the previous experiment and this was reflected in the measured parasitism. Survival (n=8, χ^2=38.1, df=4, p<0.01) and offspring production ($F_{4,35}$=24.86, p<0.001) differed significantly among the food sources.

C. marginiventris females lived longer and produced a larger number of offspring when continuously feeding on honeydew compared to unfed ones and to females that fed on honeydew or sucrose for two days only. However, they lived shorter and produced fewer offspring than females that had been continuously fed with sucrose solution (Figs. 2 and 3). Feeding on honeydew or sucrose only for the first two days resulted in a slightly higher survival probability, which was only significantly different for sucrose fed compared to unfed wasps. This increase in longevity was not reflected in the offspring production; wasps fed for only two days produced the same number of offspring as unfed wasps. This implies that *C. marginiventris* females will have to feed multiple times throughout their life in order to optimize longevity and reproductive output.

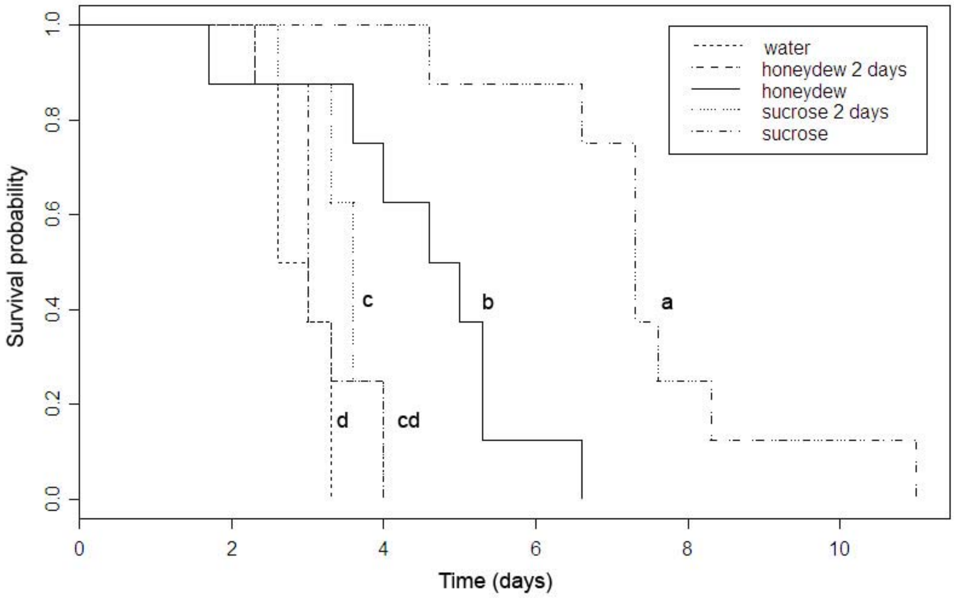

Figure 2. Survival curves showing the survival probability of *C. marginiventris*, when provided water, honeydew or sucrose solution continuously or for the two first days of their adult lives. Different letters indicate significant differences between curves (p<0.01).

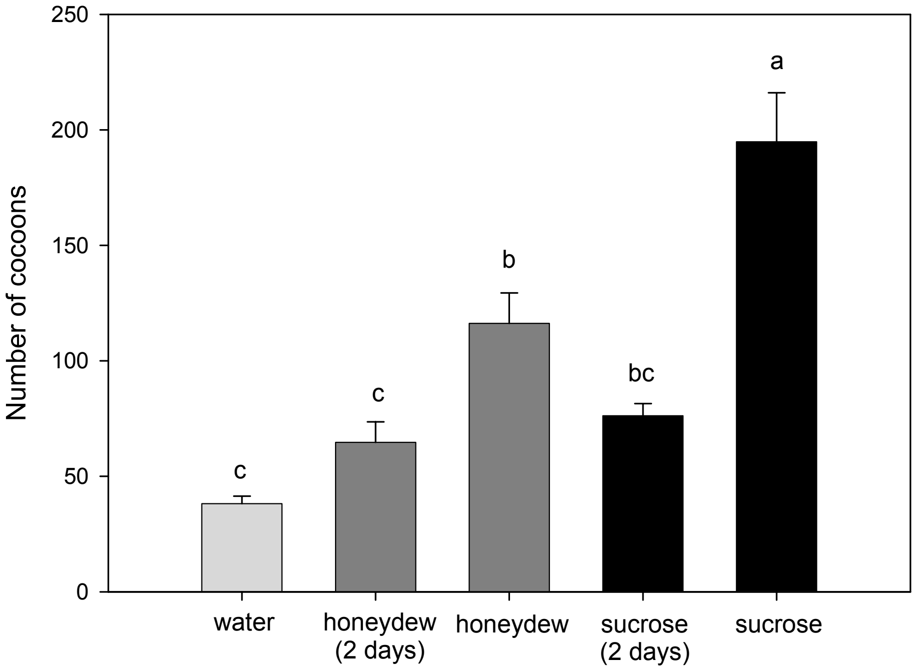

Figure 3. Total number of offspring (number of cocoons) produced by *C. marginiventris* when provided water, honeydew or sucrose solution continuously or for the two first days of their adult live. Different letters indicate significant difference between treatments (average ± SE) ($p<0.05$).

Honeydew analysis

The composition of the honeydew produced by *R. maidis* feeding on maize plants changes as a function of infestation rate and time after infestation. The honeydew was mainly composed of the phloem sugar sucrose and its hexose components, fructose and glucose, which made up between 64 and 94% of the sugars. Maltose and erlose were the most important aphid-synthesized sugars in the honeydew, trace amounts of melezitose were found in all collections and trace amounts of raffinose and melibiose were detected on the 2nd collection of the initial infestation 500 aphids. Melibiose was also found on the 4th collection of the initial infestation 10 aphids.

The two main trends that were observed are: (1) there was a decrease in the percentage of aphid synthesized sugars with increasing aphid density (e.g. in the first collection there was 21.9% of aphid-synthesised sugars for 10 aphids, 17.4% for 100 aphids, 4.5 % for 500 aphids) and time after infestation; and (2) over time there is a decrease on the percentage of sucrose excreted by the aphids, except on the 1st collection for the initial infestation of 500 aphids, where the sucrose percentage was notably low. Over time, there was also a shift from fructose to glucose.

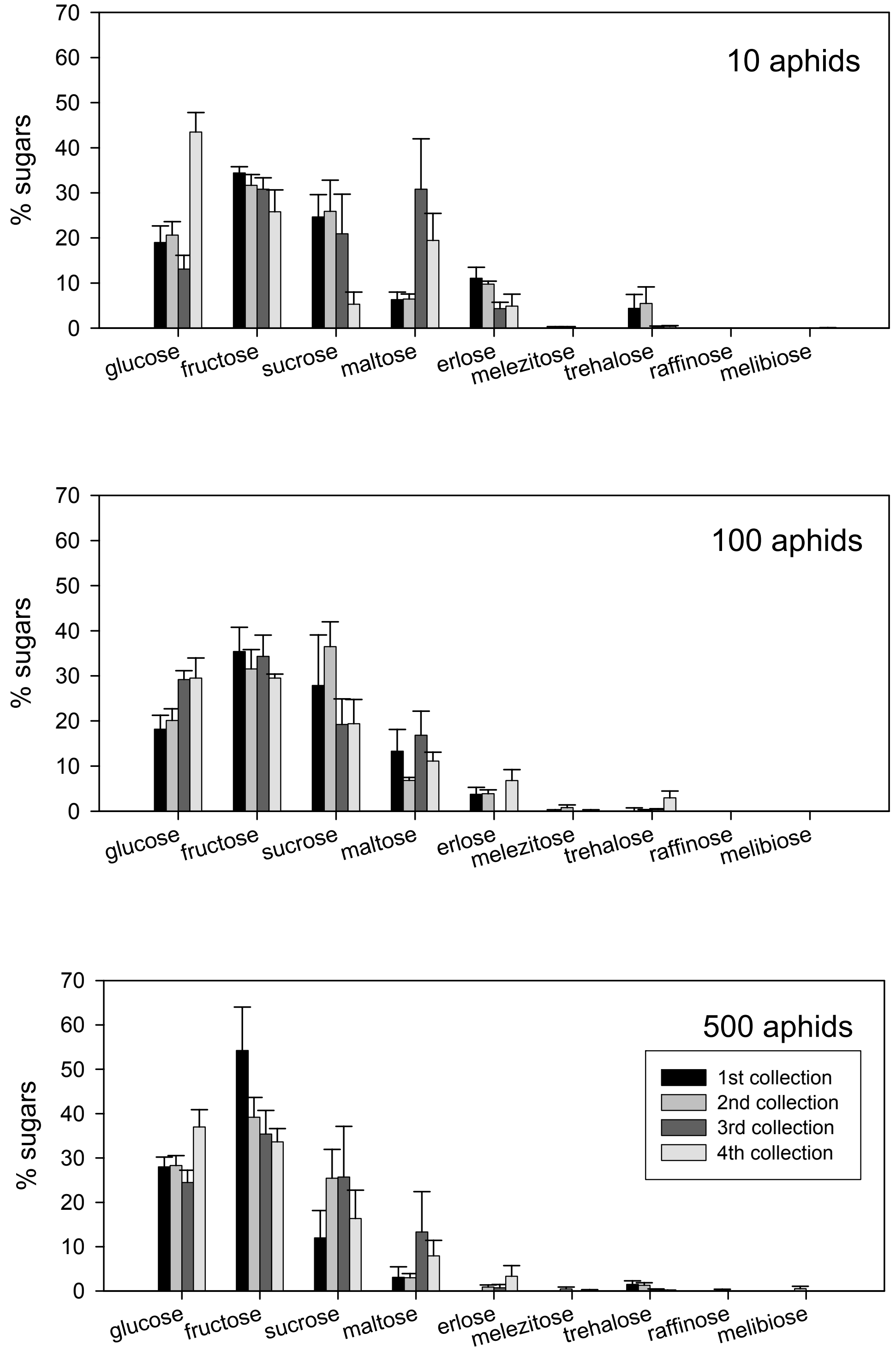

Figure 4. Sugar composition of the honeydew produced over time by different infestation rates of *R. maidis* feeding on barley plants (average ± SE). See Materials and Methods for details.

Longevity of C. marginiventris *on honeydew mimics*

There were considerable differences among the food sources with respect to their effect on parasitoid survival (n=20, χ^2=119, df=5, p<0.001) (Fig. 5), but the survival probabilities of *C. marginiventris* feeding on the different sugar solutions did not differ significantly (n=20, χ^2=1.1, df=3, p=0.77). Survival after feeding on any of the sugar solutions was significantly enhanced in comparison to honeydew (n=20, χ^2=74.6, df=4, p<0.001). Honeydew feeding, in turn, enhanced survival relative to water only (n=20, χ^2=20, df=1, p<0.001).

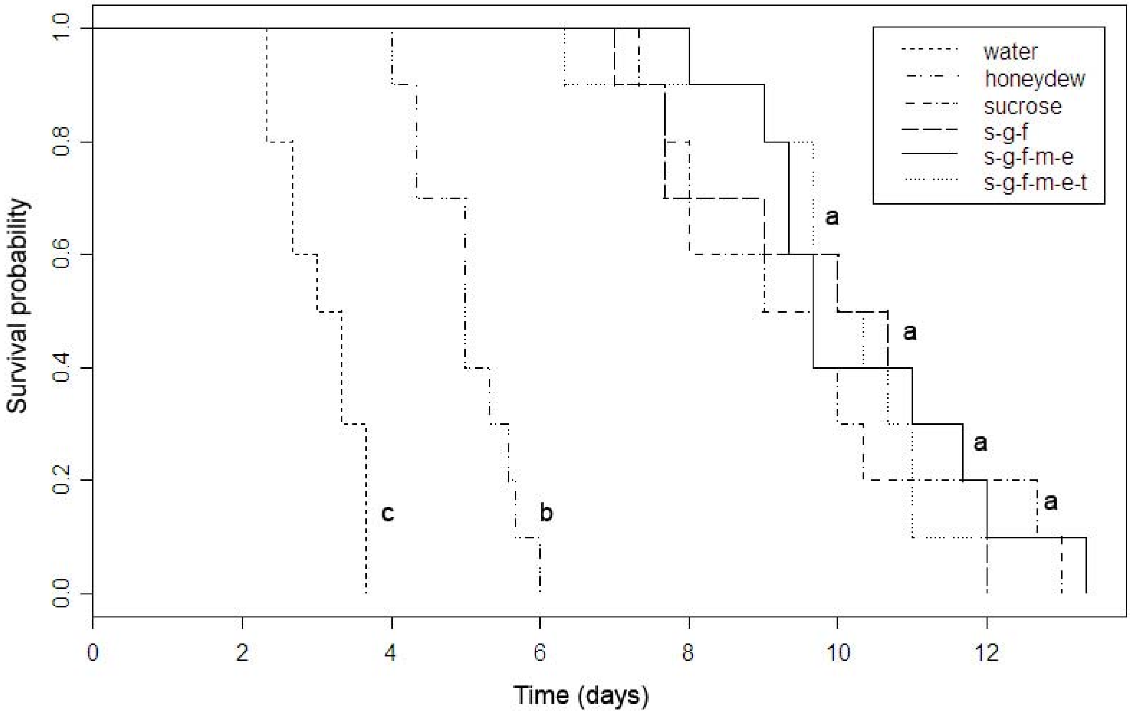

Figure 5. Survival curves showing the survival probability of *C. marginiventris* when provided 1) water, 2) honeydew, or a solution of one of the sugar mixes: 3) sucrose only; 4) sucrose, glucose and fructose; 5) sucrose, glucose, fructose, maltose and erlose; or 6) sucrose, glucose, fructose, maltose, erlose and trehalose. See Materials and Methods for details regarding the sugar proportions. Different letters indicate significant differences between curves (p<0.001).

Relationship between honeydew intake and survival

The percentage weight gain after feeding on honeydew was less than for wasps that had been feeding on sucrose (t=-6.62, df=38, p<0.001) (mean percentage of weight gain was 11.70% for wasps feeding on honeydew and 22.59% for wasps feeding on sucrose). Similarly, the average longevity of wasps fed on honeydew was shorter than for wasps fed on sucrose (T_{20}=223.5, p<0.001).

Intake alone affected longevity of *C. marginiventris* regardless of the food source ($R^2 = 0.4436$, $p = 0.001$ for honeydew and $R^2 = 0.6052$, $p < 0.001$ for sucrose; Fig. 6).

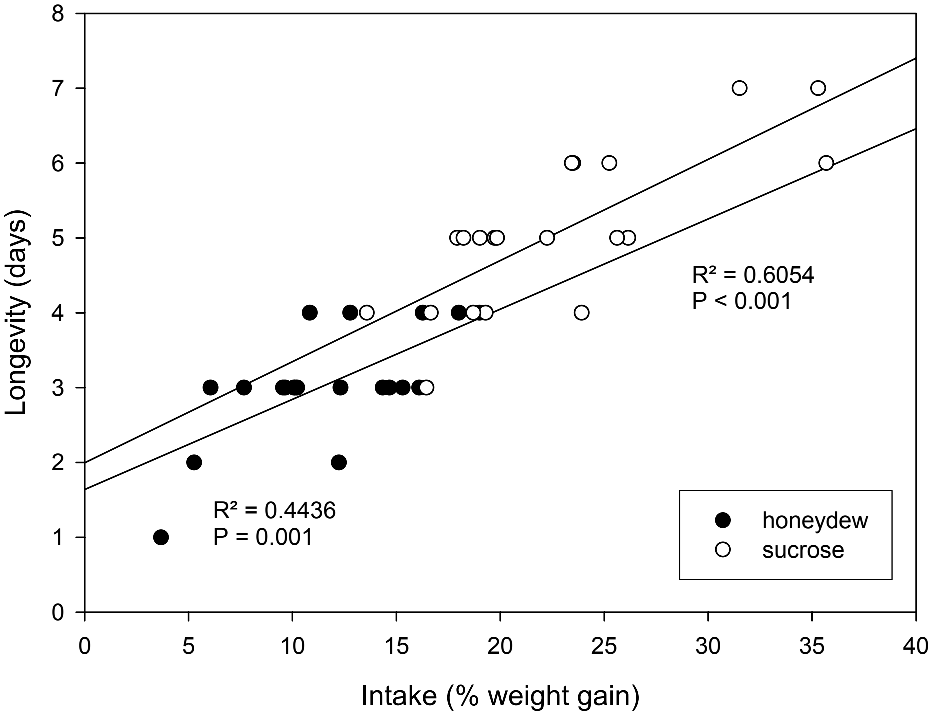

Figure 6. Regression analysis comparing the intake of honeydew or sucrose solution (in % of body weight gained) during a single meal and subsequent survival of *C. marginiventris* females.

That the observed differences in survival, in this experiment, was solely due to food intake was confirmed by the GLM, which shows that longevity increases significantly with increasing food intake and if this is taken into account then there is no effect of food source (Table 1).

Table 1. GLM for the effect of food type, percentage of weight gained after one feeding bout and their interaction on the longevity of *C. marginiventris*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	10.598	1	10.598	26.574	.000
food	.102	1	.102	.255	.616
intake	14.756	1	14.756	37.000	.000
food * intake	.048	1	.048	.121	.730
Error	14.357	36	.399		
Total	728.000	40			

Moreover, there was a significant difference (t = 570.5, df = 20, p<0.001) in the amount of time *C. marginiventris* spent feeding on the two food sources (Fig. 7). This relatively long feeding time despite of the small intake of honeydew implies that the wasps have difficulty ingesting this food source, possibly because of its high viscosity.

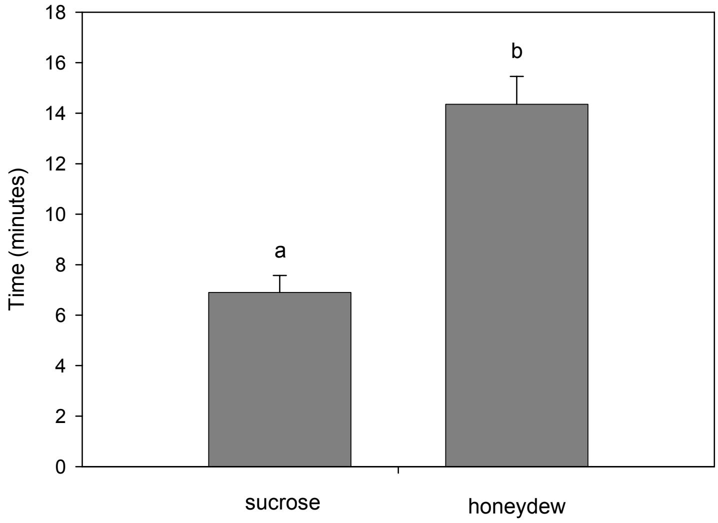

Figure 7. Time spent by *Cotesia marginiventris* feeding on honeydew or sucrose solution during one feeding bout. Different letters indicate significant differences between treatments (p<0.001).

Discussion

The outcome of studies assessing parasitoid longevity when feeding on honeydew has been inconsistent, some studies show that honeydew increases parasitoid survival but not as much as nectar or honey (Idoine & Ferro, 1988; Lee et al., 2004), others have found that honeydew has the same effect on parasitoid survival as nectar, honey or sucrose solutions (England & Evans, 1997; Singh, 2000; Hogervost et al., 2003), and it has also been found that parasitoid survival on honeydew is as low as when wasps are given no food (Avidov et al., 1970; Elliot et al., 1987). In our study, honeydew feeding increased survival probability and offspring production, but the positive effect of feeding on a sucrose solution was considerably higher. We further found that, in order to optimize longevity and reproductive output, *C. marginiventris* females need to feed throughout their life. In most agricultural monocultures food sources are spatially or temporarily separated from host patches and parasitoids will have to travel substantial distances between these resources. Experiments where food sources are provided *ad libitum* do not take this considerable investment in locating food in the field into account. Sisterson & Averill (2002), for instance, estimate that in the wild, parasitoid females spend 25% of their time searching for food.

When *C. marginiventris* had food available only as young adults, survival of honeydew fed wasps was no better than that of unfed wasps whereas wasps that had access to sucrose water during the same period showed only a marginal increase in survival. Similarly, Fadamiro & Heimpel (2001) found that *Macrocentrus grandii* life expectancy is shorter when wasps are fed on sucrose solution for one day only than when they are fed more often. Parasitoid species differ with regard to how often they need to feed to attain maximal longevity. *M. grandii* do not need to feed everyday to reach maximal longevity (Fadamiro & Heimpel, 2001), whereas for *Cotesia rubecula* food should be available once per day to avoid starvation (Siekmann et al., 2001) and the aphid parasitoid *Aphidius ervi* needs to feed twice per day to reach maximal longevity (Azzouz et al., 2004). However, all these studies were performed in the lab in absence of hosts. Under more realistic conditions, with the wasps exhibiting their foraging behaviour and parasitizing hosts, nutritional requirements can be expected to be higher.

The prolonged survival after feeding was also reflected in an increase in offspring production (Fig. 3). The fact that honeydew fed females produced fewer offspring than sucrose fed females is in contrast with findings from studies reviewed by Jervis & Kidd (1986) and Heimpel & Collier (1996). The reviewed studies suggest that, in terms of reproduction, honeydew should be a better food source than nectar, as it provides the parasitoid with higher amounts of amino acids, which are fundamental for egg production. As for survival, there was no difference between the number of offspring produced by *C. marginiventris* females that had food available for two days only and unfed wasps. Clearly, the wasps need to feed throughout their life to optimize their reproductive output. Energy reserves and consequently life expectancy and reproductive output can decline at different rates as a function of factors such as temperature (McDougall & Mills, 1997; Costamagna & Landis, 2004) and locomotory activities (Neukirch, 1982; Forsse et al., 1992; Steppuhn & Wäckers, 2004). Therefore the feeding frequency requirements of a wasp will depend on such factors. This highlights the importance of providing food sources close to the host sites, so that the parasitoids do not need to spend time and energy when switching from host to food foraging.

Several factors might be responsible for a low nutritional quality of honeydew. One of them is its sugar composition, as aphids sometimes excrete not only plant-derived sugars, but also sugars that they synthesize themselves. For the parasitoid *Cotesia glomerata* it was found that aphid-produced sugars have a lower nutritional value than glucose, fructose and sucrose, the sugars present in most flower nectars (Wäckers, 2001). Our analyses of honeydew produced by *R. maidis* when feeding on barley revealed that glucose, fructose and sucrose are the dominant sugars, but the honeydew also contained the aphid-produced sugars maltose, erlose, and trehalose, as well as trace amounts of melizitose, raffinose and melibiose. In the study on *C. glomerata* (Wäckers, 2001) maltose, erlose, melizitose and melibiose were shown to slightly increase longevity, trehalose had just a marginal effect on its survival and raffinose did not increase parasitoid longevity. If the same is true for *C. marginiventris*, the sugar composition cannot explain the much poorer performance on honeydew as compared to sucrose water.

The composition of the honeydew significantly changed over time and as a function of aphid infestation. In general, the level of aphid infestation was negatively correlated with the percentage of aphid-synthesized sugars in the honeydew. One of the expected reasons why sap feeders synthesize oligosaccharides is to reduce the osmotic pressure of the phloem sap (Kennedy & Fosbrooke, 1972; Fisher et al., 1984; Salvucci et al., 1997). If, the level and duration of aphid infestation changes the osmotic pressure of phloem sap of the plant the aphids may adapt their sugar synthesis accordingly.

In previous studies, factors such as host plant and homopteran species (Hendrix et al., 1992; Völkl et al., 1999), homopteran age (Henneberry 1999), and ant attendance (Yao & Akimoto 2001) were shown to affect the sugar composition of the honeydew. To our knowledge, this is the first time that changes in honeydew composition due to aphid infestation rate and time of infestation have been recorded. As we found that the differences in sugar compositions had no effect on parasitoid performance, it is unlikely that the observed differences for aphid densities and infestation times have important consequences for the nutritional value.

The results from the longevity tests with honeydew mimics (Fig. 5) fully ruled out the possibility that the aphid-synthesized sugars were responsible for the lower nutritional value of honeydew as compared to sugar water. The explanation for the poorer performance on honeydew is more likely the observed difference in food intake (Fig. 6).

There was a strong positive correlation between intake and longevity regardless of the food source. Intake of honeydew was on average considerably less than the intake of the sucrose solution, and in cases where they were equal, wasp survival time were very similar. Although honeydew intake was lower than sucrose intake, wasps spent more time feeding on honeydew. This finding allows us to reject the hypothesis that the lower intake of honeydew was due to a lack of feeding stimulation, but rather suggests that the high viscosity of the honeydew impairs its intake by the parasitoids. In the field, viscosity, which is largely determined by the sugar concentration in various sources, fluctuates with relative humidity (Corbert et al., 1979; Koptur, 1992). In most of our experiments the wasps were fed under conditions with high humidity. Under more realistic field conditions intake may be even more difficult.

Consequences for Biological Control

Our results confirm that parasitoids can only reach their full potential as biological control agents if they have access to suitable food sources. There are several examples where higher levels of parasitism were achieved in the field when wasps were provided with food (Evans & Swallow, 1993; Idris & Grafius, 1995; Baggen & Gurr, 1998; Cañas & O'Neil, 1998; Jacob & Evans, 1998) and the failed establishment of introduced parasitoids has been attributed to the lack of food sources for the adults (Stiling, 1993).

It should be taken into account that food sources in the field are not only exploited by natural enemies, but may also benefit the pests (Rogers, 1985; Baggen & Gurr, 1998; Romeis et al., 2005). In this context, the use of selected food sources that benefit only the natural enemies are highly desirable. Parasitoids may accept and benefit from a broader range of sugars when compared to lepidopteran pests (Wäckers, 1999; 2001; Romeis & Wäckers, 2002; Winkler et al., 2005), implying that in some situations, moderate aphid populations in the field could contribute to sucessful biological control.

Another aspect of this interaction that must be considered is the time spent feeding and the risk of predation associated with honeydew feeding. We observed that the wasps spend much longer feeding on honeydew than on sucrose. The sucrose concentration was equivalent to what can be found in flower nectar, an obvious alternative and perhaps preferred food source. In fields where there is no ready access to nectar and wasps will have to spend extended periods of time feeding on honeydew, they will be more vulnerable to predators (Morse, 1986; Maingay et al., 1991; Völkl, 1992; Heimpel et al., 1997b; Völkl & Kroupa, 1997). Limited food intake per feeding bout will increase the frequency with which the food needs to be found, as observed by Stapel et al. (1997). Despite these clear limitations to the suitability of honeydew as food for parasitoids, it is frequently consumed. Field caught parasitoids have been found to commonly contain honeydew (Wäckers & Steppuhn, 2003; Steppuhn & Wäckers, 2004) and Casas et al. (2003) showed that in a situation where homopteran honeydew seems to be the only food source available, wasps can forage for hosts and disperse within an habitat for days without running a risk of energy limitation. This suggests that this food source can be exploited in the field to enhance the efficacy of biological control agents. A better understanding of factors that determine the quality of honeydew as food may allow us to develop methods to manipulate these factors.

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Chapter 2

Learning of host-associated versus food-associated odours by a parasitoid of lepidopteran pests.

Based on: Faria, CA, Wäckers, FL, Farine ME & Turlings, TCJ. Learning of host-associated versus food-associated odours by a parasitoid of lepidopteran pests. To be submitted to *Physiological Entomology*

Abstract

To optimize their reproductive success, adult parasitoid females need not only hosts but also food sources. This means that in the field they will have to periodically interrupt host foraging to search for food. Ideally parasitoid females should maximize the time allocated to host foraging. Hence, the presence of food sources in close association with the host is highly beneficial as it decreases time and energy costs associated with switching from host to food searching. In this context aphid produced honeydew can be regarded as an important alternative food source, as even for non-aphid parasitoids it may occur in the vicinity of their hosts. One strategy that can help minimize the time lost on food foraging is the learning of food-associated odours. Olfactometer assays with females of *Cotesia marginiventris*, a parasitoid of important lepidopteran pests, showed that this species is indeed able to learn to associate the odour of barley infested by the aphid *Rhopalosiphum maidis* with with honeydew consumption. Subsequently parasitods are highly attracted to this odour when hungry. Further studies showed that *C. marginiventris* chooses between odours of barley infested by larvae of *Spodoptera littoralis* (hosts) and barley infested by *R. maidis* (honeydew-food) based on their hunger status and previous experience. The analysis of the volatiles produced by the two herbivores feeding on barley showed considerable qualitative and quantitative differences. *S. littoralis*-infested barley released many more compounds and in much higher quantities than aphid-infested barley. Evidently, *C. marginiventris* females are able to exploit these differences to optimize their foraging efficiency.

Introduction

Sugar feeding is critical for the survival of adult parasitoids, even for species of which the adults engage in host feeding (Leatemia et al., 1995; Jervis et al., 1996; Heimpel et al., 1997). Among other advantages, a regular intake of sugars increases parasitoid longevity and fertility (Leius, 1961; Syme, 1975; Schmale et al., 2001; Lee et al., 2004) and motivation to search for hosts (van Emden, 1962; Wäckers, 1994; Takasu & Lewis, 1995). Hence, in the field, parasitoid females will have to periodically switch from host to food searching. A complicating factor is the fact that host and food resources are usually located on different plants or in different patches. This is especially true in most agricultural monocultures where carbohydrate sources, such as nectar producing flowers, are usually only available for a very short period of time or not available at all.

Optimally balancing the decision whether to look for hosts or food is critical for the reproductive success of a female parasitoid (Sirot & Bernstein, 1996). Females should focus on host foraging and minimize the energy and time they spent foraging for food. As in host searching, parasitoids also use olfactory and visual cues to find nutritional resources (Wäckers & Swaans, 1993; Wäckers, 1994; 2004; Stapel et al., 1997).

It is expected that emerging parasitoid females will likely first engage in food searching rather than host searching (Lewis & Takasu, 1990; Vet et al., 1995, Jervis et al., 1996; Hegazi et al., 2000), as hunger reduces their responsiveness to host-associated odours and increases their responsiveness to food related ones. Several examples in which parasitoids switch from host to food searching when hungry support this notion (Lewis & Takasu 1990; Takasu & Hirose, 1991; Takasu & Lewis, 1993, 1995, 1996; Wäckers, 1994; Siekmann et al., 2004). Furthermore, females are known to display both innate and learned responses towards host and food related cues and choose between them according to their nutritional state (Lewis & Takasu, 1990; Takasu & Lewis, 1993; Wäckers, 1994).

The presence of nutritional resources in close vicinity of hosts can improve the parasitism rate of parasitoid females, as has been shown in several field studies (Evans & Swallow, 1993; Idris & Grafius, 1995; Baggen & Gurr, 1998; Cañas & O'Neil, 1998; Jacob & Evans, 1998). Moreover, the presence or absence of food affects the habitat preferences in parasitoids: females have a tendency to concentrate their search for hosts close to plants that provide food, thus determining their effectiveness in biological control (van Emden, 1962; Leius, 1967; Takasu & Lewis, 1993, 1995; Baggen & Gurr,1998).

In situations in which nectar is not available, aphid-produced honeydew might be an important alternative food source (Idoine & Ferro, 1988; England & Evans, 1997; Evans, 2000; Singh, 2000; Casas et al., 2003; Wäckers & Steppuhn, 2003; Nomikou et al., 2003;

Lee et al., 2004) and could help increase the levels of biological control. One added advantage is that, even for non-aphid parasitoids, honeydew may occur in close proximity of hosts. Host habitats being close to food sources will decrease travel time, energy costs and the risks associated with switching from host to food searching.

The foraging behaviour of female parasitoids, both for hosts and food is reasonably well understood. However, most food foraging studies have focused on the question of how parasitoids find floral or extra floral nectar (Jacob & Evans, 2001; Wäckers, 1994; Siekmann et al., 2004), whereas food learning studies often use arbitrary odours to show that the females can learn novel cues in association with a feeding experience (Lewis & Takasu, 1990; Takasu & Lewis, 1993; Olson et al., 2003). It is as yet unclear what cues non-aphid parasitoids might use to locate honeydew.

When searching for honeydew, parasitoids could exploit volatiles that plants emit in response to aphid feeding. It is well documented that such herbivore-induced plant volatiles are used by many parasitoids and predators to locate their hosts (e.g. Dicke & Sabelis 1988; Turlings et al., 1990; Takabayashi et al., 1991; Röse et al., 1997; De Moraes et al., 1998). Although it is often assumed that aphid parasitoids use volatile cues coming from the aphids or their by-products (Bouchard & Cloutier, 1984, 1985; Budenberg, 1990; Wickremasinghe & van Emden, 1992; Tripathi & Singh, 1994; Mackauer et al., 1996) evidence that they also use host-induced plant volatiles is increasing (Du et al., 1998; Powell et al., 1998; Gerrieri et al., 1999). These plant-provided signals may differ depending on the herbivore species, plant genotype and type of damage caused by the herbivores (De Moraes, 1998; Turlings et al., 1998a; Walling, 2000; Rodriguez-Saona, 2003). Moreover, natural enemies are able to discriminate between host and non-host herbivores (Sabelis & van de Baan, 1983; Takabayashi et al., 1995; Du et al., 1996; De Moraes et al., 1998; Powell et al., 1998).

In the current study we investigated if parasitoids of lepidopteran larvae use aphid and/or plant-provided cues to locate honeydew, if they can learn to associate such cues with the presence of food and whether they discriminate between aphid and host-infested plants. The parasitoid under study was *Cotesia marginiventris* (Cresson) (Hymenoptera:

Braconidae), which attacks larvae of numerous lepidopteran species, including many pests (Jalali et al., 1987; Riggin et al., 1992; Hoballah et al., 2004). This parasitoid exploitation of host-induced plant odours and its ability to learn to associate odours with hosts has been amply documented (e.g. Turlings et al., 1989; 1990). The wasp is also known to benefit from feeding on honeydew: in the laboratory, unfed adults live on average only three days, whereas females that have unlimited access to *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae) honeydew live on average seven days (Faria et al. in prep.). It is not known yet what cues the wasp might use to locate this food. An ability to distinguish between host and food associated cues should be adaptive, as female *C. marginiventris* will have to periodically switch from host to food foraging and using different cues for each should greatly facilitate this switch and greatly reduce the time spent foraging.

To study if *C. marginiventris* is able to make this distinction we used barley (*Hordeum vulgare*) attacked by either larvae of the potential host *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) or the corn leaf aphid *R. maidis*, which produces copious amounts of honeydew that parasitoids can use as food (J. Losey, pers. comm.). The specific questions addressed are: 1) is *C. marginiventris* able to learn to use odours associated with honeydew during a feeding experience, and 2) can females discriminate between barley odours that they have learned to associate with food and odours that they have associated with host presence?

Materials and Methods

Plants

Barley plants of the variety Lyric were individually grown from seed in a climate chamber (27±2 °C, 60% r.h., 16L:8D, and 50000 lm/m2) plastic posts (10 cm of height, 4 cm of diameter). The plants were used for the aphid rearing or experiments when 3-4 weeks old.

The evening before an experiment the exposed soil in the pots was covered with aluminium foil and three pots with plants were each placed in an odour vessel of a 6-arm olfactometer (see below). One plant was infested with 20 second instar *S. littoralis* larvae just after placing it in the odour vessel. Another of the three plants had been infested with *R. maidis* five days before the experiments. The estimated infestation rate at the time of the experiments was 8 aphids/cm^2. The third plant was left unharmed and all plants were then kept under the artificial light of the olfactometer until the actual experiments on the following day.

Insects

The corn leaf aphid *R. maidis* was provided by the Agroscope RAC Changins in Switzerland and reared in climate chambers (25°C, 70% r.h. and 14L:10D) on barley. Shipments of *Spodoptera littoralis* eggs were received weekly from Syngenta (Stein, Switzerland) once the eggs had hatched the larvae were used for the rearing of *C. marginiventris* rearing in wheatgerm based artificial diet (as described by Hoballah et al., 2002) or in experiments.

Adult *C. marginiventris* were kept in plastic Bugdorm-1 cages (30 x 30 x 30, Megaview, Taiwan) at a sex ratio of 1 : 2 (male : female) in a climate chamber (25ºC, 85% r.h. and 14L:10D). Moist cotton wool was added to the cages to provide humidity and water to drink for the wasps. Females were used for the experiments when 36 - 48h old. They were mated and were either deprived of food or fed with a 2M sucrose solution.

Training procedure

Experiment 1: Food learning

We tested five groups of females: (1) females that never had a feeding experience (naive wasps); (2) hungry females that had had a brief experience feeding on honeydew produced by *R. maidis* on barley, while perceiving the odour of aphid-infested barley; (3) hungry females that had a brief experience feeding on sucrose solution while perceiving the odour of aphid-infested barley; (4) hungry wasps that had briefly experienced the odour of aphid-infested barley, without the food reward; (5) wasps that had been provided sucrose solution throughout their life and were assumed to be food satiated.

For the experience, an aphid-infested barley plant was placed in the same type of glass odour source vessel (25.8 cm of height, 6 cm of internal diameter) that was subsequently used in the olfactometer. A glass “training” tube (3 cm of height, 2.5 cm of diameter) was connected to each vessel in which the wasps were trained. The wasps were prevented from entering the odour vessel by placing a fine mesh nylon screen between the vessel and the glass tube. For the honeydew experience, several leaves of aphid-infested barley were left overnight inside the glass tube, so that on the following day its inner surface was covered by honeydew. For the sucrose experience, 15µL of a 2M sucrose solution was placed as small droplets on the inner surface of the tube.

For the training with a food reward, honeydew or sucrose was placed in the training tube before it was connected to an odour vessel with aphid infested barley. After feeding for 15 seconds parasitoids were gently removed from the tube. Twenty minutes later the females were given the same experience one more time. The training of wasps that had the experience with odour only followed the same procedure, except that there was no food in the tube. Without food the females would actively search the whole tube and we had to prevent them from escaping by placing a Petri dish on the open side of the tube. This experience without food was added to determine if mere exposure to the odour of aphid-infested barley would increase their responsiveness to this odour (sensitization (*sensu* Turlings et al., 1993)).

After the training experiences the females were placed in plastic boxes (5cm height, 9.5 cm diameter) with moist cotton wool and there responses to odours were tested in the olfactometer 30 minutes later.

Experiment 2: Food versus host learning

Once it was confirmed that *C. marginiventris* females learn food-associated odours, an experiment was designed to test if experience and hunger state affected their preferences for host foraging cues versus food foraging cues. The training protocol for hungry females with experience feeding on honeydew associated with the odour of aphid-infested barley was the same as used in the previous experiment. To obtain control (satiated) females they were placed in the same set up as described previously, but were allowed to feed on honeydew for 20 min.

To train the wasps to associate odours with host presence, we used the same set up as for the food training, except that the source vessel contained a barley plant that had been infested on the previous evening with 20 2nd instar *S. littoralis* larvae. Another 20 *S. littoralis* larvae of the same instar were placed in the training tube and individual wasps were released in the tube and allowed to oviposit in 3 - 4 larvae. To avoid superparasitism, the larvae were replaced after every 3 females. Females that failed to parasitize 3 larvae after 10 min were discarded.

Wasps with the following combination of training experiences were used for the experiments:

1. Naive wasps: wasps that never had a feeding or oviposition experience.
2. Hungry - honeydew: wasps that had been allowed to feed twice for 15 sec on honeydew in the presence of the odour of aphid-infested barley.
3. Satiated - honeydew: females that were allowed to feed for 20 min on honeydew in the presence of the odour of aphid-infested barley.
4. Hungry - honeydew + host: wasps that were allowed to feed twice for 15 sec on honeydew in the presence of the odour of aphid-infested barley and also had 3-4 ovipositions in *S. littoralis* larvae while perceiving the odour of barley infested with *S. littoralis*.
5. Satiated - honeydew + host: females that had fed on honeydew for 20 min in the presence of the odour of aphid-infested barley and also had 3-4 ovipositions in *S. littoralis* larvae in the presence of the odour of *S. littoralis*-infested barley.

For females that received an experience with food and host, the sequence of training was changed between each trial. Analyses of the results showed that the sequence of training did not affect the choice of the females.

Bioassays

A six-arm olfactometer (Turlings et al., 2004) was used to test the attractiveness of aphid-infested and host-infested barley for *C. marginiventris* females. The olfactometer consists of six odour vessels connected to the six arms of a central chamber in which the parasitoids were released and to choose between the odours emanating from the six sources. The odours were introduced into the central chamber by pushing humidified clean air into odour source vessels at 1.2 L/min, which carried the volatiles via the arms into the chamber.

Groups of six wasps with one type of experience were released in the central chamber at a time. Wasps that entered an arm ended up in a trapping bulb where they could be counted and removed 30 min after their release. Wasps that by the end of the 30 min had not chosen an arm were considered as "no choice".

One group of each type of wasp was released per day, resulting in 5 releases per day. The experiments were repeated 6 times, whereby the positions of the odour sources were alternated.

Volatile collection and analysis

The volatiles produced by uninfested, aphid-infested and *S. littoralis*-infested barley plants were collected using similar, but larger (50 cm height with 9 cm of internal diameter) odour vessels as the ones used in the six-arm olfactometer. The larger size allowed us to place three barley plants in each vessel. The plants in each vessel were either undamaged, had been infested with *R. maidis* five days before odour collections, or had been infested with 60 *S. littoralis* larvae of 2nd instar on the night before odour collections.

Purified and humidified air was pushed into each vessel at a rate of 1.2L/min. To trap volatiles emitted by the plants 0.6 L/min of the flow was pulled through 7 cm glass tubes filled with 25 mg of 80-100 mesh Super-Q (Alltech, Deerfield, Illinois) that was inserted in a port at the top of each vessel. The rest of the air vented through a second port, thus preventing outside air from entering in the system. Before the collections each trap was rinsed with 3 mL of dichloromethane. Each collection lasted 4h, after which the traps were washed with 150 µL of dichloromethane (Lichrosolv, Merck, Switzerland).

The volatiles were analysed with a Hewlett Packard HP 6890 (Agilent 6890 Series GC system G1530A) chromatograph, coupled to a mass spectrometer operated in electron impact mode (Agilent 5973 Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33-280 amu). A 3 µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness, Alltech Associates, Inc, USA). Helium at constant pressure (18.55 psi) was used as carrier gas. Following injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min followed by a post-run of 5 min at 250°C. The detected compounds were identified by comparison of retention time with analyses from previous studies (Turlings et al., 1998b; Gouinguené et al., 2001; Hoballah et al., 2002) or by comparison of their mass spectra with those of the NIST02 library.

Statistical analysis

The odour preferences of *C. marginiventris* were examined with a log linear model (GLM), fitted for the distribution of the wasps within the olfactometer, assuming quasinormal distribution, thus allowing for overdispersion of the wasps (Turlings et al., 2004). Only wasps that made a choice were included in the analysis. The model was fitted by maximum quasilikelihood estimation and its adequacy was assessed through likelihood ratio statistics and examination of residuals using R (1.9.1). We tested the effect of the odour source on the choice of the wasps with different experiences. We also tested for release order and day effect, but none was found.

Results

Food learning

Hungry and well-fed *C. marginiventris* females did not respond to volatiles from uninfested and aphid-infested barley: the number of females choosing the arms carrying these odours was not different from the number that chose the arms that carried clean air.

Wasps that had experienced only the odours of an aphid-infested plant without the food reward, showed a weak attraction to the odour of infested and uninfested barley. However, when females had had a food reward (honeydew or sucrose) while perceiving the odour of aphid-infested plant, they subsequently had a very strong preference for this odour source.

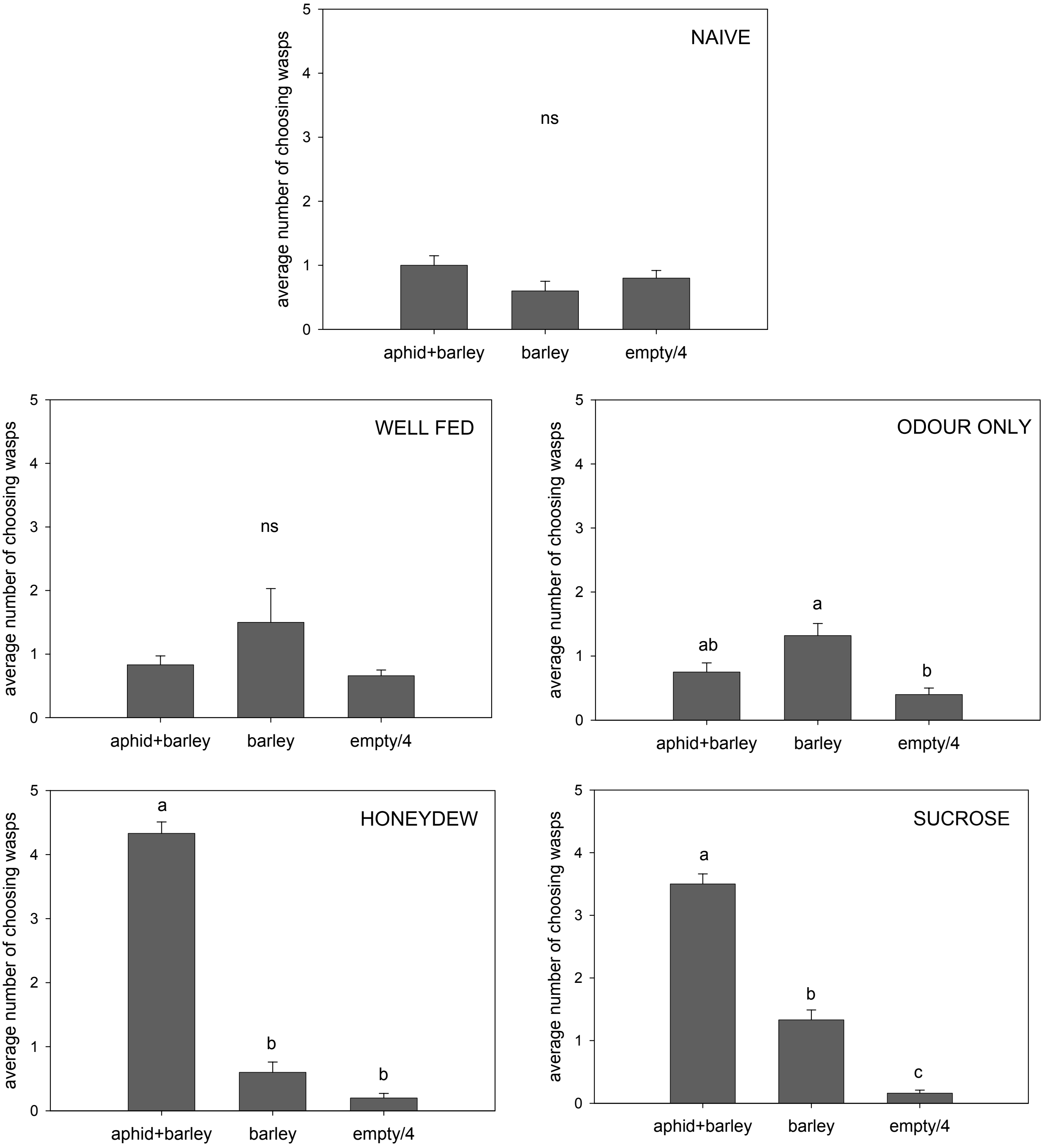

Figure 1. Responses of *Cotesia marginiventris* females that were either (1) naive (no experience with food or plant odour); (2) were well fed on sucrose solution; (3) had a brief experience with odours from barley plants infested with *Rhopalosiphum maidis*; (4) had a brief experience feeding on honeydew associated with odours from *Rhopalosiphum maidis*

infested barley; (5) had a brief experience feeding on sucrose solution associated with odours from *Rhopalosiphum maidis* infested barley. The bars represent the average number of females (per release of six) that choose for one of the odour sources or females that went into one of the empty arms (+ standard error). The letters above the bars indicate significant differences calculated from a log-linear model ($p < 0.05$).

Food and host learning

When given a choice between the odour of aphid-infested and host-infested barley, naive females that never had experience with hosts or food were only slightly attracted by these odour sources and did not distinguish between them. Satiated females that never had an oviposition experience, nor had an experience with the odour of infested plants (Satiated - honeydew) were more attracted to host-infested plants than naive females ($p< 0.01$).

As was found in the previous experiment, females that had a feeding experience associated with odour from aphid-infested plants (Hungry - honeydew) were readily attracted to aphid-infested plant. However, wasps that also had an oviposition experience while perceiving the odour of host-infested barley (Hungry - honeydew + host) were equally attracted to host and food associated odours. In clear contrast, satiated wasps with aphid-infested barley odour experience that had also had an oviposition experience (Satiated - honeydew + host) strongly preferred the odour of *S. littoralis*-infested plants.

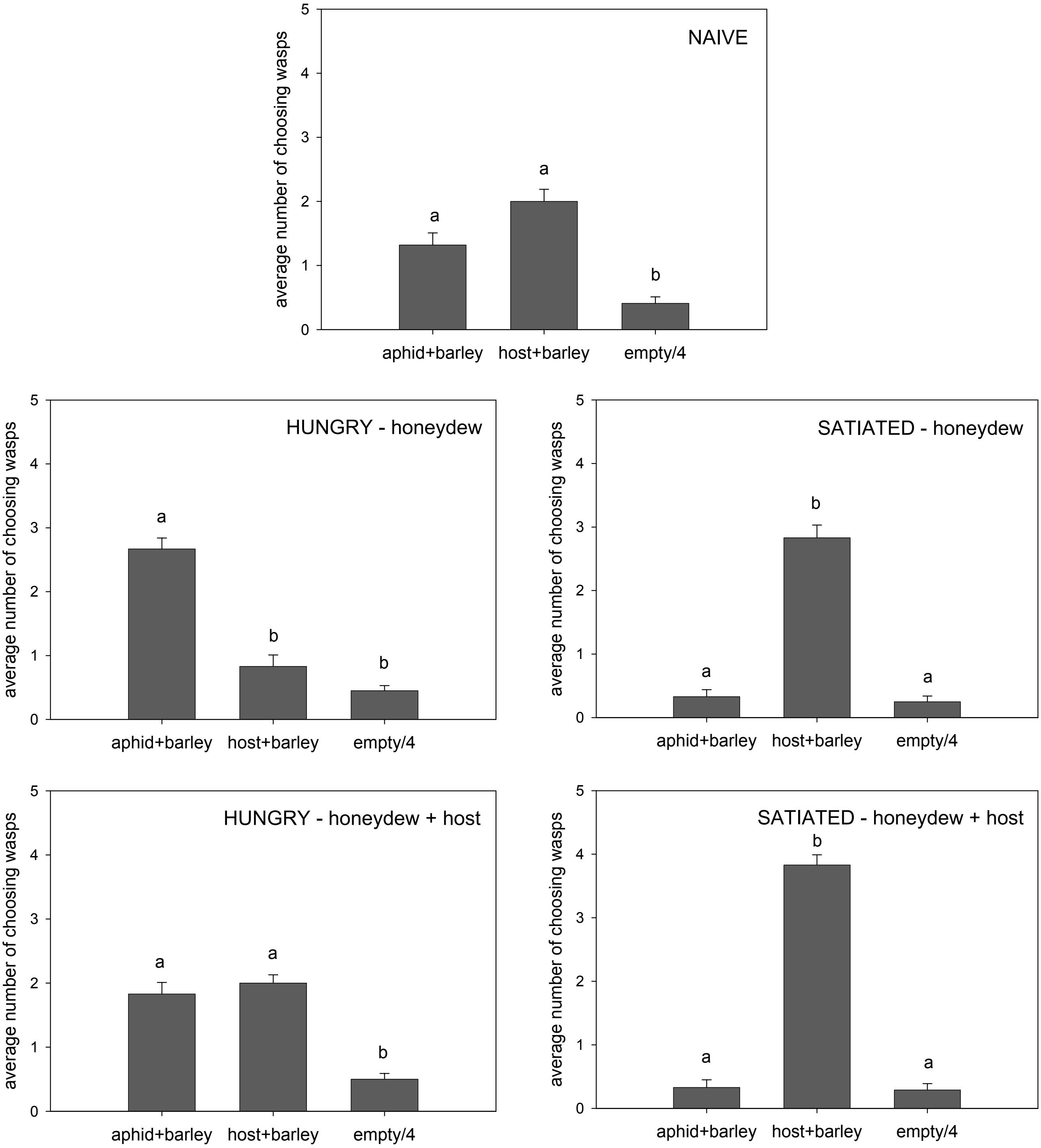

Figure 2. Responses of *Cotesia marginiventris* females that were submitted to different training procedures (see text for details) to odours of barley infested with *Spodoptera littoralis* or *Rhopalosiphum maidis*. The bars represent the average number of females attracted to one of the odour sources or females that went into one of the empty arms (+ standard error).

The letters above the bars indicate significant differences calculated from a log-linear model ($p < 0.05$).

Volatile collection

The figure 3 shows typical chromatograms of uninfested, aphid-infested and *S. littoralis*-infested barley. Uninfested barley did not emit any detectable amounts of volatiles. Aphid-infested barley emitted the monoterpene α-pinene, which was not emitted by barley infested with *S. littoralis*. The latter plants emitted large amounts of 11 compounds that are typically emitted in response to caterpillar feeding (Fig. 3).

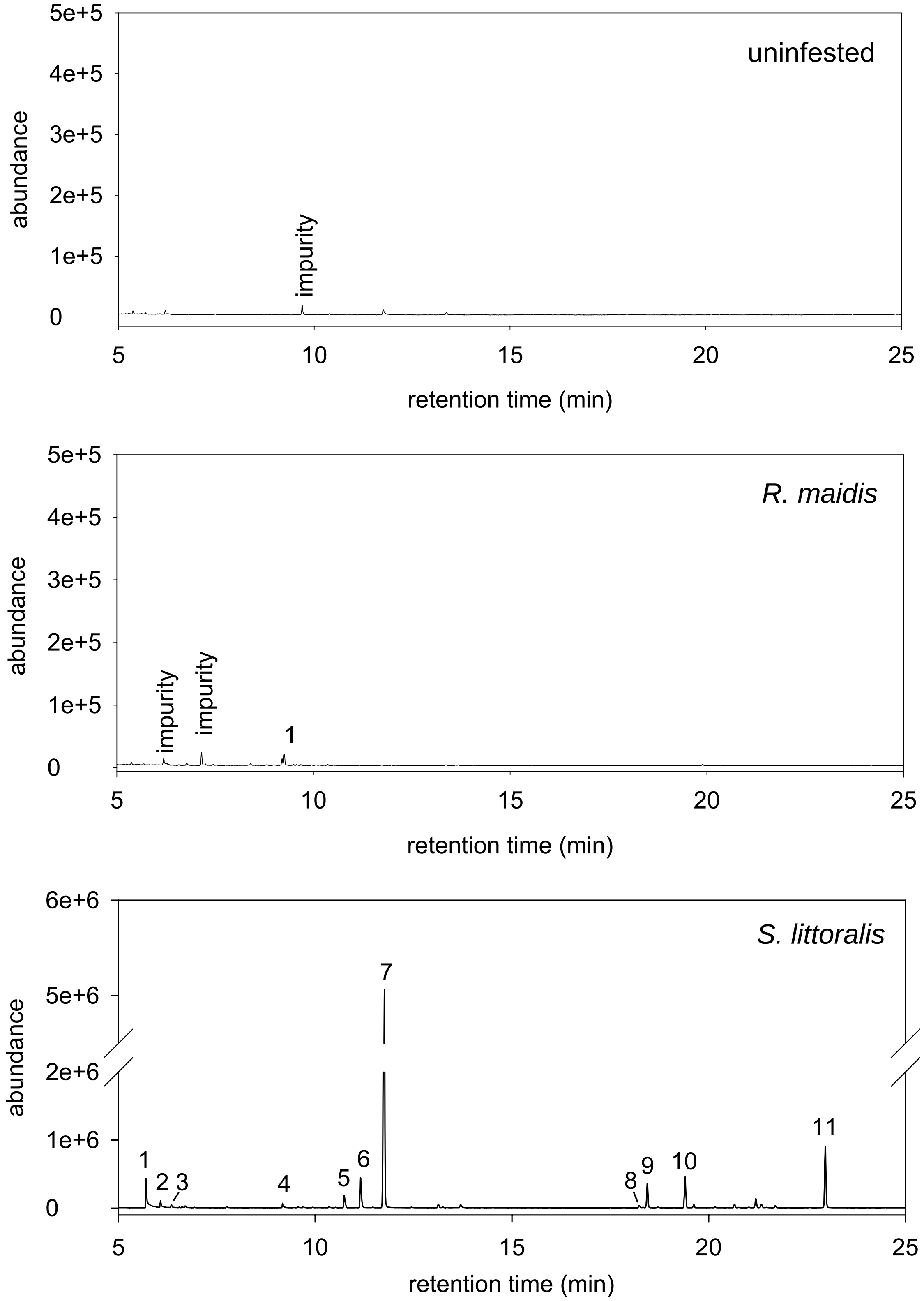

Figure 3. Typical chromatograms obtained from odour collections from barley plants that were uninfested, infested with *Rhopalosiphum maidis* or *Spodoptera littoralis*. The labelled

peaks are for *R. maidis* infested barley: 1. (+)-α-pinene. For *S. littoralis* infested barley: 1. unknown; 2. (E)-2-hexenal; 3. (Z)-3-hexen-1-ol; 4. 6-methyl-5-hepten-2-one; 5. (Z)-ocimene; 6. unknown; 7. linalool; 8. cycloisosativene ; 9. (-)-α-copaene; 10. β-caryophyllene; 11. (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

Discussion

In order to optimize its longevity and offspring production, *C. marginiventris* needs to feed several times throughout their life (Faria et al. in prep.). With such a requirement, an ability to learn to recognize food-associated and host-associated cues would help to optimize the use of time and energy when foraging for nutritional resources. The results from the current study show that the wasp is indeed able to learn to exploit odours from host- and aphid-infested plants in such a manner.

Naive *C. marginiventris* females showed no or only a marginal attraction to aphid-infested barley, whether they were hungry or not. An absence of a clear innate response has also been reported for *Cotesia rubecula* and *Brathyplectes curculionis*, two parasitoids of Lepidoptera that may also feed on aphid-produced honeydew (Wäckers & Swaans 1993; Jacob & Evans, 2000). In contrast, several parasitoids and predators that attack aphids show strong innate response to odours of aphid-infested plants and/or honeydew (van Emden & Hagen, 1976; Bouchard & Cloutier, 1984, 1985; Budenberg, 1990; Wickremasinghe & van Emden, 1992; Tripathi & Singh, 1994; Mackauer et al., 1996; Powell et al., 1998; Ninkovic et al., 2001). This difference in how aphid and non-aphid parasitoids respond to aphid-infested plants is to be expected because the former fully relies on aphids for their reproductive success, whereas the latter probably uses floral and extrafloral nectar as its main adult food sources and will only need to forage for alternative food stuffs if these nectar sources are not available.

There was a slight increase of the attractiveness of uninfested barley when wasps experienced only the odour of an aphid-infested plant without a food reward, which indicates that learning of plant odours by *C. marginiventris* could partially be the result of sensitization.

Associative learning, however, must have been the main mechanism resulting in the high attraction of wasps that had experienced food in association with the odours of aphid-infested barley.

In the field, *C. marginiventris* adults will probably first find aphids and honeydew by chance, while searching for hosts. Once honeydew has been encountered, the wasps will learn to associate it with specific cues, which probably also include spatial cues (Stapel et al., 1997).

When naive females had a choice between aphid-infested and *S. littoralis*-infested barley, they showed some attraction to both. The significant attraction to aphid-infested barley in this experiment is in contrast to what was found in the first experiment. This difference might be due to a higher responsiveness of the wasps in the second experiment or perhaps sensitization during the assay with a relatively high dose of host-infested plant odour caused an overall increase in the wasps' responsiveness.

As hypothesized, *C. marginiventris* responded differently to food- and host-associated odours depending on their hunger state and previous experience. These results agree with previous studies that show that parasitoid females are able to learn food and host associated odours and decide between food and host foraging based on their physiological state and experience (Lewis & Takasu, 1990; Takasu & Lewis, 1993, 1995, 1996; Siekmann et al., 2004).

Hungry females that had experienced odours of aphid-infested barley while feeding on honeydew (Hungry - honeydew) were attracted to aphid-infested plants and when they were satiated (Satiated - honeydew) they were readily attracted to host-infested plants. It is interesting to note that, although the satiated females had never experienced the odour of host-infested barley, they were strongly attracted to it, suggesting that the experience with the odour of aphid-infested barley odour affected their perception of and/or responsiveness to odours. Alternatively, hungry females may show a low innate responsiveness to host-associated odours until they have had an opportunity to take in sufficient food reserves. There is indeed evidence that sugar deprivation affects not only insect mobility, but also the functioning of the neural network important for information processing (Chippendale, 1978). A study by Siekmann et al. (2004) also found that unfed *C. rubecula* females exhibit reduced sensitivity to odours.

Hungry females with food and host experience (Hungry - honeydew + host) were equally attracted to plants carrying food or hosts, indicating that an oviposition experience increases attraction to host-associated odours even when wasps are hungry. This is in agreement with the findings by Takasu & Lewis (1993) who showed that intermediately fed females of the parasitoid *Microplitis croceipes* without previous oviposition experience were attracted to food odours, whereas wasps that had also had an oviposition experience preferred the odours that they had encountered during this latter experience. Attraction to host-related odours can be expected to always occur as finding host is the key to the reproductive success of parasitoids. A dramatic change in attractiveness in favour of host-related odours was observed when the females with the double experience were allowed to feed *ad lib* (Satiated - honeydew + host). The general preference for the odour of host-infested barley may also readily be explained by quantitative differences in odour emissions (Fig. 3).

There was a tremendous difference in the composition and amount of volatiles emitted by aphid- and *S. littoralis*-infested barley. This can be expected because caterpillars and aphids have very different feeding strategies. Caterpillars severely damage leaf tissue cells and probably induce a stronger reaction in the plant than aphids that can insert their stylet into a plant with almost no damage to the plant cell tissue (Pollard 1973). Moreover, *R. maidis* shows intercellular stylet penetration, particularly in young plants (Bing et al., 1991). Other studies (Turlings et al., 1998a; Bernasconi et al., 1998; Rodriguez-Saona et al., 2003) also found that plants infested by leaf chewers and sap suckers produce volatiles that differ considerably in quality and quantity. Considering these dramatic quantitative differences it is perhaps surprising that the hungry females exhibited a strong attraction to aphid-infested barley even in the presence of the much more fragrant host-infested plants. Such results confirm a strong association and exploitation of even weak odours, which they readily detect while ignoring much stronger odours.

In summary, *C. marginiventris* females learn to associate food (honeydew) with the odour of aphid-infested plants. They switch between food and host searching depending on their hunger state and previous experience. The wasps ability to discriminate between food- and host-associated odours was mostly likely based on the difference of volatiles released by plants infested with *R. maidis* and *S. littoralis*. When satiated the wasps focussed on the odours of host-infested plants, even if they had not experienced such odours previously. When hungry, however, they exhibited a strong attraction to the odour of aphid-infested plants, but only if they had had a feeding experience in the presence of this odour. This study is the first confirmation that wasps can learn odours that are naturally associated with the presence of food.

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Chapter 3

Increased susceptibility of Bt maize to aphids helps to enhance the performance of parasitoids of lepidopteran pests

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Abstract

Six Bt maize lines and their near-isogenic equivalents were evaluated for their susceptibility to the corn leaf aphid *Rhopalosiphum maidis*. Within each pair the transgenic lines were consistently more susceptible than the non-transgenic line, except for the most susceptible pair, which did not significantly differ from each other. The observed higher reproductive rates and population densities of the aphids resulted in increased honeydew production. Amino acid analyses of the phloem content in selected maize lines showed somewhat higher levels in Bt maize, which could partially explain the observed increased aphid performance. With more honeydew available as food, females of the parasitoid *Cotesia marginiventris* that were placed in cages with aphid-infested plants lived longer and parasitized more caterpillars in cages with Bt maize than in cages with their non-transgenic counterparts. This effect was merely due to the availability of larger amounts of honeydew and not to changes in honeydew nutritional quality. Susceptibility to *R. maidis* is highly variable among non-transgenic maize varieties and the increased susceptibility found for Bt maize lines tested falls well within this range of variability. The reduced presence of primary lepidopteran hosts in Bt maize fields, as well as the potential of breakouts of secondary lepidopteran pests that are less susceptible to a Bt toxin call for measures to ensure the continued presence of generalist parasitoids in maize fields. Increased aphid presence may aid in the maintenance of parasitoids and other beneficial insects in Bt maize fields. Depending on whether or not aphid densities remain below pest thresholds, an increased susceptibility of Bt maize to aphids may therefore be either a welcome or an undesirable side effect.

Introduction

With the rapid expansion of the commercial use of genetically modified (GM) plants, there is an increasing demand for information on their possible impact on non-target organisms. Of particular interests are parasitoids and predators that have an important function in pest regulation. To date several studies on the direct and indirect impact of GM plants on this group of insects have been conducted with results showing no or various degrees of detrimental effects (reviewed by Dutton et al., 2003 and by Lövei & Arpaia, 2005). In these studies, most emphasis has been on so-called Bt plants, which are crops into which a gene has been incorporated from the entomopathogenic bacterium *Bacillus thuringiensis*. The introduced genes encode for the production of specific insecticidal proteins. An impact on entomophagous insects resulting from this transformation could be due to direct effects of the toxin, indirect effects via reduction in host or prey quantity and quality, or unintended effects that result in unexpected changes in plant characteristics caused by the insertion of the transgene (e.g. pleiotropic effects). The first two potential effects have been widely investigated (Dutton et al., 2003; Lövei & Arpaia, 2005), but very few studies have specifically looked at the impact of other plant characteristics that may have unintentionally been altered as a result of transformation.

Homopteran produced honeydew can be a key alternative food source for parasitoids in the absence of plant-provided nectar (Hocking, 1966; England & Evans, 1997; Lee et al., 2004; Faria et al. in prep.), which is often the case in agricultural monocultures. Honeydew is also exploited by several other animals like honeybees, wasps, predators and vertebrates (Moller et al. 1987; Markwell et al. 1993; Evans 2000). Any changes in aphid performance that will affect the honeydew quantity or quality will reflect not only on the trophic levels directly linked with the aphid itself, but also on other animals in the food web that consume aphid honeydew.

For Bt11 and event 176 it is known that the Bt toxin is not taken up by aphids because it is not present in the phloem and therefore also does not end up in the honeydew (Head et al., 2001; Raps et al., 2001). Even when aphids are fed on artificial diets that contain high concentrations of the Bt toxin they take up only little (Head et al., 2001). Indeed, aphids seem to perform equally on Bt maize lines and their near isogenic counterparts (Dutton et al., 2002; Bourguet et al., 2002). However, there are some reports of altered performance of aphids on Bt maize, but these, intriguingly, suggest that aphids may do better on the transgenic lines (Lumbierres et al., 2004; Pons et al., 2005). We too found indications that the corn leaf aphid, *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae), does better on Bt maize. These findings from preliminary experiments prompted us to conduct the current study to test if, in general, the aphid does better on Bt events and, if so, this increased susceptibility of maize has an effect on the exploitability of honeydew by parasitic wasps.

We hypothesize that a better aphid performance can be explained by: (1) a higher lignin content in Bt plants compared to the untransformed lines (Saxena & Stotzky 2001), which might favour aphids; (2) an increased attractiveness of Bt plants to aphids, as suggested by Lumbierres et al. (2004); (3) a negative effect of the transformation on inherent plant defences; and/or (4) an unexpected change in amino acid composition in the phloem due to the transformation, as the amino acid composition and concentration in the phloem is the main factor shaping its nutritional quality for aphids (Auclair, 1963; Douglas, 1998; Karley et al., 2002). As yet, no study has evaluated these potential mechanisms.

Hence, the present study aimed to assess possible effects of the incorporation of the Bt gene in to maize on the corn leaf aphid *R. maidis* and if these effects reflect on the performance of *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), a larval parasitoid of several lepidopteran pests that can use aphid honeydew as a food source (Faria et al., in prep.). As a first step, we investigated aphid performance on six pairs of Bt maize lines and near isogenic lines, covering three different transformation events. Bt plants were indeed found to be significantly more susceptible to *R. maidis*. As a possible explanation for increased aphid performance on Bt maize, we performed analyses of phloem samples to compare amino acid composition between transgenic and near-isogenic pairs from each of the three different events. In addition, we assessed if the positive effect on the aphid performance reflected on the longevity and parasitism rate of *C. marginventris* feeding on honeydew, comparing one transgenic and near isogenic pair from each of the three different events. It was found that, within each pair, female parasitoids performed better when feeding on honeydew produced from the transgenic than on honeydew from the near isogenic line. To investigate if this enhanced performance was due to differences in the honeydew quality, the different honeydews were analysed for their sugar composition, which largely determines nutritional quality for parasitoids. As an alternative explanation, we measured if there were differences in uptake during feeding bouts on different honeydews.

Materials and Methods

Plants

All plants were individually grown from seed in a climate chamber (27±2 °C, 60% r.h., 16L:8D, and 50000 lm/m^2). For measurements of aphid performance, six pairs of hybrids from one of the three commercially available Bt maize events and the correspondent near-isogenic lines were used: Bt11 (N4640Bt/ N4640), Mon 810 (MEB 307Bt/ Monumental, TXP138 /EXP138, Novelis/ Nobilis) and Event 176 (Valmont/ Prelude, Navaris/ Antaris). For the other experiments (honeydew analysis, parasitoid performance and amino acid composition of the phloem), only one pair of each of the three events was used: Bt11 (N4640Bt/N4640), Mon 810 (MEB 307Bt/Monumental) and Event 176 (Valmont/Prelude). Barley of the variety Lyric was used for initial aphid rearing.

Insects

The corn leaf aphid *R. maidis* was used in this study because it excretes copious amount of honeydew and despite of the fact that its pest status varies in different parts of the world, they usually do not cause economical damage to the crop (Kring & Gilstrap, 1986; Kröber & Carl, 1991; Jauset et al., 2000; Waterhouse & Sands, 2001). The aphids were provided by the Agroscope RAC Changins in Switzerland and were reared in climate chambers (25°C, 70% r.h. and 14L:10D) on barley unless otherwise specified.

Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) eggs were received weekly from Syngenta (Stein, Switzerland) and once the eggs hatched, the larvae were used for parasitoid rearing or in experiments. The *C. marginiventris* colony was maintained on *S. littoralis* larvae fed with artificial wheat germ based diet. Adults were kept in plastic Bugdorm-1 cages (30 x 30 x 30 cm, Megaview, Taiwan) at a sex ratio of 1 : 2 (male : female) in climate chambers (25ºC, 85% r.h. and 14L:10D). Moist cotton wool was added to the cages to provide humidity and water for the wasps. The females used for the experiments were one day old, mated and unfed.

Aphid performance

For these experiments aphids had been reared at least for four generations on the respective variety. This was done because, as all aphid species, *R. maidis* shows telescoping generations (a parthenogenetic females may have developing embryos that on their turn have developing embryos within themselves) (Dixon, 1998). This implies that maternal effects can only be "cleaned" after three generations.

Aphid performance on the different maize lines was measured at the individual, as well as population level. In addition to the six pairs of transgenic and near isogenic lines belonging to three transformation events, we also tested the two conventional varieties Delprim and Challenger. All plants were five weeks old at the start of an experiments, at which time they were infested with the aphids and transferred to climate chambers (25°C, 70% r.h. and 14L:10D).

Mean relative growth rate (MRGR; Adams & van Emden, 1972) was used to measure the performance of individual aphids on the different varieties. For this, nymphs were individually weighed (initial weight: 40 to 60µg) on a precision scale (Mettler MX5; ±2 µg) and placed in clipcages (1.5 x 1.5 cm) that were attached to the 6th and 8th leaves of the maize plants. Four days later the aphids were removed from the clipcages and weighed again. Each plant had two clipcages and there were 15 plants from each variety. The few aphids that disappeared were replaced by new ones. Differences in MRGR [(ln initial weight - ln final weight)/number of days] within each transgenic and near isogenic pairs were compared using the Mann-Whitney test.

To measure the performance of the aphid population a group of 100 *R.maidis* individuals (50 adults and 50 nymphs of mixed ages) were placed in clipcages attached to the 6th leaf of the maize plants. Three days later, when the aphids had settled on the plant, clipcages were removed and plants enclosed in sleeve cages (Megaview, Taiwan; 30 x 70 cm). Five weeks after infestation, the stem of each plant was cut close to the soil and the whole plant in the sleeve cage was put in a plastic bag and a beaker with ether was added to kill the aphids. Once the aphids were dead, they were removed from the plant with a brush and conserved in 70% ethanol. The aphids in ethanol were then put in a Petri dish of known area and the number of aphids present in 5% of the area of the Petri dish was counted. The total number of aphids on each plant was then estimated. Differences in the number of aphids were compared within each transgenic and near isogenic pair using the t-test.

Stylectomy and amino acid analysis

In order to investigate if the enhanced aphid performance on Bt maize resulted from differences in the amino acid composition of the phloem, we collected phloem samples from one pair of transgenic/near isogenic lines belonging to the events Bt11 (N4640Bt/ N4640), Mon810 (MEB 307Bt/ Monumental) and Event 176 (Valmont/ Prelude). For this experiment we used the aphid *Rhopalosiphum padi* because this species is easier to handle for stylectomy procedure than *R. maidis*.

A maximum of ten aphids were put overnight in a clip cage (1.5 cm of diameter) that was attached on a maize plant (three to four weeks old) overnight. Stylectomy was performed on the following day using high-frequency microcautery (Unwin, 1978). When a successful cut had been made, the exuding phloem sap was immediately collected into a water filled microcapillary. The sample volume was estimated by measuring the diameter of the sap droplet formed on the stylet after one minute of exudation and this measure was multiplied by the duration of the exudation. Sap was collected for a maximum of 90 minutes. After collection the samples were stored at -20°C. Once all samples were collected, they were transferred from the microcapillaries to Eppendorf tubes and placed in a dissecator so that the water in the samples would evaporate. The Eppendorf tubes were then stored at -20°C.

The amino acids were analysed by capillary electrophoresis with a Beckman P/ACE MDQ system equipped with a 488 nm argon-ion laser module (Picometrics, France, 25mW).

The data was collected and analysed by Beckman P/ACE MDQ 1.5 or 1.2 software (Beckman-Coulter, Fullerton, CA, USA).

Half an hour before analysis, the phloem samples were put at room temperature. For the analysis, 15-45 µl of the Dissolving Matrix (Sodium phosphate monobasis, Sodium phosphate dibasis, Glycine-Glycine) were added to the sample. Thereafter, the sample was mixed with 2.5-7.5 µl 50 mM NBD-F, and heated at 60ºC for 3 min, and finally mixed with 15-45 µl DOPAC to quench the reaction and cooled down at room temperature before analysis. During capillary electrophoresis, the sample was injected by pressure at 0.5 psi for 5 s. The applied voltage for CE separation was 20.6 kV (0-16 min) and 30 kV (17-25min). CE experiments were conducted at 20ºC.

The standard amino acid solution used for comparison contained 19 amino acids (L-Arginine, L-Alanine, L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, L-Glutamine, Glycine, L-Histidine. L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tyrosine, L-Valine, DL-Ornithine). The variation in amino acid composition of the transgenic and isogenic lines of each pair was statistically compared with separate t-tests for each amino acid with the package SPSS 12.0. Additionally the distribution of amino acid concentrations was investigated by redundancy analysis (RDA), a direct gradient analysis, to explore the underlying trends in the dataset. For these statistical analyses the data (amino acid concentrations of each sample) were log-transformed.

To explore the relationship between aphid performance and amino acid concentration in the six maize varieties, we first subjected the amino acid data to a principal component analysis (PCA). The coordinates of the six varieties on the first principal axis were used as a composite variable, which expresses the most possible variation in the amino acid data. Concentrations of amino acids were standardized prior to analysis. Coordinates were used as independent variable in a linear regression to explain aphid performance.

RDA and PCA analysis were conducted using the program CANOCO 4.5

Effect of honeydew on C. marginiventris *performance*

Groups of three *C. marginiventris* females were placed in cages (50 x 50 x 100 cm) with one maize plant (5-6 weeks) from a Bt variety or the corresponding non-transgenic line, these pairs comprised events Bt11 (N4640Bt/ N4640), Mon 810 (MEB 307Bt/ Monumental) or Event 176 (Valmont/ Prelude). Two weeks prior to the tests, when plants were four weeks old, all plants were infested with around 400 *R. maidis* of mixed ages. All cages also contained two maize plants (three to four weeks old) of the conventional variety Delprim infested with around 150 *S. littoralis* larva (three to four days old) each.

The cages were sprayed with water twice per day. Mortality of the females was recorded daily and the *S. littoralis*-infested maize replaced every other day. The caterpillars from the replaced plants were collected and reared further on artificial diet until emerging parasitoids had formed cocoons, which were then counted. Differences in parasitism rate (numbers of cocoons formed) by *C. marginiventris* females kept in cages with different food sources were determined by ANOVA and differences between means compared using the Tukey's test with SPSS 12.0. In addition, effects of feeding on the honeydew from different maize varieties on survival probability of *C. marginiventris* were compared using survival analysis. Differences between survival curves of wasps feeding on honeydew from each pair of transgenic and near isogenic line were analysed with a log-rank test using S-Plus 6.2.

Honeydew collection and analysis

To test if differences in parasitoid performance were due to differences in the honeydew composition, we analysed the sugars in the honeydew from one transgenic and near isogenic pair of each of the three events. We also analysed the honeydew produced by *R. maidis* on other conventional maize varieties (Best, Byzance, Challenger, Delprim, Graf and Pactol) to access the overall variability in honeydew composition produced by *R. maidis* feeding on different maize genotypes.

All plants used for the honeydew collection were five to six weeks old and infested with around 200 *R. maidis* of mixed ages. Aphids were placed in clipcages on the 6th to the

8th leaf and one week later each clipcage was replaced by a new one, which was left on the plant for 24h. After this period, these new clipcages were removed and then placed at 100% r.h. for 24h and a micro-capillary was used to collect 1µL of honeydew, which was diluted in 50µL of 70% ethanol.

Just before analysing the samples they were diluted a further 1000x with Milli-Q water. Of each diluted 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 column (4 x 50 mm), a Dionex Carbopac PA1 analytical column (4 x 250 mm), as well as an ED 40 Electrochemical Detector for Pulsed Amperimetric Detection (PAD). 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, melezitose, raffinose, maltose and erlose by injecting calibration standards with concentrations of 2.5 ppm, 5 ppm, 7.5 ppm, and 10 ppm of these sugars. The concentrations of the individual sugars were analysed using the program PEAKNET Software Release 5.1 (DX-LAN module).

Correlation between honeydew intake and survival

As the sugar composition was similar among the different honeydews, we tested for differences in honeydew intake as an alternative explanation for the observed differences in parasitoid performance. For this we measured the honeydew intake of *C. marginiventris* females after one single feeding bout, and determined its effect on parasitoid survival.

C. marginiventris females were used when 24-30h old. To make sure that the food intake was only due to sugar need, water was provided *ad libitum*. Consumption was determined by weighting the individual females on a precision scale (Mettler MX5; ±2 µg) before and immediately after exposure to honeydew. After this the females were kept individually in vials with moist cotton and their longevity was accessed daily.

Differences in the percentage of weight gained by *C. marginiventris* after one feeding bout on honeydew produced from the transgenic and near isogenic lines of each pair were compared within a pair using the t-test. Longevity was compared using the Mann-Whitney test. The correlation between honeydew intake and survival was determined by linear regression analysis.

Results

Aphid performance

There were no differences in the MRGR of individual *R. maidis* within each pair of transgenic and near isogenic lines, except for the pair MEB 307Bt/Monumental (Fig. 1), where the aphids did not survive on the near isogenic line. This result contrasts strongly with the results for population performance (Fig. 2). For all pairs, except Navares/Antares, there were significantly more nymphs on the transgenic lines than on the respective near isogenic lines ($p < 0.05$). For adults this was only the case for the pairs N4640Bt/N4640 and Valmont/Prelude. As in the tests for individual performance, in the population study no aphids survived on the variety Monumental. The few winged aphids observed were present only on the pair Navares/Antares and there was no difference in their number.

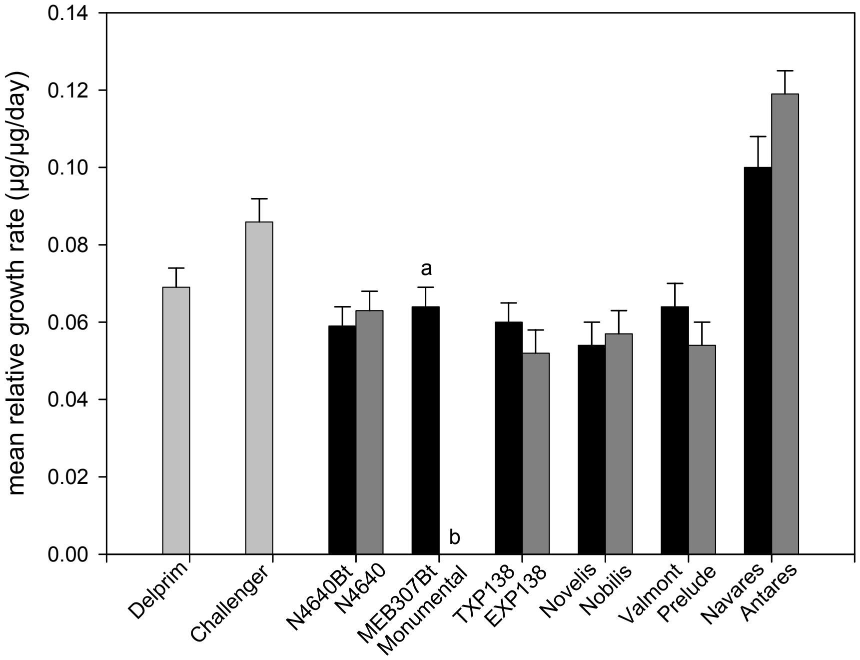

Figure 1. Mean relative growth rate of individual *R. maidis* on six pairs of transgenic and near isogenic varieties and on two conventional varieties (+SE). Different letters indicate significant difference within each pair of transgenic and near isogenic line (p < 0.001).

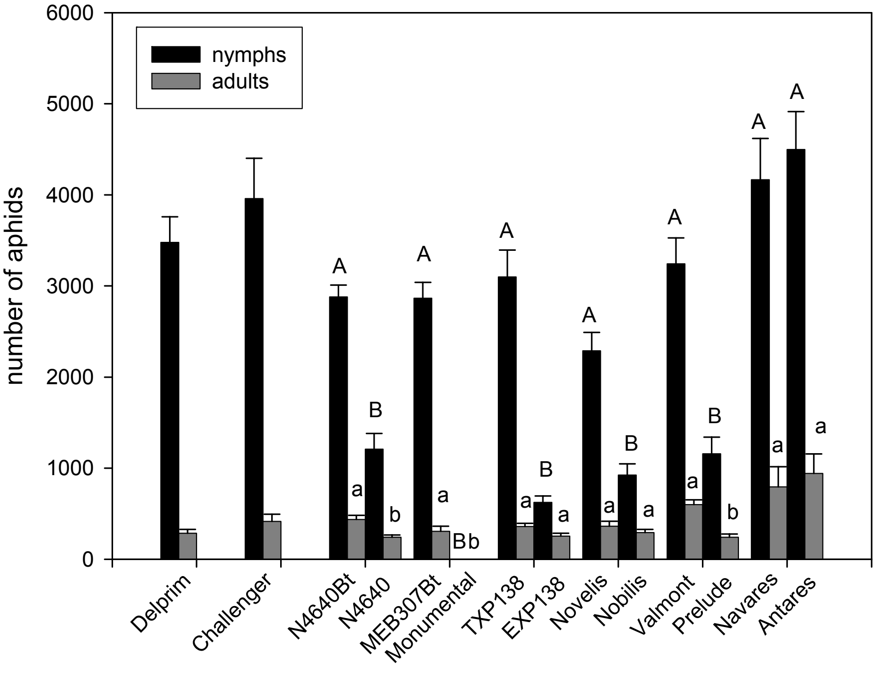

Figure 2. Average number of *R. maidis* adults and nymphs on six pairs of transgenic and near isogenic varieties and on two conventional varieties (+SE). All comparisons are

performed within each transgenic and near isogenic pair. Different capital letters indicate significant difference for nymphs and minuscule letters for adults within each pair of transgenic and isogenic genotype ($p < 0.05$).

Stylectomy and amino acid analysis

Eighteen amino acids were detected in phloem sap samples: arginine, tyrosine, lysine, ornithine, phenylalanine, leucine, isoleucine, histidine, valine, glutamine, proline, threonine, alanine, serine, asparagine, glycine, glutamate, aspartate. Methionine was present in the standards but was not found in any of the phloem samples.

Phloem sap from all varieties was dominated (81 - 87%) by non essential amino acids. Differences in amino acid concentration within transgenic and isogenic pairs were found for two of the three events tested (Fig. 3). For the pair N4640Bt/N4640, the concentration of the amino acids ornithine and alanine was higher in the transgenic than in the isogenic variety ($p < 0.05$) and the difference between these lines in total amino acid concentration was marginally significant ($t=2.08$, $df = 13$, $p = 0.057$). For the pair Valmont/Prelude, the concentration of the amino acids arginine and proline was higher in the transgenic than in the isogenic line ($p < 0.05$).

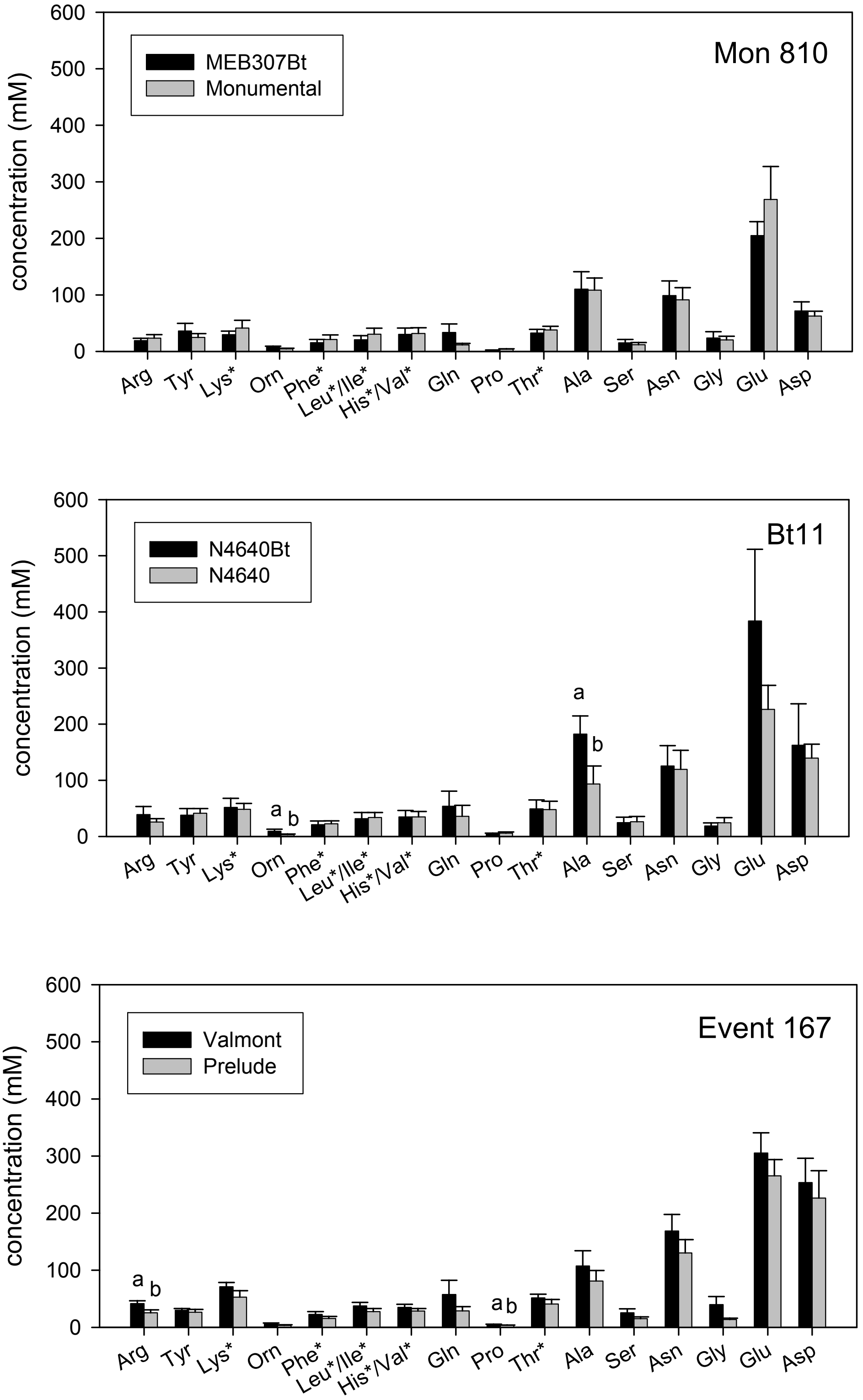

Figure 3. Amino acid concentration (mM) of phloem samples from three transgenic varieties belonging to three transformation events and their correspondent near isogenic lines. Different letters indicate significant differences between amino acid concentration within one

transgenic/isogenic pair ($p<0.05$). Aminoacid abbreviations: arg, arginine; tyr, tyrosine; lys, lysine; orn, ornithine; phe, phenylalanine; leu/ile, leucine/isoleucine; his/val, histidine/valine; gln, glutamine; pro, proline; thr, threonine; ala, alanine; ser, serine; asn, asparagine; gly, glycine; glu, glutamate; asp, aspartate. Asterisks indicate the essential amino acids. Different letters indicate significant differences between treatments within one transgenic/isogenic pair ($p<0.05$).

The RDA (Fig. 4) indicates that the distribution of investigated amino acids differs between transgenic and isogenic lines. Most amino acids (except proline, serine and leucine/isoleucine) were positively linked to the vector “trans” corresponding to the transgenic varieties, meaning that their concentrations tend to be higher in the transgenic lines.

Moreover, there were quantitative and qualitative differences that separate the three transgenic and isogenic pairs from each other. Different groups of amino acids correlate to different transgenic/isogenic pairs. Few amino acids (ornithine, alanine and glycine) were linked to (higher in) the pair belonging to event Mon 810, whereas the pair belonging to event 167 was positively correlated to almost all amino acids. In contrast, the pair belonging to event Bt11 was negatively correlated to most amino acids. The fact that the eigenvalues were small (0.054 for RDA-axis 1 and 0.018 for RDA-axis 2) indicates that variables other than maize line explain the variability in amino acid composition.

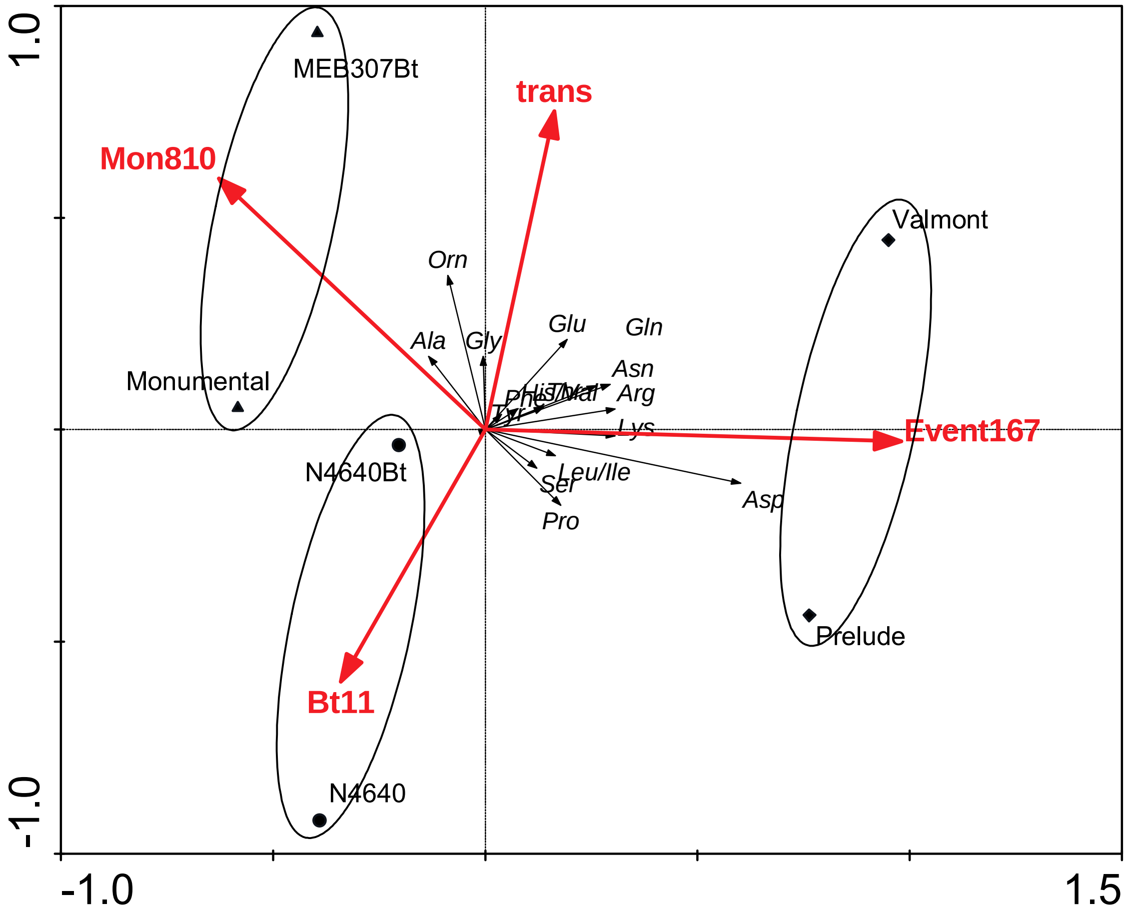

Figure 4. Distribution of amino acid concentrations (plotted as vectors) in samples of transgenic/isogenic pairs of maize plants belonging to three transformation events (vector Mon 810 denotes the pair MEB307Bt/Monumental; vector Bt11 denotes the pair N4640Bt/N4640; vector Event 167 denotes the pair Valmont/Prelude, vector trans indicates transgenic varieties belonging to all three transformation events) in the ordination biplot of an RDA. Axis 1 (EV = 0.054) and axis 2 (EV = 0.018) are presented. The ellipses group the different transgenic/isogenic pairs. For amino acid abbreviations see figure 3.

In the PCA of amino acid distribution two clusters were evident, one comprising the three isogenic lines and a second one comprising the three transgenic lines (Fig. 5). The insertion of the vector for the population performance of the aphids indicated that all amino acids are positively linked to aphid performance and show higher concentrations in the transgenic varieties. Among all amino acids, glutamine and ornithine seem to best explain the higher population performance of the aphids. The high eigenvalues indicate that these variables explain 75% of the variability. The coordinates of the varieties on this axis were consequently an adequate description of the amino acid data.

Although the above results indicate that a higher amino acid concentration in the transgenic lines may explain the enhanced aphid performance, a regression analysis between the coordinates and aphid performance gave a non-significant result (R^2=0.51, F=4.19, P=0.11). However, given the very small sample size (n = 6) this result is still an indication of an amino acid effect.

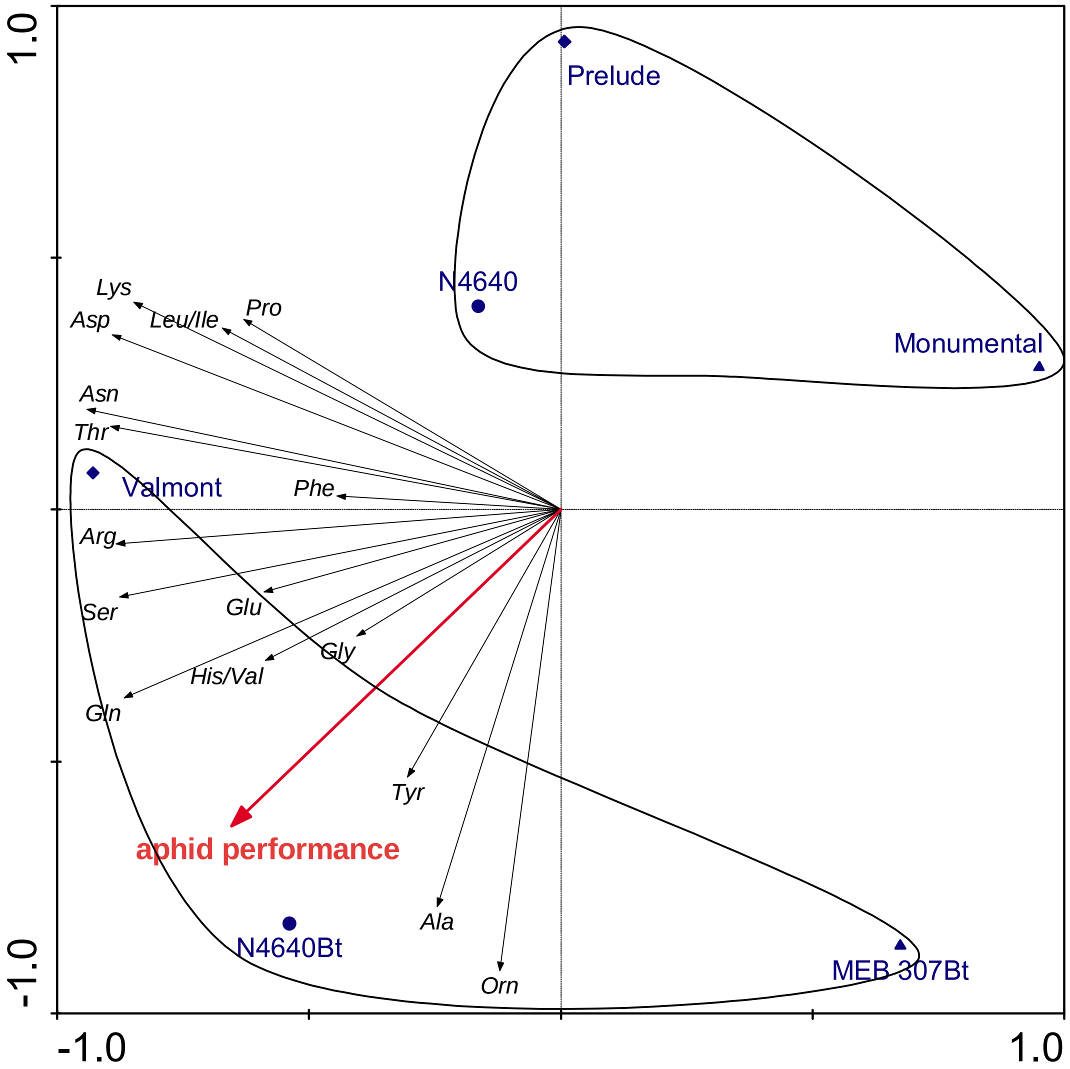

Figure 5. Distribution of amino acid concentrations (plotted as vectors) in samples of transgenic/isogenic pairs of maize plants in the ordination biplot of a PCA. Axis 1 (EV = 0.56)

and axis 2 (EV = 0.19) are presented. The the two clusters formed by either the transgenic or the isogenic varieties are indicated. The vector aphid performance indicates the population performance of aphids (for details see figure 2). For amino acid abbreviations see figure 3.

Effect of honeydew on C. marginiventris *longevity and performance*

Survival and offspring production of the parasitoid differed between the transgenic and isogenic lines within each event. *C. marginiventris* that fed on honeydew produced by aphids on the transgenic lines had higher probability of prolonged survival than the ones that fed on honeydew produced by aphids on the respective isogenic lines (for the pair N4640Bt/N4640 n=9, χ^2=7.3, df=1, p=0.006; for the pair MEB307Bt/Monumental n=9, χ^2=14.6, df=1, p<0.001; for the pair Valmont/Prelude n=9, χ^2=9.9, df=1, p=0.001) (Fig. 6).

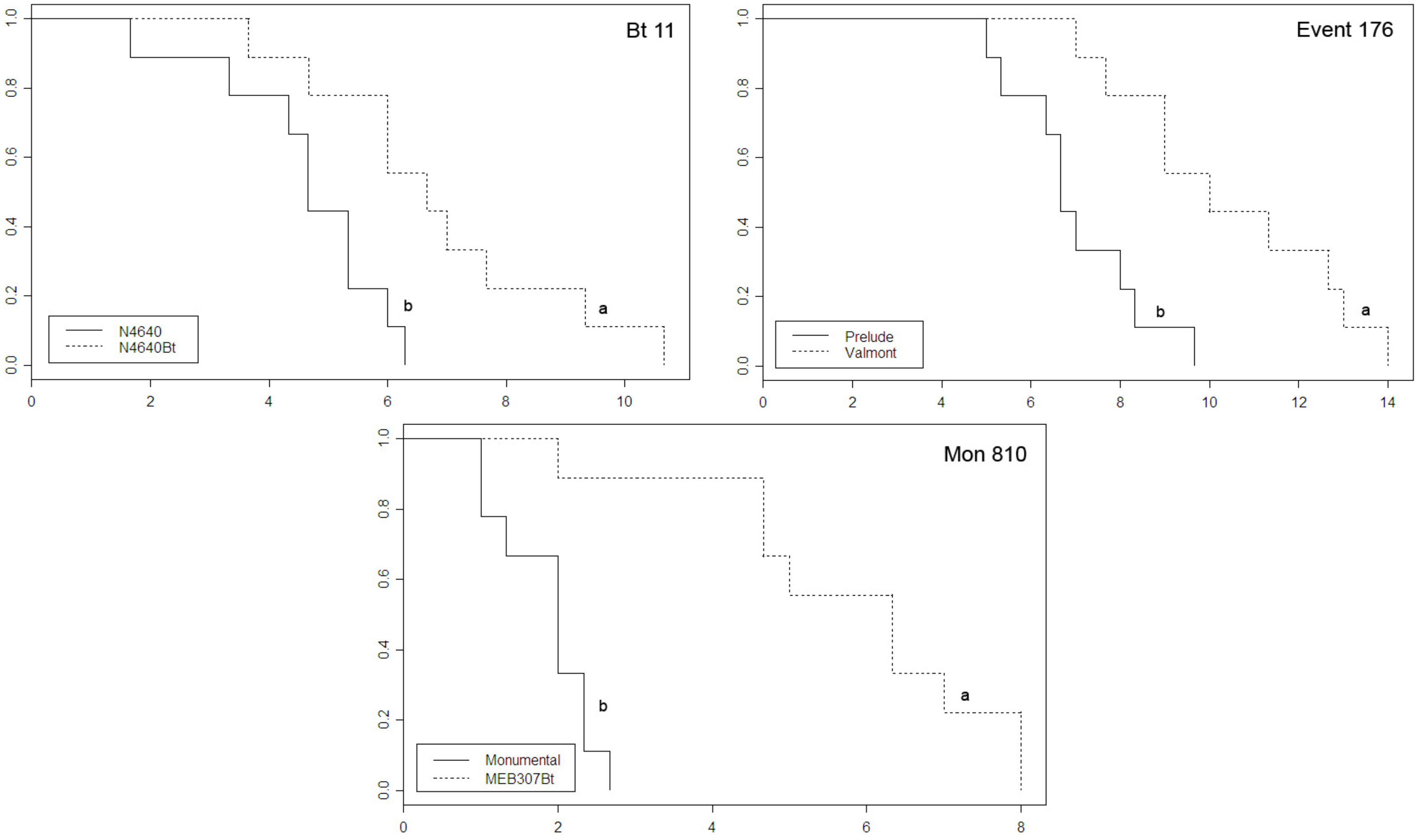

Figure 6. Survival curves showing the survival probability of *C. marginiventris* when feeding on honeydew produced by *R. maidis* on maize of transgenic/isogenic pairs belonging to three different events. Different letters indicate significant differences between curves (p<0.01).

Similarly, parasitoid females that had fed on honeydew produced by *R. maidis* on transgenic plants produced more offspring than the females that had fed on honeydew produced by aphids on the respective isogenic lines (for the pair N4640Bt/N4640: t=2.55, df = 16, p = 0.02; for the pair MEB 307Bt: t = 3.79, df = 16, p = 0.002; for the pair Valmont/Prelude: t=2.93, df = 16, p = 0.002) (Fig 7).

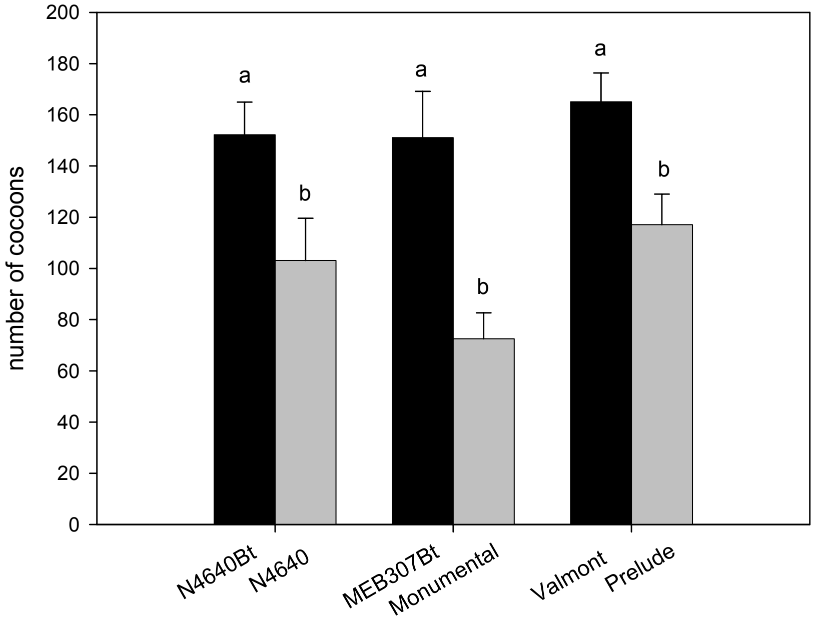

Figure 7. Total number of offspring (number of cocoons) produced by *C. marginiventris* when feeding on honeydew produced by *R. maidis* on different maize varieties. Different letters indicate significant differences between treatments within one transgenic/isogenic pair (p<0.05).

Honeydew analysis

There were no obvious differences in sugar composition between the honeydew produced by *R. maidis* when feeding on the transgenic and isogenic lines of each event, and the composition of honeydew from the transgenic lines fell well within the overall variability among the different maize genotypes (Fig. 8). It was not possible to collect honeydew produced from the variety Monumental, as it was highly resistant to the aphid and it was not possible to establish a colony.

The typical phloem sugar sucrose and its hexose components, fructose and glucose, made up between 81 and 88% of the sugars present in the honeydew produced on the varieties tested. The maltose found in the honeydew might be at least partially plant derived as this sugar has been found in maize plants (Ferguson et al., 1979; Shawn & Dickinson, 1984). Erlose was the most important aphid-synthesized sugar and trehalose was also present in all varieties. Traces of melibiose were detected for the varieties N4640Bt, Challenger and Byzance; traces of melezitose were detected for N4640, N4640Bt, MEB 307Bt, Prelude, Valmont, Delprim; and traces of raffinose detected for Best, Challenger and Byzance.

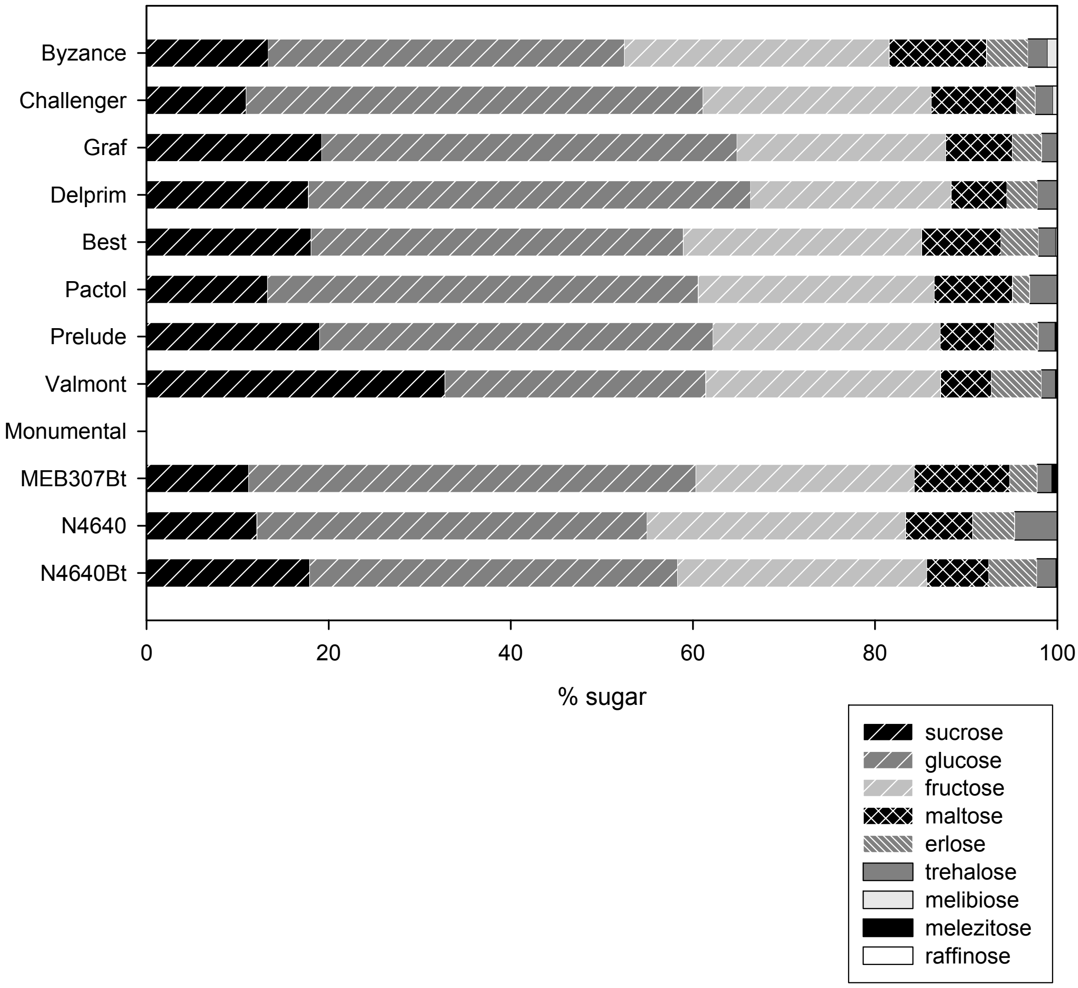

Figure 8. Sugar composition of the honeydew produced by the corn leaf aphid *Rhopalosiphum maidis* feeding on different maize genotypes.

Correlation between honeydew intake and survival

There was a positive effect of honeydew intake on the longevity of *C. marginiventris* (Fig. 9). The percentage weight gain of wasps that fed on honeydew produced by aphids on the respective transgenic and isogenic lines was very similar (for the pair N4640Bt/N4640 p = 0.42; for the pair Valmont/Prelude p = 0.30). Similarly, there was no difference in longevity between wasps feeding on honeydew from the transgenic and isogenic pairs (for N4640Bt/N4640 p = 0.62; for Valmont/Prelude p = 0.50). As the variety Monumental was resistant to *R. maidis* for the pair MEB307Bt/Monumental the results are only available for the transgenic line (Fig. 9 MEB 307Bt).

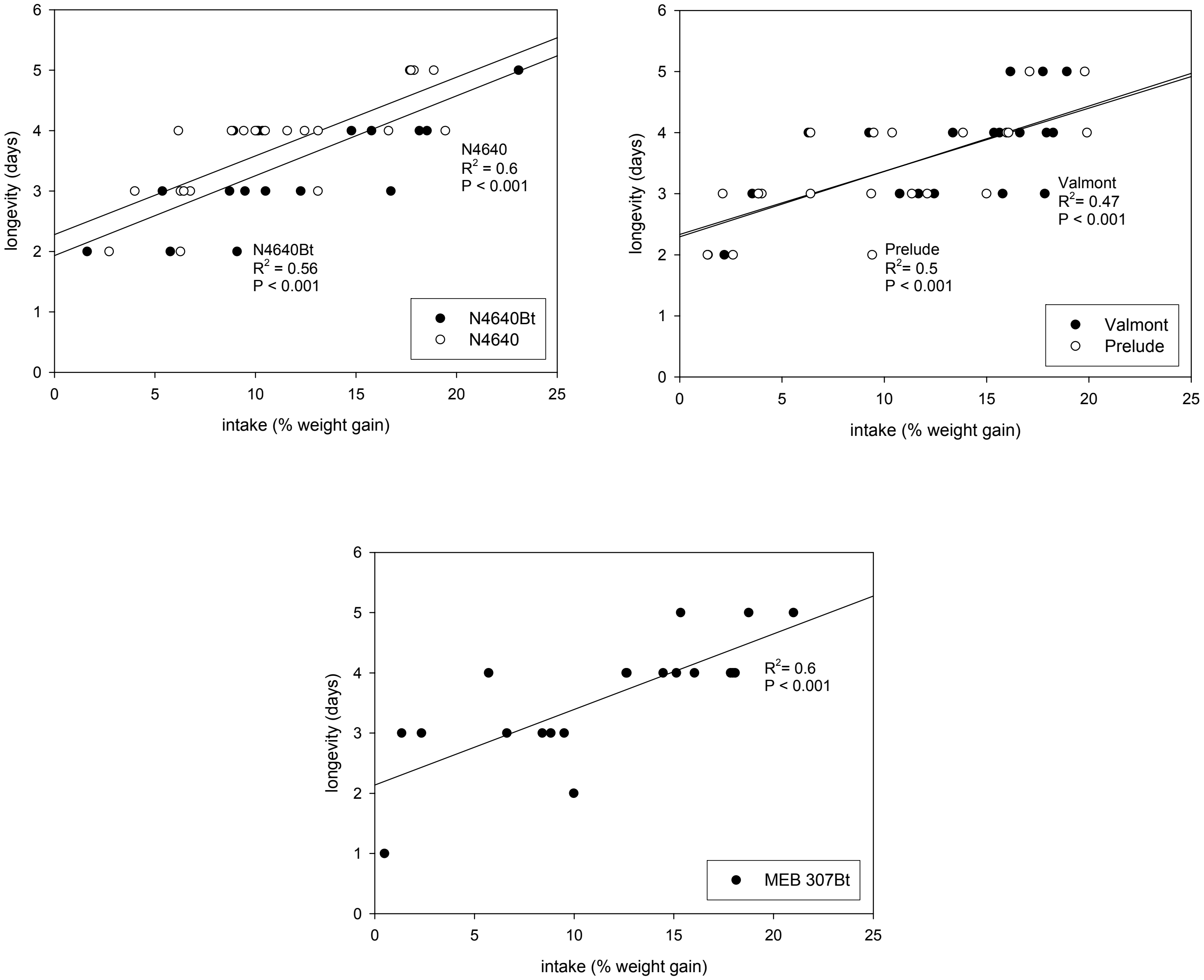

Figure 9. Regression analysis comparing the intake (in % of body weight gained) of honeydew from transgenic/isogenic maize lines during a single meal and subsequent survival of *C. marginiventris*. Different types of honeydew produced by *R. maidis* feeding on maize varieties from three different transgenic/isogenic pairs were tested (no honeydew was obtained from the isogenic line Monumental because aphids did not survive on it).

Discussion

For all combinations tested, *R. maidis* colonies were found to perform considerably better on Bt maize than on the near isogenic correspondent lines. The only exception was for the Navaris-Antaris combination, both of which were found to be highly susceptible, much more so than any of the other lines. Enhanced performance on transgenic maize was reflected in the population sizes, but was not measurable at the individual level. A similar discrepancy between individual and population performance has been reported for the aphid *Cepegillettea betulaefoliae* by Awmack et al. (2004). This could explain why previous studies concluded that there is no difference in performance of aphids infesting Bt plants and their correspondent near isogenic lines (Dutton et al., 2002; Lozzia et al., 1998). That aphids do indeed better on Bt maize also follows from the studies by Pons and colleagues who, in a first series of experiments, found a significantly higher rate of offspring production by colonizing alate mothers of *R. padi* and consequently higher densities of this species on Bt maize (Lumbierres et al. 2004; Pons et al., 2005). Unlike our results for *R. maidis*, the laboratory studies of Lumbierres et al. (2004) showed a poorer performance of the offspring of the first generation of *R. padi* apterous mothers on Bt maize. No such effect was found for the subsequent generations. In a follow-up farm scale experiment they confirmed an increased presence of aphids on Bt maize and, depending on the stages of the aphids, this was found for three of four species of aphids that were observed on the plants, as well as for a species of leafhopper (Pons et al., 2005). It therefore appears that, in general, sucking insects do better on Bt than on non-Bt maize. The observed differences in aphid numbers are unlikely only the result of a difference in attractiveness of the plants. In our experiments, the colonizing aphids were directly placed on their respective plants and did not have an option to move away. We rather think that the higher population densities on Bt maize were caused by differences in chemical constituents that rendered the plants less defended and/or more nutritious for the aphids. The nutritional value is almost entirely determined by the amino acid content and composition of the plant phloem (Auclair, 1963; Douglas, 1998; Karley et al., 2002).

Although we could not find strong statistical differences in amino acid composition between the lines, explorative data analyses (RDA) did indicate some significant correlations between different maize lines and the concentrations of individual amino acids. Transgenic lines in general had higher amino acid concentrations than the corresponding isogenic lines. This was especially true for the essential amino acids, which were all positively linked to the transgenic varieties with the exception of leucine/isoleucine (Fig. 4). The PCA exploring the relationship between aphid performance and amino acid concentration showed, in addition, that these two parameters were positively linked. However, this was only marginally significant. This suggests that the differences in susceptibility levels between transgenic and isogenic varieties can partially be explained by the differences in the amino acid composition of the phloem. Furthermore, each transgenic/isogenic pair from the three events had quantitatively and qualitatively characteristic typical amino acid compositions. Several studies have shown the key role of phloem amino acid concentration and composition in the phloem in determining aphid performance (e.g. Weibull, 1987; Kazemi & van Emden, 1992; Sandström & Petterson, 1994; Karley et al., 2002). However, this relationship is not always apparent (Girousse & Bournoville, 1994; Wilkinson & Douglas, 2003).

Our results agree with previous studies showing that the phloem is dominated by non-essential amino acids (Sasaki et al., 1990; Douglas 1993, 2003a; Girousse & Bournoville 1994; Sandström & Petterson, 1994; Winkinson & Douglas, 2003). The lack of the essential amino acid methionine in the maize phloem is especially interesting because the endosymbiotic bacteria *Buchnera*, which provides aphids with essential amino acids (Douglas, 1998) appears to lack the capacity to synthesize methionine (Shigenobu et al., 2000; Tamas et al., 2002). If this is also true for *Buchnera* from *R. maidis*, this aphid species might have other bacteria ("secondary symbionts") with biosynthetic capability (Douglas et al., 2003b).

Several other factors can influence the ability of an aphid species to exploit a host plant, such as physical characteristics (Roberts & Foster, 1983; Webster, 1994; Tosh et al., 2001) and secondary plant metabolites (Niraz et al., 1985; Niemeyer, 1988; Givovich et al., 1994; Leszczynski et al., 1995). Our results suggest that the dramatic difference in susceptibility to *R. maidis* between MEB 307Bt and Monumental is due to factors other than differences in the amino acid composition of the phloem and it cannot be ruled out that this difference arose as a consequence of breeding procedures after the transformation (Conner 2003; Kok & Kuiper 2003).

R. maidis is usually a minor pest of maize (Kring & Gilstrap, 1986; Kröber & Carl, 1991; Jauset et al., 2000; Waterhouse & Sands, 2001). Its poor performance on maize makes it a good study subject for research into maize susceptibility. The other reason why *R. maidis* was chosen for the current study is that it produces large amounts of honeydew, thus supplying numerous organisms with an additional source of sugars. A change in quantity and quality of the honeydew may therefore have consequences for these organisms. Indeed, the larger numbers of *R. maidis* found on the Bt plants resulted in an increase of honeydew production, which posed a significant advantage for *C. marginiventris* females that were allowed to feed on the honeydew. Such females lived significantly longer and produced more offspring (i.e. parasitized more host larvae on neighbouring plants) than females that had access only to aphid-infested near isogenic lines. Two experiments strongly suggest that this benefit was merely due to the increased honeydew quantity and not to a higher nutritional quality. Firstly, analyses of honeydew for sugar composition showed it to be similar for Bt maize and non-Bt maize. Sugar composition is one of the key factors determining the nutritional value of honeydew (Wäckers, 2000). Of most value to parasitoids are the plant-derived sugars sucrose, glucose and fructose, whereas the aphid-synthesized sugars lower the honeydew nutritional value (Wäckers, 2000; 2001). The analysis of the honeydew produced by *R. maidis* feeding on several maize varieties showed that only a small proportion of the sugars were aphid-produced. There were some differences in the composition of the honeydew produced by aphids feeding on the transgenic and the correspondent near isogenic lines, but these small differences fell well within the overall variability of the composition of the honeydew produced by *R. maidis* on maize.

That a difference in honeydew quality was not responsible for the observed increase in performance of wasps on Bt maize was also evident from the experiment whereby females had a single feeding bout on different honeydews. They consumed comparable amounts of honeydew and after such a bout they survived just as well on honeydew from Bt maize as on honeydew from non-Bt maize, apparently honeydew quality was not affected by transformation of the maize lines. From a previous study we know that for optimal survival and reproduction, *C. marginiventris* needs to feed repeatedly on a sugar source (Faria et al., in prep.). Here we find that an increase in availability and accessibility of such a source facilitates this need.

In at least one other study, Bt plants provide food of altered quality to natural enemies: females of the predatory mite *Neoseiulus cucumeris* live longer, but have significantly lower fecundity when feeding on pollen of transgenic plants compared to females feeding on pollen of the correspondent untransformed line (L. Obrist pers. comm.). The authors argue that, as the toxin content in the Bt pollen was negligible, the most probable explanation is that concentrations of other compounds in the pollen from the transgenic were altered. A. Dutton (pers. comm.) found that the amino acid content of pollen indeed tends to be slightly lower (but not statistically different) in pollen from Bt maize than the corresponding near isogenic line. Our study illustrates the importance of including a range of conventional crop varieties for a realistic assessment of the potential consequences of introducing GM crops (see also Turlings et al., 2005). Most other studies only compare one GM variety versus its (near) isogenic line. If we had done so here, one could have concluded that a particular Bt maize poses a risk because of increased susceptibility to aphids, but by including several varieties it can indeed be concluded that the insertion of the Bt gene renders maize more susceptible to aphids, but that the Bt lines tested here all fall within the susceptibility range of conventional varieties. The range of susceptibilities appears to be extremely broad, as is the case for other maize traits (e.g. Saxena & Stotzky 2001; Degen et al., 2004; Lundgreen & Wiedenmann, 2004). It is essential to take this variability among maize lines into account in future studies into the risk assessment of transgenic maize and there is perhaps reason to invalidate some studies that have not done so in the past. Because of this very high variability in resistance traits, this study can neither claim that Bt maize increases the risk of aphid infestations, nor can it claim that parasitoids will perform better on Bt maize than on conventional maize.

It should not be assumed that for other plant species or other maize constructs, our results hold true. For instance, *Macrosiphum euphorbiae* had reduced growth and fecundity when reared on Cry3A potatoes, but it has an improved performance on transgenic potatoes producing rice cystatin I (Ashouri a al., 2001). Furthermore, in other insects the Bt toxin may end up in the honeydew as is the case for the planthopper *Nilaparvata lugens* when it feeds on different varieties of transgenic rice containing different promoters, including CaMV 35S, the same promoter used in the events Mon 810 and Bt11 (Bernal et al., 2002). The planthopper shows no difference in performance on Bt and control lines, but they were found to produce more honeydew on Bt lines, and the Bt derived honeydew was more acidic (derived from feeding on xylem and other non-phloem sources) than the one from the control non Bt lines (Bernal et al., 2002). Unlike for aphids, the honeydew from planthoppers is therefore a new route of exposure of non-target organisms to Bt toxin.

For the maize lines studied here it can be concluded that increased susceptibility to aphids is advantageous to parasitoids that feed on aphid honeydew. This finding has important implications for the effectiveness of parasitoids as biological control agents; increased honeydew production not only helped to increase parasitoid longevity, but it also resulted in a significantly enhanced rate of parasitism. In maize monoculture parasitoids will have no or very limited access to plant-provided sources of sugar and aphids may be the only providers of these essential nutrients. Aphids may also supply food to various generalist predators and thus maintain higher numbers of them in cropping systems and, in cases where they cause no yield-reducing harm to the crops, aphids should be considered beneficials rather than pests. Reduced availability of lepidopteran hosts on Bt maize will negatively affect parasitoid population densities. Increased availability of aphid honeydew may compensate for some of this negative effect. *R. maidis* is normally not an important pest of maize (Kring & Gilstrap, 1986; Kröber & Carl, 1991; Jauset et al., 2000; Waterhouse & Sands, 2001). In fact, their presence may, by being and/or producing an additional food source help to sustain beneficial natural enemies of pest insects in a maize field. As long as aphid numbers do not reach pest status, the unexpected and unintended increase of aphid susceptibility of Bt maize may therefore pose an advantage in maintaining a beneficial insect fauna in Bt maize fields.

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Conclusions and Outlook

In this thesis I addressed three general questions concerning the use of aphid honeydew as a food source by non-aphid parasitoids:

1. Are larval parasitoids able to use honeydew produced by *R. maidis* as food?

2. Is *C. marginiventris* innately attracted by aphid-produced honeydew and can it learn to distinguish between honeydew-associated cues and host-associated cues?

3. Does Bt maize affect the performance of *R. maidis* and, if so, does this effect translate in the performance of *C. marginiventris* after honeydew feeding?

In Chapter 1 it was shown that aphid-produced honeydew indeed increases parasitoid longevity, but not as much as a sucrose solution. Furthermore, *C. marginiventris* needs to feed several times throughout their life in order to achieve optimal longevity and reproductive output. The honeydew produced by *R. maidis* infesting barley was mainly composed of the plant-derived sugars, sucrose, fructose and glucose, but also contained several aphid-synthesized sugars. The composition of the honeydew changed over time and as a function of aphid infestation. In general, the higher the aphid infestation, the smaller the percentage of aphid-synthesized sugars in the honeydew. Experiments using a sugar mixture mimicking the sugar composition of the honeydew showed that the observed differences in the honeydew composition had no effect on nutritional value. This result rules out the possibility that the aphid-synthesized sugars were responsible for the relatively low performance of the parasitoids that had fed on honeydew. Additional experiments showed that this poorer performance of *C. marginiventris* on honeydew is readily explained by differences in food intake due to the physical properties of the honeydew.

In Chapter 2 it was found that, as expected, *C. marginiventris* females did not display an innate attraction towards odours of aphid-infested plants. However, after experiencing these odours in association with a food reward the females were highly attracted to them. Females were also shown to respond more strongly to these food-associated odours when hungry and to exhibit a higher responsiveness to host-associated odours when satiated. Thus, they switched between host and food foraging based on their physiological state (hungry or fed) and previous experiences with hosts and food. Volatile collections showed that barley-infested with the aphid *R. maidis* released small amounts of α-pinene, whereas barley infested with larvae of *S. littoralis* released large amounts of various other volatiles that are know to be released by plants in response to caterpillar attack. Evidently, the wasp is capable of distinguishing the two odours and uses this ability to optimize its foraging efficiency.

Chapter 3 focused on how the exploitation of honeydew by *C. marginiventris* might be affected by genetic transformation of plants. Using several genotypes of Bt maize, it is shown that the aphid *R. maidis* performs better on transgenic maize than on their respective untransformed counterparts. Analysis of the amino acid composition of the phloem showed that there are slight differences between the amino acids in the phloem from transgenic plants and their correspondent near-isogenic lines, which could be one of the explanations for the differences observed in aphid performance. Further experiments showed that the enhanced performance of the aphid on Bt maize lines was reflected in the performance of the parasitoid when it was allowed to feed on honeydew. *C. marginiventris* females lived longer and produced a higher number of offspring in the presence of transgenic plants infested with *R. maidis*, when compared to females that were kept with the correspondent near-isogenic lines infested with aphids. Analyses of the honeydew revealed that there were some small differences in sugar composition between the transgenic plants and the correspondent untransformed variety, but these differences fall within the variability that was observed among several conventional maize varieties. Based on honeydew intake tests and the findings presented in Chapter 1 we may conclude that differences in parasitoid performance are likely an effect of larger quantities of and better access to honeydew on transgenic plants due to higher aphid infestation.

In summary, the studies presented in this thesis revealed that (1) despite of its relatively low nutritional value, honeydew can increase parasitoid longevity and reproductive output. (2) *C. marginiventris* is able to learn to respond to odours that are associated with honeydew and chooses between host and food foraging based on their physiological state and previous experience. (3) Enhanced aphid performance, caused by unexpected changes due to genetic transformation of maize has changed the availability of honeydew for non aphid parasitoids, thus positively affecting their performance.

Outlook

This work highlights the importance of providing parasitoids with food sources close to the host sites, so that the wasps do not need to spend time and energy when switching from host to food foraging. One should consider that the presence of food sources in the field may benefit not only the natural enemies, but also the pests (Baggen & Gurr, 1998), so the use of food sources that benefit only the natural enemies is desirable. In this context, honeydew may be of higher value than other naturally occurring or artificial food sources as parasitoids accept and benefit from a broader range of sugars than some Lepidopteran pests (Wäckers, 1999, 2001, Romeis & Wäckers, 2002, Winkler et al., 2005). This implies that in some situations, moderate aphid infestation of a crop is likely to improve biological control of other pests. Despite of the fact that the use of honeydew by parasitoids has been shown in nature (Wäckers & Steppuhn 2003; Steppuhn & Wäckers 2004; Casas et al., 2003) to our knowledge only one field study has assessed the effect of honeydew feeding on the performance of a larval parasitoid (Stapel et al.,1997). Unlike our results, the study by Stapel et al. (1997) found that honeydew fed females performed as poorly as unfed females. It seems therefore pertinent that more studies of this type should be carried out and to also investigate if the honeydew-promoted benefits found in the lab are translated in the field performance of parasitoids.

It was further shown that *C. marginiventris* females are able to learn honeydew-associated odours. Further studies should investigate if this learning ability indeed results in higher retention in patches with hosts and honeydew, and if the same is true when food sources of higher quality and detectability (such as floral nectar) are present in the vicinity of the host patches. As nectars are usually of superior nutritional quality it can be expected that the wasps have an innate preference for cues that may lead them to such food sources. Indeed, parasitoids are highly responsive to flower odours and colours (Wäckers, 1994; Wäckers, 2004). A preference for such cues would allow the wasps to still locate high quality food even after learning the cues associated with poor quality food such as honeydew. The results from the experiments with transgenic plants reinforce our notion that it is important to include a range of conventional crop varieties for a realistic assessment of the potential consequences of introducing GM crops. Moreover, it highlights the importance of investigating unexpected changes due to the insertion in the plant of a foreign gene and how these changes might affect the food web. Future studies should determine, in the field, the exact implications of the higher susceptibility levels of Bt maize on aphid infestation levels and also on the performance of parasitoids and predators. It can be expected that in regions were aphids are not a serious economical problem an increase in aphid density (and consequently higher honeydew production) will help to sustain natural enemies of pests that are not fully controlled by the Bt toxin.

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