The importance of phenology in studies of plant-herbivore-parasitoid interactions

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To my mother

致我的母亲

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Abstract

As food resources of herbivorous insects, the quality and quantity of plants can directly affect the performance of herbivorous insects and indirectly affect the performance of natural enemies of the herbivorous insects. In nature, plant quality and quantity are dynamic and can change in individual plants over the course of a single growing season. Many multivoltine insects are known to attack short-lived annual plants that are present for only 2 or 3 months in the field. These short-lived plants may germinate and grow at different times and locations during the growing season. In this situation, each generation of insects is obligated to search for potentially new species of food plants across the growing season, which may differ in qualitative and quantitative traits. The aim of this thesis was to explore how seasonal phenology of potential food plants effects a multivoltine herbivore-parasitoid interaction. In particular, I examined potential qualitative and quantitative constraints imposed by the seasonal phenology of several food-plant species on the development and survival as well as on oviposition decisions of a gregarious specialist herbivorous insect and its natural enemy that both have multiple generations per year. As a model system, I used a multivoltine specialist herbivorous insect associated with different plant species, the large cabbage white butterfly, *Pieris brassicae* L., and its specialized multivoltine endoparasitoid, *Cotesia glomerata* L.. *Pieris brassicae* primary feed on plants in the large family Brassicaceae. I used three annual brassicaceous plants, *Brassica rapa* L., *Sinapis arvensis* L., and *Brassica nigra* L., which grow rapidly and exhibit differing phenologies, each growing within a short period of time and with little temporal overlap amongst them. These plants are known to serve as food plants for successive generations of *P. brassicae* and related species.

In bioassay experiments under controlled greenhouse and semi-field conditions, I found that *P. brassicae* and *C. glomerata* were marginally affected by seasonal-related and plant species-specific differences in food-plant quality. *Pieris brassicae* was also marginally affected by the ontogenetic variations in food-plant quality. In addition, food-plant shifts in different generations had small effects (both positive and negative depending on plant species) on the performance of *P. brassicae* and *C. glomerata*. Survival and performance of *P. brassicae* was much more constrained by quantitative than qualitative aspects of the food plant. The survival

and performance of *C. glomerata* was also affected by similar quantitative constraints as that of its host.

In behavioural experiments under controlled greenhouse and semi-field conditions, I found that female *P. brassicae* oviposition preference order for food plants declined with plant age of different plant species (*S. arvensis* and *B. nigra*). Female *P. brassicae* butterflies may 'anticipate' future quantity or quality potential of the food plants when choosing oviposition sites. Pre-adult experience had minor effects on *P. brassicae* butterfly oviposition preference and had no effect on *C. glomerata* landing preference. *Pieris brassicae* also did not exhibit consistent preference for any of the plant species, whereas *C. glomerata* had a clear preference on *B. rapa*. Further studies on trophic interactions need to incorporate more spatial and temporal realism, i.e. plant species shifts (temporally dynamic interactions) as well as to 'track' insect foraging behaviour in the field (spatially dynamic interactions). Thus far, virtually nothing is known about these areas or as to the success of naïve insects in locating new patches of food plants or hosts in different habitats.

Chapter 1

General introduction

Introduction

In terrestrial ecosystems, most phytophagous organisms are insects, both in absolute numbers and in the number of species (Schoonhoven et al. 2005). On the other hand, green plants make up the largest part of the total biomass in terrestrial ecosystems. Interactions between plants and insects are therefore the most common in these ecosystems (Schoonhoven et al. 2005). However, these interactions cannot be completely understood without incorporating the third trophic level, i.e. the natural enemies of herbivorous insects, such as predators and parasitoids (Price 1980, Price 2003). Natural enemies can have large top-down effects on the structure and dynamics of herbivore communities via predator-prey or parasitoid-host interactions and as a consequence of this they indirectly influence the abundance of plants (Schmitz et al. 2000, McGovern et al. 2006). From the bottom up, plant-mediated effects on the herbivores can ‘cascade up’ the food web and influence species diversity and population dynamics at higher trophic levels (Hunter and Price 1992, Bukovinszky et al. 2008).

Plants serve as food resources for insects

Plant resistance/defence traits

Plants have evolved a diverse array of physical and chemical resistance traits to prevent or reduce damage by herbivorous insects. These defence strategies can be divided into direct and indirect defences. Direct defences can directly negatively impact the development and behaviour of herbivorous insects and include morphological defences such as sticky glands, thickened leaves and trichromes, and various types of chemical defences such as secondary plant compounds (Karban and Baldwin 1997, Scriber 2002). Spines or trichromes on the leaf surface can hamper herbivorous insect movement or prevent them from consuming plant tissues. Thickened leaves can reduce the palatability of plants and thus negatively influence the feeding efficiency of herbivorous insects (Schoonhoven et al. 2005). Secondary plant compounds are chemicals that do not apparently have a major function in plant metabolism but instead function as defences against plant antagonists such as pathogens and herbivores (Karban and Baldwin 1997). They vary phylogenetically (e.g. among different plant families) and include glucosinolates in the Brassicaceae, furanocoumarins in hogweeds and related umbellifers, iridoid glycosides (IG) in *Plantago* spp. and pyrrolizidine alkaloids in *Senecio* and *Jacobea* spp. (Schoonhoven et al. 2005). Resistance based on secondary plant compounds may involve deterrence of oviposition or feeding and interference with the insect's physiology once plant tissues have been ingested (Harvey et al. 2005, Berger et al. 2007). Indirect defences enhance the ability of natural enemies to exploit their herbivorous hosts or prey on the plant. For instance, the plant can attract, nourish or house other organisms, which as a result of their presence can reduce enemy pressure (Price 1980, Dicke and Sabelis 1987, Vet and Dicke 1992). Examples of indirect defences are the production of attractive volatile organic compounds, extra floral nectar, food bodies and structures that can be used as domatia or nesting space (Heil 2008). Among all of these indirect defences, the production of plant volatiles, especially those emitted in response to herbivore feeding, and their attractiveness to natural enemies has been intensively studied (Vet and Dicke 1992). Plants can biosynthesize and release volatiles in response to egg deposition and herbivore damage, and these volatiles are not only released from the damaged tissues but also from undamaged tissues (Vet and Dicke 1992, Hilker and Meiners 2006, Agerbirk et al. 2008).

Plant nutrition and quantity

The nutritional quality of food plants is determined by concentrations of primary metabolites, such as proteins, particular carbohydrates, and fatty acids that are essential for development and reproduction of all living organisms. Among these primary metabolites, nitrogen and amino acids are considered to be of critical importance for growth and development of herbivorous insects (Schoonhoven et al. 2005). However, these compounds are often found limiting in plant tissues and as a consequence many plants are often of poor nutritional quality for insects (Lawton and McNeil 1979, Mattson 1980, Scriber and Slansky 1981).

Plant quantity is another constraint for survival and performance of insects. Generally, large plants (i.e. woody plants) provide more resources than small plants (i.e. weedy plants) to insects for their development, but often harbour many more insects as a result of their greater biomass.

In nature, qualitative and quantitative plant characteristics are dynamic over the course of a growing season (Schoonhoven et al. 2005). Dynamics in plant quality are, to a large extent, determined by plant ontogeny (Mattson 1980, Coley 1983, Boege 2005, Barton and Koricheva 2010). Barton and Koricheva (2010) using a meta-analysis, reviewed whole-plant ontogenetic changes in plant secondary metabolites in both weedy and woody plants and found that concentrations of secondary metabolites tend to increase from the seedling to the adult stage. Additional seasonal variation in plant quality traits may be caused by variation in biotic, e.g. herbivore and pathogen damage (Karban and Baldwin 1997), and abiotic factors, e.g. rainfall, temperature and soil nutrients (Schoonhoven et al. 2005).

Temporal patterns of plant availability

Plant availability is determined by the biology and phenology of the plants. The timing of plant phenological events such as leaf budburst or first flowering is often highly sensitive to ambient environmental conditions (Cleland et al. 2007). Phenology is defined as the study of periodic or episodic plant and animal life cycle events and how these are influenced by seasonal and inter-annual variations in abiotic factors such as temperature (Schoonhoven et al., 2005). Different plant species exhibit particular seasonal phenology and biology events during the growing season. Many long-lived plants, such as deciduous trees, typically grow new leaves every year that mature during the course of the growing season. In these woody plants, leaf tissue quality often decreases whereas their quantity increases during the season. On the other hand many short-lived annual plants (e.g. forbs) grow at specific times during the growing season. In these plants, quality and quantity as a result of ontogeny and environment may change dramatically within a very short time. There are also annual and perennial plants that develop and mature leaves more slowly, encompassing an entire growing season.

Insect life-history traits that constrain interactions with food plants

Food-plant specialization

Although plants have evolved defensive mechanisms that enable them to resist herbivory, insects have evolved behavioural and physiological adaptations to counter these defences enabling them to better exploit certain plant types. For example, insects can avoid feeding on toxic plant tissues (Dussourd 1993), and some insects can excrete, or detoxify plant toxins (Nishida 2002, Ratzka et al. 2002). Herbivorous insects differ in their degree of dietary specialization, and many insects feed on only one or a few plant species that are restricted to a specific family (Schoonhoven et al. 2005, Loxdale et al. 2011). The performance of specialized herbivores is often influenced by the presence of specific chemicals in the food plant which stimulate oviposition and/or feeding, balanced against the negative effect of inhibitors (van Loon and Schoonhoven 1999, Renwick 2002). For example, the buckeye butterfly *Junonia coenia* is specialized on *Plantago lanceolata* and related native plants in North America and can deal with the negative effects of iridoid glycosides in *P. lanceolata* when concentrations of these compounds are high (Adler et al. 1995). In addition, some specialized insects have not only evolved adaptations to cope with plant toxins, but they can also sequester plant toxins and use them for their own defence (Müller et al. 2001). For instance, some insects specialized on brassicaceous plant species sequester GS in their haemolymph to deter their natural enemies (Müller et al. 2002, Müller and Brakefield 2003, Vlieger et al. 2004). However, in other species, such as the sawfly *Athalia rosae*, sequestration does not protect against all natural enemies but instead appears to also be an alternate way of dealing with secondary metabolites found in their brassicaceous food plants (van Geem et al. 2014). Generalist herbivores are usually more sensitive to plant toxins than specialist herbivores, which are adapted to feed on plants containing specific types of allelochemicals (Ali and Agrawal 2012).

Diet specialization of herbivorous insects can also influence the performance of their natural enemies (Sznajder and Harvey 2003), especially endoparasitoids that develop within the body of their host (Godfray 1994), where larval parasitoid development is closely integrated with host physiology (Askew and Shaw 1986). The performance and survival of parasitoids depends on resources that are obtained from hosts and is also determined by the host’s defences against parasitism. Therefore, there is generally a correlation between host and parasitoid performance (Harvey 2005). Unsurprisingly, plant allelochemicals do not only influence the performance of the herbivore host, but may also affect parasitoid performance as mediated through the host. Studies have found that parasitoids attacking generalist herbivores are more strongly affected by the herbivore’s diet than parasitoids that attack more specialized hosts who only feed on a small range of plant species (Sznajder and Harvey 2003, Gols et al. 2008, Reudler et al. 2011).

Gregarious development in insects

Many phytophagous insects are known to lay their eggs in batches on their food plants and the immature (and sometimes adult) stages live and feed in groups (Lemasurier 1994, Hunter 2000, Fordyce 2003). Gregariousness has evolved in 23 families of the Lepidoptera (Sillentullberg and Leimar 1988). There are various benefits of a gregarious lifestyle, including group defence (Stamp 1980, Gagliardo and Guildford 1993, Hunter 2000, Tullberg et al. 2000), thermoregulation (Porter 1982, Fordyce 2003) and increased reproductive potential (McEvoy 1979). However, disadvantages include increased apparency to natural enemies (Hunter 2000), the increased risk of sharing pathogens (Hochberg 1991, Brown et al. 2001) and an increased chance of intraspecific competition for food resources (Andrewartha and Birch 1954, Damman 1991, Lemasurier 1994, Konvicka et al. 2003). Therefore, gregarious species often need to attack plants with excess biomass that can support large numbers of conspecifics such as trees, while other gregarious species must associate with small plants that grow in large, densely clustered stands to reduce the risk of intense competition and starvation.

As with gregarious herbivorous insects, gregarious parasitoids have higher quantitative nutritional requirements than solitary parasitoids. The resource requirements of hosts parasitized by koinobiont parasitoids may sometimes exceed those of healthy larvae, and lead to parasitized hosts being larger than healthy ones (Harvey 2005). When a gregarious koinobiont parasitoid attacks a herbivorous host that is gregarious itself, both organisms may potentially experience significantly negative effects on their survival and fitness due to insufficient food availability in terms of plant food.

Voltinism in insects

Insects are known to exhibit significant inter-specific (and even intra-specific) variation in their seasonal patterns of activity (e.g. number of generations, temporal variation in the presence of different stages, diel rhythms). The number of generations an insect has per year is described by the term 'voltinism'. Univoltine herbivorous insects have a single generation per year and often grow at specific periods during the growing season where their development is closely synchronized with the availability of their food plant(s) (Darquenne et al. 1993, Sobhian and Fornasari 1994). In nature, when univoltine insects associate with their food plants, plant quality is often predictable. As a result, some insects species are found only early in the season, whereas others appear later in the season (Wolda 1988). For instance, monophagous insects that have one generation per year are active only when suitable food plant tissues are available (van Asch et al. 2010). Multivoltine insects, on the other hand, have two or more generations per year (Gullan and Cranston 2010). Each generation grows at different periods during the growing season with little overlap among them.

When multivoltine herbivorous insects associate with their food plant, the quality of these plants is often less predictable, because plant quality is variable during the growing season. Multivoltine insects that feed on long-lived plants that produce large amounts of leaf tissue (i.e. trees) can spend several consecutive generations on the natal plant without leaving them. Different generations of these insects are only confronted with changes in the quality of the food. By contrast, many multivoltine insects feed on short-lived annual plants that are only present for 2 or 3 months during the growing season. These plants may not only vary in their temporal distribution but also in their spatial distribution. Thus, each generation of these insects needs to leave the natal patch in search of a new food-plant species, which may grow at a different location some distance away from the natal patch (Chapter 2, Fig. 1). Moreover, plants that serve as food for the second generation may also differ in various traits, including nutritional quality, from those that served as food for the first generation (Gols and Harvey 2009). Multivoltine parasitoids of these herbivores are confronted with the same constraints of tracking their hosts from the natal plant patch to a new patch.

Insect foraging behaviour for nutrition and reproduction

Female adult herbivorous insects that are searching for food plants in the field rely on different sensory inputs, including visual, olfactory, mechano-sensory and gustatory cues (Andrewartha and Birch 1954, Traynier 1986, Vet and Dicke 1992, Bernays and Chapman 1994, Vinson 1998, Schoonhoven et al. 2005, van Dam and Raaijmakers 2006). The two main phases during the process of food-plant selection are searching for the food plant from long distances and contact-tasting to test the suitability of the food plant after locating one (Schoonhoven et al. 2005).

During the searching phase, adult herbivorous insects rely on visual and olfactory cues, whereas mechano-sensory and gustatory cues become more important in deciding to accept or reject the food plant (Schoonhoven et al. 2005). Studies have shown that the presence or absence of plant secondary metabolites can influence food-plant recognition of herbivorous insects (Fraenkel 1959). For instance, glucosinolates play a decisive role for food-plant acceptation by gravid *Pieris brassicae* and *P. rapae* females (van Loon et al. 1992). The black swallowtail (*Papilio polyxenes*) is specialized on plants in Apiaceae family and also recognizes suitable food plant based on the presence of specific terpenes in essential oils of suitable food plants (Dethier 1941).

As with herbivorous insects, their natural enemies, in particular parasitoids searching for their hosts, also utilize volatiles for host location. However, parasitoids use volatiles that are produced by the plant in response to feeding damage by their host. These so-called 'herbivore induced plant volatiles' (HIPVs) have been intensively studied (Dicke et al. 1990, Turlings et al. 1990, Vet and Dicke 1992, Fatouros et al. 2012). The HIPV blend is specific to the host-plant species, and also varies depending on the identity of the herbivorous insects that is causing the damage. Therefore, the HIPV blend provides fairly reliable signals to the parasitoids revealing the presence of its host (Dicke 1999, Vet 1999a). Parasitoids are often specialized in attacking only one or a few related host herbivore species and have therefore evolved complex mechanisms that allow them to find their hosts efficiently (Godfray 1994, Vet 1999a, Wertheim et al. 2005).

In addition to the cues mentioned above, experiences in the natal habitat could further influence food-plant acceptance or host herbivore location of adult herbivorous insects and their parasitoids, respectively (Immelmann 1975, Davis and Stamps 2004). This phenomenon is also referred to as the 'Hopkins' host selection principle' (HHSP) (Barron 2001). To what extent natal experience of both parasitoid and herbivorous insects affects foraging behaviour depends on the developmental stage at which the experience happened. Studies have shown that experience during the adult stage strongly influences successive foraging behaviour of both herbivorous insects and their parasitoids. For instance, for herbivorous insects, experience that is gained during the adult stage in butterflies has been shown to affect the phase of searching for food plants and then in tasting the food plant for oviposition (Raysher 1978, Papaj and Prokopy 1989, Allard and Papaj 1996, Weiss and Papaj 2003). Parasitoids also change their responses following experience and learn to recognize HIPVs when they have had a successful oviposition experience in the presemce of HIPVs and this reduces the time spent foraging for new hosts (Vet and Groenewold 1990, Quicke 1997, Vet et al. 1998). The latter phenomenon is also referred to as 'associative learning' (Papaj and Prokopy 1989, Vet et al. 1990).

The HHSP pertains to experience during the larval period as this affects habitat preferences during the adult stage but is widely considered to be controversial (Emden et al. 1996). This is because insects undergo significant physiological changes during metamorphosis that can impede the retention of 'conditioning' from the larvae through the pupae to the adult (Chapman 2003). Although evidence supporting the HHSP is scarce (Barron 2001), in both herbivorous insects and parasitoids it has been observed that natal experience can affect foraging behaviour of naïve adult insects (Davis and Stamps 2004). For instance, the preference of *Papilio glaucus*, which feeds on tree species from several taxonomic families, has been shown to be affected by larval feeding experience (Bossart and Scriber 1999). The parasitoid wasp, *Pachycrepoideus vindemiae*, reared on large *Musca domestica* pupae or small *Drosophila melanogaster* pupae are better in handling the pupal-size class on which they had been reared when offered different-size pupae for oviposition (Morris and Fellowes 2002). Insects can also be conditioned at eclosion: an insect that emerges from its pupa will immediately encounter odors associated with its food plant. This may affect an individual's subsequent foraging behaviour and oviposition decisions (Vet 1983, Kester and Barbosa 1992, Bjorksten and Hoffmann 1998, Henniges-Janssen et al. 2011).

Research objective

The effect of differences in food-plant quality of perennial and annual plant species on the performance and survival of herbivorous insects and their natural enemies have been extensively studied (Awmack and Leather 2002). However, the importance of effects of temporal variation in short-lived annual plant availability and quality on behaviour and performance of multivoltine insects has received less attention. In particular, the effect of obligatory food-plant shifts in multivoltine herbivorous insects is virtually unstudied. Many experimental studies (both lab and field) focusing on plant-insect interactions select plants of optimal quality for the insects and assume that food-plant quality is static, or else that the insects are always associated with a certain species of plant, whereas the effect of temporal arrangement of food plants on foraging behaviour and performance of herbivorous insects and their specialized natural enemies has been rarely considered. Temporal realism is thus often missing in studies of the mechanisms of tri-trophic interactions.

In this thesis, I focus on qualitative and quantitative constraints on interactions involving a multivoltine gregarious lepidopteran herbivorous insect, its gregarious endoparasitoid wasp and the short-lived food plants that they are associated with in the field. Thus, my research examines important constraints in a tri-trophic interaction in a naturally occurring system. In particular, I studied constraints imposed by the seasonal phenology of several potential food-plant species on development, survival and oviposition decisions in these insects.

Model system

In this thesis I studied multitrophic interactions involving a multivoltine herbivore, the large cabbage white butterfly, *Pieris brassicae*, its endoparasitoid wasp, *Cotesia glomerata*, and three short-lived naturally occurring cruciferous food-plant species, *Brassica rapa, Sinapis arvensis* and *Brassica nigra* that are known from previous work to serve as food plants for these insects. I examined the effect of temporal changes in plant quality (phenological and ontogenetic), the effect of food availability, and the effect of food-plant shifts on the behaviour and performance of the herbivore and its parasitoid.

Insect species

Pieris brassicae L. (Lepidoptera: Pieridae) (Chapter 2, Fig. 1D) is a gregarious herbivorous insect of which the larvae are specialized to feed on plant species in the order Brassicales, and primarily in the family Brassicaceae. *Pieris brassicae* is bi- or tri-voltine in the Netherlands with butterflies emerging from overwintering pupae in early-mid spring producing the first generation, the second generation occurring in mid-to-late summer and a potential third generation in autumn in warm years. A realistic sequential order of food-plant species used by three generations of *P. brassicae* in the Netherlands is a spring generation on *B. rapa*; an early summer generation on *S. arvensis* and a late summer/early autumn generation on *B. nigra*.

Cotesia glomerata L. (Hymenoptera: Braconidae) (Chapter 2, Fig. 1E) is a specialized gregarious endoparasitoid that primarily attacks early caterpillar stages of *P. brassicae*. It is a koinobiont species (Askew and Shaw 1986) that allows the host to feed and grow after it is parasitized. The parasitoid larvae do not kill the caterpillars until late in the final instar, and parasitized caterpillars often grow as large or even larger than healthy ones depending on how many eggs were laid into the host by the female parasitoid (Harvey 2000, Gu et al. 2003). This parasitoid also has two or three generations in the Netherlands, depending on temperature.

Plant species

The three annual plants studied here belong to the Brassicaceae family. They grow rapidly and exhibit different seasonal phenologies, each growing within a short period of time and with little temporal overlap amongst them. Wild turnip, *B. rapa*, grows early in the season (typically from April to May) (Chapter 2, Fig. 1A), charlock mustard, *S. arvensis* grows in late spring and early summer (from May to July) (Chapter 2, Fig. 1B) and black mustard, *B. nigra* grows in mid-late summer (from July to August) (Chapter 2, Fig. 1C). These annual plants tend to grow in large stands and are, therefore, likely to be the most important wild food plants for the gregarious *P. brassicae* caterpillars. In the Netherlands and elsewhere in Western Europe, only 6 or 7 brassicaceous plant species grow in large enough aggregated populations to support a typical brood of *P. brassicae* of 30-100 eggs (Feltwell 1982).

Brassicaceous plant species, like species in many other plant families, have evolved secondary metabolites as a defence against their antagonists including herbivorous insects. Glucosinolates (GS), secondary metabolites typically produced by brassicaceous plant species are among the best studied of secondary plant metabolites (Fahey et al. 2001). In response to tissue damage, GS that are stored in the vacuoles are exposed to the enzyme myrosinase, which itself is stored in specific cells. Because of myrosinase activity, GS are hydrolysed into e.g. (iso)thiocyanates and nitriles (Fahey et al. 2001). These GS breakdown products play an important role in defence against herbivorous insects (Rask et al. 2000). For instance, GS breakdown products have been shown to be toxic to a wide variety of generalist herbivores (Bones and Rossiter 2006, Halkier and Gershenzon 2006). However, specialist herbivores of brassicaceous plants have evolved specific adaptations to detoxify GS (Ratzka et al. 2002), sequester GS (Müller 2009), and some species even use GS and their hydrolysis products as oviposition or feeding stimulants (van Loon et al. 1992, Miles et al. 2005). However, high levels of these toxic compounds can also have a negative effect on the development of specialist herbivores (van Dam and Raaijmakers 2006, Agerbirk et al. 2008). GS can also affect the natural enemies of herbivorous insects (Gols and Harvey 2009). For example, GS in plants have been shown to negatively correlate with the performance of parasitoids and predators that feed on specialist herbivores of brassicaceous plants (Francis et al. 2001, Sznajder and Harvey 2003, Gols and Harvey 2009, Hopkins et al. 2009). Moreover, volatile breakdown products of GS act as attractive compounds to several specialist parasitoids of herbivores specialized on brassicaceous plants (Blande et al. 2007, Gols et al. 2011). Brasscaceous plants also emit other classes of plant volatiles that are involved in the attraction of natural enemies, such as green leaf volatiles and terpenoids (Mumm and Dicke 2010, Shiojiri et al. 2010).

Thesis outline

Chapter 2

When multivoltine insects associate with short-lived annual plants, including *B. rapa*, *S. arvensis* and *B. nigra*, different generations of the insects must find and exploit different plant species that may differ in their quality. In Chapter 2, I examine whether rearing history, i.e. growing on the same or different food-plant species in successive generations, affects the performance of *P. brassicae* and *C. glomerata*. The effect of species- and season-related differences in plant quality and food-plant shifts on the performance of insects is discussed.

Chapter 3

When adult females of multivoltine herbivorous insects that feed on short-lived herbaceous plant species are searching for oviposition sites in nature, they are faced with unpredictable variations in traits of the food plant such as size and quality, which can potentially have huge effects on the survival and fitness of their progeny. In this chapter, I studied the effects of plant ontogeny in *B. nigra* and *S. arvensis* on female oviposition preference and offspring performance of *P. brassicae*. Chemical analyses of plant tissues collected from plants in different developmental stages were performed to reveal variation in primary (sugars, amino acids) and secondary metabolites (GS) to correlate these with larval performance.

Chapter 4

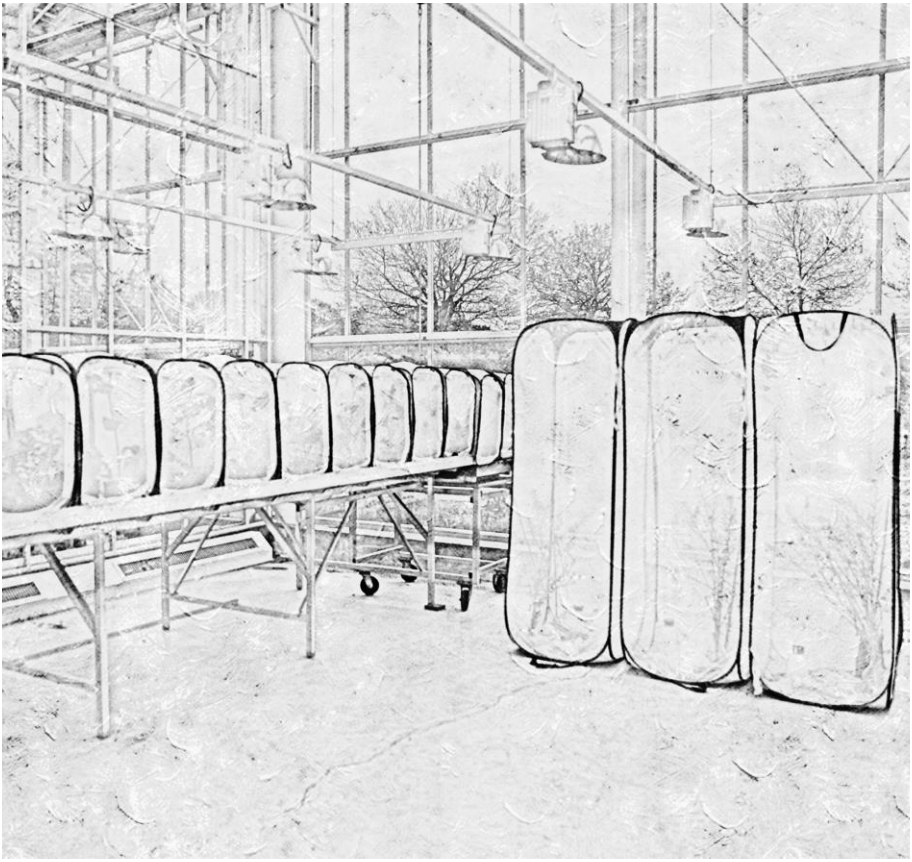
Multivoltine gregarious insects are known to associate with short-lived annual plants, which have limited biomass. In this chapter, using *B. nigra* as the food-plant species, I studied the constraints of food availability on behaviour and performance of *P. brassicae* and *C. glomerata* both in semi-field experiments and in the greenhouse where we manipulated food availability thus leading to competition and potential starvation.

Chapter 5

Experience of insects in their natal habitat may affect an individual's likelihood of accepting a similar habitat or food plant/herbivore during dispersal. However, when multivoltine insects exploit short-lived annual plants for their development, the food plant on which the caterpillars had fed during their larval stage may no longer be present, or are dying and are unsuitable, at eclosion. This means that the insects are obligated to leave the natal patch to find new resources (plants or hosts) elsewhere. In this chapter, I examined whether rearing history (i.e. growing on a specific food-plant species in one generation) affects oviposition preference in *P. brassicae* and foraging behaviour in *C. glomerata*.

Chapter 6

In chapter 6, I summarize and discuss the main findings of my thesis and discuss future research directions in the study of multitrophic insect-plant interactions, thereby focusing on the importance of understanding how temporal effects of plant identity and quality may influence the behaviour and development of herbivorous insects and their natural enemies higher up the food chain.



Chapter 2

Seasonal phenology of interactions involving short-lived annual plants, a multivoltine herbivore and its endoparasitoid wasp

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Abstract

Spatial-temporal realism is often missing in many studies of multitrophic interactions. Most studies are conducted at a single time frame and/or involving interactions between insects with a single species of plant. In this scenario, an underlying assumption is that the host-plant species is ubiquitous throughout the season and that the insects always interact with it. We studied interactions involving three naturally occurring wild species of cruciferous plants, *Brassica rapa*, *Sinapis arvensis* and *Brassica nigra*, that exhibit different seasonal phenologies, and a multivoltine herbivore, the large cabbage white butterfly, *Pieris brassicae*, and its gregarious endoparasitoid wasp, *Cotesia glomerata*. The three plants have very short life cycles. In central Europe, *B. rapa* grows in early spring, *S. arvensis* in late spring and early summer, and *B. nigra* in mid to late summer. *Pieris brassicae* generally has three generations per year, and *C. glomerata* at least two. This means that different generations of the insects must find and exploit different plant species that may differ in quality and which may be found some distance from one another. Insects were either reared on each of the three plant species for three successive generations or shifted between generations from *B. rapa* to *S. arvensis* to *B. nigra*. Development time from neonate to pupation and pupal fresh mass were determined in *P. brassicae* and egg-to-adult development time and body mass in *C. glomerata*. Overall, herbivores performed marginally better on *S. arvensis* and *B. nigra* plants than on *B. rapa* plants. Parasitoids performance was closely tailored with that of the host. Irrespective as to whether the insects were shifted to a new plant in successive generation or not, development time of *P. brassicae* and *C. glomerata* decreased dramatically over time. Our results show that there were some differences in insect development on different plant species and when transferred from one species to another. However, all three plants were of generally high quality in terms of insect performance. We discuss ecological and evolutionary constraints on insects that must search in new habitats for different plant species over successive generations.

Introduction

The concept of co-evolution has long underpinned our understanding of plant-insect interactions in natural communities (Ehrlich and Raven 1964, Scriber 2002, Leppanen et al. 2012). In this scenario, insects evolve behavioural and physiological adaptations that enable them to better exploit certain plant types, whereas the plants then evolve defensive counter mechanisms that enable them to resist the herbivores and vice versa sequentially. As a result, plants have evolved a diverse array of physical and chemical resistance traits to prevent or reduce damage by insect herbivores. These include morphological defences such as sticky glands, thickened leaves and trichomes, and various types of chemical defences including phytotoxins and digestibility reducers (Karban and Baldwin 1997, Scriber 2002, Johnson 2011). At the same time, insect herbivores are known to employ various strategies to avoid, excrete, detoxify or harmlessly sequester plant toxins (Dussourd 1993, Muller et al. 2001, Nishida 2002, Ratzka et al. 2002). Price et al. (1980) took the co-evolutionary concept further and argued that a complete understanding of the reciprocal effects of interactions between plants and herbivores needs to incorporate natural enemies (predators and parasitoids) which can profoundly affect evolutionary trajectories of plant-herbivore interactions via top-down regulation. Since the publication of that article, the study of multitrophic interactions has become a discipline at the heart of community and population ecology. Moreover, it is important to stress that the effects of plant quality may directly or indirectly influence the performance of natural enemies (Harvey et al. 2003, Van Nouhuys and Lei 2004, Harvey et al. 2005, Ode 2006) thus increasing the complexity of these trophic interactions.

Food-plant suitability for herbivore (and possibly natural enemy) development are determined by both quality, for example concentrations of primary and secondary metabolites and morphological traits, and quantity (Schoonhoven et al. 2005). In nature, these characteristics are dynamic and can change within individual plants over the course of a growing season (Barton and Koricheva 2010). For instance, plants can exhibit profound variation in nutritional quality over time regardless of whether the species is a long-lived perennial or an ephemeral, short-lived annual (Gols et al. 2007, Zehnder et al. 2009). Different species of univoltine insects, which have only a single generation per year but which feed at different times during the growing season on the same plant species, may therefore encounter quite significant temporal variations in plant quality. This variation may be caused by seasonal changes in biotic and abiotic factors and conditions, such as herbivore and pathogen damage (Runyon et al. 2010), rainfall, or temperature gradients (Schoonhoven et al. 2005). Seasonal changes in nitrogen and lignin levels in striped maple *Acer pensylvanicum* foliage were found to be more important in determining plant quality for the associated insect community, than spatial heterogeneity (Zehnder et al. 2009). Gols et al. (2007) found that even under strictly controlled greenhouse conditions, plant secondary chemistry and insect performance varied on mustard and cabbage plants grown at different time points over the course of a single growing season. Moreover, different growth stages of plants also exhibit variation in plant quality (Mattson 1980, Coley 1983, Boege 2005, Barton and Koricheva 2010).

Univoltine insects may also grow at specific periods during the growing season because their development is intimately correlated with the production of certain plant tissues, such as seeds or young shoots (Darquenne et al. 1993, Sobhian and Fornasari 1994). As a result, some herbivore species are found only early in the season, whereas others appear later in the season (Wolda 1988). Alternatively, multivoltine insects have two or more generations per year (Gullan and Cranston 2010). The relationship between insect voltinism and plant phenology can generate two main outcomes. Multivoltine insects that feed on perennial or annual plant species whose growth phase encompasses an entire growing season are only faced with temporal changes in food-plant quality. Thus, the progeny of the first generation can immediately mate and lay their eggs on the same plant species or even individual (provided sufficient resources remain) without the need of having to leave the natal patch to look for a new food plant elsewhere. However, many species of multivoltine insects are known to attack short-lived annual plants that are present for only 2 or 3 months in the field. These short-lived plants may germinate and grow at different times and/or locations during the growing season. In this situation, each herbivore generation is faced with the challenge of leaving the natal patch to find and lay eggs on a different plant species that may be growing some distance from where they themselves developed. At the same time, the quality of the different food-plant species on which they feed and grow over the spring and summer seasons may also be highly variable (Gols and Harvey 2009).

Although the effects of differences in food-plant quality of perennial and annual plant species on the performance of insect herbivores has been well studied, the effects of season-related temporal dynamics in ephemeral plants has thus far received little attention. Moreover, the effects of obligatory host shifts in multivoltine insects that primarily feed on short-lived annuals that differ in their phenology have not been explored. This is not only true for the herbivore, but also for species in the third trophic level, such as parasitoids, that are intimately associated with these insect herbivores. Some parasitoids also have two or more generations per year, and these different generations exploit the same host species. Therefore, in this scenario, plant-related qualitative and spatial constraints will also affect the parasitoid. Studies investigating tritrophic interactions involving plants, insect herbivores, and their natural enemies are often based on experiments performed during a short time frame using a single plant species, thus measuring the performance of only one generation of the insect herbivore and its natural enemy. Spatial-temporal realism is therefore often missing in studies elucidating the mechanisms underlying tri-trophic interactions.

In this study, we examine developmental interactions involving three species of wild cruciferous plants that are native to central Europe, a multivoltine herbivore and its multivoltine endoparasitoid. Many species in the family of the Brassicaceae are short-lived annual plants. The three annual plants studied here are large, leafy crucifers that grow rapidly and exhibit differing phenologies, each growing within a short period of time and with little temporal overlap amongst them. In western Europe, the wild turnip, *Brassica rapa* L., grows early in the season (typically April-May), the charlock mustard, *Sinapis arvensis* L., grows in late spring and early summer (May-June) and the black mustard, *Brassica nigra* L., grows in mid-late summer (July-August) (Fig. 1). A much smaller second generation of *S. arvensis* grows in late autumn into winter but usually after *Pieris brassicae* and *Cotesia glomerata* have entered winter diapause. These annuals are likely to be the most important wild food plants for caterpillars of the large cabbage white butterfly, *P. brassicae* L. (Lepidoptera: Pieridae). Caterpillars of *P. brassicae* only feed on plants containing glucosinolates, a group of secondary plant metabolites characteristic of plants in the Brassicaceae family (Feltwell 1982). In the Netherlands, *P. brassicae* is trivoltine, with butterflies emerging from overwintering pupae in early to mid spring, and separate generations occurring in spring through late summer and even into autumn in warm years. *Cotesia glomerata* L. (Hymenoptera: Braconidae) is a specialized gregarious endo-parasitoid that primarily attacks early caterpillar stages of *P. brassicae*. The parasitoid has two to three generations in the Netherlands, depending on temperature. In spring and early summer, the most abundant food plant for first generation of *P. brassicae* larvae is likely to be *B. rapa*; in the second generation *S. arvensis*; and in the third generation, *B. nigra* (Fig. 1). Larvae and pupae of *P. brassicae*, as well as cocoons of *C. glomerata*, have also been found on plants of all three species in the Netherlands (J. Harvey, personal observations).

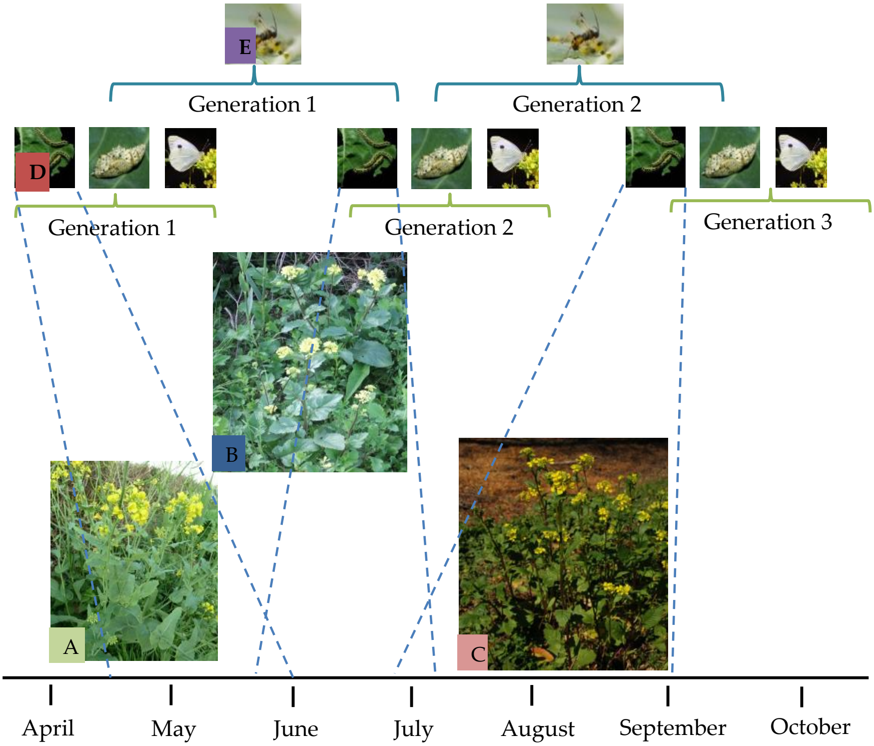

Figure 1. Phenology of wild Brassicaceous plant species that are potential food plants for different generations of *Pieris brassicae* and its endoparasitoid *Cotesia glomerata*, in The Netherlands. (A) *Brassica rapa*, (B) *Sinapis arvensis*, (C) *Brassica nigra*. As shown, the three species grow at different times of the spring and summer with little temporal overlap amongst them. (D) *Pieris brassicae* and (E) *Cotesia glomerata* have at least 2, and often 3, generations per year. The food plants described are likely to be the most important species for the herbivore and its parasitoid.

The major aim of this study is to determine whether rearing history, that is, growing on the same or different food-plant species in successive generations, affects the performance of *P. brassicae* and *C. glomerata*. Insects were reared in separate cohorts on one of the three plant species: *B. rapa*, *S. arvensis* or *B. nigra* for three consecutive generations. A fourth cohort of both the herbivore and the parasitoid was transferred in each generation to a new food plant according to their phenology in the Netherlands. We tested the following hypotheses: (i) herbivore performance is similar on all three plant species which are known to serve as important food plants for *P. brassicae*; (ii) herbivore performance will increase on plant with which they have a rearing history (e.g. those on which the herbivores are reared for successive generations), whereas it will remain static in the herbivores that are transferred to a new plant species in successive generations. This is because the herbivores become adapted to the natal plants; (iii) differences in insect performance related to food-plant quality and food-plant shifts are similar for the herbivore and the parasitoid, given that the development of *C. glomerata* is intimately correlated with that of *P. brassicae* (Harvey 2000, Gols et al. 2008). Our results are discussed in the context of the phenology of mutitrophic interactions in natural communities, an aspect that is often ignored in studies of plant-insect interactions.

Materials and methods

Plants

Seeds were collected from large individual *Brassica rapa*, *Sinapis arvensis* and *Brassica nigra*, populations growing naturally in Gelderland in the Netherlands. For each population, seeds were collected and pooled from 15-20 plants per population.

Insect performance was followed for three successive generations. Seeds for the three consecutive trials were germinated on 2 March 2012, 4 May 2012 and 10 May 2012, respectively, but were otherwise grown using the same protocol. Plants were grown in a climate-controlled greenhouse at 22±2℃ (day) and 16±2℃ (night) 50% relative humidity, and a photoperiod of ~16h. If the light dropped below 225 μmol photons $m^{-2}s^{-1}$ during the 16-h photoperiod, supplementary illumination was applied by sodium lamps. Seeds were germinated in white plastic boxes (5x 11x 16cm) filled with moist cutting soil (‘Lentse stekground’ no.0513; Lent, the Netherlands). After 5 days, seedlings were transferred to 1.5-L pots filled with potting soil (‘Lentse potgrond’ no. 4; lent, the Netherlands). The plants were watered once every 2 days.

When plants were three weeks old, they were fertilized once a week with Kristallon Blauw (16N: 6P: 20K: 3Mg) at a concentration of 1 gL^{-1}. Plants were approximately 4 weeks old at the start of each experiment. Watering and fertilization continued during the experiments. The experiments were conducted in the same greenhouse and therefore under the same conditions as where the plants were grown.

Insects

The herbivore *Pieris brassicae* and its gregarious endoparasitoid *Cotesia glomerata* were originally collected from agricultural fields near Wageningen, the Netherlands. Cultures of *P. brassicae* were maintained in a climate room at 22±2°C, 50-70% r.h. and a photophase of 16L: 8D and were reared on cultivated Brussels sprouts plants (*B. oleracea* var. *gemmifera,* cv. Cyrus) for many generations at Wageningen University (WU). Cultures of *C. glomerata* were reared on *P. brassicae* feeding on Brussel sprout plants, which were maintained in a climate room under the same conditions as the host at the Netherlands Institute of Ecology (NIOO-KNAW).

Experimental protocol

Development of Pieris brassicae *and* Cotesia glomerata *on three cruciferous plants*

Insects were reared on each of the three plant species (*B. rapa*, *S. arvensis* or *B. nigra*) in two treatments. In the first, the insects were reared on each plant for three successive generations; in the second, different generations were shifted from shifted from *B. rapa* to *S. arvensis* to *B. nigra,* reflecting the natural seasonal phenology of these plants in the Netherlands (Fig. 1). To compare insect performance, we measured development time from egg hatching to pupation and pupal fresh mass for the herbivore and development time from oviposition to adult eclosion and adult fresh mass for the parasitoid. For the parasitoid, the performance parameters were measured for each sex, as previous work has shown this parameter to vary significantly between male and female wasps (Harvey, 2000). In addition, brood sizes, that is, the number of offspring produced by single hosts, were recorded as previous studies have demonstrated a negative correlation between brood size and adult mass (Harvey 2000, Gu et al. 2003).

Generation 1

To obtain healthy (= unparasitized) caterpillars and caterpillars to be used for parasitism by *C. glomerata,* 3-week-old plants were placed in the rearing cage with adult *P. brassicae* butterflies at the entomology department at WU. Females were allowed to oviposit on these plants for 24 h. The following day, the plants were removed from the butterfly cage and transferred to the experimental greenhouse. Once the eggs hatched the larvae were allowed to develop into the 2nd instar (L2), which takes 5-7 days, depending on the food plant. Cohorts of 20 randomly selected 1-day-old L2 larvae were transferred to cages (40x 40x 55cm) containing five plants of the same species and six cages (= replicate) per plant species. Larvae were transferred to the same plants species as the one on which they had hatched and been feeding previously. A second cohort of 120 larvae was randomly selected from each plant species for parasitism by female *C. glomerata* wasps. Mated females used for parasitism originated from the general culture maintained at the NIOO-KNAW. Larvae were parasitized by presenting them individually at the end of a small brush to a female wasp in a plastic vial. A larva was considered to be parasitized when the female wasp had inserted her ovipositor into the host for at least 10 s. The parasitized larvae were then transferred to cages containing five plants of the same species, 30 larvae per cage and four cages (= replicate) per plant species. Larvae were transferred to the same plants species as the one on which they had hatched and been feeding previously. Healthy and parasitized caterpillars were allowed to move and feed freely on the plants within a cage until they pupated (healthy caterpillar treatment) or formed cocoons (parasitized caterpillar treatment). Pupae were collected daily and weighed, and the pupation date was recorded as well. Pupae were transferred to large cages (60x 60x 180cm, one cage per plant species). At adult emergence, the butterflies were provided with honey and cotton wool saturated with 10% sugar water, which was offered in small blue containers and served as an energy source. These butterflies were used to produce offspring that were used in the second generation (see below).

Cocoons of *C. glomerata* were collected and transferred to Petri-dishes containing one brood per dish. When several adult wasps had emerged, the time of emergence was recorded and the Petri-dish was placed in a refrigerator at 4°C for 10 minutes to immobilize the wasps. The first five males and five females to emerge were collected from each dish, anaesthetized using CO_2 and weighed individually on a Mettler-Toledo (Columbus, OH USA) microbalance. All emerged wasps were transferred to one of three cages (24x 24x 24cm) according to the origin of their food plant. Adult wasps were provided with honey as food and were kept in climate chambers at 7°C to extend their longevity.

Second and third generations

The experimental protocol for the second and third generation was the same as for the first, with the exception that the insects, butterflies and wasps from the previous generation were used to initiate the following generation. Thus, to obtain eggs for the second generation, plants of each species were placed in the cages for 24 h with mated butterflies that had also developed on that species in the previous generation. These butterflies were 4-6 days old following adult emergence. For the host-shift cohort in the second generation, an additional set of *S. arvensis* plants was placed in the cage with butterflies that had developed on *B. rapa* in the previous generation. Both healthy caterpillars and those that were parasitized by wasps in the host-shift cohort that had developed on *B. rapa* in the first generation were reared on *S. arvensis* in the second generation. To initiate the third generation, the emerging butterflies in the host-shift cohort were used to oviposit on *B. nigra.* For parasitism, the wasps from the host-shift group in generation two were used. Performance parameters were determined as described before.

It was not possible to ascertain mating in *C. glomerata*, and unmated females thus produce all male broods. In nature, however, all male broods are very rarely observed. In these experiments, approximately only 10% of broods produced males only. These data are excluded in the statistical analysis (see below).

Effects of flower-feeding by Pieris brassicae *on herbivore development and size*

S. arvensis plants were already flowering when they were 4 weeks old, whereas the other two plant species did not flower during the experiment. Caterpillars of *P. brassicae* prefer to feed, and attain faster growth rates, on the flowers than on the foliage of *B. nigra* plants (Smallegange et al. 2007). To determine the influence of the flowering status of the plants on the performance of the herbivore, an additional treatment was included. Here, *S. arvensis* plants were infested with herbivores as described above, but all the flower buds were removed using scissors just prior to herbivore infestation. This treatment included three replicates and was performed simultaneously with the third generation. Herbivore performance parameters were determined as described previously.

Statistical analysis

In the statistical analyses, cages served as experimental units. The performance data (development time and biomass) of the herbivore were statistically analyzed using a general linear mixed model that included the fixed factors, plant species (*B. rapa, S. arvensis*, and *B. nigra*), generation (1, 2 and 3) and their interaction terms and the random factor cage nested within plant species. The variance components were estimated using restricted maximum likelihood (REML). Tukey-Kramer multiple comparisons between means were conducted when any of the fixed factors in the REML models were significant. In an additional analysis, the performance on flowering and non-flowering *S. arvensis* plants was compared using the data generated in the third generation.

The data on food-plant shifts were split into two data sets: in the first analysis, we compared the performance of the transferred insects with the performance of conspecifics that remained on the original food plant (comparison previous host). In the second analysis, we compared the performance of the transferred insects with the performance of conspecifics that had been feeding on this new plant already for two (*S. arvensis* comparison) or three generations (*B. nigra* comparison). Data for the two plant shifts were compared in one analysis.

Because *C. glomerata* is gregarious, multiple individuals egress from a single caterpillar host. Moreover, the brood size, here number of parasitoids emerging from a single host, has been demonstrated to affect the size and development time of the emerging adult wasps (Harvey 2000). Therefore, host individual was included as a second random variable nested within cage, and the brood size was included as a covariate. In the analyses, we only included the data resulting from mix broods (90% of the broods produced male and female offspring), but data were analyzed separately for males and females. Development times of *P. brassicae* were log transformed to meet assumptions of normality and homoscedasticity. Analyses were performed using the statistical package SAS version 9.2 (SAS Institute Inc., Cary NC, USA).

Results

Food-plant quality in relation to phenology

Herbivore. Survival of the herbivore was high (> 90% of the larvae developed into pupae) irrespective of plant species and generation. Both food-plant species and generation had a significant effect on the fresh mass of *P. brassicae* pupae (food-plant species, $F_{2,41.9} = 3.94$, $P = 0.03$; generation, $F_{2,41.9} = 4.12$, $P = 0.02$). Pupae were heavier when reared on *S. arvensis* than on *B. rapa* plants (Tukey-Kramer test: $P < 0.05$), whereas the masses of pupae reared on *B. nigra* were not significantly different compared with those reared on the other two plant species (Fig. 2A). Plant species affected insect generation similarly (plant species-generation interaction term is not significant: $F_{2,41.9} = 0.25$, $P = 0.91$). Butterfly pupae attained the highest biomass in the first generation and lower biomasses in the second and third generation; moreover, pupal masses in the second and third generation were not significantly different (Tukey-Kramer test: $P > 0.05$, Fig. 2A). Egg-to-pupa development time also varied significantly with generation ($F_{2,40.8} = 666$, $P < 0.001$) and plant species ($F_{2,40.8} = 55.5$, $P < 0.001$). Development time decreased in each successive generation on each of the different food-plant species (Tukey-Kramer test: all within species comparisons were statistically significant, Fig. 2B). Larval development was fastest on *B. nigra*, slower on *S. arvensis*, and slowest on *B. rapa* (Tukey-Kramer: all plant species comparisons $P < 0.05$, Fig. 2B). The interaction between food-plant species and generation was also significant ($F_{2,40.8} = 2.83$, $P = 0.04$); the effect of generation on the larval development was slightly more pronounced when the caterpillars were feeding on *S. arvensis* and *B. rapa* than on *B. nigra* (Fig. 2B).

In an additional experiment, we investigated whether the flowering status of *S. arvensis* plants affected food-plant quality. Pupae of *P. brassicae* that had been feeding on *S. arvensis* plants from which the flowers had been removed were significantly smaller than the pupae that had developed on intact plants with flowers

($F_{1,5.8} = 11.9$, $P = 0.01$). However, flower-feeding had no effect on egg-to-pupa development time ($F_{1,5.9} = 0.30$, $P = 0.60$).

Parasitoid. Male and female wasp data were analyzed separately, but overall, the fresh mass of adult female wasps was greater ($F_{979,3671} = 31.7$, $P < 0.001$, Fig. 3A) and the development longer than for male wasps ($F_{979,3671} = 480$, $P < 0.001$, Fig. 3B). Both generation (females, $F_{2,25.5} = 4.01$, $P = 0.03$; males, $F_{2,24.9} = 4.04$, $P = 0.03$) and food-plant species (females, $F_{2,24.5} = 15.7$, $P < 0.001$; males, $F_{2,24.1} = 38.5$, $P < 0.001$) had a significant effect on male and female adult mass although the direction of the effects was sex-dependent (Fig. 3A). Females were significantly heavier in the third than in the first and second generation, regardless of the species on which they had developed; the biomass of the females did not differ significantly between generation 1 and 2 (Tukey-Kramer test, $P > 0.05$, Fig. 3A). Females were larger when reared on *S. arvensis* than on the other the other two plant species (Tukey-Kramer test, $P < 0.05$) on which females obtained similar masses (Tukey-Kramer test, $P > 0.05$, Fig. 3A). In males, there was a strong species-generation interaction ($F_{4,23.2} = 8.70$, $P < 0.001$). As with females, males were larger on *S. arvensis* plants than on the other two plant species. However, the effect of plant species was differentially affected by generation. For example, there was no effect of generation when the wasps were reared on *B. nigra* (all Tukey-Kramer test within species comparisons, $P > 0.05$), whereas males were significantly smaller in the third compared to the first generation when reared on *S. arvensis* (Tukey-Kramer test, $P < 0.05$). The patterns for development time in relation to food-plant species and generation (female, $F_{2,25.6} = 9.59$, $P < 0.001$; male, $F_{2,25.1} = 17.9$, $P < 0.001$) and generation (females, $F_{2,26.2} = 503$, $P < 0.001$; male, $F_{2,25.5} = 439$, $P < 0.001$) were similar for male and female wasps and to a large extent reflected the pattern found for the host (Fig. 3B). Development times decreased in successive generations irrespective of food-plant species. Both male and female wasps developed faster on *B. nigra* plants than on the other two plant species (Tukey-Kramer test, $P < 0.05$), whereas development time on the other two populations did not differ significantly (Tukey-Kramer test, $P > 0.05$).

Rearing history: effect of food-plant shift

Herbivore. Development time of *P. brassicae* differed significantly for conspecifics that were transferred to a new food-plant species (Table 1). The extent of this effect depended on the identity of the new food plant (food-plant-shift interactions were significant). Second generation insects that were transferred from *B. rapa* to *S. arvensis* developed faster than conspecifics that were reared on the same food plant (*B. rapa* or *S. arvensis*) continuously (Fig. 4B). However, herbivores that were transferred in the third generation from *S. arvensis* to *B. nigra* tended to develop slower than conspecifics reared on the same food plant for three generations, but this effect was not statistically significant. The effect of food-plant shift on biomass was less pronounced and only affected the herbivores that were transferred from *S. arvensis* to *B. nigra* in the third generation (Table 1, Fig. 4A). The pupal size of *P. brassicae* was negatively affected by this plant shift.

Parasitoid. Food-plant shift influenced primarily adult body mass (Table 1, Fig. 4C and 4E) and the effects were similar for male and female wasps. The direction of the effect of food-plant shift depended on the identity of the previous and the new food plant. Adult body masses of the parasitoids that were transferred from *B. rapa* to *S. arvensis* in the second generation were similar with those measured for conspecifics that were not transferred, but they were lower compared to those recorded for wasps that were reared on *S. arvensis* for two generations already. Moreover, when the wasps were transferred to *B. nigra* in the third generation or were reared on *B. nigra* for three successive generations, they were smaller than the wasps that had been reared on *S. arvensis* continuously.

Discussion

One of the critical requirements for multivoltine insects that obligatorily develop on different plant species in successive generations over the course of a growing season is that each of these food plants is suitable for the development of insect progeny and preferably that each plant represents a high-quality resource. For gregarious herbivores that produce large broods, and additional constraint is that several, and perhaps many, food plants must grow in close proximity to support the nutritional requirements of the immature insects. Any disruption in this sequence, whether by being forced to develop on nutritionally poor plants or if the plants grow in small, scattered populations, can have potentially serious consequences on the persistence of the herbivore and its parasitoid and on their population dynamics.

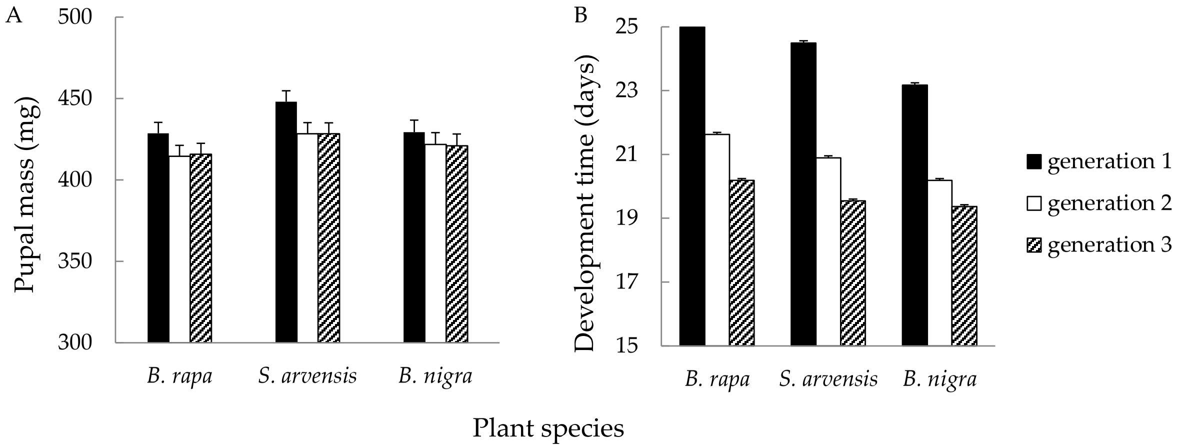

Figure 2. Pupal fresh mass (A) and egg-to-pupa development time (B) of *Pieris brassicae* reared on *Brassica rapa*, *Sinapis arvensis* or *Brassica nigra* for three successive generations. Bars present the means + SE.

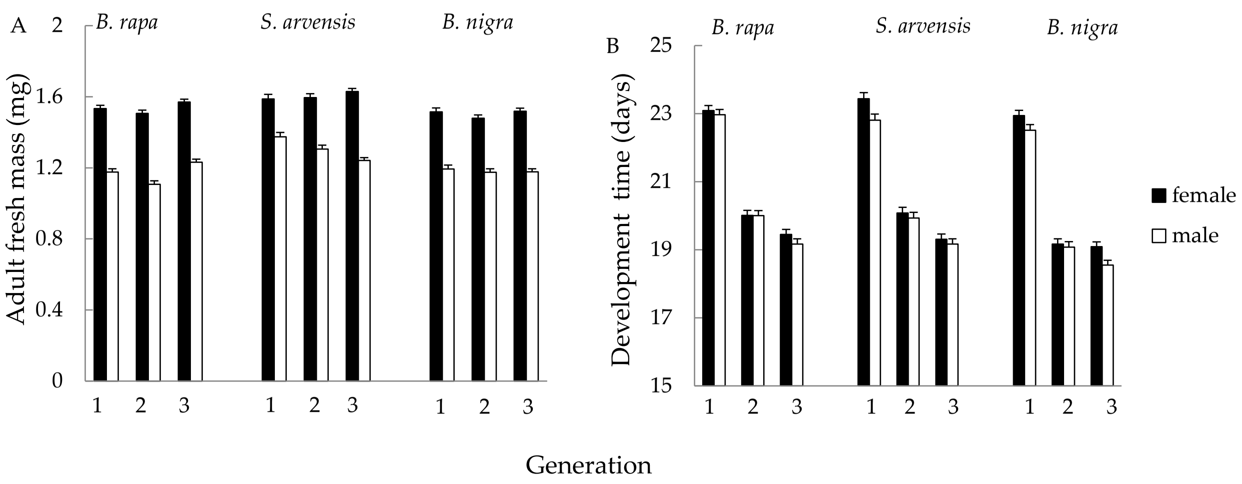

Figure 3. Ault fresh mass (A) and egg-to-adult development time (B) of *Cotesia glomerata* females (black bars) and males (white bars) reared on *Brassica rapa, Sinapis arvensis* or *Brassica nigra* for three successive generations. Bars present the means + SE.

Table 1: Statistical analyses of biomass and development time data of *Pieris brassicae* and *Cotesia glomerata* after being transferred to new food-plant species. The left-hand panels show comparisons of insect development on the previous food-plant species, whereas the right-hand panels show comparisons of insect development on the new food-plant species.

Factors	ndf/ ddf	*F*-value	*P*-value	ndf/ ddf	*F*-value	*P*-value
Pupal/adult fresh mass						
(*P. brassicae*)						
Food-plant(1)	1/17.7	0.07	0.79	1/17.4	5.42	0.03
Food-plant shift(2)	1/17.7	0.56	0.46	1/17.4	1.53	0.23
(1) x (2)	1/17.7	8.1	0.01	1/17.4	0.73	0.4
(Female of *C. glomerata*)						
Brood size	1/203	5.9	0.02	1/208	19.6	<0.001
Food-plant(1)	1/14.1	12.5	0.003	1/17.9	2.72	0.12
Food-plant shift(2)	1/14.2	22.1	<0.001	1/18.4	14.4	0.001
(1) x (2)	1/14.5	5.06	0.04	1/18.3	8.68	0.009
(Male of *C. glomerata*)						
Brood size	1/203	10.7	0.001	1/222	42.3	<0.001
Food-plant(1)	1/12.6	17	0.001	1/12.1	8.65	0.01
Food-plant shift(2)	1/12.6	6.1	0.03	1/12.5	24.4	<0.001
(1) x (2)	1/12.8	12.4	0.004	1/12.4	10.8	0.006
Development time						
(*P. brassicae*)						
Food-plant(1)	1/17.9	42.9	<0.001	1/17.4	25.4	<0.001
Food-plant shift(2)	1/17.9	8.29	0.01	1/17.4	0.25	0.62
(1) x (2)	1/17.9	27	<0.001	1/17.4	13.7	0.002
(Female of *C. glomerata*)						
Brood size	1/202	0.1	0.76	1/205	13.8	<0.001
Food-plant(1)	1/12.7	21.9	<0.001	1/12.7	50.5	<0.001
Food-plant shift(2)	1/12.6	1.23	0.29	1/12.9	2.33	0.15
(1) x (2)	1/12.7	0.48	0.5	1/12.8	0	0.99
(Male of *C. glomerata*)						
Brood size	1/201	1.7	0.19	1/908	1228	<0.001
Food-plant(1)	1/11.7	48.6	<0.001	1/12.6	68.3	<0.001
Food-plant shift(2)	1/11.6	0.62	0.45	1/12.6	4.6	0.05
(1) x (2)	1/11.7	3.8	0.08	1/12.6	0.08	0.78

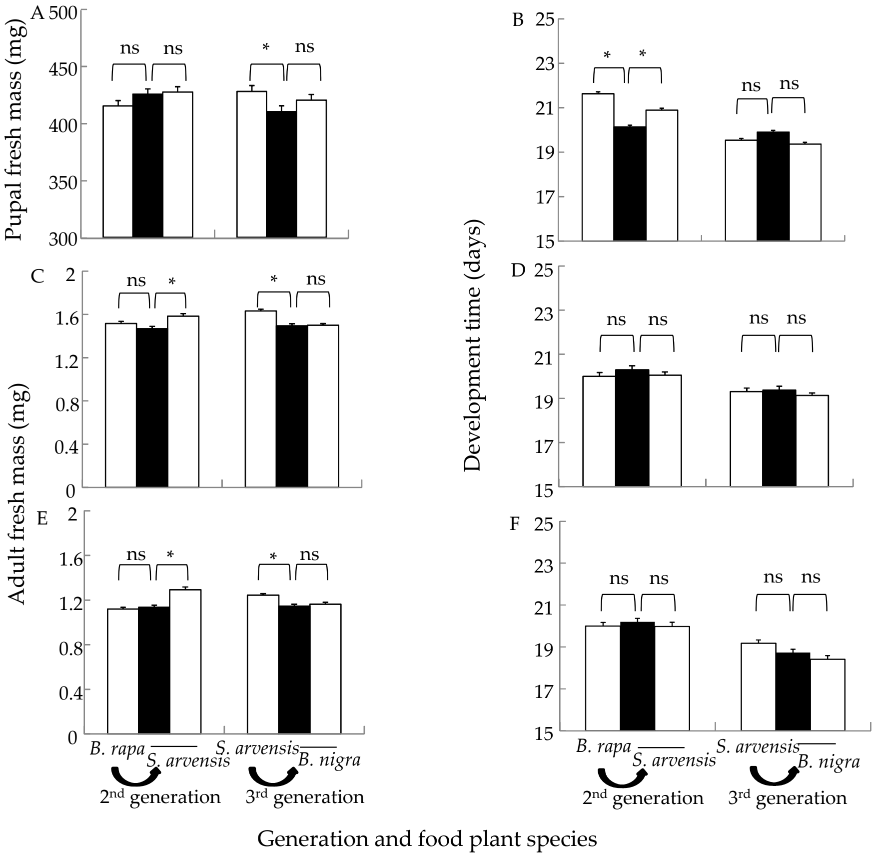

Figure 4. Comparisons of herbivore and parasitoid performance after food-plant shifted. Pupal fresh mass (A) and development time (B) of second generation (left panel) and third generation (right panel) *Pieris brassicae* that were transferred to a new food plant (black bars) with those that were not transferred (white bars). Similarly, adult fresh mass of female (C) and male (E) *Cotesia glomerata* and egg-to-adult development time (D) and (F) for females and males, respectively. Bars (means +SE) with * are significantly different from each other (Tukey Kramer multiple comparisons).

Such processes can occur if one (or more) of the plants is decimated by pathogenic infection or declines as a result of some biotic and abiotic forces.

Here, we found that *P. brassicae* and *C. glomerata* performed well on all three plant species, although *S. arvensis* and *B. nigra* were of higher quality than *B. rapa*. Moreover, food-plant shifts also affected the performance of the insects to some extent. The effects of food-plant species depended on the identity of the previous plant and new plant on which the insects were reared, although these effects did not affect herbivore and parasitoid development similarly. Importantly, although differences in plant quality were reflected statistically in terms of insect performance, the experiments were conducted in the laboratory where abiotic conditions were controlled. In the field, abiotic conditions are often dynamic and hash, and statistically, significant effects on various plant or insect-related traits may not be ecologically significant when this is taken into account. Importantly, many studies correlate statistical and ecological significance when in reality they may be artefacts. In the context of the present study, differences in pupal mass and development time of the insects on the three plants partially obscures the fact that all three species are indeed highly suitable for *P. brassicae* (and by association its parasitoid *C. glomerata*).

The results thus only partly support our first hypothesis, in which plant interspecific quality was not expected to differ. This may be because of differences in the frequency with which *P. brassicae* and *C. glomerata* interact with the three plants in nature. *B. rapa* grows early in the season (e.g. germinates in March and reaches optimal growth phase by late April-mid May) before many specialist herbivores have emerged from winter diapause. Consequently, it may be less heavily attacked by *P. brassicae* which does not normally emerge as an adult from winter diapause until late May. On the other hand, the insects exhibit strong phenological overlaps with *S. arvensis* and *B. nigra*. Most importantly, however, *P. brassicae* and *C. glomerata* are almost certainly associated with different plant species in different generations over the course of a single year. This suggests that the insects may exhibit partial ontogenetic ‘memory’ with certain plant types with which they regularly develop over many years, but not annually.

Insect performance was much less positively affected by rearing history on the same plant than by temporal (=seasonal) changes for different generations. In particular, development times of both *P. brassicae* and *C. glomerata* changed over three successive generations, irrespective of plant species. This reveals that plant quality increased during the experimental period, that is, from March to July. Although the experiments were conducted under controlled greenhouse conditions, it was not possible to exclude the effects of seasonal changes in day length. Early in the experiment, there was less access to sunlight and thus, plants were supplemented through the use of sodium lamps. However, as the experiment progressed, day length increased and access to natural sunlight increased as well. The significance of these temporal affects cannot be understated. In our experiments, we controlled for every abiotic factor but access to natural sunlight. In the field, we believe that a combination of increasing day length, greater access to direct sunlight, and warmer temperatures may all combine to greatly amplify the effects on insect performance that we have shown here. Future studies are planned in which this is tested.

Discussions of the nutritional quality of food plants for insect herbivores has often focused on a combination of concentrations of primary metabolites (e.g. carbon, nitrogen, amino acids) versus concentrations of secondary plant metabolites, such as glucosinolates (characteristic of the Brassicales) in plant tissues (Slansky 1993, Schoonhoven et al. 2005). However, this has hampered our understanding of other important processes, such as spatial and/or temporal variation in the life cycles of short-lived plants and their herbivore/natural enemy complexes. In much of Western Europe, all of the major natural brassicaceous food plants attacked by several species of pierid butterflies are short-lived annual species with life-cycles that rarely exceed 2-3 months, or biennials with short temporal periods in which suitable shoots are available (Carter and Hargreaves 1994). Few perennial crucifers have been described in the literature, and the only one which retains significant amounts of foliage over the course of an entire growing season is wild cabbage, *B. oleracea*, which has a very limited distribution and is confined to the rocky coastlines of Great Britain, France, Ireland and Denmark (Mitchell and Richards 1979). Even where it grows naturally wild cabbage is considered to be rare, although wild populations occasionally harbor large number of *P. brassicae* and other specialist herbivores (Moyes et al. 2000).

Feltwell (1982) described brassicaceous plants that are suitable for laboratory rearing of *P. brassicae*, but actual and realized dietary breaths of herbivores (and by association their parasitoids) are often strikingly different. In the Netherlands, for instance, there are few wild brassicaceous plant species that can support the development of a single brood of *P. brassicae* caterpillars. Large broods of *P. brassicae* can contain up to 150 caterpillars or more (Lemasurier 1994), and this many larvae would require several to many plants to provide sufficient food to complete their development. Most nutritionally suitable plants are either too small, or do not grow in aggregated populations which would allow the caterpillars to move from one plant to another when the natal plant is exhausted (Lemasurier, 1994). Therefore, this greatly restricts the number of plant species on which butterflies will oviposit. Andrewartha and Birch (1954) described food limitation as one of the major constraints on the evolution of gregariousness in animals, including insects. Insects feeding on plants containing super-abundant resources, such as trees, are largely (though not entirely) relieved of this burden, but for insects feeding on very small plants with limited resources optimal host-plant selection in the field can be a serious problem. Besides the plants used in this study, the only other wild crucifers that might serve as food plants for *P. brassicae* in the Netherlands are the wintercress, *Barbarea vulgais*, the garlic mustard, *Alliaria petiolata*, the wild radish, *Raphanus raphanistrum*, and the hedge mustard, *Sisymbrius officinale* (Carter and Hargreaves 1994).

The developmental pattern of *C. glomerata* closely mirrored that of its host over the successive generations. Herbivores that performed better thus produced fitter parasitoids. This is unsurprising, because many studies have found that there is a strong correlation between host and parasitoid performance (Harvey 2005). In the field, *C. glomerata* is under similar spatial, ecological and physiological constraints as those faced by its host, *P. brassicae* (van Nouhuys et al., 2004). Parasitized caterpillars generally grow as large as healthy caterpillars (Harvey, 2000), although this is to some extent correlated with parasitoid load. The parasitoid is able to regulate host growth in accordance with the nutritional requirements of its offspring, and this increases with the number of parasitoids developing inside of individual caterpillars. In some instances, heavily parasitized caterpillars (e.g. containing 70 or more wasp larvae) may attain terminal masses some two to three times higher than healthy cohorts. Therefore it is anticipated that the amount of plant biomass necessary to support the development of both healthy and parasitized broods of *P. brassicae* is similar.

In nature, different generations of multivoltine insect herbivores (and, by association, their specialist natural enemies) are faced with constraints imposed by changing plant quality and (in the case of short-lived plants) spatial shifts in plant availability (Awmack and Leather 2002). How these insects deal with food-plant shifts in successive generations has been little studied. In our system, different generations of *P. brassicae* and *C. glomerata* almost certainly develop on different food-plant species in different generations, based on the short-lived seasonal phenology of their food plants. A final major constraint on food-plant shifts is that the distance between the natal patch and new food plant may be anywhere from a few meters to 10km or more. Whereas the butterflies possess strong wings and the capacity to cover large areas of landscape in the search for new plants, it will be interesting to determine what enables their comparatively small parasitoid, *C. glomerata* to locate new hosts under these same conditions.

It has long been known that intimate plant-insect interactions can drive co-evolutionary adaptations in both parties (Ehrlich and Raven 1964, Wittstock et al. 2004, Zangerl et al. 2008). However, less is known about the importance of diffuse co-evolution in herbivores exploiting multiple plants in different generations of a single growing season. The study of epigenetics, which has been used to study the importance of heritable traits in insects such as honeybees (Maleszka 2008), may provide useful insights into the evolution of traits that may benefit subsequent generations of herbivores (and their specialist natural enemies) that are associated with specific types of plants. The degree to which *P. brassicae* is locally adapted to the plants studied here is open to question, because although the insects had been collected in the field they had been reared for many generations on cultivated cabbage. It is possible, however, that the insects retain some sort of ontogenetic 'memory' that persists over an extended period of time and enables them to exploit plants with which they have a recent evolutionary history. Alternatively, cultivated cabbage is closely related to the naturally occurring food plants, and therefore, a successful switch requires little preadaptation.

In summary, our study has revealed that plant species identity, seasonal phenology and food-plant shifts all may affect the performance of multivoltine insect herbivores and their parasitoids. Most existing studies have been conducted under largely 'static' conditions using single plant species grown at specific times of the year (e.g. not replicated over longer periods of time). We argue that more studies are needed incorporating spatial and temporal reality into them. This would include plant species shifts as well as studies manipulating abiotic characteristics such as light regimes. Field studies are also essential, because these will enable us to see how the insects cope with season shifts in plants at a landscape level. If possible, it would be interesting to track the movement of individual adult butterflies and their parasitoids after emerging from the natal patch. Work with bees using harmonic radar has shed considerable light on orientation in flight behaviour of honeybees over several days in learning to exploit profitable flower patches (Capaldi et al. 2000). If it was possible to utilize similar technology with herbivores and their specialist natural enemies, we may gain novel insights into the entire foraging repertoire, from emergence, through dispersal to host-plant (or host) patch location. At present, our knowledge is largely confined to behaviour within patches. Furthermore, the effects of anthropogenic stress may affect the phenology of multitrophic interactions. The loss of habitat, invasive plants or rapid climate warming may increase extinction risks and unravel trophic webs or by desynchronizing the optimal developmental period of insects with the most nutritious stages of their food plants (van Asch and Visser 2007). It may also increase the distance between suitable patches of plants, thus making it harder for insects to locate their food plants (and parasitoids their hosts) in successive generations. Moreover, if warming increases the number of insect generations per year, it may also reduce the length of time that short-lived annual plants are available, adding an extra layer of complexity to our understanding of cause-and-effect relationships in multitrophic interactions.

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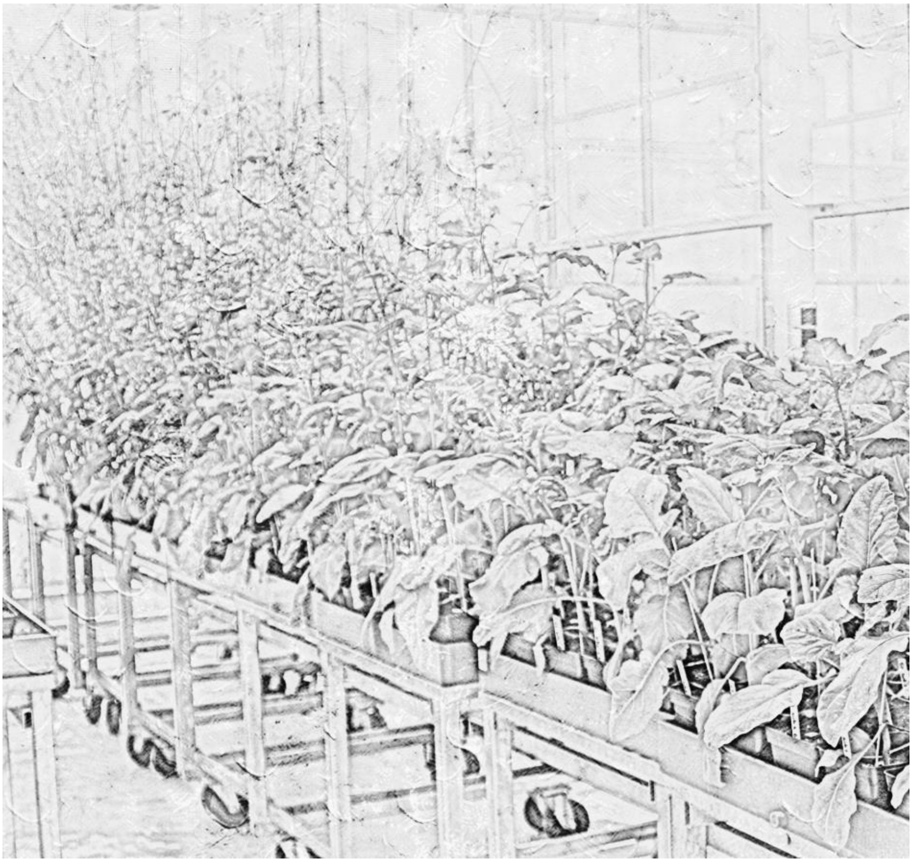

Chapter 3

Cabbage butterfly mothers are choosy when selecting plants that differ in age and size

Minghui Fei, Jeffrey A. Harvey, Yi Yin, and Rieta Gols

Submitted in a slightly different form

Abstract

The effects of temporal variation in plant quality of short-lived annual plants on oviposition preference and larval performance of insect herbivores has received little attention. This study examines the effects of plant age on female oviposition preference and offspring performance in the large cabbage white butterfly *Pieris brassicae*. Adult female butterflies lay variable clusters of eggs on the underside of short-lived annual species in the family Brassicaceae including *Brassica nigra* and *Sinapis arvensis*. Both plants have short life cycles, exhibit little phenological overlap during the growing season and are important food plants for different generations of *P. brassicae*. Here, we compared oviposition preference and larval performance in *P. brassicae* on three age classes (young, mature, and pre-senescing) of *B. nigra* and *S. arvensis* plants. Oviposition preference by *P. brassicae* declined with plant age in both plant species whereas their larvae performed well on all three plant age classes of both plant species. Oviposition preference was correlated with larval performance on *S. arvensis,* but less so on *B. nigra*. Analysis of primary and secondary chemistry in the plant shoots revealed that differences in their quality and quantity were more pronounced with respect to tissue type (leaves vs flowers) than among different developmental stages in both plant species. Butterflies clearly select plants for their progeny not on the basis of size at oviposition or visual acuity, potentially anticipating that future plant growth and size is optimally synchronized with the final larval instar, which contributes >80% of insect growth.

Introduction

The suitability of a food plant for growth and development of herbivorous insects is generally determined by a combination of sufficiently high concentrations of primary metabolites and low concentrations of secondary metabolites (Schoonhoven et al. 2005). Primary metabolites, such as proteins, particular carbohydrates, and fatty acids are essential for the growth, development, and reproduction of all living organisms (Berenbaum 1995). Given its central role in most metabolic processes, nitrogen is considered to be of critical importance for the growth and development of insects and is often limiting in plant tissues reducing the quality of plants as food (Lawton and McNeil 1979, Mattson 1980, Scriber and Slansky 1981). Secondary metabolites, or allelochemicals, often play a defensive role in providing resistance against insect herbivores (Adler et al. 2014). Resistance may involve deterrence of oviposition or feeding and interference with the insect's physiology once tissues have been ingested (Harvey et al. 2005, Berger et al. 2007). However, most insect herbivores are dietary specialists and thus feed on only a few species or closely related plants that produce phylogenetically conserved allelochemicals (Schoonhoven et al. 2005, Loxdale et al. 2011). Consequently, many insect herbivores have evolved various adaptations to deal with plant allelochemicals (Despres et al. 2007, Winde and Wittstock 2011).

The production of primary and secondary metabolites is largely genetically controlled and often varies with plant age (Barton and Koricheva 2010), although concentrations of phytochemicals may also change in response to biotic and abiotic factors such as rainfall, temperature and insect feeding and pathogen attack (Karban and Baldwin 1997; Schoonhoven et al. 2005). Nitrogen demands (and N levels) are often higher in younger plants, but then decline with age (Mattson 1980). Concentrations of allelochemicals in plant tissues are also dynamic and can change with time. Using meta-analysis, Barton and Koricheva (2010) reviewed whole-plant ontogenetic changes in plant secondary metabolites across a range of plant taxa and found that constitutive concentrations of all types of secondary metabolites significantly increased from the seedling to the adult stage in both herbaceous and woody plants.

Adult females of insect herbivores have to find suitable food plants for their offspring often in habitats that vary in their structural and chemical complexity. When the temporal availability and quality of suitable food plants is highly predictable, searching for oviposition sites is often closely synchronized with these parameters. For example, larval feeding stages of monophagous insect that have only one generation per year (e.g. are univoltine) are usually active only when suitable tissues are available (van Asch et al. 2010). In contrast, when adult females of multivoltine herbivorous insects that feed on short-lived herbaceous plant species are searching for oviposition sites, they are faced with different challenges. For example, different generations have to find suitable food-plant species that may differ in quality due to differences in plant age, which can potentially have huge effects on the development, survival and fitness of their progeny.

According to the preference-performance hypothesis, females will maximize their fitness by laying eggs on plants on which the offspring perform the best (Mayhew 1998). However, this theory ignores the fact that the relationship between female preference and offspring performance may also be influenced by other ecological factors such as predation risk and the dietary breadth of the herbivores (Gripenberg et al. 2010). For instance, the effect size of the correlation between preference and performance was found to be larger in oligophagous than in monophagous and polyphagous herbivore species (Gripenberg et al. 2010). The authors argued that polyphagous insects may be neurophysiologically constrained when processing complex information from the environment when searching for suitable food plants, whereas for monophagous herbivores variation in quality among the small range of suitable food plants may be simply too small for a preference-performance relationships to be detected (Gripenberg et al 2010). When the quality of food plants varies unpredictably over time, females may fail to evolve an ability to choose the plant type that would be most suitable for development of their offspring (Cronin et al. 2001, Gripenberg et al. 2007). Multivoltine insect herbivores may also be forced to use different food-plant species over the course of a growing season, depending on what is available at a specific time and their performance may be constrained by temporal and species-specific variations in food-plant quality (Gols et al. 2007, Fei et al. 2014).

In this study, we examine female preference and offspring performance in the large cabbage white butterfly *Pieris brassicae* L. (Lepidoptera: Pieridae) in relation to plant ontogeny. Larval stages of this herbivore feed only on plants that contain glucosinolates, defensive secondary metabolites characteristic of species in the plant family Brassicaceae (Fahey et al. 2001). Adult females and larvae of *P. brassicae* use glucosinolates as oviposition and feeding stimulants, respectively (Renwick 2002). Most of the food-plant species of *P. brassicae* are short-lived annual plant species that are present in the field for only 2-3 months (Feltwell 1982). These include cabbage crops and mustard oil species on which *P. brassicae* is considered an important pest species across much of Eurasia (Feltwell 1982). In the Netherlands, *P. brassicae* has two to three generations per year. In relation to the phenology of the herbivore and its potential food plants, amongst others, the charlock mustard *Sinapis arvensis* L., which grows in late spring and early summer and the black mustard, *Brassica nigra* L., which grows in middle to late summer, are considered important food plants for this herbivore. These ephemeral plant species often grow in dense populations, which are an important feature, as *P. brassicae* lays eggs in clutches of 30-100 eggs on single plants. For brood survival it is generally necessary for the natal plant to be embedded in a larger population where the plants are clustered, given that a single brood can devour up to several plants during their larval development (Chapter 2).

In the field, when female *P. brassicae* butterflies are searching for oviposition sites, they are likely to encounter plant individuals of variable age and thus condition. These plants may also vary considerably in size, as *B. nigra* plants grow as high as 2 meters or more. The aims of this study were to (i) investigate whether female butterflies distinguish between different developmental stages of the two food-plant species for oviposition and (ii) to determine whether the female butterflies lay their eggs on plants in the developmental stage that is best for growth and development of their offspring. Concentrations of primary metabolites (nutrients) are usually higher in younger than in older tissues (Mengel et al. 2001). Similarly glucosinolate concentrations are generally higher in newly developed leaves than in older leaves (Gols et al. 2007). As *P. brassicae* are specialized feeding on brassicaceous plants, we predict that larval development will not be compromised by high concentrations of glucosinolates. Consequently larval performance will be better on younger plants that are of higher nutritional quality in terms of primary metabolites than on older plants. To link larval performance with qualitative characteristics of the plant tissues, we analyzed and quantified primary (amino acids and sugars) and secondary metabolites (glucoinolates) in tissues collected from plant in different developmental stages and correlated these with larval performance variables using comprehensive multivariate statistics.

Materials and Methods

Plants and insects

Sinapis arvensis and *Brassica nigra* seeds were collected from several plants (>15) in wild populations growing naturally near Wageningen, the Netherlands. Seeds were germinated and seedlings were subsequently transferred to 1.1-L pots filled with peat soil ('Lentse potgrond' no.4; lent, the Netherlands). Plants were grown in a greenhouse at 21±2 °C (day) and 16±2°C (night), 50% r.h., and a photoperiod of at least 16h. If the light dropped below 225 μmol photons/m^2/s during the 16-h photoperiod, supplementary illumination was applied by sodium lamps. The plants were watered twice a week during the first 3 weeks of development and watering was then gradually increased to once a day. When the plants were 3 weeks old, they were fertilized once a week with Hoagland solution, which was applied to the soil. Watering and fertilization continued during the experiments.

To investigate the effect of the plant's developmental stage (and size) on insect preference and performance, we reared plants of different age classes by sowing the seeds and growing the plants at different time points. Because *S. arvensis* has a shorter life cycle compared to *B. nigra*, we used different age classes for the two species. Seeds of *S. arvensis* were germinated at two-week intervals and plants used in the experiment were 3, 5 and 7 weeks old, respectively. In the greenhouse, three-week old *S. arvensis* plants are still developing leaves, but flower buds are starting to develop as well (in the transition between the late-vegetative/early-reproductive developmental phase). Five week old *S. arvensis* plants are flowering and the leaves are still green (mid-reproductive phase), whereas seven-week old plants are still flowering but the leaves are starting to senescence (late-reproductive phase). Seeds of *B. nigra* were germinated at three-week intervals and plants used in the experiments were 3, 6 and 9 weeks old, respectively. Three-week old *B. nigra* plants are still in the vegetative developmental phase, six-week old plants are in the late-vegetative/early-reproductive phase, whereas nine-week old plants are fully flowering and still have green leaves (=mid-reproductive phase).

Cultures of *Pieris brassicae* were maintained in a climate room at 22±2 °C, 50-70% r.h. and a photoperiod of at least 16h. *Pieris brassicae* were reared on Brussels sprout plants (*B. oleracea* var. gemmifera, cv. Cyrus) for many generations at the Laboratory of Entomology, Wageningen University.

Experiment 1. Butterfly oviposition preference for plants in different stages of development

Pieris brassicae larvae were reared on Brussels sprouts and pupae were collected from the rearing cages and transferred to a clean cage (60 x 60 x 180 cm) placed in the experimental greenhouse. Eclosing butterflies were provided with a (20%) honey solution applied to cotton wool in blue plastic caps to promote feeding activity. The butterflies were allowed to mate and were 3-5 days old when used in the choice bioassays.

Oviposition preference was determined in three-choice experiments in tents (2 x 2 x2 m) placed outside in the garden of the Netherlands Institute of Ecology on bare soil. Plants of the three different age classes, one plant from each class, were prepared as described in the *Plants and insects* section. Bioassays were performed for each plant species separately.

Single plants from the three different age classes were randomly placed in one of 3 corners of the tent. One female and one male butterfly were released in the middle of the tent. A bioassay was terminated when a female butterfly had laid the first egg clutch which was checked every hour. The age class of the plant on which the eggs were laid was recorded. The bioassay was repeated at least 60 times for each plant species. Bioassays were conducted from June to August. New plants and butterflies were used for each replicate. Plants of the three age classes differed in size. To determine whether differences in size of the plants influenced oviposition decisions, we also measured total leaf surface area and plant height of at least ten plants per age class. For leaf surface area measurements, all the leaves from single plants were collected and scanned using a photo scanner (Perfection4990; Epson, Japan). The leaf surface area was determined using the software WinFOLIA (Regent Instruments, Sainte-Foy, Canada).

Experiment 2a. Ontogenetic effects of plant quality on herbivore performance

To obtain larvae for the performance bioassay, additional plants of the various developmental stages were placed in the rearing cage with *P. brassicae* butterflies. Since *P. brassicae* eggs need about one week to hatch under the greenhouse conditions used in this study, *B. nigra* and *S. arvensis* plants were prepared one week before they were used in the experiments. Female butterflies were allowed to oviposit on these plants for 24 h. The following day, the plants were removed from the butterfly cage and transferred to the experimental greenhouse. The eggs were allowed to hatch and the larvae were allowed to feed and develop into second instar (L2) caterpillars on these plants before they were used in experiments. For each plant developmental stage, cohorts of 20 randomly selected 1-day old L2 larvae were transferred to cages containing four plants that were in the same developmental stage as the one on which they had hatched and had been feeding previously. Per age class we used 5 cages (=replicate) each with 20 caterpillars.

Depending on the developmental stage of the plants, we used differently sized cages (40 x 40 x 60 cm and 60 x 60 x 180 cm, respectively, Vermandel, Hulst, the Netherlands). In order to exclude a possible effect of temperature differences in the two differently-sized cages, we measured the temperature in the middle of the cages twice a day. There was no significant difference in temperatures between the two cage sizes. Caterpillars were allowed to move and feed freely on the plants within a cage until they pupated. Pupation was recorded daily and pupae were collected and weighed on an analytical balance (0.1 mg accuracy). During the experiment, extra plants of the same age as the plants in the cage were added when needed. As proxies for insect performance, we measured insect development time from egg hatching to pupation and pupal fresh mass. The conditions were the same as for plant growth.

Experiment 2b. Plant ontogenetic changes in chemical profiles

During the larval performance bioassays, plant tissues were collected for analysis of primary (sugars and amino acids) and secondary metabolites (glucosinonates) from both *B. nigra* and *S. arvensis* plants in the three age classes. Tissues were sampled from the plants when the caterpillars had been feeding on them for 9 days. Caterpillars were removed before sampling. Leaf tissues were collected by excising five 7 mm discs from each of four fully developed green leaves of two plants within each cage. As *P. brassicae* caterpillars feed on both leaves and flowers (Smallegange et al. 2007), we also collected flowers for chemical analysis. Eight stems with flowers were cut off the same plants from which leave tissues had been collected. Leaf discs and flowers, respectively, were pooled per cage in tinfoil and frozen in liquid nitrogen immediately after sampling and stored at -80°C until further processing. Samples were freeze-dried and pulverized.

For quantification of soluble sugars, amino acids, and glucosinolates, one global extraction was conducted. In a 2-ml Eppendorf tube, approximately 50 mg finely ground plant material was suspended in 1.0 ml 70% MeOH in water (vol/vol), vortexed, and immediately boiled for 5 min to inactive enzymes, especially myrosinase that is involved in the hydrolysis of glucosinolates (Textor and Gershenzon 2009). Tubes were placed in an ultrasonic bath for 15 min and centrifuged (10 min at 10000g). The extraction was repeated with the pellet, omitting the boiling step. Both supernatants were combined per sample and supplemented with 70% MeOH to a final volume of 2 ml. This ‘stock’ extract was stored at -20°C until further analysis.

To analyze soluble sugar content, a 10 ml aliquot of the stock extract was diluted in 990 ml MilliQ water. Soluble sugar were analyzed on Ion-exchange HPLC using standard injection volumes of 5µl. A “10 ppm” reference solution containing 54.9 µM sorbitol and manitol, 29.21 µM trehalose, sucrose and melibiose, and 55.51 µM glucose and fructose, was diluted to obtain 2.5 ppm, 5 ppm and 7.5 ppm calibration standards for construction of a reference curve. Quantification was based on these reference curves (van Dam and Oomen 2008). Sugar levels were divided by the dry mass of the original tissue sample.

For amino acid analysis, 20 ml of the stock extract was diluted in 980 ml MilliQ water. Amino acid concentration was analyzed on an Ion-exchange HPLC with an injection volume of 25µl. For quantification and identification we used a reference sample that contained 20 of the most common amino acid. For preparation of the reference sample and the standard curves, we used the Sigma AA-S-18 amino acid standard (Sigma, St Louis, MO, USA), containing 17 amino acid, supplemented with aspargine, glutamine and tryptophane (2.5 mM/ml each). The reference sample was diluted to obtain a calibration curve ranging from 1-8 mM for each amino acid, except for cysteine, which had a range of 0.5-4 mM (van Dam and Oomen 2008). Amino acid levels were divided by the dry mass of the original tissue sample.

Half (1.0 ml) of the stock was used for glucosinolate analysis and applied to a DEAE-Sephadex column. Further extraction and purification followed the protocol as described in van Dam et al. (2004). Glucosinolates were separated on a reverse phase C-18 column on HPLC with an acetonitrile water gradient. Glucosinolates detection was performed with a photodiode array detector set at 229 nm as the integration wavelength. For quantification, sinigrin was used as an external standard. The different glucosinolates were identified based on their retention times and the UV spectra were compared to those of pure compounds standards provided by M. Reichelt (Max Planck Institute for Chemical Ecology, Jena, Germany) and a certified rapeseed standard (Community Bureaus of Reference, Brussels, Belgium, code BCR-367 R). Glucosinolate levels were divided by the dry mass of the original tissue sample.

Statistical analyses

Data were analyzed for the two plant species separately. To statistically analyze butterfly oviposition preference, we used Chi-square tests comparing the observed oviposition counts on the three age classes with an expected distribution of 1:1:1. When the test result was significant, we conducted pairwise Chi-square tests with α=0.05/3 to correct for type I errors (Bonferroni correction). Data on plant height and leaf area were analyzed using one-way ANOVA with plant developmental class as a fixed factor, followed by Tukey-Kramer multiple pair-wise comparison tests to reveal differences between means.

In the experiments measuring larval performance on plants belonging to different age classes, cages, each containing 20 caterpillars, served as experimental units. Therefore, we first calculated the mean values per cage for the two performance variables pupal mass and egg-to-pupa development time. These mean values were then subjected to one-way ANOVA with plant developmental class as a fixed factor. Tukey-Kramer multiple comparison tests between means were conducted when the ANOVA models were significant. The ANOVA’s were carried out using SPSS (IBM SPSS, statistics version 19).

We used a multivariate statistical approach, i.e. constrained redundancy analysis (RDA), to determine differences in plant chemistry among the three age classes and the two tissue types (leaves and flowers). The data were 'constrained' by classifying them according to tissue type and developmental stage. RDA was also carried out to analyze chemical profiles differences among the three age classes of a certain plant tissue (leaves or flowers). RDA was also used to analyze whether there is a significant correlation between compounds and herbivore fitness parameters (biomass and development time). For analysis of the chemical data we used the concentrations of the various sugars, amino acid, and glucosinolates, expressed as amount of chemical per unit dry weight of tissue and for the herbivore performance data we used the corresponding mean pupal weight and developmental time. Analyses were performed on data that were log-transformed and mean-centered. Data were analyzed for the two plant species separately. RDA analysis on all chemical data was performed in Canoco version 5.03 (Ter Braak and Šmilauer 2002, The Netherlands).

Results

Experiment 1: Butterfly oviposition preference for plants in different stages of development

All eggs were laid on the leaves of the plant and not on the flowers when present. Female *P. brassicae* butterflies clearly discriminated between the three age classes of *S. arvenis* ($\chi^2_2 = 19.7$, $P < 0.001$) and *B. nigra* ($\chi^2_2 = 22.8$, $P < 0.001$), respectively, when deciding where to oviposit (Fig. 1). Oviposition preference order declined with plant age for both plant species (Fig. 1). Females preferred to lay eggs on plants in the vegetative or early-reproductive developmental phase over plants that are in the mid- to late-reproductive phase.

Plant height differed among the three age classes in both plant species (*S. arvensis*, $F_{2,35} = 147$, $P < 0.001$, Fig. 2A; *B. nigra*, $F_{2,44} = 101$, $P < 0.001$; Fig. 2C). Plant height correlated positively with plant age. In addition, there was an effect of age class on total leaf surface area (*S. arvensis*, $F_{2,77} = 17.7$, $P < 0.001$, Fig. 2B; *B. nigra*, $F_{2,57} =$

21.7, $P < 0.001$, Fig. 2D). In *S. arvensis*, total leaf surface area was higher in the mid-reproductive phase than in plants that were in the late-vegetative/early-reproductive phase (leaves on these plants were still expanding) and late-reproductive phase (leaves on these plants started to wilt and die). In *B. nigra*, vegetative plants were still developing new leaves and total leaf area was lowest in these plants. Total leaf area was intermediate in plants in the mid-reproductive phase on which the older leaves started to wilt and die, and highest was in plant in the late-vegetative/early-reproductive phase.

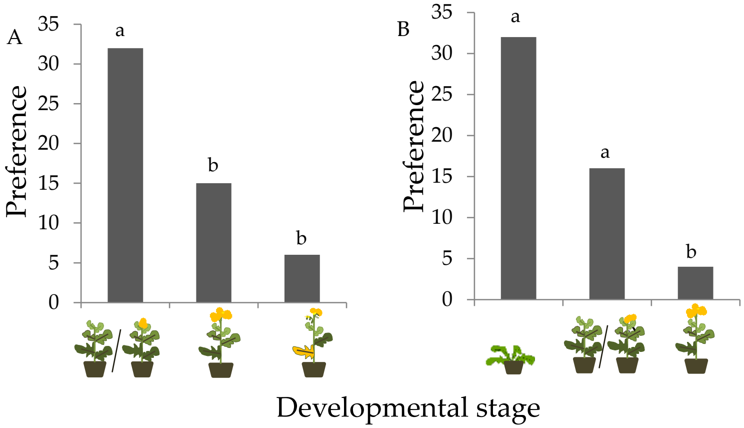

Figure 1. Oviposition preference of *Pieris brassicae* butterflies for *Sinapis arvensis* (A) and *Brassica nigra* (B) that were in different stages of development.  Represents vegetative phase,  represents late-vegetative to early-reproductive,  represents mid-reproductive phase,  represents late-reproductive phase. Bars present the cumulative counts of plants on which eggs were laid (n=57 for *S. arvensis* and n=53 for *B. nigra*), bars with the same letter are not significantly different (pairwise χ^2-tests with a Bonferroni correction for multiple comparisons).

Experiment 2a: Ontogenetic effects of plant quality on herbivore performance

Both egg-to-pupa development time ($F_{2,14} = 11.8$, $P = 0.001$) and pupal mass ($F_{2,14} = 5.07$, $P = 0.025$) were affected by the age class of the *S. arvensis* plants on which the insects had been reared. Insects developed slower and developed into smaller pupae with increasing age of the plants (Fig. 3A and 3B). The effect of plant developmental stage on food-plant quality for *P. brassicae* was less pronounced in *B. nigra* than in *S. arvensis*, and affected only pupal mass ($F_{2,14} = 18.3$, $P < 0.001$, Fig. 3C) and not egg-to-pupa development time ($F_{2,14} = 20.95$, $P = 0.17$ Fig. 3D). Pupae were heavier on plants in both the vegetative and mid-reproductive developmental phase whereas pupal mass was lower when plants were in the late-vegetative/early-reproductive phase (Fig. 3C).

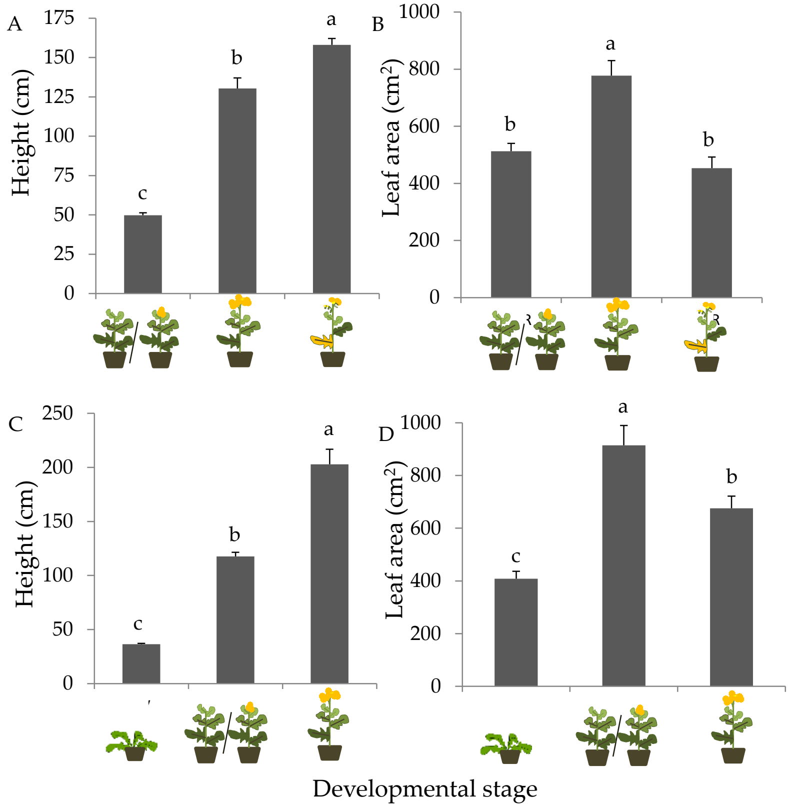

Figure 2. Effect of developmental stage on plant height (A, C) and total leaf surface area (B, D) in *Sinapis arvensis* (A, B) and *Brassica nigra* (C, D). Represents vegetative phase, / represents late-vegetative to early-reproductive phase, represents mid-reproductive phase, represents late-reproductive phase. Bars present the means + SE and bars with the same letter are not significantly different (Tukey-Kramer tests for multiple comparisons among means.

Experiment 2b: Plant ontogenetic changes in chemical profiles

In leaf and flower tissues of *S. arvensis* 12 different glucosinolates, 7 sugars and 16

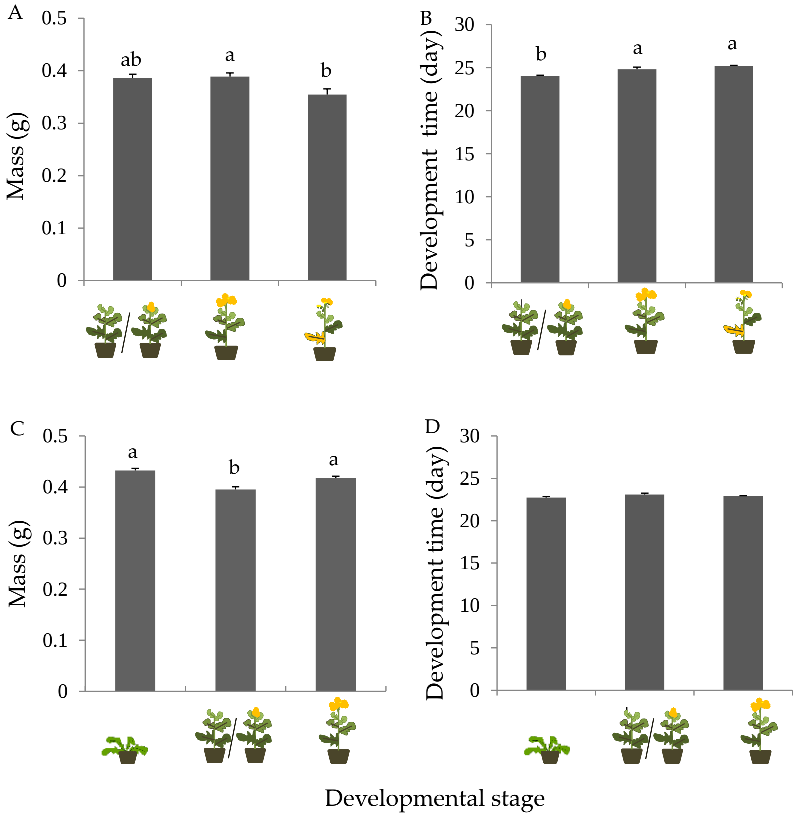

Figure 3. Pupal fresh mass (A, C) and egg-to-pupa development time (B, D) of *Pieris brassicae* reared on three developmental stages of *Sinapis arvensis* (A, B) and *Brassica nigra* (C, D). Represents vegetative phase, / represents late-vegetative to early-reproductive phase respectively, represents middle-reproductive phase, represents late-reproductive phase. Bars present the means + SE and bars with the same letter are not significantly different (Tukey-Kramer tests for multiple comparisons among means).

amino acids were detected, whereas in *B. nigra* 5 glucosinolates, 6 sugars and 16 amino acids were detected. In both plant species, amino acid, sugar and glucosinolate concentrations clearly separated the samples based on whether chemical compounds were measured in the leaves or in the flowers (RDA: *S. arvensis*, F = 105, P = 0.002, Fig. 4A; *B. nigra*, F = 92.8, P = 0.002, Fig. 4B), but did not differ among the developmental phases of the plant (RDA: *S. arvensis*, P > 0.05, Fig. 4A; *B. nigra*, P > 0.05, Fig. 4B). The first principal component (PC) explained 79.5% of the variation in *S. arvensis* and 83.4% of the variation in *B. nigra*, and separated the samples according to tissue type, i.e. flowers and leaves. The second PC separated the samples according to the developmental phase of the plants but only explained an additional 4.2% of the variation in *S.arvensis* and 2.5% in *B. nigra* (Fig. 4A and 4B).

In both plant species, separation of flower and leaf tissues could be largely attributed to relatively high amino acid concentrations in the flowers with the exception of alanine, which was higher in the leaves. Concentrations of some sugars were higher in flower tissues (glucose, fructose, and sucrose), whereas those of other sugars were higher in leaves tissues (sorbitol, trehalose and melezitose). Glucosinolate concentrations also tended to be higher in the flowers than in the leaves, especially concentrations of the most dominant glucosinonates. In *B. nigra*, sinigrin (SIN), contributed > 98% to the total glucosinolate content in both the flowers and leaves, and in *S. arvensis* sinalbin (SNALB) contributed on average 66% and 91 % to the glucosinolate content of the flowers and the leaves, respectively. No consistent patterns could be detected in chemicals that separated the samples along the second PC associated with age class of the plants (Fig. 4A and 4B). To reveal the more subtle differences among plants in the three age classes, we analyzed the chemical profiles of the three age classes of leaves and flowers separately.

S. arvensis. Chemical profiles of leaf tissues in different stages of development significantly different (RDA, P = 0.002). Phytochemicals in leaf tissues clearly separated early and late developmental stages of the plant along the first PC which explained 42.0% of the variation, whereas the second PC separated the mid-developmental stages from the other two and explaining an additional 3.5% of the variation (Fig. 5A). With the exception of sorbitol, sugar concentrations in leaf tissues tended to increase with age of the plants. Some of the amino acids tended to be higher in leaf tissues of younger plants (Pro, Gln, Glu and Ser), whereas foliar concentrations of other amino acids were higher in older plants (Thr, Ile, His, Asn, and Ala). The contribution of the glucosinolates in the separation of samples according to age class was less clear, although Sinalbin (SNALB), the dominant glucosinolate in *S. arvensis* tended to be higher in leaf tissues of younger than of older plants.

Redundancy analysis (RDA) of phytochemicals in flower tissues also separated the samples according to age class (RDA, P = 0.002, Fig. 5B). As was found in leaf tissues, concentrations of some of the amino acids were higher in younger than in older flower tissues, whereas for other amino acids this pattern was reversed. Sugars, especially concentrations of fructose and glucose, as well as concentrations of the dominant glucosinolate sinalbin tended to be higher in older than in younger flower tissues.

B. nigra. Chemical profiles of leaf tissues in different stages of development were significantly different (RDA, P = 0.004). Phytochemicals in leaf tissues clearly separated early and late developmental stages of the plant along the first PC explaining 43.36% of the variation, whereas the second PC separated the mid-developmental stages from the other two explaining an additional 2.84% of the variation (Fig. 6A). Some of the amino acids and sugars tended to be higher in younger than in older leaves, whereas for other amino acids and sugars this pattern was reversed. All four glucosinolates were higher in leaf tissues of younger than of older plants.

Redundancy analysis (RDA) of phytochemicals in flower tissues also separated the samples according to age class (RDA, P = 0.006, Fig. 6B). As was found in leaf tissues, concentrations of some of the amino acids were higher in younger than in older flower tissues, whereas for other amino acids this pattern was reversed. Sugar levels tended to be higher in older than in younger flower tissues, whereas glucosinolate concentrations especially that of singrin were higher in the younger flower tissues.

Linking insect performance to plant chemistry

For *S. arvensis*, significant correlations were found between insect performance variables and plant chemistry variables for both the leaves (RDA, P = 0.004) and the flowers (RDA, P = 0.048) (Figure S1). For *B. nigra*, significant correlations were found between insect performance variables and plant chemistry for the flowers (RDA, P = 0.034) but not for the leaves (RDA, P = 0.70) (Figure S2). However, no consistency in chemical profiles explaining insect performance could be detected when comparing the two plant species or intra-specifically comparing leaves and flowers.

Discussion

The results of this investigation reveal that, when confronted with different developmental stages of *S. arvensis* or *B. nigra* plants, the order of ovipositional preference of female *P. brassicae* butterflies decreased with plant age, i.e. younger plants were preferred over older plants. The selection of plants was clearly not based on their size at oviposition, because the plants preferred were the smallest offered to the female butterflies. However, the overall effects of plant age and size on food-plant quality for *P. brassicae* were relatively small. In *S. arvensis*, ovipositional preferences and offspring performance were positively correlated, though the effect sizes were relatively small, whereas in *B. nigra* there was no correlation between these two variables. Chemical analyses of leaf and flower tissues collected from the two plant species in different developmental stages revealed that the differences in plant chemistry were more evident with respect to tissue type (leaves vs flowers) than among different developmental stages for both plant species. Although for both plants species correlations were found between insect performance and plant chemistry variables, we did not detect clear consistencies in phytochemistry characteristics and insect performance.

Food-plant selection for oviposition sites by adult insect herbivores is a complex behavioural process that is guided by various sensory inputs (Papaj and Vet 1990; Byers 1996; Finch and Collier 2000; Bergman and Wiklund 2009). Female butterflies select plants for oviposition based on visual, olfactory and gustatory information (Schoonhoven et al. 2005). In some species, the concentrations or proportions of particular chemical compounds determine oviposition preference of female butterflies (Thompson and Pellmyr 1991). Caterpillars of P. brassicae are specialist feeders on plant containing glucosinolates, mainly species in the family Brassicaceae (Feltwell 1982). Studies have shown that glucosinolates stimulate oviposition by adult females and feeding by the larvae, respectively, although not all glucosinolate compounds do this to the same extent (Chew and Renwick 1995). Previous studies have shown that food-plant recognition for oviposition by *P. brassicae* butterflies requires physical contact with the plant and is based on chemoreception of glucosinonates on the leaf surface (van Loon et al. 1992). On leaves, where *P. brassicae* females habitually lay their eggs, concentrations of most glucosinolates decreased with plant age in both plant species and this could explain

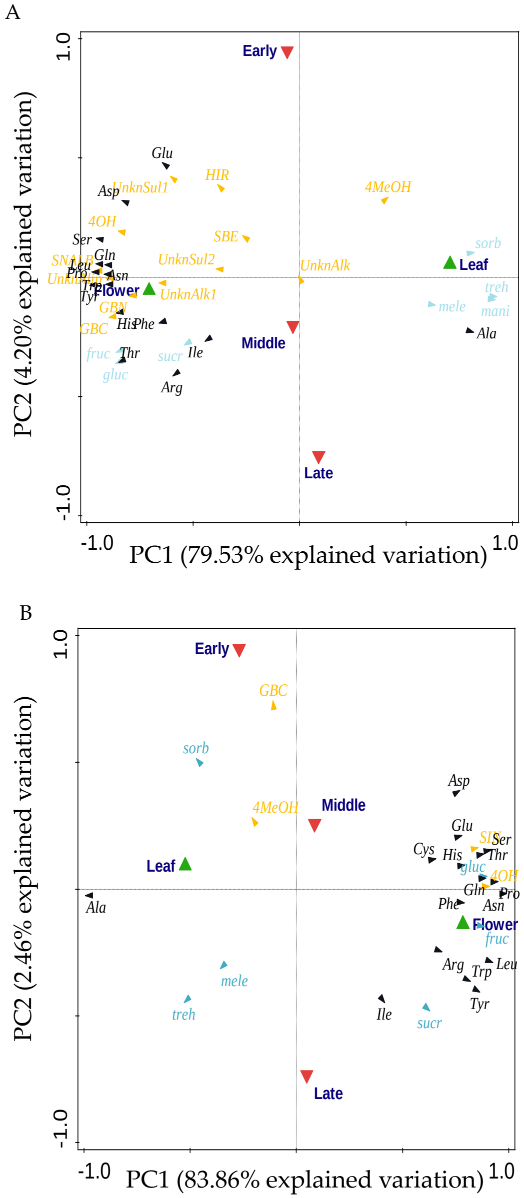

Figure 4: Redundancy analysis (RDA) on the chemical profiles (sugar, amino acid, and glucosinolate concentrations) in leaf and flower tissues (green triangles) from plants in three developmental stages (red inverted triangles) of *Sinapis arvensis* (A) and *Brassica nigra* (B) represented in bi-plots of scores (sample group centrioles, green triangles and red inverted triangles) and loadings (chemical variables). The distance from the axes origins to each of the arrows indicates the magnitude of the contribution of the variable to the model and its direction. For amino acids (black triangles) we used the international IUPAC three letter codes starting with a capital letter. Sugars (blue triangles): gluco= glucose, gluc= gluctose, sucr= sucrose, sorb= sorbitol, mele= melezitose, mani= mannitol, treh= trehalose, sucr= sucrose. For abbreviations of glucosinolates (yellow triangles): SIN= sinigrin, GBN= glucobrassicanapin, SBE= glucosiberin, SNALB= sinalbin, 4OH= 4-hydroxyglucobrassicin, GBC= glucobrassicin, 4MeOH= 4-methoxyglucobrassicin, HIR= glucohirsutin, UnkSul= unkown sulfinyl 1, UnknAlk1= unkown alkenyl, Unksul2= unkown sulfinyl, UnkAlk= unkown alkyl, UnkAli= unkown aliphatic. In 4A, early represents late-vegetative/early-reproductive phase, middle represents mid-reproductive phase, and late represents late-reproductive phase. In 4B, early represents vegetative phase, middle represents late-vegetative/early-reproductive phase, and late represents middle-reproductive phase.

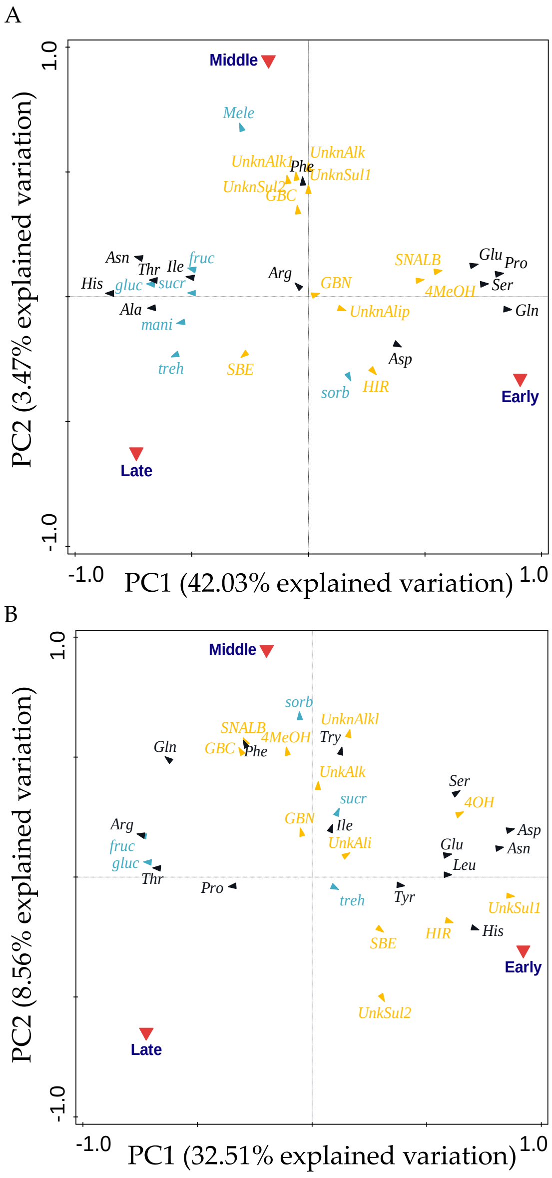

Figure 5. Redundancy analysis (RDA) on the chemical profiles (sugar, amino acid, and glucosinolate concentrations) in leaf (A) and flower (B) tissues from plants in three developmental stages (red inverted triangles) of *Sinapis arvensis* represented in bi-plots of scores (sample group centrioles, red inverted triangles) and loadings (chemical variables). The distance from the axes origins to each of the arrows indicates the magnitude of the contribution of the variable to the model and its direction. For amino acids (black triangle) we used the international IUPAC three letter codes starting with a capital letter. Sugars (blue triangle): gluco= glucose, gluc= gluctose, sucr= sucrose, sorb= sorbitol, mel= melezitose, mani= mannitol, treh= trehalose. For abbreviations of glucosinolates (yellow triangle): SIN= sinigrin, GBN= glucobrassicanapin, SBE= glucosiberin, SNALB= sinalbin, 4OH= 4-hydroxyglucobrassicin, GBC= glucobrassicin, 4MeOH= 4-methoxyglucobrassicin, HIR= glucohirsutin, UnkSul= unkown sulfinyl 1, UnknAlk1= unkown alkenyl, Unksul2= unkown sulfinyl, UnkAlk= unkown alkyl, UnkAli= unkown aliphatic. Early represents late-vegetative/early-reproductive phase, middle represents middle-reproductive phase, and late represents late-reproductive phase.

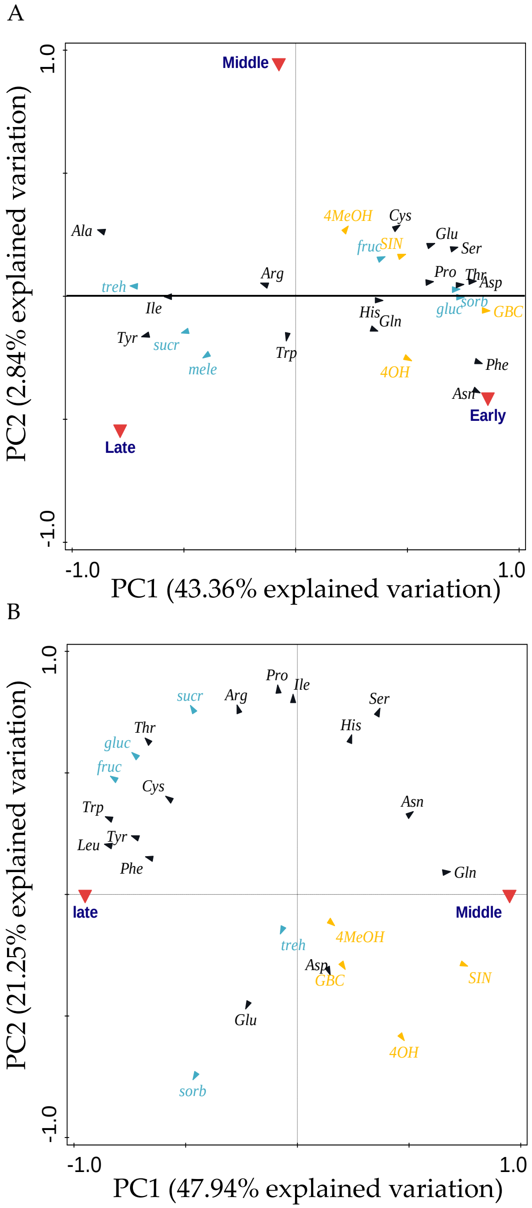

Figure 6. Redundancy analysis (RDA) on the chemical profiles (sugar, amino acid, and glucosinolate concentrations) in leaf (A) and flower (B) tissues from plants in three developmental stages (red inverted triangle) of *Brassica nigra* represented in bi-plots of scores (sample group centrioles, red inverted triangles) and loadings (chemical variables). The distance from the axes origins to each of the arrows indicates the magnitude of the contribution of the variable to the model and its direction. For amino acids (black triangle) we used the international IUPAC three letter codes starting with a capital letter. Sugars (blue triangle): Sugars (blue triangle): gluco= glucose, gluc= gluctose, sucr= sucrose, sorb= sorbitol, mele= melezitose, treh= trehalose. For abbreviations of glucosinolates (yellow triangle): SIN= sinigrin, GBN= glucobrassicanapin, SBE= glucosiberin, SNALB= sinalbin, 4OH= 4-hydroxyglucobrassicin, GBC= glucobrassicin, 4MeOH= 4-methoxyglucobrassicin, HIR= glucohirsutin, UnkSul= unkown sulfinyl 1, UnknAlk1= unkown alkenyl, Unksul2= unkown sulfinyl, UnkAlk= unkown alkyl, UnkAli= unkown aliphatic. Early represents vegetative phase, middle represents late-vegetative/early-reproductive phase, and late represents middle-reproductive phase.

why younger plants are preferred over older plants for oviposition.

Females of *P. brassicae* lay eggs in clutches varying between 30 to more than 100 eggs per clutch. Depending on the number of caterpillars and the size of the food plants, several plants growing in close proximity are needed for successful development of *P. brassicae* larvae from a single egg clutch. Thus both quantitative and qualitative characteristics of the food plant determine development and survival of *P. brassicae* caterpillars. One area that is rarely considered in studies of oviposition behaviour and plant preference in herbivores, however, is that the performance of offspring is not necessarily determined by current plant quality and quantity but by the ways in which these parameters are affected in the future. It must be borne in mind that the plant is not static during insect development, but that it is growing and developing as well. The eggs of many insects, including *P. brassicae*, take up to a week or longer to hatch in the field. Each larval instar must then feed on the growing plant, with each instar punctuated by a transient molting phase. Importantly, the plant is not a static but a dynamic resource. Depending on local abiotic conditions like temperature, this might mean that the entire egg-to-pupa development period may cover a month or more. Furthermore, as in most holometabolous insects, >85% of insect feeding and growth in *P. brassicae* occurs during the final (= L5) instar. This stage can occur some 3-4 weeks after oviposition, meaning that the mother may have to be able to roughly predict plant quality (and quantity) several weeks in advance when selecting a plant on which to oviposit. Given that their food plants may be present for only 2-3 months in the field, this represents a large fraction of the plant's lifetime. Our results thus suggest that *P. brassicae* may select plants not on the basis of present quality or quantity but more importantly on anticipating future quality and quantity coinciding with the final instar of their larvae. Laying eggs on plants in the mid to late reproductive phase may have serious consequences for offspring fitness and survival as plant tissues may be senescing by the time the larvae reach their final instar.

In butterflies that associate with long-lived plants, the relative growth rate of caterpillars is often faster compared with those feeding on plants in the reproductive stages of perennial plants (Quintero et al. 2014). In our experiment, the developmental stage of the plants had relatively small effects on insect performance variables such as development time and pupal mass as well as on larval survival which was high across all treatments. This may be due to the fact that *P. brassicae* has evolved to respond to rapid temporal changes in plant quality that occur in the short lived annual species upon which it feeds. Moreover, it is known that, whereas young larvae of *P. brassicae* feed only on leaf tissues, older caterpillars move up the plant and prefer to feed on the flowers (Smallegange et al. 2007). Flowers of *B. nigra* were shown to be of higher nutritional quality than the leaves in bioassays were caterpillars where constrained to feed on either leaves or flowers (Smallengange et al. 2007). Availability of flowers may explain some of the variation in larval performance in this study; however, our results also showed that leaves of *B. nigra* plants in the vegetative phase are of similar nutritional quality as the flowers of *B. nigra* plants in the early reproductive phase.

Plant chemicals such as primary and secondary metabolites vary with plant ontogeny (Barton and Koricheva 2010) and variation in these chemicals may also influence the performance of insects feeding on these plants (Scriber and Slansky 1981). For example, primary plant compounds, such as proteins and amino acids are critical for herbivore growth and development, whereas qualitative or quantitative variation in plant secondary metabolites often negatively influence herbivore behaviour and development (Olofsson et al. 2013). For instance, the ontogenetic stage of long lived trees influences herbivore density, with more herbivores found on saplings than on young reproductive trees (Waltz and Whitham 1997; Boege 2005). When we compared chemical profiles of different developmental stages of the two plant species, we found that the differences in plant chemistry were more pronounced with respect to tissue type (leaves vs. flowers) than to developmental stage. Compared to the leaves, flowers contained relatively higher concentrations of most amino acids. Younger stages of plant tissues contained more glucosinolates and fewer sugars compared to more mature plants. The better performance of *P. brassicae* on younger plants of *S. arvensis* supports the prediction that development in this species is not hampered by high glucosinolate concentrations. However, correlation analysis of insect performance and plant chemistry variables did not reveal consistent patterns explaining insect performance in relation to plant chemistry. In our experiments, we allowed the insect to move and feed freely on the leaves and the flowers. The mixed diet of the caterpillars may further explain the poor correlation between phytochemistry and insect performance. Nevertheless, it is notable that despite the large differences in chemistry between the leaves and the flowers, the performance of the caterpillars was relatively little affected by this. For example, performance of the caterpillars was similar on young *B. nigra* plant that had not produced flowers yet, and old *B. nigra* plants that had.

According to the ‘preference-performance’ hypothesis, insect herbivores should lay their eggs on food plants that are most suitable for development and survival of their offspring (Jaenike 1978). However, the results of studies on the relationship between butterfly oviposition preference and larval performance only partially corroborate this theory (Thompson 1988; Jaenike 1990; Mayhew 2001; Gripenberg et al. 2010). Adult preference and larval performance were positively correlated in *S. arvensis* but less so in *B. nigra*. As we have shown, oviposition decisions may be based on future, and not present, plant quality; the biology of the plant, in terms of the rate of growth and dynamic changes in primary and secondary chemistry may be association specific (e.g. differ in long-lived perennials and short-lived annuals). Thus preference-performance studies need to account for differences in the biology of the food plants. Furthermore, optimal larval performance is not the only criterion for selection of oviposition sites by female butterflies. Ecological factors, such as reduced risk of attack by the herbivore’s natural enemies, or enemy free space (Mira and Bernays 2002; Oppenheim and Gould 2002; Pöykkö 2011), interactions with mutualists preventing predation (Atsatt 1981; Pierce and Elgar 1985), and the availability of optimal food plants (Fenner 1998; Danks 2001) may all play important roles when selecting plants for oviposition. Moreover, optimization of survival of the eggs rather than the larvae (Tammaru et al. 1995) and female physiology (age, egg loads) may further explain why adults preference and offspring performance are not always positively correlated.

In summary, our study reveals that adult *P. brassicae* female butterflies distinguish between different developmental stages of their food plants and preferred younger stages of both *S. arvensis* and *B. nigra* for oviposition, although the consequences of these preferences for larval growth and development were relatively small. In the Netherlands *S. arvensis* and *B. nigra*, which often grow in large stands, are important food-plant species for *P. brassicae*. For a multivoltine insect herbivore like *P. brassicae* that primarily feeds on short-lived annuals, it is a challenge for different generations to locate suitable food plants. Preference for younger food plants may allow the plant to grow additional resources to support larval growth during their voracious feeding final instar phase. For a multivoltine insect that feeds on short-lived, fast growing plants it is also adaptive to be able to exploit a broad range of food plants and to be little affected by ontogenetic changes in plant quality.

Acknowledgments

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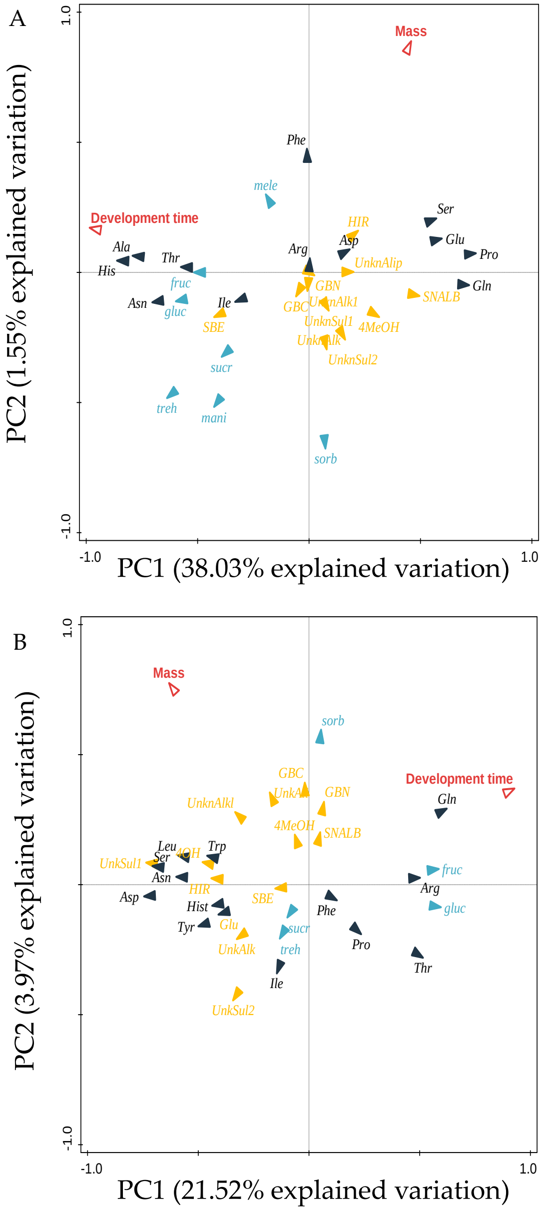
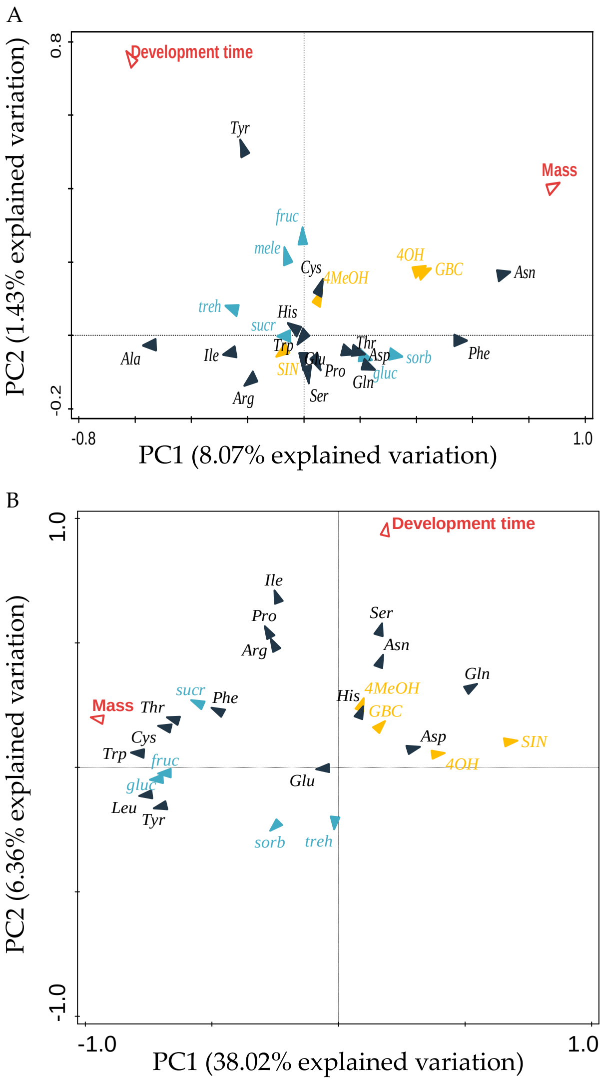
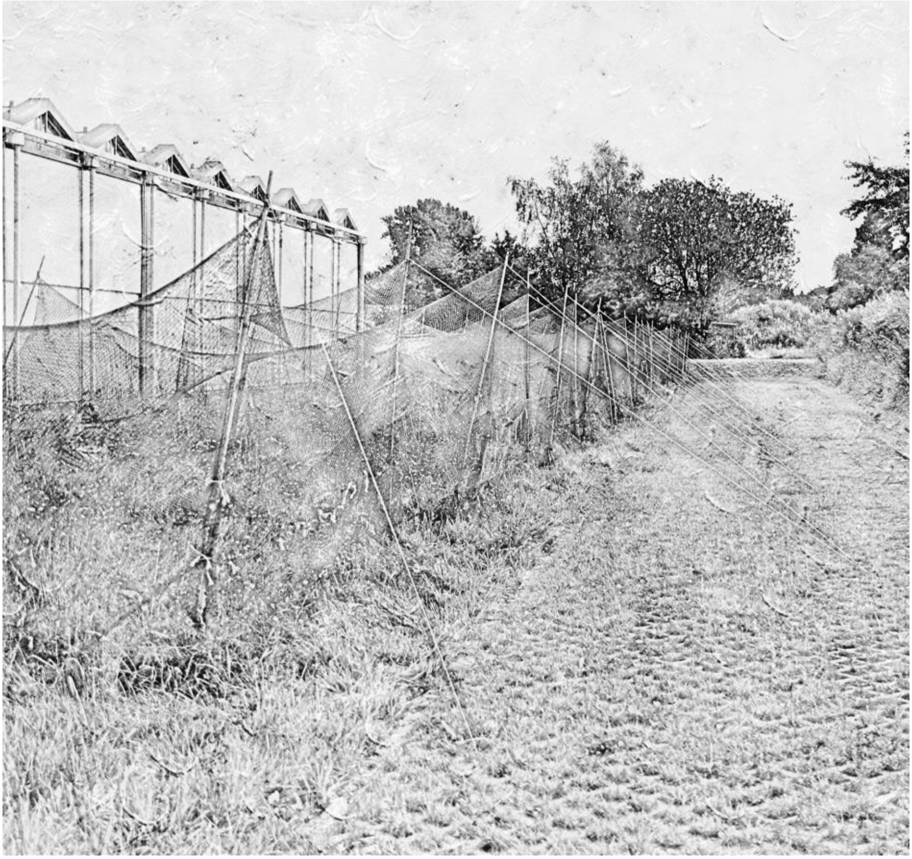
Supplementary material



Supplementary Figure 1: Redundancy analysis (RDA) on the chemical profiles (sugar, amino acid, and glucosinolate concentrations) in leaf (A) and flower (B) tissues from plants of *Sinapis arvensis* and insect performance variables (mass and development time, red triangles), represented in bi-plots of scores (sample group centrioles, red inverted triangles) and loadings (chemical variables). The distance from the axes origins to each of the arrows indicates the magnitude of the contribution of the variable to the model and its direction. For amino acids (black triangle) we used the international IUPAC three letter codes starting with a capital letter. Sugars (blue triangle): Sugars (blue triangle): mani= mannitol, gluc= gluctose, sucr= sucrose, sorb= sorbitol, mele= melezitose, treh= trehalose, gluco= glucose. For abbreviations of glucosinolates (yellow triangle): SIN= sinigrin, GBN= glucobrassicanapin, SBE= glucosiberin, SNALB= sinalbin, 4OH= 4-hydroxyglucobrassicin, GBC= glucobrassicin, 4MeOH= 4-methoxyglucobrassicin, HIR= glucohirsutin, UnkSul= unkown sulfinyl 1, UknnAlk1= unkown alkenyl, Unksul2= unkown sulfinyl, UnkAlk= unkown alkyl, UnkAli= unkown aliphatic.



Supplementary Figure 2: Redundancy analysis (RDA) on the chemical profiles (sugar, amino acid, and glucosinolate concentrations) in leaf (A) and flower (B) tissues from plants of *Brassica nigra* and insect performance variables (mass and development time, red triangle), represented in bi-plots of scores (sample group centrioles, red inverted triangles) and loadings (chemical variables). The distance from the axes origins to each of the arrows indicates the magnitude of the contribution of the variable to the model and its direction. For amino acids (black triangle) we used the international IUPAC three letter codes starting with a capital letter. Sugars (blue triangle): gluco= glucose, gluc= gluctose, sucr= sucrose, sorb= sorbitol, mele= melezitose, treh= trehalose. For abbreviations of glucosinolates (yellow triangle), SIN= sinigrin, GBN= glucobrassicanapin, SBE= glucosiberin, SNALB= sinalbin, 4OH= 4-hydroxyglucobrassicin, GBC= glucobrassicin, 4MeOH= 4-methoxyglucobrassicin, HIR= glucohirsutin, UnkSul= unkown sulfinyl 1, UnknAlk1= unkown alkenyl, Unksul2= unkown sulfinyl, UnkAlk= unkown alkyl, UnkAli= unkown aliphatic.



Chapter 4

Plant quantity represents a greater constraint than quality for a gregarious insect herbivore and its endoparasitoid wasp

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Abstract

Virtually all studies of plant-herbivore-natural enemy interactions focus on plant quality as the major constraint on development and survival. However, for many gregarious feeding insect herbivores that feed on small or ephemeral plants, the quantity of resources is much more limiting, yet this area has received virtually no attention. Here, in both lab and semi-field experiments, we studied the effects of periodic food deprivation in a tri-trophic system where quantitative constraints are profoundly important on insect performance. The large cabbage white *Pieris brassicae*, is a specialist herbivore of relatively small wild brassicaceous plants that grow in variable densities, with black mustard (*Brassica nigra*) being one of the most important. Larvae of *P. brassicae* are in turn attacked by a specialist endoparasitoid wasp, *Cotesia glomerata* that requires its host to grow as large as or even larger than unparasitized caterpillars. Increasing the length of food deprivation of newly molted final instar caterpillars significantly decreased herbivore and parasitoid survival and biomass, but shortened their development time. Moreover, the ability of caterpillars to recover when provided with food again was negatively correlated with the length of the food deprivation period. In outdoor tents with natural vegetation, we generated conditions faced by *P. brassicae* broods in nature by manipulating plant density. Low densities of *B. nigra* lead to potential starvation of *P. brassicae* broods and their parasitoids, replicating nutritional conditions of the lab experiments. The ability of both unparasitized and parasitized caterpillars to find corner plants was similar but decreased with central plant density. Survival of both the herbivore and parasitoid increased with plant density and was higher for unparasitized than for parasitized caterpillars. Our results, in comparison with previous studies, reveal that quantitative constraints are far more important that qualitative constraints on the performance of gregarious insect herbivores and their gregarious parasitoids in nature.

Introduction

In nature, there is often considerable variation in the ways in which a species distributes itself within its habitat relative to conspecifics. Many organisms exhibit solitary lifestyles except when they are mating and/or raising their offspring. Still others live in groups of several to many thousands of individuals, although this may vary according to season. For group living to be favored, the overall benefits must outweigh the costs, and the resulting fitness pay-off to each group member must be greater than for a solitary individual (Keeling 2001). In vertebrates, an advantage of group living is the development of social interactions among group members, which is often based on a shared genetic background (Rona 1981). Similar patterns may be observed within invertebrate groups, e.g. insects, where gregarious living is often based on the genetic relatedness of the individuals in the group (Queller et al. 1988). This is certainly true in the social Hymenoptera, such as ants, bees and wasps, which reproduce via haplodiploidy and where distinct castes perform specific functions that optimize colony fitness (Hedrick and Parker 1997). Many phytophagous insects also lay their eggs in batches on their food plants and the immature (and sometimes adult) stages live in groups (Davies and Gilbert 1985, Le Masurier 1994, Fordyce 2003). In the Lepidoptera (butterflies and moths), for example, large clutch size and aggregative feeding by the larvae has evolved independently in up to 23 families (Sillen-Tullberg and Leimar 1988).

There are many obvious advantages of living in a group, (described as the Allee effect) which include the production of more offspring in a single reproductive event, faster development and more effective group-based defences against natural enemies (Allee et al. 1949, Riipi et al. 2001, Liebhold and Boscampte 2003, Reader and Hochuli, 2003). However group living involves costs that include higher levels of disease transmission amongst closely assembled individuals (Hochberg 1991, Brown et al. 2001), and increased apparency to natural enemies that have the capability of attacking multiple prey (Hunter 2000). Another important factor is that there is an increase in the level of intraspecific competition for potentially limiting food resources (Andrewartha and Birch 1954, Damman 1991, Konvicka et al. 2003, Reader and Hochuli 2003). Starvation of entire broods of insect herbivores and even population crashes may be mediated by the depletion of local food supply (Shorrocks and Swingland 1990). However, studies examining the evolution of gregarious development in insects rarely examine this parameter, and instead assume that later instars can always disperse to find new food plants once the natal plant is exhausted of tissues (e.g. Reader and Hochuli, 2003).

The vast majority of insect herbivores are specialized on plants producing phylogenetically conserved secondary metabolites (Loxdale et al. 2011). Food deprivation is particularly problematical for immature feeding stages of gregarious specialist herbivores that feed on small, fast growing and/or ephemeral plants that are present for only a short time in the field. However, this constraint is rarely discussed in studies which instead focus primarily on plant quality as the major determinant of insect performance (e.g. Awmack and Leather, 2002). However, if the natal food plant is exhausted before the insects have reached maturity, then they are forced to disperse in search of new suitable plants, which may or may not be located nearby. This can have severe repercussions on their survival and/or fitness. For instance, when they are not feeding they expend metabolic energy, do not grow, and are thus susceptible to starvation and precocious death. Le Masurier (1994) reported that only 16% of final instar *Pieris brassicae* (large cabbage white butterfly) larvae located fresh food plants 2.5 meters from release sites over the course of 72 hours when they were released from a central point. For gregarious herbivores that produce large broods on small plants the mother should optimally choose plants that grow in large aggregations. However, eggs and larvae of gregarious herbivores like the peacock butterfly, *Inachis io* and *P. brassicae* are occasionally laid on isolated plants and the larvae rapidly exhaust the plant and starve as a result.

Larval stages of Lepidoptera harbour many natural enemies, including parasitic wasps or ‘parasitoids’. These are insects whose larvae feed on, or in the host body whereas the adults are free living (Godfray 1994). One group of parasitoids called ‘koinobionts’ attack hosts that continue feeding, growing and defending themselves during parasitism (Harvey 2005). Solitary koinobiont parasitoids, in which only a single parasitoid larva develops inside the host, generally have lower nutritional requirements than gregarious parasitoids (Harvey 2005). As a result, solitary parasitoids generally greatly reduce host growth, relative to that of unparasitized larvae, whereas gregarious parasitoids have little effect or may even stimulate increased host food consumption and growth (Harvey 2000). Consequently, when a gregarious endoparasitoid attacks a gregarious-feeding herbivore host that in turn feeds on a small plant with insufficient biomass to support an entire brood, both organisms can potentially experience significantly negative effects on their survival and fitness. Thus far, however, this area has been little studied.

Here, we examine development of *P. brassicae* L. (Lepidoptera: Pieridae) and its specialist gregarious endoparasitoid, *Cotesia glomerata* L. (Hymenoptera: Braconidae) under variable periods of food deprivation in the final instar. The final instar is critical for the development of most holometabolous insect herbivores, as 80% or more of larval biomass is accrued during this stage (Slansky 1976). *Pieris brassicae* is a specialist herbivore of plants in the family Brassicaceae. One of the most important food plants in the Netherlands for *P. brassicae* is the black mustard, *Brassica nigra* (Brassicaceae), a small, fast growing annual weed which grows along rivers and roadsides (Fig. 1). *Pieris brassicae* lays broods of 30-100 eggs on the underside of mustard leaves and the larvae complete 5 instars before pupation. Instars 1-3 (hereafter L1-L3) feed in tight aggregations on the plant whereas the later instars disperse to some extent. *C. glomerata* females lay 20-40 eggs into L1-L2 *P. brassicae* larvae (Harvey 2000). Caterpillars parasitized by *C. glomerata* are not killed by the parasitoid until the final instar, and often grow as large as unparasitized caterpillars (Harvey, 2000).

In the field we have found *P. brassicae* broods growing on both isolated, solitary plants and plants growing in large populations (Fig. 1). This tri-trophic system is both natural and a model system for studying nutrition-related constraints on herbivore (and natural enemy) survival and development. Thus far, studies with *P. brassicae* (with or without *C. glomerata*) have focused almost exclusively on plant quality effects on growth, development and survival (Mattiaci et al. 2001, Harvey et al. 2003, Sznajder and Harvey, 2003, Soler et al. 2005, Maskato et al. 2014, Pashalidou et al. 2015a, b) with only scant attention paid to plant size and/or quantity (Davis and Gilbert 1985, Le Masurier 1994). Indeed, *P. brassicae* has also been recently studied on *Arabidopsis thaliana*, where a single plant is too small to support the development of even a single caterpillar, let alone 50 or more (Geiselhardt et al. 2013, Hilfiker et al. 2014, Rasmann et al. 2015). Clearly studies ignoring quantitative constraints are lacking in ecological realism (Harvey et al., 2015).

Figure 1. Clustered population of *Brassica nigra* plants (upper left), a solitary plant (upper right) and feeding *Pieris brassicae* caterpillars (below).

In lab experiments, we determined how periodic food deprivation of unparasitized and parasitized final-instar caterpillars affected survival and development (body mass and development time) of *P. brassicae* and *C. glomerata*. We also investigated the ability of herbivores and their parasitoids to recover from variable periods of food deprivation when reacquainted with plant diet. We then mimicked the starvation experiments in a semi-field experiment by placing parasitized and unparasitized caterpillars in groups of 50 onto plants grown in differing densities to determine how variation in plant biomass and natural barriers to dispersal -such as that provided by neighbouring vegetation- affects the survival of both parasitized and unparasitized larvae. We hypothesize that (i) food deprivation reduces herbivore and parasitoid survival and fitness, (ii) both unparasitized and parasitized caterpillars are able to recover from food deprivation depending on the length of the food deprivation period, and that (iii) in the field herbivore and parasitoid survival are negatively affected by a reduction in the density of focal plants, even when other mustard plants are placed nearby. As parasitism induces stress to the caterpillars, parasitized caterpillars will find new plants less successfully than unparasitized ones. We discuss our results in the context of food quantity and the spatial distribution of food plants as a major constraint on dietary breadth and development in gregarious herbivorous insects.

Materials and methods

Plants and insects

Seeds were collected from large populations of *Brassica nigra* which grow naturally near Wageningen in the Netherlands and were pooled from 15-20 plants. Plants were grown from seeds in 1.1-L pots filled with peat soil (‘Lentse potgrond’ no. 4; Lent, the Netherlands) in a climate-controlled greenhouse at 21±2°C (day) or 16±2°C (night), 50% relative humidity, and 16h day length. If light dropped below 225μmol photons $m^{-2}s^{-1}$ during the day, supplementary illumination was applied by sodium lamps. Plants were watered twice a week. When plants were 3 weeks old, they were fertilized weekly with Hoagland solution. Plants were approximately 4 weeks old at the start of each experiment. Watering and fertilization continued during the experiments.

Pieris brassicae and *Cotesia glomerata* were obtained from a culture maintained at the Laboratory of Entonology, Wageningen University (WU), The Netherlands. *Pieris brassicae* were reared on Brussels sprout plants (*B. oleracea* var. *gemmifera,* cv. Cyrus) in a climate room at 22±2°C, 50-70% relatively humidity, and a photophase of 16:8D at WU. Cultures of *C. glomerata* were reared on *P. brassicae* feeding on Brussels sprout plants, which were maintained under the same conditions as the host at the Netherlands Institute of Ecology (NIOO-KNAW).

General experimental protocol

To obtain *P. brassicae* caterpillars for the experiments, over two successive days, 3-week old *B. nigra* plants were placed in the rearing cage with adult *P. brassicae* butterflies at the Entomology Laboratory for 24h. Plants containing egg clusters were removed from the butterfly cage and transferred to the experimental greenhouse. Once the eggs hatched the larvae were allowed to develop into the early 2nd instar (L2).

Mated *C. glomerata* females used for parasitism originated from the general culture maintained at the NIOO-KNAW. Early L2 instar *P. brassicae* larvae were parasitized by presenting them individually to female parasitoids at the end of a small brush in a plastic vial. Previous studies (Harvey, 2000, Sznajder and Harvey, 2003) showed that virtually 100% of stung caterpillars produce parasitoids (encapsulation does not occur).

As proxies for insect performance, we measured herbivore development time from egg hatching until pupation and pupal fresh mass, which was weighed on a Mettler-Toledo (Columbus, OH USA) microbalance (accuracy±1µg). For the parasitoid, we determined the development time from oviposition to adult eclosion, adult fresh mass and secondary brood size (number of egressing parasitoid larvae that successfully spun cocoons). From each brood, a sub sample of five males and or five females were weighed on the microbalance. Parasitoids were anaesthetized with CO_2 before weighing.

Experiment 1. Effect of food deprivation duration of L5 Pieris brassicae *on the performance of herbivores and parasitoids*

Insect herbivores obtain 80% or more of biomass in the final instar, therefore, we examined temporal food deprivation in L5 *P. brassicae* on the performance of herbivores and parasitoids. Five cohorts of 150 1-day old L2 larvae were randomly selected, and each cohort was transferred to cages (40 x 40 x 55 cm) containing five *B. nigra* plants (five cages in total). In addition, five cohorts of 80 1-day old L2 larvae were randomly selected for parasitism by female *C. glomerata* wasps. Following parasitism, larvae were transferred in groups of 80 to cages containing five *B. nigra* plants (five cages in total). Unparasitized and parasitized caterpillars were allowed to move and feed freely on the plants within a cage. Additional plants were added when necessary. Larvae were reared in their respective cages with excess food until they molted into L5.

Pieris brassicae caterpillars were deprived of food at variable times during L5 beginning on the first day. When caterpillars molted into L5 they were transferred to new cages in densities of 130 unparasitized or 60 parasitized caterpillars, with 5 separate cages for unparasitized caterpillars and 5 cages for parasitized caterpillars. Each cage contained 5 *B. nigra* plants. Five cohorts of 20 unparasitized and 8 parasitized from early L5 caterpillars were not transferred to new cages, but were instead placed in 9.5-cm Petri-dishes (1 caterpillar per dish) without food. On consecutive days thereafter 20 unparasitized caterpillars and 8 parasitized caterpillars were randomly taken from each of the cages and reared without food in Petri-dishes (as above); this was continued until either pupation or parasitoid emergence in cages with food plants.

The survival of both unparasitized and parasitized caterpillars in Petri-dishes was recorded daily. The pupae of surviving caterpillars were weighed and the day of pupation was recorded. For parasitized caterpillars, when several adult wasps had emerged, the time of eclosion was recorded and the wasps were weighed.

Experiment 2. Insect recovery ability after variable food deprivation periods of L5 Pieris brassicae

As the field experiment shows, some caterpillars are able to leave the natal plant when it is exhausted and find new plants nearby. Therefore, we set up an experiment to test the recovery ability of insects after variable food deprivation periods.

Unparasitized and parasitized caterpillars were prepared as described for *Experiment 1* until molting into L5 and transferred into new cages. Most caterpillars in *Experiment 1* died when deprived of food during the first 3 days of their final (L5) instar. Therefore, three groups of 20 unparasitized and 15 parasitized caterpillars were randomly selected from the cages on the first day of L5 (4 replicates cages for unparasitized caterpillars, 5 replicates cages for parasitized caterpillars). These larvae were thereafter deprived of food for either 1, 2 or 3 days after which time they were once again provided with food plants. A second group of unparasitized caterpillars (4 replicate cages) was allowed to feed until day 3 since molting into L5. Then three groups of 20 unparasitized were randomly selected from the cages that were deprived of food for either 1, 2, or 3 days before they were re-provided with food plant. Because there were insufficient numbers of parasitized caterpillars, the second treatment was done with unparasitized caterpillars only.

During the food deprivation period caterpillars were maintained individually in labeled Petri-dishes (1 caterpillar per dish), and status (alive or dead) was monitored daily. After the food deprivation periods, insects from each replicate were placed in a new cage with 5 *B. nigra* plants. The survival and performance of these insects was followed until pupation of unparasitized caterpillars, or adult parasitoid eclosion. Performance parameters were measured using the same methods as described in *Experiment 1*.

Experiment 3. Effect of natal plant density on survival of unparasitized and parasitized caterpillars in semi-field set-up

To investigate the effect of food plant availability on survival of unparasitized and parasitized caterpillars, and on their ability to find a new food plant away from the natal patch, a semi-field experiment was conducted (for plant species composition see Table S1) adjacent to the NIOO-KNAW. Fifteen 3 x 3 m experimental plots were enclosed in meshed tents that prevented predation by birds (5 cm mesh). In the center of each tent potted plants were placed together in densities of 1, 5 or 8 *B. nigra* plants (5 replicates each), while in each corner of the tents 1.5 m away 2 potted *B. nigra* plants were placed.

Cohorts of 25 randomly selected 1-day old unparasitized and parasitized L2 larvae were transferred to each of 15 *B. nigra* plants. Caterpillars were kept on mustard plants in the greenhouse until they molted into the L3, when they are unsuitable for parasitism by *C. glomerata* (Harvey 2000). Plants with L3 caterpillars were then placed in the center area of the tents. Plants in the corner of each tent were thereafter checked daily for *P. brassicae* caterpillars which were then removed from the plants and dissected in the lab to determine whether they had been parasitized or not. When caterpillars reached late L5 just prior to pupation, they were collected, both from center and corner plants, to ensure that they did not enter the wandering phase when they would be hard to find. The experiment was replicated three times in the summer of 2013 (from July to early September) generating a total of 15 replicates per plant density.

Statistical analysis

For statistical analyses, cages (experiment 1 and 2) or tents (experiment 3) served as experimental units. The performance variables, development time and biomass, in experiment 1 and 2 were analyzed using a general linear mixed model (GLMM) where food deprivation period was entered as a fixed factor and cage as a random factor. The variance components were estimated using restricted maximum likelihood (REML) and the degrees of freedom in the approximated F-tests were adjusted according to the Satterthwaite method (Littell et al. 2002). Tukey-Kramer multiple comparison tests between means were conducted when the model was significant.

For the parasitoid, the data were analyzed separately for males and females. Being gregarious, multiple *C. glomerata* individuals egress from a single caterpillar host. Thus, caterpillar host was included as a second random factor. The brood size has been demonstrated to affect the size/mass and development of the emerging adult wasps (Harvey 2000). Therefore, brood size was included as a covariate in the model. The GLMM analyses were performed using the statistical package SAS version 9.3 (SAS Institute Inc., Cary NC, USA).

Logistic regression, i.e. a generalized linear model (GLM) was employed with a binomial distribution and logit link function, to analyze survival of unparasitized and parasitized caterpillars in relation to those subjected to variable periods of food deprivation. In the case of over-dispersion, we corrected for this by allowing the variance functions of the binomial distribution to have a multiplicative over-dispersion factor. The response variable in this analysis was the number of caterpillars that developed into pupae (unparasitized caterpillars) or produced parasitoid wasps (parasitized caterpillars) out of the total number of caterpillars that were initially collected for each food deprivation period. Caterpillar treatment (unparasitized or parasitized) was entered as a fixed factor and food deprivation period as a covariate. For the data on recovery ability following-food deprivation, we used a similar approach. In this analysis the time of initial food deprivation (early L5 or mid L5 caterpillar stages) was entered as a fixed factor and food deprivation period as a covariate (unparasitized caterpillars only). In a second analysis, we compared survival of unparasitized and parasitized caterpillars that were deprived of food as early L5 for variable time periods.

The data on survival in the semi-field experiment were also analyzed using logistic regression. We first investigated whether plant density of the central plant patch in the center of the tent had an effect on the proportion of the surviving caterpillars that were recovered on the additional plants placed in the corners of the tent at 1.5 meters distances from the central patch. As a measurement of overall survival, we also compared the recovery rate of the 25 released caterpillars either on the central or distant *B. nigra* plants. In both analyses caterpillar treatment was entered as a fixed factor and plant density as a covariate. Logistic regression analyses were performed in Genstat 17 (VSN International Ltd., Hemel Hempstead, UK).

Results

Experiment 1. Effect food deprivation duration of L5 Pieris brassicae *on the performance of herbivores and parasitoids*

Herbivore pupal mass and development time. Food deprivation period had a significant effect on pupal mass of *P. brassicae* ($F_{4, 25.43} = 134$, $P < 0.001$) and development time ($F_{4, 18.54} = 44.6$, $P < 0.001$). Pupal mass was negatively correlated with the duration of the food deprivation period (Fig. 2A). Moreover, the longer the food deprivation period, the faster the larvae developed (Fig. 2B). However, the longest development time was found for caterpillars that were deprived of food one day prior to control caterpillar pupation (Fig. 2B).

Parasitoid adult biomass and development time. Male and female wasp data were analyzed separately, but overall, the fresh mass of adult female wasps was higher (Fig. 2C) and their development longer, than in male wasps (Fig. 2D). The length of the food deprivation period had a significant effect on adult fresh mass (GLMM: males, $F_{4, 142.6} = 11.6$, $P < 0.001$; females, $F_{4, 132.6} = 14.2$, $P < 0.001$). However, the effect of food deprivation duration on adult mass also depended on the brood-size (GLMM: brood size-food deprivation interaction: males, $F_{4, 150} = 2.94$, $P = 0.023$; females, $F_{4, 144.4} = 3.27$, $P = 0.013$). Similar as for unparasitized hosts, wasps weighed less the longer they were deprived of food (Fig. 2C).

Food deprivation treatment, and brood size and their interaction had a significant effect on male development time (GLMM: treatment, $F_{4,\ 133.2} = 2.60$, $P = 0.039$; brood size, $F_{1,\ 153.6} = 18.98$, $P < 0.001$; interaction, $F_{4,\ 145.3} = 3.10$, $P = 0.018$). In general, development time decreased with brood size, however, the slope of this relationship depended on the food deprivation treatment. Here no consistent pattern could be discerned in relation to food deprivation duration. For females, only brood size had a significant effect on development time ($F_{1,\ 146.6} = 8.69$, $P = 0.004$), whereas both the effect of the food deprivation treatment and the interaction with brood size-treatment were not significant (GLMM: treatment, $F_{4,\ 133.3} = 1.87$, $P = 0.12$; brood size-treatment interaction, $F_{4,\ 143.7} = 1.17$, $P = 0.33$). As it is, there was no clear pattern for both male and female wasp development time with treatment (Fig. 2D).

Parasitoid secondary brood size. Food deprivation treatment also had a significant effect on secondary wasp brood size ($F_{5,\ 14.78} = 41.98$, $P < 0.001$). Brood size decreased dramatically from approximately 30 in host caterpillars that were not deprived of food or were deprived of food for only 24-48 h prior to parasitoid larval egression to approximately 5 in host caterpillars that were deprived of food from the first day following the final molt (Fig. 3).

Herbivore and parasitoid survival. Food deprivation duration dramatically reduced survival of the insects (GLM: $\chi^2_1 = 717$, $P < 0.001$, Fig. 4). The effect of food deprivation duration on survival was not significantly different for unparasitized *P. brassicae* caterpillars and those parasitized by *C. glomerata* (GLM: $\chi^2_1 = 2.89$, $P = 0.09$), neither was the interaction between density and species significant (GLM: $\chi^2_1 = 1.21$, $P = 0.27$).

Experiment 2. Insect recovery ability after variable food deprivation periods of L5 Pieris brassica

Herbivore pupal mass and development time. The ability to recover from food dependedon the duration of food deprivation both in terms of pupal mass ($F_{7,\ 29.74} = 91.98$, $P < 0.001$) and development time ($F_{7,\ 27.55} = 26.4$, $P < 0.001$). For pupal mass, this ability decreased more strongly with the duration of food deprivation for caterpillars that were deprived of food as early L5 compared to mid L5. However, caterpillars that were deprived of food for only one day when they were mid L5 did not fully recover from this, whereas caterpillars that were deprived of food for one day when they were early L5 could fully recover (Fig. 5A).

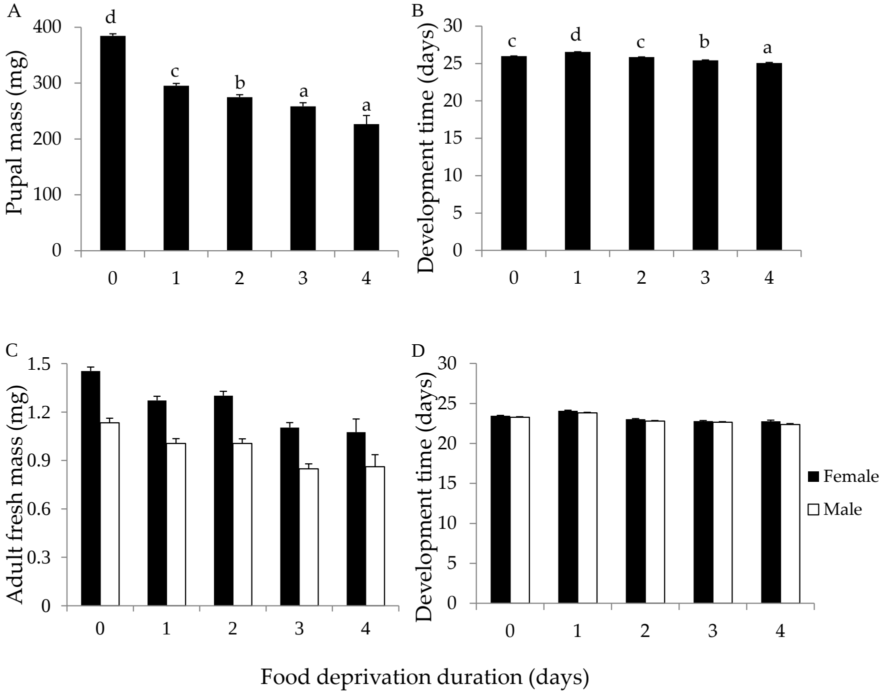


Figure 2. Effect of food deprivation of L5 *Pieris brassicae* on the performance of herbivores and parasitoids. Pupal fresh mass (A) and egg-to-pupa development time (B) of *Pieris brassicae*, and adult fresh mass (C) and egg-to-adult development time (D) of *Cotesia glomerata* females (black bars) and males (white bars). Bars (means + SE) with different letters are significantly different from each other (Tukey multiple comparisons). Sample sizes per cage (five cages per food deprivation treatment) were for *P. brassicae*: undeprived of food controls: 20; deprived of food for one day: 18-19; two days: 12-20; three days: 6-11; four days: 0-5, and for *C. glomerata*-parasitized hosts: undeprived of food controls: 8; deprived of food for one day: 7-8; two days: 6-8; three days: 4-8; four days: 3-6. For *C. glomerata*, adult fresh mass and development time was determined for the first 5 males and 5 females eclosing from each host.

When the food deprivation period was initiated one day following the final molt, development time increased with the length of the food deprivation (Fig. 5B), whereas the effect of the length of food deprivationwas only minor orabsent when this was initiated when the larvae were already in the mid L5 stage (Fig. 5B).

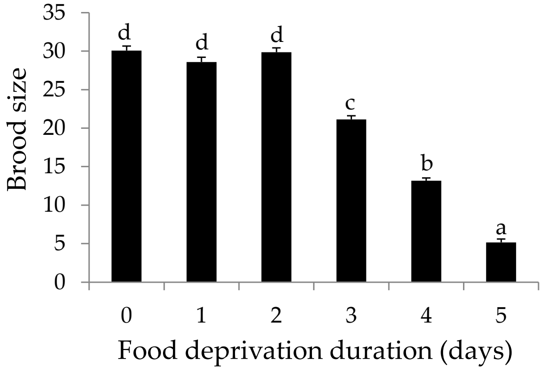


Figure 3. Effect of food deprivation duration of L5 *Pieris brassicae* on secondary brood size of *Cotesia glomerata*. Bars (means + SE) with different letters are significantly different from each other (Tukey multiple comparisons). Sample sizes were per cage (five per food deprivation treatment): undeprived of food controls: 8; deprived of food for one day: 7-8; two days: 7-8; three days: 6-8; four days: 4-6; five days: 1-2.

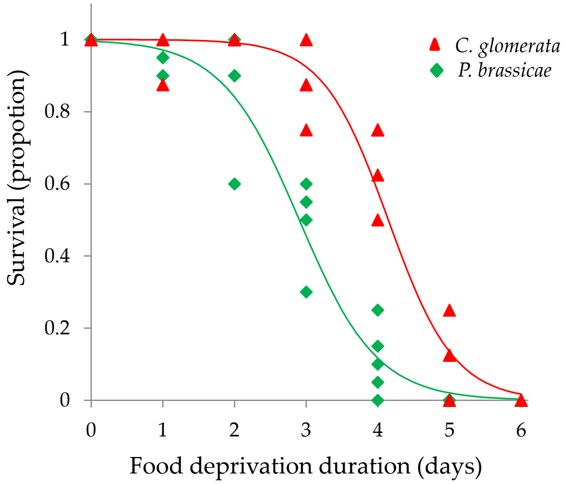


Figure 4. Effect of food deprivation duration of L5 *Pieris brassicae* on survival rate of herbivore (green diamond, *Pieris brassicae*) and parasitoid (red triangle, *Cotesia glomerata*).

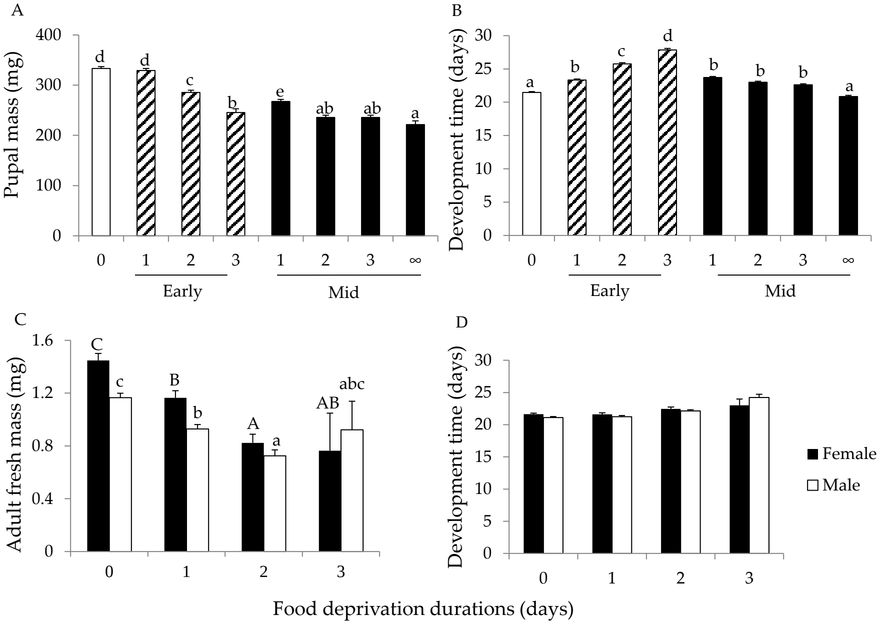


Figure 5. Insect recovery ability after variable food deprivation period of L5 *Pieris brassicae*. Pupal fresh mass (A) and egg-to-pupa development time (B) of *Pieris brassicae*, and adult fresh mass (C) and egg to adult development time (D) of *Cotesia glomerata* females (black bars) and males (white bars). Unparasitized *P. brassicae* caterpillar were deprived of food for variable periods as early L5 (dashed bars) or mid L5 (white bars), whereas hosts parasitized by *C. glomerata* were deprived of food only as early L5. Bars (means + SE) with different letters are significantly different from each other (Tukey multiple comparisons). Sample sizes for *P. brassicae* were per cage (four cages per food deprivation treatment): without food deprivation controls: 20; deprived of food for one day as early L5: 20; two days as early L5: 15-19; three days as early L5: 5-8; deprived of food for one day as mid L5: 14-20; two days as mid L5: 15-19; three days as early L5: 15-18; food deprivation deprived of food as mid L5 until pupation: 5-7. Sample sizes for *C. glomerata* were per cage (five cages per food deprivation treatment): without food deprivation controls: 12-15; deprived of food for one day as early L5: 9-14; two days as early L5: 3-12; three days as early L5: 1-3. For *C. glomerata*, adult fresh mass and development time was determined for the first 5 males and 5 females eclosing from each host.

Parasitoid adult biomass and development time. The ability to recover from food deprivation in terms of adult fresh mass depended on food deprivation duration for both male and female wasps (GLMM: male, $F_{3, 79.35} = 18.4$, $P < 0.001$; female, $F_{3, 58.28} = 8.79$, $P < 0.001$) and was not affected by brood size (GLMM: male, $F_{1, 138.7} = 0.34$, $P = 0.56$; female, $F_{1, 94} = 1.46$, $P = 0.23$). Adult fresh mass of both sexes declined the longer the wasps were deprived of food before they were provided with food again, except for the male wasps with the longest (3 days) food deprivation periods (Fig. 5C). Both male and female development time was longer in wasps that were deprived of food the longest before being provided with food again but this was only statistically significant for the males (Fig. 5D). For males, the ability to recover in terms of development was affected by food deprivation period (GLMM: $F_{3, 44.02} = 26.3$, $P < 0.001$) and brood size (GLMM: $F_{1, 122.1} = 10.0$, $P = 0.002$), but also by the interaction between brood size and food deprivation period (GLMM: $F_{3, 163.1} = 4.68$, $P = 0.004$). The development time of female wasps was neither effected by food deprivation period (GLMM: $F_{3, 31.23} = 1.03$, $P = 0.39$), brood size (GLMM: $F_{1, 70.5} = 0.75$, $P = 0.39$) nor was the interaction between these parameters significant (GLMM: $F_{3, 108} = 0.22$, $P = 0.88$).

Parasitoid secondary brood size. Parasitoid brood size was affected by the duration of host deprivation before hosts were re-provided with food (GLMM: $F_{3, 10.28} = 15.4$, $P < 0.001$) and decreased the longer that hosts were deprived of food (Fig. 6).

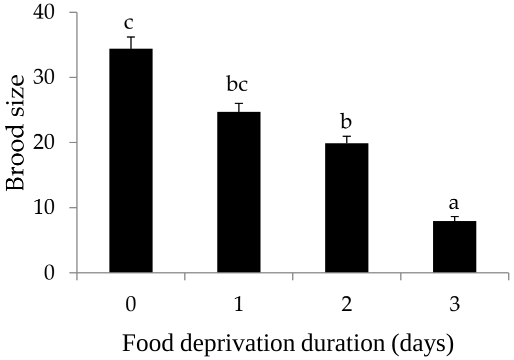


Figure 6. Effect of variable food deprivation periods of early L5 *Pieris brassicae* on secondary brood size of *Cotesia glomerata*. Bars (means + SE) with different letters are significantly different from each other (Tukey multiple comparisons). Sample sizes were per cage (five cages per food deprivation treatment): without food deprivation controls: 14-15; deprived of food for one day: 12-14; two days: 4-12; three days: 1-3.

Herbivore and parasitoid survival. Food deprivation period affected recovery of unparasitized *P. brassicae* caterpillars that were deprived of food as early L5 more strongly that those deprived of food as mid L5 in terms of survival (GLM: onset of food derivation duration interaction, $\chi^2_1 = 76.5$, $P < 0.001$; Fig 7A). Recovery of unparasitized and parasitized caterpillars that were deprived of food were similarly negatively affected by the length of the food deprivation period (GLM: species, χ^2_1 = 4.64, P = 0.031; food deprivation duration, χ^2_1 = 700, P < 0.001; species-food deprivation duration interaction, χ^2_1 = 2.96, P = 0.085; Fig. 7B). The survival rate decreased dramatically with the length of food deprivation (Fig. 7B).

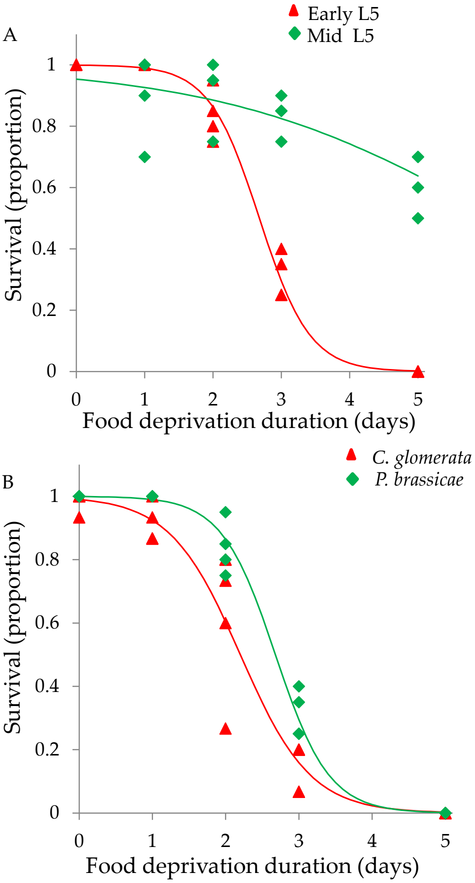


Figure 7. Effect of variable food deprivation period from early (red triangle) or mid (green diamond) L5 on survival rate of *Pieris brassicae* (A) and effect of variable food deprivation period of early L5 *Pieris brassicae* (green diamond) or *Cotesia glomerata* (red triangle) (B).

Experiment 3. Effect of natal plant density on survival of unparasitized and parasitized caterpillars in semi-field set-up

The density of the central food-plant patch significantly affected dispersal to distant plants at 1.5 m distance (GLM: χ^2_1 = 104, P < 0.001) and this response was similar for unparasitized and parasitized caterpillars (GLM: species, χ^2_1 = 0.88, P = 0.35; species-density interaction, χ^2_1 = 2.74, P = 0.10; Fig. 8A). Fewer caterpillars were found on distant *B. nigra* plants (=corner plants) when the density of the central food-plant patch (=center plants) increased. As an indication of mortality we also measured total recovery of the insects on any plant in the tent. The recovery of caterpillars increased with the density of plants in the central food patch (GLM: $\chi^2_1 = 67.2$, $P < 0.001$) and was higher for unparasitized caterpillars than for the parasitized ones (GLM: species, $\chi^2_1 = 20.3$, $P < 0.001$; species-density interaction, $\chi^2_1 = 1.04$, $P = 0.31$; Fig. 8B).

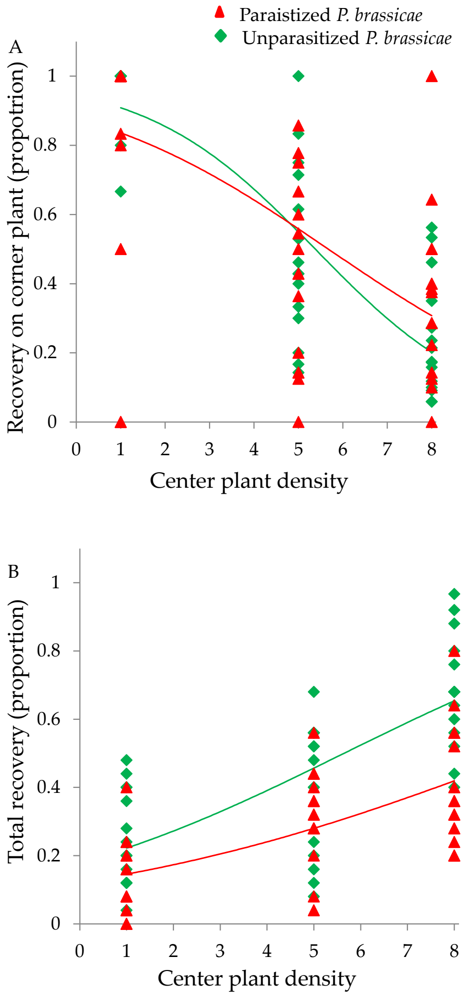


Figure 8. Relative recovery rate of unparasitized *Pieris brassicae* (green triangle) and parasitized *Pieris brassicae* (red triangle) on corner plants (A) with different center plant densities, and total recovery of *Pieris brassicae* (green diamond) and *Cotesia glomerata* (red triangle) out of a total of 25 released insects (B).

Discussion

The results of this investigation show that depriving *P. brassicae* caterpillars of food for variable periods in the final instar significantly affected the survival and development of both the herbivore and its endoparasitoid, *C. glomerata*. Mortality of unparasitized caterpillars that were deprived of food one day after molting to the final (L5) instar was almost 100%, revealing that they did not reach a minimum viable size for pupation. However, when larvae were deprived of food for a shorter duration i.e. from 3rd day of the final instar onwards, survival increased rapidly. Pupal mass was negatively correlated with the duration of food deprivation; caterpillars deprived of food from the second day of molting only attaining 60% of the pupal mass as controls that were fed *ad libitum* until pupation. The final day of feeding is critical for *P. brassicae,* as larvae that were deprived of food for just one day prior to pupation were still some 25% smaller in terms of pupal mass than controls. By contrast, food deprivation had quite different effects on development time. Caterpillars that were deprived of food from the second day after molting to L5 developed significantly faster than those deprived of food for shorter periods or controls. On the other hand, larvae deprived of food just one day prior to control caterpillars took longer to develop than in any other treatments.

The effect of food deprivation on development in *C. glomerata* was similar to that of its host, with higher mortality, smaller brood size and smaller body mass of adult wasps developing in hosts deprived of food for longer periods. Larvae of *C. glomerata* feed primarily on hemolymph and fat body and consume about 80% of the fat content of their hosts (Harvey et al. 2008). As brood size increases, however, more fat is consumed, increasing potentially antagonistic interactions amongst siblings. A significant reduction in brood survival may have occurred through asymmetrical contest competition enabling a small number of 'winners' to exploit more *per capita* host resources. If competition had been more symmetrical amongst the siblings under pure scramble competition there would not have been enough food to facilitate survival of all siblings meaning all would have perished. Thus, under conditions of periodic food deprivation in the host, contest competition amongst parasitoid siblings increases the chance that at least a few wasps survive, revealing some level of adaptation to resource-related constraints in *C. glomerata*. Starvation has been shown to affect the survival of other solitary endoparasitoids (Dover &

Vinson, 1990; Harvey et al., 1995) but few studies have examined how starvation affects gregarious endoparasitoids (Segoli et al., 2010).

When unparasitized and parasitized caterpillars of *P. brassicae* were initially deprived of food for variable periods and then resupplied with *B. nigra* plants again, there were also significant effects on survival and development of both the herbivore and parasitoid. Caterpillars that were deprived of food for one day as early L5 almost totally recovered, but this declined sharply as the duration of food deprivation increased. Importantly, even short term food deprivation (e.g. 24 h) negatively affected insect performance, even when these individuals were given access to plants again. Thus, any kind of interruption in the feeding behaviour of the herbivore can have significant fitness related effects on both itself and its endoparasitoid, and in the herbivore at least, these effects are correlated with the age (in L5) of the caterpillar.

In the semi-field experiments, we found that the density of clustered *B. nigra* plants significantly affected survival and dispersal of both unparasitized and parasitized *P. brassicae* caterpillars. The larger the density of centrally placed plants in tents the less likely the larvae were to emigrate in search of new mustard plants and the more likely they survived to late L5. The survival of parasitized caterpillars was somewhat lower then unparasitized caterpillars, suggesting that the former group is under greater physiological stress (e.g. nourishing two trophic levels instead of one) than the latter. Le Masurier (1994) found that whereas 80% of released L4-L5 *P. brassicae* caterpillars located fresh cabbage plants placed 0.5 meters away from the release point, only 16% of larvae were able to locate fresh cabbage plants 2.5 meters away. However, in that study the larvae did not disperse voluntarily based on food availability but were released presumably as well fed caterpillars. Moreover, the background consisted largely of bare ground, which does not replicate a natural situation. In our experiments the caterpillars were allowed to choose when to leave the central plant(s) and had to navigate through dense stands of natural vegetation to reach new mustard plants 1.5 meters away. This vegetation not only represents a serious impediment to movement, but is also likely to harbor many natural enemies that pose a threat to dispersing caterpillars.

The progeny of gregarious butterflies developing on isolated plants may survive if nutritionally suitable plants are located in close proximity to the natal plant. However, there are both costs incurred as a result of energy expended in searching during the feeding phase of the life cycle and an increase in the risk of predation during the search for new food plants. Moreover, if new plants are not located then the entire brood may starve. For example, starving L4-L5 caterpillars of the peacock butterfly, *Inachais io*, and *P. brassicae* have been observed on their respective food plants, *Urtica dioeca* and *B. nigra* in nature that were tens of meters away from other suitable plants of the same species (Fig. 9A). However, some gregarious Lepidoptera do adjust clutch size when choosing isolated plants with limited biomass. For instance, the cinnabar moth, *Tyria jacobeae* (Fig. 9B) typically lays no more than 10-15 eggs on tansy ragwort (*Jacobea vulgaris*) plants (Myers 1980). However, in this association, plants never grow in clusters but as individuals that are always separated from neighboring conspecifics by up to several meters. This suggests that spatial distribution of ragwort plants is predictable and has been a major selective force in the evolution of clutch size in the cinnabar moth. Interestingly, *P. brassicae* has been shown not to adjust its clutch size in the field (Courtney, 1986).

Food limitation is clearly a potential threat to the survival and fitness of many gregarious-feeding insect herbivores and their parasitoids in nature, especially those associated with fast-growing, ephemeral plants like *B. nigra* and other weedy species. In much of Europe *P. brassicae* is restricted to feeding on wild brassicaceous plants that only grow in large enough clusters to support the development of a single brood (Chapter 2). This limits the number of quantitatively (but not qualitatively) suitable plant species on which they can develop and survive to around 6. By contrast, the solitary congener *P. rapae* has been recorded on over 40 brassicaceous species in Europe alone (Feltwell 1982), including tiny species such as *A. thaliana*. This clearly shows that quantitative resource-related constraints on solitary specialist herbivores are considerably less than in gregarious species.

Thus far, most experiments studying plant-herbivore-natural enemy interactions in the lab as well as in the field select plants of optimal age and/or quality for the insects and, tacitly at least, assume that resources are not limiting (Awmack & Leather 2002). Although broods of *P. brassicae* caterpillars depend on multiple plants growing together to ensure sufficient food is available to complete their development, focus thus far has been primarily on qualitative differences in plant tissues mediated by, for example, plant direct defence responses to herbivore damage (Awmack & Leather, 2002; Soler et al., 2005; Pashalidou et al., 2015a,b). However, as we have shown here, the amount of food available to gregarious insect herbivores may be a much more serious constraint on their development and survival than quality. Optimality theory suggests that gregarious herbivores with large food requirements should always choose clustered plant resources or at least patches where the amount of food is more than necessary to support the successful development of the entire brood (Mayhew 1998). Studies by Pilson and Rauscher (1988) and Bergstrom et al. (2006) showed that clutch size decisions in two gregarious butterfly species are based on plant quality and not size. Neither study, however, measured constraints imposed by plant size and population structure on the development and survival of large broods, a major omission.

In summary, we have shown that food limitation exerts significant costs on the development and survival of a specialist butterfly and its endoparasitoid wasp. Our study reflects conditions faced by these insects in nature but quantitative constraints on development have thus far received little attention. Importantly, the spatial arrangement (and/or size) of food plants has rarely been considered in empirical studies with gregarious herbivores. Moreover, limitation in food supply is not only based on plant density, but on other factors as well, such as age, and on the abiotic environment i.e. temperature, moisture etc. that may also affect plant quality (Chaves et al. 2002). We stress that future studies with both solitary and gregarious herbivores and their natural enemies - in particular, specialists - that are associated with small or ephemeral plants need to acknowledge the profound importance of quantitative constraints, and how these may be more important factors than quality in determining their success.

Acknowledgments

The authors thank Roel Wagenaar for rearing cultures of *C. glomerata* and the Laboratory of Entomology at WUR for supplying *P. brassicae*. Miriama Malcicka is thanked for helping with experiment. Louise Vet and members of Terrestrial Ecology Department are thanked for discussion. This work is supported by a grant from the China Scholarship Council.

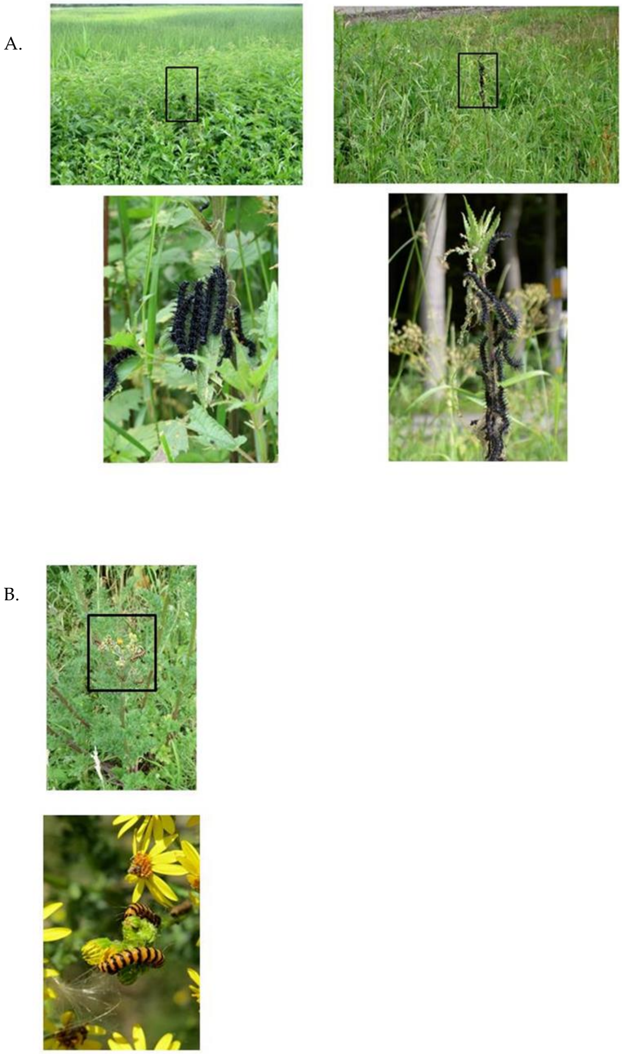
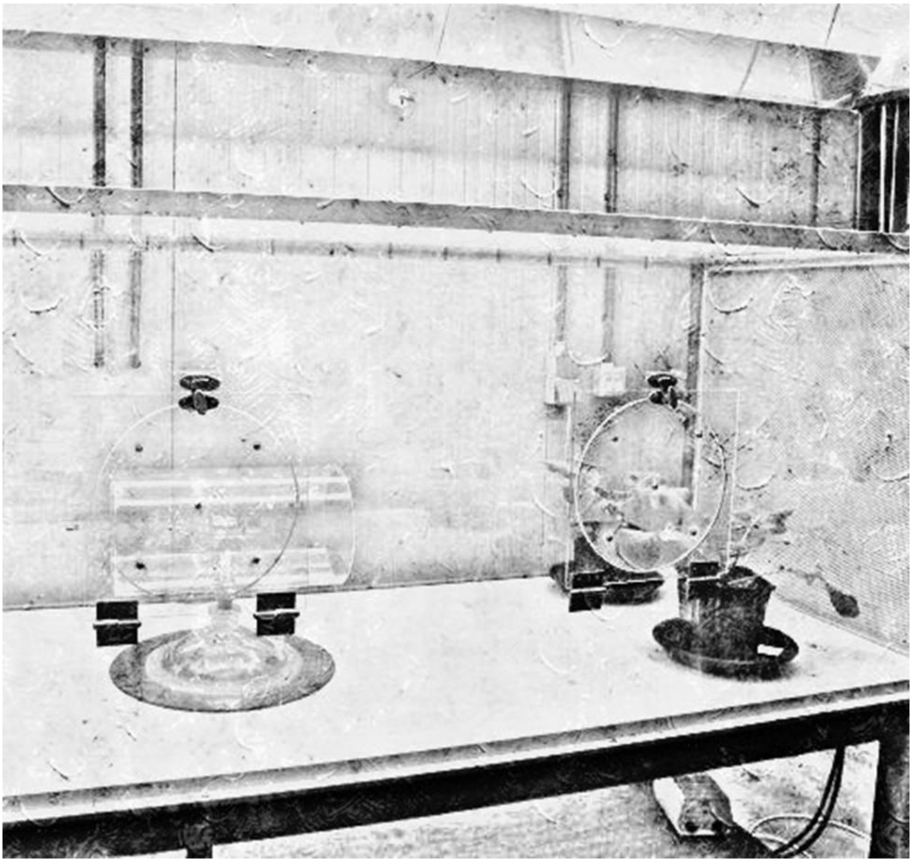


Figure 9. (A) Larvae of the Peacock Butterfly (inset and below), *Inachais io*, on aggregated plants (top) and an isolated plant (below) of the Nettle, *Urtica dioeca*. (B) Larvae of the Cinnabar Moth, *Tyria jacobaea* (inset and below) on leaves of their food plant, the Tansy Ragwort *Jacobaea vulgaris*.

Supplematary material

Table 1 Plant species of vegetation background in the semi-field experiment:

1.	*Plantago lanceolata*
2.	*Trifolium repens*
3.	*Taraxacum officinale*
4.	*Tripleurospermum inodorum*
5.	*Melilotus officianalis*
6.	*Melilotus albus*
7.	*Jacobaea vulgaris*
8.	*Lolium perenne*
9.	*Rumex acetosa*
10.	*Festuca rubra*
11.	*Cirsium arvense*
12.	*Solidago gigantea*
13.	*Ranucanlus acris*
14.	*Conyza canadensis*
15.	*Equisetum telmateia*
16.	*Elipobium hirsutum*
17.	*Urtica dioeca*
18.	*Tetragonolobus maritimus*
19.	*Lythrum salicaria*
20.	*Helichrysum orientale*
21.	*Medicagosativa*
22.	*Artemisia biennis*
23.	*Ononis spinosa*
24.	*Tephroseris integrifolia*
25.	*Symphytum officinale*



Chapter 5

Integrating insect life history and plant phenology: offspring are not restricted by maternal choice

Minghui Fei, Rieta Gols, Berhane Weldegergis, Huang Tzeyi, Kimmy Reijngoudt, Louise E.M. Vet, Jeffrey A. Harvey

Submitted in a slightly different form

Abstract

The experience of insect herbivores and their specialized natural enemies in their natal habitat is considered to affect their likelihood of accepting a similar habitat or plant/host during dispersal. Important biological traits of the different species, such as seasonal growing phenology in plants and the number of generations in the insects will play pivotal roles in determining how labile insect behavioural responses are at eclosion. Here, we studied the effect of rearing history on oviposition site selection in a herbivore (*Pieris brassicae*) of which the larvae primarily feed on short-lived plant species in the Brassicaceae, and volatile-mediated foraging behaviour in the endoparasitoid (*Cotesia glomerata*) which is specialized on *P. brassicae*. Both species have two or even three generations per year and each generation, most likely, have to find and exploit a different food/host plant species. In the Netherlands, wild turnip (*Brassica rapa*), black mustard (*Brassica nigra*), and charlock mustard (*Sinapis arvensis*), which exhibit different seasonal phenologies, are considered important successive food plants of *P. brassicae* across the growing season. Insects were reared on each of the three plant species for one generation after which their behavioural preferences were monitored. Rearing history only marginally affected oviposition preference of *P. brassicae* butterflies, and had no effect on *C. glomerata* foraging behaviour. *P. brassicae* also did not exhibit consistent preference for any of the plant species, whereas *C. glomerata* had a clear preference on *B. rapa*. The total amount of volatile production and the high emission of the glucosinolate breakdown product 3-butenyl isothiocyanate measured in the headspace of *B. rapa* plants could explain the higher attractiveness of the *B. rapa* blend for the parasitic wasp. Our results reveal the potential importance of flexible preference for female multivoltine insects in habitat choice in nature.

Introduction

The majority of insect herbivores are specialists and feed on only a few related plant species in nature (Loxdale et al. 2011). These specialist herbivorous insects and their co-evolved natural enemies, such as parasitic wasps or ‘parasitoids’, have to locate their food plants and hosts/prey, respectively, on plants that are often embedded in habitats that may be species-rich and structurally and chemically complex (Meiners 2015). Location of these resources during foraging is often characterized by a gradual narrowing down of the area in which these resources can be found (Vinson et al. 1998), and is described as a ‘reliability-detectability’ problem (Vet et al. 1990, Dicke 1999). For example, herbivorous insects first have to find the proper habitat and then locate a suitable food plant within this habitat; their parasitoids must also overcome these same challenges to locate hosts that are often small and feeding in cryptic locations on the food plant. Once potential food plants/hosts have been located in the case of the parasitoids of the herbivores, these can be accepted as oviposition sites or rejected, which is largely determined by differences in the suitability and quality of the resources for their development (Vinson et al. 1998, Schoonhoven et al. 2005). The first steps, i.e. habitat and host location, of this sequential process leading to successful insect development primarily rely on visual and olfactory cues utilized by the insects (Prokopy and Owens 1983, Thompson and Pellmyr 1991, Renwick and Chew 1994, Bruce and Pickett 2011). In particular, volatiles, which are released by plants in response to herbivore feeding (so-called ‘herbivore induced plant volatiles’ or HIPVs) are attractive to parasitoids, and have been extensively studied over the past 25 years (Turlings et al. 1990, Vet and Dicke 1992, Tumlinson et al. 1993, Arimura et al. 2009, Dicke et al. 2009, McCormick et al. 2012).

Because most insect herbivores and their parasitoids have a narrow diet-breadth, they are also expected to rely on specific cues that are related to their food plants or hosts (Fraenkel 1959, van Loon et al. 1992, Vet and Dicke 1992). Larvae of the cabbage butterflies *Pieris brassicae* or *P. rapae*, for instance, primarily feed on plant species in the family Brassicaceae that produce glycoside compounds called ‘glucosinolates’ (Hopkins et al. 2009). Gravid female butterflies of these two species use these glucosinolates to recognize suitable food plants for their offspring (Renwick et al. 1992, van Loon et al. 1992).

Once they are in the appropriate habitat containing suitable plants or hosts, learning and subsequent experience may further affect the foraging behaviour of herbivorous insects and their natural enemies when they are searching for resources. However, the strength of this effect often depends on the developmental stage at which the experience occurs. For example, oviposition experience of adult female parasitoids in the presence of characteristic volatiles blends often enhances the parasitoid's response to these volatiles when these are offered in the absence of hosts (Vet et al. 1995, Hoedjes et al. 2010). This behavioural adaptation is referred to as 'associative learning' and has been observed in both insect herbivores and their natural enemies (Papaj and Prokopy 1989, Vet et al. 1995). Natal experience, which is obtained during larval feeding and growth, may also affect habitat preferences later in life during the adult stage. Known as the 'Hopkins' host selection principle' (HHSP), it is, however, more controversial as it implies some form of imprinting that persists through metamorphosis (Emden et al. 1996, Barron 2001). Another major problem with the HHSP is that it does not take into account constraints imposed by temporal changes in diet that may be predicated by life-history characteristics of the consumer and its resource. For insects that must switch diets from one generation to another, it is clear that larval imprinting on a plant may be maladaptive if it hinders the ability of the insects to find and locate new resources that are chemically different from those on which they developed.

Some studies have shown that pre-adult experience can affect later foraging behaviour in insects for oviposition sites, if these sites are the same or at least very similar to those on which the offspring developed (Immelmann 1975, Davis and Stamps 2004). Preference for the natal habitat type can be adaptive when it improves the ability of insects to find and exploit similar habitats (Gotthard and Nylin 1995) thereby reducing the costs associated with exploring multiple habitats in assessing their suitability (Davis and Stamps 2004). However, the strength of adaptation also depends on the degree to which the environment changes across space and time, in relation to the generation time of the insects.

Many species of insect herbivores are 'multivoltine' and thus have two or more generations per year (Gullan and Cranston 2010). Moreover, some of these herbivores are known to feed on short-lived annual plants that are present in the field for only 2 or 3 months during the growing season (Chapter 2). Under these conditions, adult female herbivores in successive generations are obligated to leave the natal plant patch to locate and oviposit on a different plant species that may be growing some distance from the food-plant species on which they developed themselves. In addition, their specialist multivoltine parasitoids are faced with the same constraints related to habitat and host location as their herbivorous hosts and thus must track them from one patch to another.

In this study, we investigate oviposition preference for different related food-plant species in a multivoltine herbivore *Pieris brassicae* L. (Lepidoptera: Pieridae) and host-infested plant preference behaviour in its endoparasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae) in relation to rearing history. In the Netherlands, *P. brassicae* butterflies that emerge from overwintering pupae in early to mid-spring mate and search for suitable food plants, and generally have three generations per year depending on temperature. *C. glomerata* is a specialized gregarious endoparasitoid that primarily attacks early caterpillar stages of *P. brassicae* and also has two to three generations in the Netherlands. Caterpillars of *P. brassicae* are specialized on brassicaceous plant species of which most native species in its range are short-lived annuals. The three annual plants studied here, the wild turnip, *Brassica rapa* L. growing early in the season, the charlock mustard, *Sinapis arvensis* L. growing in late spring to early summer, and the black mustard, *Brassica nigra* L. growing in mid to late summer, are important wild food plants for successive generations of *P. brassicae* in the Netherlands (Chapter 2). These plant species tend to grow in dense stands, which is a prerequisite for survival of *P. brassicae* because females lay eggs in clusters that need several plants to sustain their larval development (Chapter 4).

The main aim of this study is to determine whether rearing history (i.e insects reared on the different food-plant species [*B. rapa*, *S. arvensis*, *B. nigra*] in one generation) affects preference of *P. brassicae* butterflies and attraction of *C. glomerata* wasps to host-infested plants when given the choice between the three different plant species. We hypothesize that the rearing history of the two insects will not affect preference for food-plant species (herbivore) or volatile-mediated foraging (parasitoid) of future generations, because pre-adult conditioning on the natal plant (invoking the HHSP) may confer ecological costs.

Methods and Materials

Plants

Brassica rapa, *Sinapis arvensis* and *Brassica nigra* seeds were collected from natural growing populations in Gelderland, the Netherlands. Seeds were germinated and seedlings were subsequently transferred to 1.1-L pots filled with peat soil ('Lentse potgrond' no.4; lent, the Netherlands). Plants were grown in a greenhouse at the Netherlands Institute of Ecology (NIOO-KNAW) under the following conditions: 21±2°C (day) and 16±2°C (night), 50% relative humidity, and a photoperiod of at least 16h. The plants were watered twice a week during the first 3 weeks of development. When the plants were 3 weeks old, they were fertilized once a week with Hoagland solution, which was applied to the soil. Watering and fertilization continued during the experiments.

As the insects have been reared on Brussels sprout plants, *Brassica oleracea* (Brassicaceae) var. gemmifera cv. Cyrus for many (>10) generations, this plant was used as a control. Brussels sprout plants were grown from seeds in peat soil in 1.1-L plastic pots in a greenhouse (50-70% relative humidity, 20-25°C, and a photoperiod of 16h) and were 4 to 5 weeks old when used in the experiments.

Insects

Pieris brassicae and *Cotesia glomerata* were collected in fields near Wageningen, the Netherlands. *Pieris brassicae* caterpillars were reared on Brussels sprout plants for many generations in a greenhouse at 50-70% relative humidity, 20-25°C, and a photoperiod of 16h at Wagenigen Universtiy (WU). *Cotesia glomerata* was reared on young *P. brassicae* caterpillars feeding on Brussels sprouts. Once the fully developed larvae of *C. glomerata* emerged from *P. brassicae* hosts and had spun cocoons, they were collected for further rearing or experimental purposes. Approximately five days after cocoon formation, adult wasps emerged at which point they were provided with 10% sugar solution, i.e. a mix of water and honey.

Conditioning of insects

Herbivore. *Pieris brassicae* were reared from egg-to-adult for one generation on one of the three food-plant species: *B. rapa, S. arvensis* or *B. nigra*. We also determined oviposition preference of butterflies reared on *B. oleracea* on which they had been reared for many generations. Four-week-old *B. oleracea* and three-week-old *B. rapa*, *S. arvensis*, or *B. nigra* plants were placed in the rearing cage with adult *P. brassicae* butterflies for 24h. Plants with egg clusters were transferred to a cage with additional plants of the same species as the one on which the eggs were laid. Eggs were allowed to develop into pupae on their respective food plants. Eclosing butterflies were provided with a (20%) honey solution and were allowed to mate. Butterflies were 3-5 days old when they were used in the choice bioassays.

Parasitoid. *Cotesia glomerata* were reared for one generation on *P. brassicae* caterpillars feeding on one of the four food-plant species: *B. oleracea, B. rapa, S. arvensis* or *B. nigra*. Caterpillars of *P. brassicae* were obtained and reared as described in the previous section until they reached the mid first instar stage. For parasitism, female wasps were collected from the general culture. First instar *P. brassicae* caterpillars were parasitized by presenting them individually to a female wasp. After parasitism by *C. glomerata*, caterpillars were introduced onto one of the four food-plant species (*B. oleracea, B. rapa, S. arvensis* or *B. nigra*), which were maintained in separate cages until the larvae of the parasitoids emerged and formed cocoons. Parasitoid cocoons were collected in Petri-dishes and were maintained in an incubator at 21±1°C until adult eclosion at which point they were transferred into 30x 30x 30cm (Bugdorm, MegaView Science Co., Ltd., Taiwan) plastic cages and provided with 10% sugar, water, and honey. Female wasps used in the bioassays were 2-8 days old.

Oviposition preference of Pieris brassicae *butterflies*

Oviposition preferences were assessed in three-choice experiments in six outdoor tents (2x 2.5x 2.2m) placed on bare soil in an experimental field adjacent to WU. Plants from each species were prepared as described in the *Plants* section. Single plants of each of the three plant species were randomly placed in a triangle, approximately 1.5 m apart in the experimental tents. One female and one male butterfly were released in the middle of the tent. A bioassay continued until a female butterfly had laid the first egg clutch, which was checked three times a day. The plant on which the female had laid her eggs was recorded. Females were used only once. The bioassay was repeated at least 30 times with butterflies reared on the same plant species. Bioassays were conducted from June to August. New plants were used for each replicate and the positioning of the plant species in the tent was randomized.

Landing preference of Cotesia glomerata *wasps*

Volatile-mediated foraging behaviour of female *C. glomerata* wasps was studied in a wind tunnel set-up which is described in detail in Geervliet et al. (1994). The environmental conditions were set as follows: wind speed, 0.1 ms^{-1}; light intensity: 518-962 Lux; temperature: 25±1°C; r.h. 60±5%. To stimulate foraging, female wasps were exposed to a damaged Brussels sprout leaf from which *P. brassicae* caterpillars had been removed. Wasps were collected in 7-ml glass vials and released individually in a "release cylinder" located in the middle of the wind tunnel. Two test plants were placed approximately 60-70 cm up-wind from the release cylinder. When a wasp did not land on one of the two plants within the 15-min observation time, it was recorded as non-responding and this data point was excluded from the statistical analysis. Each plant pair was tested with 10 responding wasps, which served as a single data point. Each test-plant combination in relation to the wasp's rearing history was tested 8-10 times with a new set of plants and new wasps.

In the wind tunnel, plant pairs, i.e. all three combinations of host-infested *B. rapa*, *S. arvensis* and *B. nigra* plants were offered to parasitoids reared on the four different food plants. Individual plants were infested with 20 first instar *P. brassicae* or 10 second instar *P. brassicae* caterpillars, depending on caterpillar availability, and incubated in a greenhouse for 24 h at 50-70% relative humidity and 20-25°C with a photoperiod L: D of 16:8h. Plant combinations used in single choice bioassays were always infested with the same number of caterpillars of the same instar. The response of a total of 1270 wasps was recorded in the bioassays examining the effect of rearing history.

To determine whether differences in the amount of feeding damage affected landing preference, we determined for each of the three plant species (n=19 per plant species) the amount of leaf tissue consumed from plants infested by 20 first-instar *P.*

brassicae larvae for 24 h. Damaged areas were calculated using millimeter paper on transparent plastic sheets.

The plant species differed in their leaf architecture, which could affect landing preference of the wasps. For instance, *S. arvenis* and *B. nigra* grow taller than *B. rapa,* which has a shorter main stem and leaves that initially expand horizontally. In an additional wind tunnel experiment, we examined the architectural influence on HIPV preference using single detached leaves from *B. nigra* and *B. rapa* instead of intact plants. The rearing history of the wasps used in this experiment was *B. nigra*. Leaves infested by 20 first-instar *P. brassicae* larvae for 24 h were cut and put into vials with water, and were allowed to recover for 2-4 hours before they were used in a wind tunnel experiment. This comparison was tested in 10 replicate bioassays with a total of 100 responding wasps.

Furthermore, wasps can also be conditioned by exposure to HIPVs when they emerge from the host caterpillars for pupation in the presence of plant material (Kester and Barbosa 1991, Bjorksten and Hoffmann 1998). As described above, one group of wasps was collected and separated from its host and the host-infested plants during the cocoons stage, whereas another group of wasps was left with its host and host-infested plants during the cocoons stage until adult eclosion. Landing preference (n=10) was compared when wasps of these two groups were offered a host-infested *S. arvensis* and *B. rapa* plant. The rearing history of the wasps used in this experiment was *S. arvensis*.

Headspace collection and analysis of herbivore infested plants

Volatiles emitted by *B. rapa*, *S. arvensis*, and *B. nigra*, which had been exposed to feeding by 20 first instar *P. brassicae* caterpillars for 24 h were collected and analyzed. Plants were treated similarly as described for the behaviour bioassay. Volatiles were collected from individual plants, 10 plants per species. The potting soil of the plants were wrapped in aluminum foil to reduce the release of plastic- and soil-related volatiles before the plants with the caterpillars remaining on them were transferred to 30-L glass jar containers. Glass jars were sealed with viton-lined glass lids equipped with an air inlet and outlet. Compressed air filtered through charcoal was led into the glass jars, and the plants were allowed to acclimatize for 40 min.

Dynamic headspace volatile collection was carried out in a laboratory at 20±2°C, by sucking air out of the jar at a rate of 200 ml min^{-1} for 2 h through a stainless steel cartridge containing 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA). Immediately after volatile collection, foliar fresh weight of each plant was measured and the Tenax TA cartridges containing sample volatiles were dry-purged under a flow of nitrogen (50 ml min^{-1}) for 10 min at 21±2 °C to remove moisture. Periodically, volatiles from just pots with soil wrapped in aluminum foil were collected and the compounds recorded, together with the volatiles originating from the Tenax TA adsorbent and the analytical instruments were excluded as artefacts from the data obtained for the plant samples as a correcting measure. Headspace volatile samples were analyzed using a Thermo Trace Ultra Gas Chromatography (GC) for separation coupled to a Thermo Trace DSQ quadrupol mass spectrometer (MS) for detection, both from Thermo (Thermo Fisher Scientific, Waltham, MA, USA). For details of the analytical protocol please refer to Cusumano et al. (2015).

Statistical analysis

To analyze *P. brassicae* female butterfly oviposition preference we used chi-square tests comparing the observed oviposition preference counts for the three plant species with an expected distribution of 1:1:1. When the test result was significant, we conducted pairwise chi-square tests with α=0.05/3 to correct for type I errors (Bonferroni correction).

The response variable in the statistical analyses of the *C. glomerata* wind tunnel bioassays is the fraction of wasps out of the total of 10 responding wasps choosing one of the plant species that was set to be the focal odour source. We used logistic regression, generalized linear model with binomial error distribution and a logit link function, to test the effect of rearing history on wasp landing preference for each plant pair combination. In the case of over-dispersion, we corrected for this by allowing the variance functions of the binomial distribution to have a multiplicative over-dispersion factor. The response variable was the fraction out of 10 wasps that chose one plant species over the other. Plant species on which the wasps were reared was entered as the explanatory variable in the regression model. We used a similar approach to test the effect of plant architecture and early HIPVs exposure on wasps landing preference. In addition, we used one-sample t-tests to determine whether there was a preference for one of the two odour sources offered in the wind tunnel with H_0: mean preference fraction is 0.5 (=no preference). One-way ANOVA was used to examine differences in leaf damage on the three food plants.

Principal component analysis (PCA) was used to visualize whether volatile profiles could be separated according to different plant species and determine which volatile compounds contribute the most to the separation. Kruskal-Wallis tests were employed to reveal significant differences in the emission of each volatile and the total amount of emitted volatiles among the three plant species. When the Kruskal-Wallis test was significant, post-hoc tests between total amounts of emitted volatiles was performed using "posthoc.kruskal.nemenyi.test" function in R. PCA on all volatile data was performed in Canoco version 5.03 (Ter Braak and Smilauer 2002). All other analyses were performed using R statistical language, version 3.2.0 (R Development core team 2015).

Results

Oviposition preference of Pieris brassicae *butterflies*

Pieris brassicae butterflies preferred specific food-plant species for oviposition over others when they were reared on *B. oleracea* ($\chi^2_2 = 6.2$, P = 0.045) and *B. nigra* ($\chi^2_2 = 7.1$, P = 0.029), whereas, this was not the case when the butterflies were reared on *B. rapa* ($\chi^2_2 = 3.8$, P = 0.15, Fig. 1B) and *S. arvensis* ($\chi^2_2 = 2.4$, P = 0.31, Fig. 1C). When reared on *B. oleracea,* butterflies preferred *B. rapa* over *S. arvensis* ($\chi^2_1 = 6.37$, P = 0.012, corrected α = 0. 0167, Fig. 1A), whereas, there was no significant difference in oviposition preference for *B. rapa* and *B. nigra* ($\chi^2_1 = 0.62$, P = 0.43, Fig. 1A), or for *S. arvensis* and *B. nigra* ($\chi^2_1 = 3.27$, P = 0.07, Fig. 1A). When reared on *B. nigra*, *P. brassicae* marginally preferred *B. rapa* over *S. arvensis* ($\chi^2_1 = 5.54$, P = 0.019, corrected α = 0. 0167, Fig. 1D), and there was no significant difference in oviposition preference for *B. rapa* and *B. nigra* ($\chi^2_1 = 3.57$, P = 0.06, Fig. 1D) or between *S. arvensis* and *B. nigra* ($\chi^2_1 = 0.25$, P = 0.62, Fig. 1D).

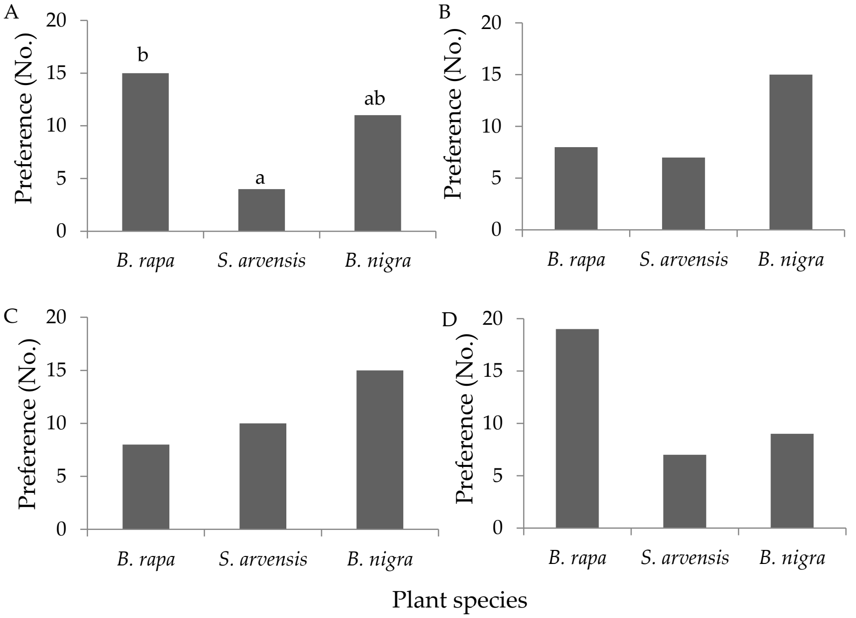


Figure 1. Oviposition preference of female *Pieris brassicae* butterflies that had been reared on *Brassica oleracea* (A), *B. rapa* (B), *Sinapis arvensis* (C), or *Brassica nigra* (D) when given a three-way choice of single plants of the three plant species. Bars represent the total number of butterflies ovipositing on each plant species, and bars without letter or with the same letter are not significantly different from each other (pairwise χ^2-tests with a Bonferroni correction for multiple comparisons).

Landing preference of Cotesia glomerata *wasps*

The overall response rate of the wasps was 92%. Rearing history had no effect on volatile-mediated foraging behaviour (Fig. 2; *B. nigra* vs *S. arvensis*, $^2_3 = 6.70$, $P = 0.23$; *S. arvensis* vs *B. rapa*, $\chi^2_3 = 5.13$, $P = 0.45$; *B. nigra* vs *B. rapa*, $\chi^2_3 = 8.49$, $P = 0.24$). However *C. glomerata* clearly preferred host-infested *B. rapa* plants over the other two plant species (*B. rapa* vs. *S. arvensis*, $t_{37} = 7.0$, $P < 0.001$; *B. rapa* vs. *B. nigra*, $t_{39} = 6.2$, $P < 0.001$) and also preferred host-infested *B. nigra* over host-infested *S. arvensis* plant ($t_{38} = 2.7$, $P = 0.01$; Fig. 2). Furthermore, both early exposure to HIPVs ($\chi^2_1 = 3.77$, $P = 0.16$, Fig. 3) and plant architecture ($\chi^2_1 = 0.34$, $P = 0.71$) had no effect on wasp landing preference (Fig. 4).

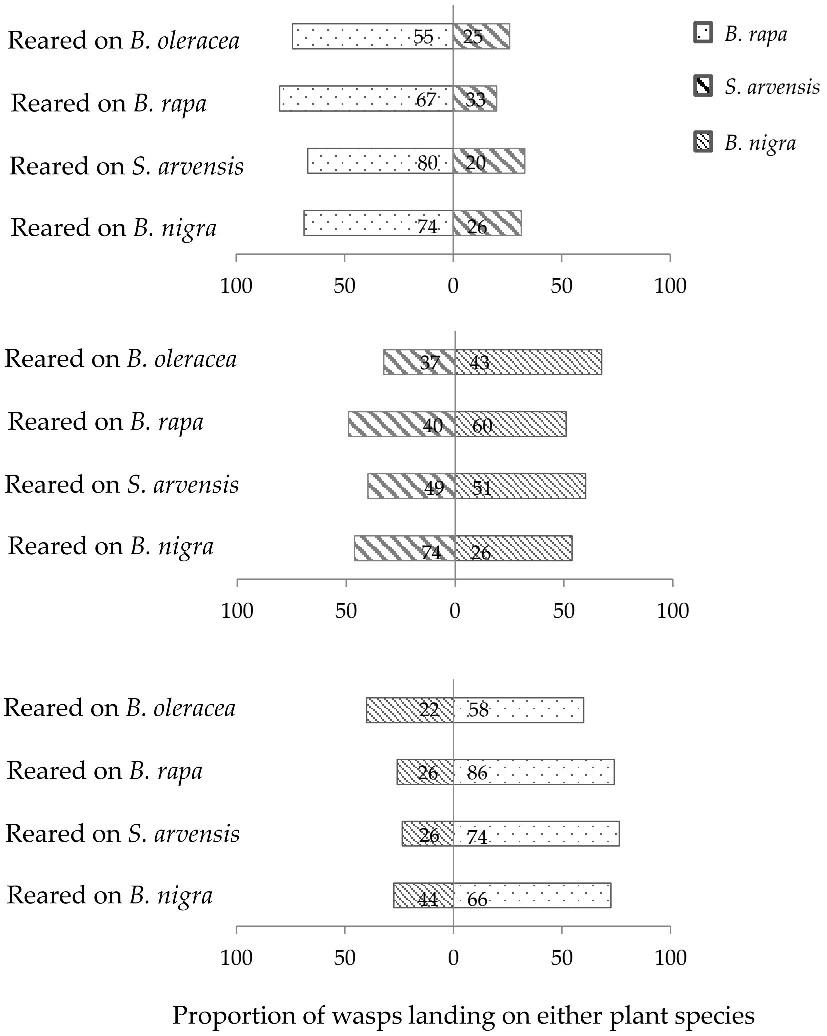


Figure 2. Landing preference of female *Cotesia glomerata* parasitoids that had been reared on *Brassica oleracea*, *B. rapa*, *Sinapis arvensis*, or *B. nigra*, when offered all pair-wise combinations of the three plant species *B. rapa*, *S. arvensis*, and *B. nigra* infested with *Pieris brassicae* host caterpillars for 24 h. Bars represent the mean proportion of choice. Numbers in bars indicate the total number of tested females.

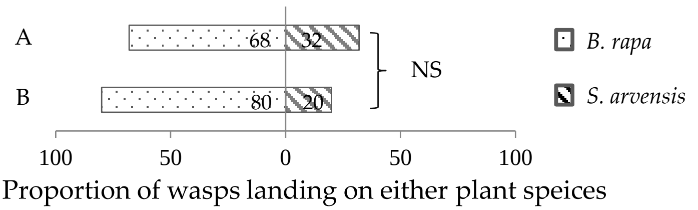


Figure 3. Landing preference of female *Cotesia glomerata* parasitoids reared on *Sinapis arvensis* that (A) remained with the host infested plants during the pupal and early adult eclosion phase or (B) that were collected and separated from the host-infested plant immediately after pupation. Wasps were given the choice between a *Brassica rapa* and a *S. arvensis* plant that had been infested with 20 first instar *P. brassicae* caterpillars for 24h. Bars represent the mean proportion of choice and numbers in bars are the total number of responding females. NS indicates that results are not significantly different (logistic regression, χ^2_1=3.77, P=0.16).

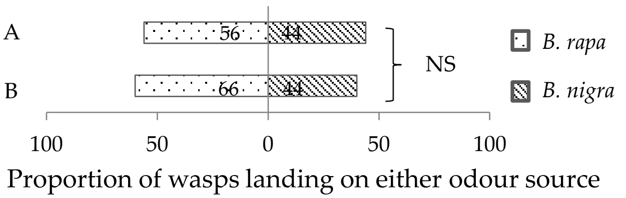


Figure 4. Landing preference of female *Cotesia glomerata* wasps reared on *Brassica nigra* when offered leaves (A) or entire plants (B) of *B. nigra* and *B. rapa* that had been infested with 20 first instar *P. brassicae* caterpillars for 24 h. Bars represent the mean proportion of choice and numbers in bars are the total number of responding females. NS indicates that results are not significantly different (logistic regression, χ^2_1 = 0.34, P = 0.71).

There were significant differences in the amount of consumed leaf tissues by *P. brassicae* larvae among the three plant species ($F_{2,54}$ = 3.38, P = 0.04; Fig. 5). The damage inflicted to *S. arvensis* plants was marginally larger than the damage inflicted to *B. rapa* (Tukey-kramer tests: P = 0.064) and *B. nigra* (P = 0.08), and was similar on *B. rapa* and *B. nigra* (P = 0.99, Fig. 5).

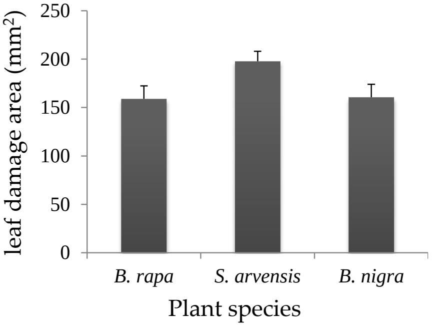


Figure 5. Leaf damage (mean + SE mm^2) caused by 20 first instar *Pieris brassicae* caterpillars feeding for 24 h on *Brassica rapa*, *B. nigra* or *Sinapis arvensis*.

Headspace analysis

In the headspace of *B. rapa*, *S. arvensis*, and *B. nigra* that had been fed upon by *P. brassicae* larvae for 24 h, 33 different compounds were detected, of which 29 were present in the HIPV blend of all three food-plant species (Table 1).

Based on PCA analysis of the volatiles, samples from the three plant species clearly separated (Fig. 6A). The first PC, explaining 28.94% of the variation, separated *S. arvensis* samples from *B. rapa* and *B. nigra*, whereas the second PC, explaining an additional 24.76% of the variation, further separated *B. rapa* and *B. nigra* plants (Fig. 6A). This means that the volatile blends emitted by *B. rapa* and *B. nigra* were more similar to each other than they were to the blend emitted by *S. arvensis* plants. There was a significant difference in the total amount of volatiles emitted by each of the three plant species (Kruskal-Wallis: $\chi^2_2 = 15.98$, $P < 0.001$). *B. rapa* emitted a larger volume of volatiles than *S. arvensis* (Nemenyi Test test, $P < 0.01$), whereas, there was no significant difference in the volume of volatile emission between *B. rapa* and *B.nigra* ($P = 0.28$), or between *B. nigra* and *S. arvensis* ($P = 0.06$). In the PCA, compounds that were emitted relatively high by *B. rapa* were the two nitriles: 2-methylbutanenitrile (ID 1), and 3-methyl-3-butenenitrile (ID 2); the glucosinolate hydrolysis product: 3-butenyl isothiocyanate (ID 9); the two green leaf volatiles (*Z*)-3-hexen-1-ol (ID 4) and (*Z*)-3-hexen-1-ol-acetate (ID 11) and a sesquiterpene (E,E)-alpha-farnesene (ID 29) (Fig. 6B, Table 1). *B. nigra* plants were characterized by the relatively high emissions of the glucosinolate breakdown product allyl isothiocyanate (ID 5) and sesquiterpenes of silphiperfolene isomers (ID 19, 20, 22), which were absent or only emitted in very small amounts by the other two plant species (Fig. 6B, Table 1). *S. arvensis* plants produced relatively larger amounts of the sesquiterpernes α-and β- caryophyllene (ID 24 and 28) (Fig. 6B, Table 1).

Table 1. Volatile emissions[a] by plants from *Brassica rapa*, *Sinapis arvensis*, and *B. nigra* in response to *Pieris brassicae* caterpillars feeding

	Plant species →	*B. rapa*	*S. arvensis*	*B. nigra*
ID[b]	Compound ↓	(n=13)	(n=10)	(n=9)
1	2-Methylbutanenitrile ***	91.8±24.4	3.7±0.8	3.8±0.4
2	3-Methyl-3-butenenitrile ***	6.1±2.0	ND	ND
3	(*E*)-2-Hexenal	0.7±0.1	0.6±0.2	0.3±0.07^{7}[!]
4	(*Z*)-3-Hexen-1-ol ***	20.3±3.0	6.7±1.4	7.5±2.0
5	Allyl isothiocyanate ***	0.2±0.1^{7}	1.4±0.6^{7}	135.2±22.6
6	Butane, 1-isothiocyanato ***	14.7±5.3	0.5±0.2^{8}	0.5±0.08^{8}
7	(*E*)-4-Oxo-2-hexenal *	16.5±2.7	14.9±6.5^{8}	5.6±2.4^{7}
8	Sabinene	0.9±0.1	1.1±0.2^{9}	1.0±0.1
9	3-Butenyl isothiocyanate ***	20.1±5.1	0.05±0.08^{2}	0.7±0.1
10	Myrcene	3.5±0.3	2.9±0.4	3.0±0.3
11	(*Z*)-3-Hexen-1-ol, acetate	165.4±20.8	49.7±9.4	21.9±7.9
12	Hexanoic acid, 2-ethyl-, methyl ester ***	1.6±1.2	3.6±2.7	0.1±0.09^{6}
13	(*E*)-DMNT *	26.4±11.0	4.3±1.2	28.8±7.7
14	Unknown	0.4±0.02	0.3±0.07^{8}	0.4±0.04
15	Menthol	2.7 ±0.9	2.0±0.3	3.7±0.8
16	Unknown	0.5±0.1	1.1±0.3	0.8±0.3
17	Unknown	0.7±0.2	1.6±0.6	0.8±0.3
18	Methyl salicylate ***	2.6±0.7	0.3±0.04	0.7±0.2^{8}
19	Presilphiperfol-7-ene ***	ND	ND	0.4±0.1^{7}
20	7-beta-H-Silphiperfol-5-ene ***	ND	ND	0.5±0.2^{7}
21	alpha -Terpinyl acetate *	0.3±0.05	0.4±0.06^{9}	0.2±0.06^{7}
22	Silphiperfol-6-ene ***	ND	ND	0.2±0.1^{7}
23	alpha -Funebrene **	0.1±0.05^{11}	0.09±0.02^{8}	0.6±0.1
24	beta -Caryophyllene **	2.2±1.4^{6}	9.3±3.7	0.02±0.05^{1}
25	(*E*)- alpha -Bergamotene	0.09±0.06^{6}	0.1±0.04^{7}	0.2±0.08^{7}
26	alpha-Guaiene	0.1±0.03	0.1±0.03^{8}	0.09±0.03^{8}
27	trans-beta-Bergamotene,	0.2±0.2^{5}	0.05±0.2^{1}	0.2±0.1^{5}
28	alpha -Caryophyllene	0.5±0.3^{5}	1.1±0.6^{6}	0.03±0.08^{1}
29	(*E*,*E*)- alpha -Farnesene ***	22.8±10.6	0.3±0.2^{4}	0.9±0.3^{7}
30	alpha -Bulnesene	0.1±0.04^{8}	0.06±0.1^{2}	0.04±0.1^{1}
31	Methyl cis-dihydrojasmonate	7.1±1.3	9.5±1.3	12.0±1.1
32	Unknown *	20.0±2.7	30.0±6.7	43.1±6.3
33	Unknown *	4.7±0.6	6.8±1.3	10.0±1.3
	Total***	433.5±50.4	152.4±25.9	283.2±32.7

[a] olatile emissions are given as mean (± SE) peak areas per g fresh weight of foliage divided by 10^5 with the number of samples between brackets.

[b] ID corresponds with the number presented in the loading plot (Fig. 6B)

[!] Numbers in superscript following emission quantities represent the number of samples in which a given compound was detected and measured, and if the compound was not found in all the samples of a given plant species, it is presented as ND refering to Not Detected.

[*] Compounds with asterisks indicate significant differences in emission quantities of volatiles among the three plant species (*Kruskal-Wallis* one-way ANOVA, * P<0.05; ** P<0.01; *** P<0.001).

(*E*)-DMNT: (*E*)-4,8-dimethylnona-1,3,7-triene

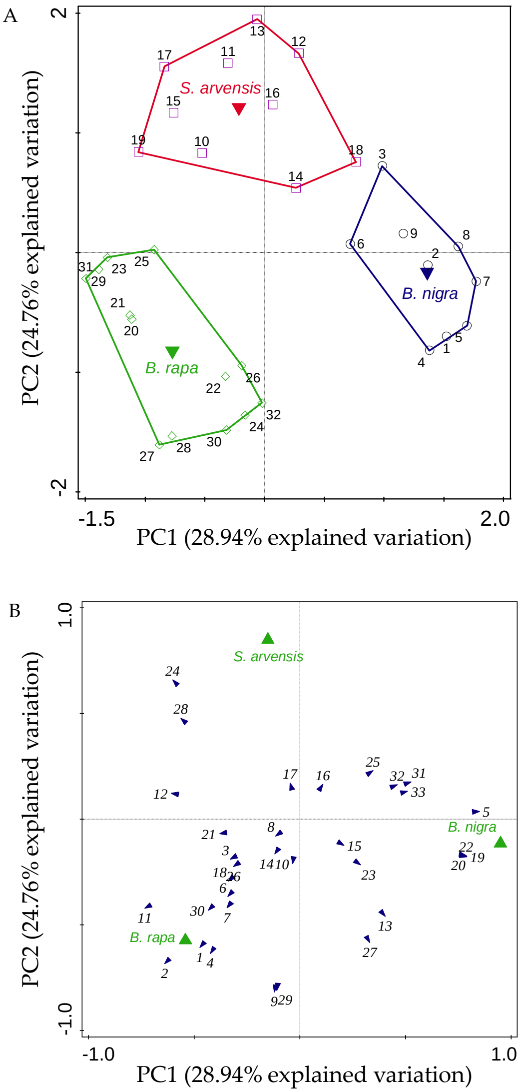


Figure 6. Principal component analysis (PCA) on the quantitative data of volatile compounds detected in the headspace of *Brassica rapa*, *B. nigra* and *Sinapis arvensis* plants that had been damaged by *Pieris brassicae* feeding for 24 h. Score plot of the samples (A) presenting group separation of the different plant species, and its corresponding loading plot of variables (B), showing the contribution of each volatile compounds to the separation of the plant species based on the first two principal components. The identity of the compounds presented as numbers in the loading plot (B) is given in Table 1.

Discussion

In this study we show that rearing history only marginally affected oviposition preference of *P. brassicae* butterflies and had no effect on HIPV-mediated foraging behaviour (e.g. plant preference) of its parasitoid, *C. glomerata.* These results thus show that natal imprinting is not applicable in this multitrophic association. Whereas *P. brassicae* butterflies reared on the different food plants did not exhibit any consistencies in oviposition preference behaviour, *C. glomerata* clearly preferred to alight on herbivore-damaged *B. rapa* plants. Preference of *C. glomerata* for *B. rapa* could not be explained by plant architecture. In addition, this preference was also no affected when the wasps remained with the plants during the pupal stage until adult eclosion. Headspace analyses revealed significant quantitative and qualitative differences among the HIPVs blends emitted by the three plant species.

Natal experience has been reported to affect adult habitat selection by insects (Davis and Stamps 2004, Liu et al. 2005, Anderson and Anton 2014). For example, larval feeding experience with a feeding deterrent modified oviposition responses of subsequent adults in the moth species, *Ephestia cautella* and *Plodia interpunctella* (Olsson et al. 2006), *Trichoplusia ni* (Akhtar et al. 2009), *Spodoptera littoralis* (Anderson et al. 2013), and *Lobesia botrana* (Moreau et al. 2008). To affect choices made during oviposition, preferences learned in the natal habitat must be 'memorized' during larval feeding and carried through pupation to the adult stage. However, in holometabolous insects, the nervous system is profoundly reorganized during metamorphosis (Armstrong et al. 1998, Ray 1999), which makes it unlikely that experience learned during the larval stage is easily retained at adulthood (Barron 2001). This could explain why rearing history had no effect on *P. brassicae* butterfly oviposition preference.

In nature, *P. brassicae* has two to three generations across much of its natural range in Western and Central Europe (Feltwell, 1982). Furthermore, *P. brassicae* larvae require many food plants to support the successful development of a single brood, a requirement that limits the number of suitable plant species as oviposition sites in nature. These plants, including the three species studied here, all must grow in large tightly assembled populations that enable the caterpillars to disperse from the natal plant to adjacent plants later during larval development (Lemasurier 1994, Chapter 4). Importantly, qualitatively and quantitatively suitable food plants are annuals or biennials with short life cycles, and many of these plants also exhibit discrete periods of growth, with some species growing in early spring, others later in spring and early summer and others during summer only. This means different generations of *P. brassicae* must search for different food plants that generally grow at different locations, often some kilometers away from the natal patch. Therefore, it is adaptive for *P. brassicae* that oviposition preference is not affected by larval rearing history; otherwise the adults would risk wasting time searching for food plants that are no longer present (or nutritionally suitable) in the natal habitat. Natal experience is only expected to affect plastic traits when it benefits animal fitness (Gotthard and Nylin 1995). The fact that *P. brassicae* is multivoltine and a specialist on short-lived, clustered brassicaceous plant species may explain why the effect of natal imprinting on adult oviposition preference is weak or non-existent.

Natal imprinting is not only found in lepidopteran species, but has also been observed in some species of Hymenoptera (Morris and Fellowes 2002, Davis 2008). Some studies have shown that the response of parasitic wasps to HIPVs differ depending on the diet on which the insects have been feeding during the immature developmental period (Hérard et al. 1988, Gols et al. 2012). For example, the ectoparasitoid *Hyssopus pallid* were more attracted to frass from the fruit-feeding host *Cydia pomonella* when the wasps had developed on hosts fed on apples compared to wasps reared on hosts fed on artificial diet (Gandolfi et al. 2003). Studies have also showed that the length of rearing history might play a role in the wasp's future preferences for plant volatiles. For instance, when *Plutella xylostella*, parasitized by its parasitic wasp *Diadegma semiclausum*, feed on snow pea for three successive generations, wasps show a relatively higher preference to snow pea volatiles in the third than in first generation (Rossbach et al. 2006). In our study, the wasp was only reared on hosts feeding on a specific plant species for a single generation, reflecting conditions found in nature where different generations generally must find hosts on different plant species. Consequently, we found that natal experience had no effect on volatile-mediated foraging behaviour in *C. glomerata*.

The adaptive potential of natal imprinting clearly depends on such factors as the reliability of being associated with the same plant species or the degree of chemical and structural similarity of different plant species that may be used in successive generations by the herbivore and its parasitoid. An effect of natal experience is suggested to be favored when the environment is predictable within the lifespan of an insect. For multivoltine parasitic wasps that also need to search for hosts on different plant species in different habitats, it is important for their host searching to be efficient and, therefore, natal experience should exert little effect on their landing preference.

The early exposure to HIPVs at eclosion had no effect on wasp landing preference in our experiment. By contrast, host plant stimuli have been reported to increase a parasitoid's attraction to that natal host plant (Bjorksten and Hoffmann 1998). The attraction of the parasitoid wasp *Trichogramma brassicae* to tomato plants only increased when the wasps were allowed to emerge from their hosts in the presence of these plants. Similarly, attraction of *C. congregata* to cherry volatiles increased when the parasitoid experienced intimate associations with the host plant at eclosion Therefore, the importance of conditioning at eclosion appears to be association-specific and even differs amongst closely related taxa (e.g. *C. glomerata* and *C. congregata*).

Female parasitoids clearly preferred *B. rapa* over the other two cruciferous species. Several factors could contribute to this preference. *B. rapa* grows early in the season and may therefore be one of the few plant species available in the Netherlands for *P. brassicae* when they emerge from winter diapause. Consequently *C. glomerata* may have evolved a strong sensitivity to (volatiles) cues related to the first available food plant of its host. Alternatively, some of the volatiles in the headspace of *B. rapa* may trigger a stronger sensory response because of their physical characteristics than volatiles in the blend of *B. nigra* and *S. arvensis*. Little is still known as to the identity of specific volatiles or volatile blends that are most attractive to parasitoids (Gols et al. 2012), although some compounds produced by species in the Brassicaceae have been shown to play an important role in enhancing attractiveness of the blend (Bradburne and Mithen 2000, Blande et al. 2007).

When HIPV blends induced by different treatments of the same plant species are compared, quantitative aspects of these blends may to a large extent determine parasitoid attraction (Geervliet et al. 1998, Bradburne and Mithen 2000). However, when parasitoid attractiveness to HIPV blends emitted by different plant species is compared, qualitative rather than quantitative aspects may be more important (Vet 1999b, Gols et al. 2012). It is known that blends produced by species in the Brassicaceae vary dramatically (Bukovinszky et al. 2005, Gols et al. 2009).

All brassicaceous plant species produce glucosinolates (Fahey et al. 2001), which function as defensive compounds against a range of attackers including pathogens and insect herbivores (Rask et al. 2000, Hopkins et al. 2009). Deterrent or toxic activity only emerges after tissue damage, e.g., by caterpillar feeding, and concomitant release of the enzyme myrosinase, which is stored in specialized cells. This enzyme catalyzes the conversion of glucosinolates into toxic hydrolysis products of which many are volatiles (Halkier and Gershenzon 2006). These volatile breakdown products could serve as reliable signals for parasitoids of hosts that feed restrictively on brassicaceous plant species (Bradburne and Mithen 2000, Blande et al. 2007, Gols et al. 2011). If breakdown products of glucosinolates play a role in host plant selection of *C. glomerata*, the high amounts of 3-butenyl isothiocyanate, which is the breakdown product of gluconapin, the dominant glucosinolate in *B. rapa*, may explain its enhanced attraction to *B. rapa*. However, this does not explain why *B. rapa* is more attractive than *B. nigra* which emits allyl isothiocyanate in even larger amounts than *B. rapa* emits 3-butenyl isothiocyanate (allyl isothiocyanate is a hydrolysis product of sinigrin, the dominant glucosinolate in *B. nigra*). *Diaeretiella rapae*, which is a parasitoid of the aphid *Brevicoryene brassicae*, a specialist of brassicaceous plants, was shown to be more attracted to synthetic 3-butenyl isothiocyanate than to 4-pentenyl isothiocyanate (Bradburne and Mithen 2000, Blande et al. 2007), although it is also attracted to synthetic allyl isothiocyanate (Read et al. 1970) suggesting that these isothiocyanates are differentially attractive to parasitoids. Interestingly, *B. nigra* was only slightly more attractive to *C. glomerata* than *S. arvensis*, whereas the latter species does not emit volatiles characteristic for glucosinolate hydrolysis. The low volatility of hydrolysis products of sinabin, the dominant glucosinolate in *S. arvensis*, may be responsible for the absence of these compounds in the headspace of *S. arvensis*. These results suggests that other blend characteristics than glucosinolate hydrolysis products contribute to the attractiveness of the HIPV blends emitted by brassicaceous plant species for *C. glomerata* and most other parasitoid of herbivores specialized on plants in this family (Gols and Harvey 2009, Gols et al. 2012).

In summary, our study reveals that rearing history has little or no effect on oviposition preference of *P. brassicae* butterflies or HIPV-mediated foraging of *C. glomerata*. *Pieris brassicae* did not exhibit a consistent preference for any of the three plant species that are considered as important food plant of this herbivore in the Netherlands, whereas *C. glomerata* had a clear preference for host-infested *B. rapa* plants. For multivoltine insects, like *P. brassicae* and *C. glomerata* that primarily rely on short-lived annuals for immature development it is a challenge for different generations to locate suitable food plants, given that they are forced to leave the natal habitat to do so. Therefore it is adaptive that these insects are not too strict in the cues that they use in food-plant location behaviour and this behaviour is not affected by natal imprinting. In effect, there is a benefit for the herbivore and its parasitoid to exhibit a nomadic lifestyle during their adult lives and not to develop a strong innate preference for their 'home turf'. Furthermore, our study also shows that the herbivore and the parasitoid use different cues when searching for food or food plants. To better understand the mechanisms that underline these interactions, it is important to examine an array of ecophysiological constraints on the insects and the traits the insects exhibit to counter them. Clearly, the biology and phenology of the food plant(s) leave an indelible mark on their insects.

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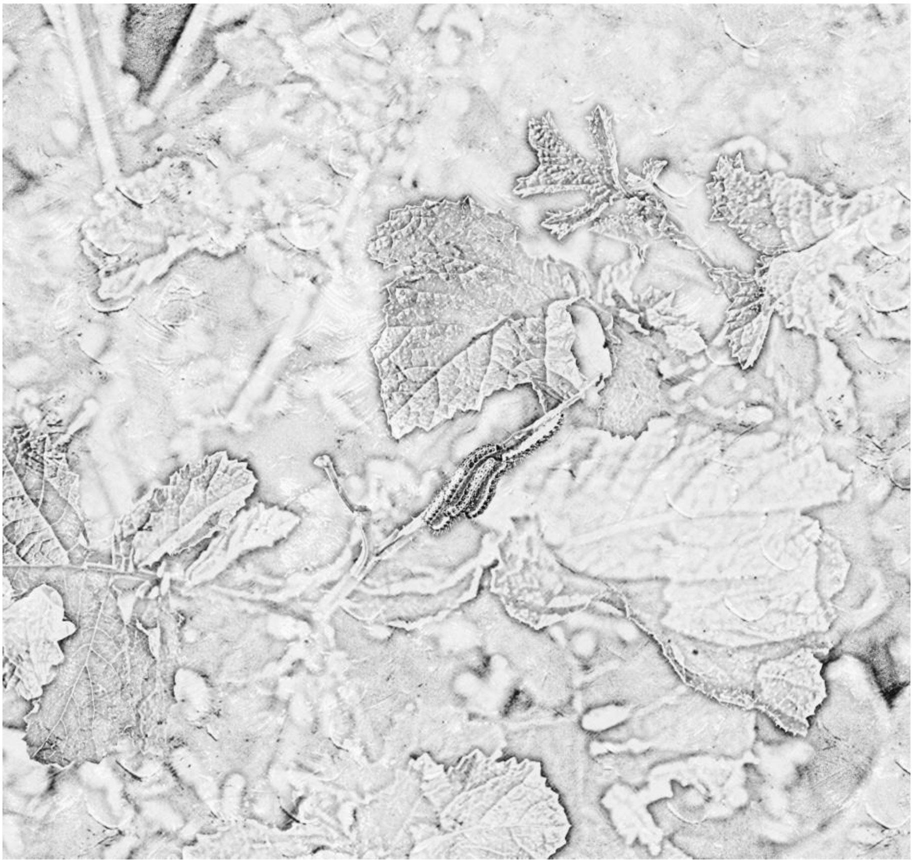

Chapter 6

General discussion

Introduction

In my thesis, I explored how seasonal phenology of potential food plants effects a multivoltine herbivore-parasitoid interaction. I examined potential qualitative and quantitative constraints imposed by the seasonal phenology of several food-plant species on the development and survival, as well as on oviposition decisions of a gregarious specialist insect herbivore and its natural enemy that both have multiple generations per year. My model system consisted of the gregarious lepidopteran insect herbivore *Pieris brassicae* and its gregarious endoparasitoid wasp *Cotesia glomerata*. Food plants of *P. brassicae* are mainly short-lived annual plant species in the Brassicaceae family. In my thesis, I studied the interaction between these insects and three natural food-plant species: *Brassica rapa*, *Sinapis arvensis* and *Brasscia nigra*, which grow at different time points during the growing season and potentially serve as the natural food plant sequence for the different generations of the two insects in the Netherlands. In Chapter 2, I examined the effect of seasonal and plant-species specific variation in food-plant quality on the performance of successive generations of *P. brassicae* and *C. glomerata*. In Chapter 3, I explored the effect of ontogenetic variation in food-plant quality on the performance and oviposition preference behaviour of *P. brassicae.* In Chapter 4, I tested the quantitative constraints of the plants on the performance and the behaviour of both healthy *P. brassicae* caterpillars and caterpillars parasitized by *C. glomerata*. In Chapter 5, I studied whether pre-adult experience can have an effect on oviposition preference of *P. brassicae* and HIPV-mediated foraging behaviour of *C. glomerata*. In this chapter I discuss the significance of these results and place them into a novel contextual framework of multitrophic interactions.

Seasonal phenology of plant-insect interactions

Univoltine insects and their food plants

Univoltine insects, which have one generation per year, have two strategies to interact with their food plants. Some univoltine insects feed on foliage of which growth encompasses almost the entire growing season (e.g. deciduous trees, some annual and perennial plant species), and are only faced with changes in food-plant quality over time. For instance, *Melitaea cinxia* feed on the leaves of *Plantago lanceolata*, which is a perennial plant species. Adult butterflies are active in June, and females lay eggs in large clutches of 100-200 eggs after mating (Kuussaari et al. 1998, Haikola 2003). Larvae hatch in July and live in sib-groups in silken tents until the autumn when they enter diapause as L5; they break diapause the following spring and complete their final two instars (L6, L7) on plantain. During the winter, the larvae live in tight clusters in their web to maintain a higher temperature, in which they diapause (Kuussaari et al. 1998). However, many species of univoltine insects are known to feed on specific plant tissues (buds or seeds) or plant species that are only present at a specific time during the growing season. In this situation, insects are faced with the challenge of 'being on time' when these plant tissues or species are available. For example, the larvae of the winter moth (*Operophtera brumata*) feed on very young buds of Oak (*Quercus robur*) (van Asch et al. 2010). As a result, the feeding window for the larvae is narrow, i.e. from March to April every year. To feed on the bursting buds on time, the winter moth diapauses in the egg stage, and when the temperature is warm enough in spring, the eggs hatch and the larvae start feeding on the bud burst immediately (Visser and Holleman 2001). The life cycle of the parsnip webworm (*Depressaria pastinacella*) is also closely linked with the production of certain plant tissues, i.e. the seeds of hogweeds and related plants (Berenbaum and Zangerl 2006) on which the larvae feed. Because the parsnip webworm larvae feed on the developing seeds of their food plant, which are produced in the middle of the growing season, the parsnip webworm diapauses in the adult stage. Thus, the adults have plenty of time to emerge from winter diapause, mate, search for and oviposit on their preferred food plants. Overall, the life cycle of univoltine insects are closely synchronized with that of their food plants, because availability and the quality of suitable food plants are highly predictable.

Multivoltine insects and their food plants

When multivoltine herbivorous insects interact with their food plants, they are faced with less predictable variation in food-plant quality. Multivoltine insects have also evolved strategies to link their life cycle with the availability of suitable resources. Some multivoltine insects feed on biennial or perennial plant species whose growth covers the entire growing season. Therefore, each generation feeds on a certain plant tissue of the same plant, under the condition that the plant provides enough resources to sustain more than one generation. For instance, various species of psyllids associate with different ages of *Eucalyptus camaldulensis* across a growing season in Australia (Collett 2001). Some multivoltine insects also feed on short-lived annual plant species. In that case, each generation needs to leave their natal patch and find a different food-plant species, as is the case for *P. brassicae,* the insect herbivore used in this study.

Phenology of multitrophic interactions involving multivoltine insects and short-lived annual plants

The effects of variable food-plant quality on the performance of herbivorous insects and their parasitoids have been well explored (Mattiacci et al. 2001, Soler et al. 2005, Quintero and Bowers 2012, Pashalidou et al. 2015). However, the effects of temporal variation in food-plant quality have received much less attention. Moreover, the effects of host shifts of successive generations of multivotline insects that primarily associate with short-lived annual plant species have not been studied. I believe that this is a major omission in our understanding of multitrophic interactions.

Food-plant quality (inter- and intra-specifically) and insect performance

One of the critical requirements for multivoltine insects that obligatorily develop on different short-lived plant species in successive generations is the availability of food plants that are suitable for the development and survival of the insects.

In Chapter 2, I determined whether the quality of the natal food-plant species affects the performance of the following generation of both the herbivore *P. brassicae* and its parasitoid *C. glomerata*. I hypothesized: (i) naïve insect performance is similar on all three plant species, because these plant species are closely related and are known to be important food plants for different generations of *P. brassicae* in the Netherlands; (ii) herbivore performance will be better on plant species with which they have a rearing history, i.e. herbivores that have been reared for successive generations on the same food-plant species, because food resources remain static for herbivores and they adapt to the natal plants; (iii) differences in insect performance related to food-plant quality and food-plant shifts are similar for the herbivore and the parasitoid. Previous studies have shown that the development of *C. glomerata* is intimately correlated with that of *P. brassicae* (Harvey, 2000; Sznajder & Harvey 2003;

Gols et al. 2008). I found that food-plant shift only marginally affected the performance of both *P. brassicae* and *C. glomerata*. All three plant species were of high quality in terms of survival, irrespective of whether the insects shifted to a new food-plant species or not. *Sinapis arvensis* and *B. nigra* were of slightly higher quality than *B. rapa* in terms of the fitness correlates development time and pupal or adult biomass. Although the experiments were conducted in the laboratory where the abiotic conditions were well controlled, food-plant quality increased over the growing season (Gols et al. 2007). As a consequence, the development time of *P. brassicae* decreased in successive generations irrespective of food-plant species. Season-related increases in day length (from March to July), and stronger sun light in the greenhouse compartments may have resulted in improved conditions for plant growth and may have enhanced its quality as food for the insects. Considering that the experiments were conducted in a greenhouse we cannot exclude the possibility that seasonal variation in abiotic factors may have more profound effects on plant-insect interactions in the field.

In addition to interspecific variation, intraspecific variation in plant quality can also affect plant-insect interactions (Barrett and Agrawal 2004, Boege and Marquis 2005). For instance, studies have shown that plant-induced defence responses to herbivory may also be modified by plant ontogeny (Barrett and Agrawal 2004). Adult females of multivoltine herbivorous insects that feed on short-lived annual plants are not only faced with seasonal variation in food-plant quality, but they are also confronted with variations in plant quality related to plant development and aging. In Chapter 3, I examined offspring performance of *P. brassicae* caterpillars in relation to plant ontogeny in *B. nigra* and *S. arvensis*. We found that *P. brassicae* is little affected by ontogenetic variation in plant quality.

Many potential natural food plants for *P. brassicae* are short-lived brassicaceous annual species with a life cycle of about 2-3 months, or biennials with short periods in which suitable shoots are available (Carter and Hargreaves, 1994). Therefore, for a multivoltine herbivore like *P. brassicae*, it is adaptive for survival and larval growth to be little affected by temporal variations in plant quality, both within (Chapter 3) and among food-plant species (Chapter 2).

Survival and development of multivoltine parasitoid species is also constrained by the quality of the food plant of their herbivorous hosts, as they indirectly also obtain nutrients from plants (Harvey 2005, Ode 2006, Gols and Harvey 2009). The developmental patterns of *C. glomerata* on different host plant species were similar as for *P. brassicae* (Chapter 2), which are also advantages for them to deal with the obligatory host shifts.

Food-quantity and insect performance

One of the costs of being a gregarious herbivore is an increasing chance of intraspecific competition for resources (Andrewartha and Birch 1954, Damman 1991, Lemasurier 1994, Konvicka et al. 2003). Gregarious herbivores that feed on short-lived annual plants producing limited biomass are faced with an additional quantitative constraint because the biomass of a single plant may not nutritionally support the development of many siblings, as is the case for *P. brassicae* which lays clutches of 30-100 eggs on a single plant. Therefore, many food plants must grow closely together to support the development of a whole brood of *P. brassicae* caterpillars. There are around 40 possible plant species on which *P. brassicae* can feed in Europe (Feltwell 1982). However, most of these plant species either produce too little biomass, or do not grow in aggregation, and therefore cannot support an entire egg clutch of *P. brassicae* to complete their larval development (Lemasurier 1994). Besides the plant species used in my thesis, there are only a few other wild brassicaceous plant species that might serve as food plants for *P. brassicae* e.g. *Barbarea vulgaris*, *Alliaria petiolata*, *Raphanus raphanistrum* and *Sisymbrium officinale* (Carter and Hargreaves 1994).

In Chapter 4, in both lab and semi-field experiments, I manipulated the availability of food on the development and survival of both unparasitized *P. brassicae* and *P. brassicae* parasitized by *C. glomerata*. I found that even periodic starvation can significantly affect the development of *P. brassicae* and *C. glomerata*, and that the density of the natal plants has profound effects on the survival of unparasitized and parasitized *P. brassicae*. These results suggest that quantitative aspects of the food supply (Chapter 4) often represent bigger constraints in terms of survival and growth of a gregarious insect herbivore and, by association, its endoparasitoid, than qualitative aspects (Chapter 2 and 3).

Preference for food plant (herbivores) and host-infested plants (parasitoids)

When female butterflies or parasitoids are searching for hosts in the field, they need to decide where to lay their eggs and, in the case of gregarious insects, how many eggs. According to the 'preference-performance' hypothesis, adult herbivorous insects should lay their eggs on hosts that are most suitable for development and survival of their offspring (Jaenike 1978). In Chapter 3, I examined female butterfly *P. brassicae* preference in relation to plant ontogeny (*S. arvensis* and *B. nigra*) and whether the mother's preference positively correlated with offspring performance. We found that oviposition preference of *P. brassicae* declined with plant age in both plant species, whereas the size of the plants increased with the age of the plants. These results indicate that butterfly oviposition choice may be based on future quality or quantity of the food plant rather than on the current size/quality of the plant. This could be a strategy of gregarious insects to deal with the potential resource constraints. Similarly, Smallegange et al. (2006) found that *P. brassicae* prefers to deposit eggs on plants growing in aggregated patches over single food plant growing in isolation. In Chapter 3, I also found that oviposition preference of *P. brassicae* butterflies was correlated with larval performance on *S. arvensis*, but less so on *B. nigra*. Relationships between adult oviposition preference and offspring performance may also be influenced by factors such as predation risk and dietary breadth of the herbivores, which may explain why adult preference and offspring performance are not always positively correlated (Gripenberg et al. 2010).

Some studies have shown that pre-adult experience can affect their later foraging behaviour, for example when they are searching for food plants for oviposition (herbivores) (Immelmann 1972, Davis and Stamps 2004) or hosts (parasitoids) (Kester and Barbosa 1994, Davis and Stamps 2004). Furthermore, if the natal habitat experience influences later choices, it always increases the insect acceptance of a habitat that is similar to the natal one (Davis 2008). For herbivorous insects, as well as their parasitoids, that obligatory develop on different short-lived plant species in successive generations, each generation often needs to leave the natal patch to locate a different food-plant species than the one on which they developed themselves. In Chapter 5, I examined whether growing on the different food-plant species (*B. rapa*, *S. arvensis*, *B. nigra*) from egg to pupation/cocoon, which is a form of pre-adult experience (or conditioning), affected oviposition site selection of *P.*

brassicae butterflies and preference for host-infested plants of *C. glomerata* wasps. We found that pre-adult experience had only minor effects on oviposition preference of *P. brassicae* butterflies, and had no effect on *C. glomerata* preference for host-infested food plants. Preference of the natal plant could be advantageous for insects, because natal experience can influence the plasticity of insect traits, which make them better adapted to similar environments (Gotthard and Nylin 1995). Also it may reduce the costs associated with locating new habitats and determining their quality (Davis and Stamps 2004). However, for multivoltine insects that feed on short-lived annuals plants, it is unlikely for them to use the same host plant in successive generations. Therefore, it is important for multivoltine insects that associate with short-lived food plants to be not too strict in the cues that they use for host location and that this behaviour is little affected by natal experience.

Benefits and constraints of life-history traits in relation to plant-insect interactions

'Life-history theory' in evolutionary biology deals with how the lives of organisms have been influenced by natural selection, i.e the way that they develop, reproduce, and die (Stearns 1992). Under natural selection, insects have evolved various life histories to deal with temporal variations in food-plant availability, quality and quantity. Insect life-history traits that are important for their interactions with plants include host resource specialization (specialist or generalist), feeding strategies within habitats (solitary and gregarious), and the number of generations per year (univoltine and multivoltine). Insects exhibit variation in life-history traits based on evolutionary trade-offs and costs and benefits, which generate optimal phenotypes.

Specialists and generalists

Insects vary in their degree of food (herbivore) or host (parasitoid) specialization. Many herbivorous insects are specialized on plants producing particular groups of allelochemicals. For example, larvae of *P. brassicae* are specialized feeders on plants producing glucosinolates (Gols et al. 2008) and Larvae of many species of checkerspot (fritillary) butterflies are restricted to plants producing iridoid glycosides (Wahlberg 2001). Specialized herbivores can often deal with high levels of specific phytotoxins in their diet (Nishida 2002). On the other hand, generalist herbivores that have a broader dietary range are more sensitive to plant allelochemicals in terms of their development, survival and fitness (Harvey et al. 2005), although they benefit in having more potential food plants to use. In general, food-plant quality represents a greater constraint on the development of generalist herbivores than specialists, whereas food-plant quantity may represent a greater constraint for specialist herbivores. This is because specialists may be restricted to the natal plant whereas some generalists, such as the larvae of woolly bears, may feed on several different species of plants during their development (Singer 2001).

Solitary versus gregarious lifestyles

There are benefits of having a solitary lifestyle. For example, being solitary potentially reduces intraspecific competition for food resources. Solitary insects that feed on perennial or annual plants species that produce large amounts of biomass are not constrained by food-plant quantity, and are only faced with temporal changes in food-plant quality. For example, the solitary butterfly *Pieris rapae* is known to associate with many cultivated and wild species in the Brassicaceae, and lay eggs individually on the food plant (Mallet 1986). A single food plant usually provides enough leaf tissues to support the development of *P. rapae* caterpillars, even in the case of a tiny plant like *Arabidopsis thaliana* (Harvey et al. 2007). Food limitation is considered to be one of the major constraints on the development of gregarious animals (Andrewartha and Birch, 1954), including insect herbivores that feed on plants producing limited biomass (Andrewartha and Birch 1954, Damman 1991, Lemasurier 1994, Konvicka et al. 2003). In the case of *P. brassicae*, several to many plants are necessary to support the successful development of a typical brood; this includes even large plants like those studied in my thesis. If plants are not aggregated, the caterpillars are forced to leave the natal plant and to search for new plants in habitats that may contain other plants that hinder dispersal. Moreover, if new plants are not located, the larvae may starve or pupate when they are very small (Chapter 4). Lastly, the caterpillars use up valuable metabolic energy when searching for new plants, and are exposed to an increased risk of predation.

Univoltine and multivoltine lifestyles

The active period of univoltine insect herbivores is often closely synchronized with the availability or quality of their food plants (van Asch et al. 2010). Multivoltine insects that feed on long-lived plants whose growth phase covers an entire growing season are only faced with temporal changes in food-plant quality. Multivoltine insects that feed on short-lived annual plant are obliged to feed on different food-plant species where the nutritional quality is potentially highly variable. However, specialist multivoltine insects like *P. brassicae* are well-adapted to shifting between food-plant species across the growing season (Chapter 2), as well as to the variation in quality depending on the developmental stage of the food plant (Chapter 3). Overall, I may conclude that the food-plant quality is more of a constraint for univoltine insects that have life cycles with critical timing requirements for certain types of food plants or plant structures (e.g. young buds or seeds) compared with multivoltine insects that have the potential of more choices of food plants.

Climate change effect on phenology of multitrophic interactions

The effects of climate change on the seasonal activity of species in terrestrial ecosystems are significant and well documented (Peñuelas and Filella 2001, Walther et al. 2002, Root et al. 2003). The IPCC reported that the global average surface temperature shows an increase of 0.85 °C from 1880 to 2012 (IPCC 2014). The three ways in which plant and animals species respond to climate change are: 1) by shifting their distributions to track suitable climatic conditions; 2) by changing their phenology; 3) through in situ adaptation to altered climate conditions (Walther 2010, Bellard et al. 2012). However, the responses of individual species to climate change are also influenced by their interactions with other species (Voigt et al. 2003, Tylianakis et al. 2008), which generates uncertainty in predicting changes in the abundance and distribution of species over time under climate change scenarios (van Vliet et al. 2003, Walther 2010).

Plants are at the base of virtually all terrestrial ecosystems in providing resources for higher trophic level organisms (=consumers). Climate warming, precipitation changes and rising CO_2 concentrations will alter the nutritional quality of plants and their availability for herbivorous insects (Bezemer and Jones 1998, Jun Chen et al. 2007, Tylianakis et al. 2008). Temperature is the main driver of plant phenology and there is strong evidence that recent climate warming affects plant phenological events. Khanduri et al (2008) explored some of these phenological changes in more than 650 temperate species, and found the average length of the growing season has extended by 3.3 days per decade (Khanduri et al. 2008).

The effect of climate change on herbivorous insects can be direct by affecting their physiology and behaviour, or it can be indirect, through climate-induced changes in their food plants (Collinge and Louda 1989, Bale et al. 2002, Singer and Parmesan 2010). Various studies have suggested that direct effects of temperature on herbivorous insects are likely to be more important than other factors such as increased CO_2 concentrations and UVB levels (Bale et al. 2002). Temperature may induce changes in insect development time, biomass, voltinism, and the extent of food-plant exploitation as well as in their geographical distributions (Bale et al. 2002). However, the responses of insect life histories to climate change are likely to be both complex and variable, depending on insect life history and the biology of the food plant. Changes in plant phenology may influence the insects associated with them depending on life-history traits that are important for establishing or affecting interactions with plants (Bale et al. 2002).

Climate change may result in phenological asynchrony between univoltine insect herbivores and their food plants (Singer and Parmesan 2010). A classic example is the winter moth (*Operophtera brumata*) and its only food plant, the oak tree (*Quercus robur*). Newly hatched caterpillars of winter moths are only able to chew and ingest soft tissues in oak buds that are about to ‘burst’ and produce leaves. Asynchrony between the time of egg hatching and bud burst has been documented in the Netherlands as a result of climate warming, especially during a critical period in early spring when winter moth eggs are hatching and oak buds are forming (van Asch and Visser 2007). Multivoltine insects feeding on short-lived annuals, like *P. brassicae*, may be less affected by climate change because they have many potential food-plant species that they can exploit.

The fate of parasitoids is often intimately tied to that of their hosts. Compared to their herbivorous hosts, parasitoids may be more sensitive to climate change because of their trophic position higher up in the food chain. Moreover, their relatively high degree of specialization also makes them more susceptible to increased variability in host population dynamics mediated by climate change

(Stireman et al. 2005, Hance et al. 2006). Host specificity and dispersal ability in parasitoids could have a strong influence on how host-parasitoid interactions respond to climate change (Berg et al. 2010). Little is known about the dispersal abilities of parasitoids (Jones et al. 1996, Elzinga et al. 2007). However, because of the more patchy distribution of their hosts, many specialized parasitoid will have difficulty in tracking their hosts (van Nouhuys and Ehrnsten 2004, Roy et al. 2011, Harvey 2015). Hosts and parasitoids may have different thermal preferences and different capacities to survive extreme temperatures under climate change scenarios (Hance et al. 2006). Therefore, climate change can affect host and parasitoid asymmetrically (in terms of development and phenology).

Climate change could alter phenological relations among tritrophic interactions if species in these trophic links respond differently in terms of their life cycles. For instance, in my study system, interactions involving *P. brassicae*, its natural short-lived food plants and a specialist endoparasitoid, *C. glomerata*, are complex in the context of life-history interactions. The consequences of climate warming on the phenology of these trophic interactions depends on how the plants and insects each respond to increasing temperatures, and how this in turn affects the availability and suitability of the resources which they exploit as food. Climate warming may change the growing time of the food plants and can increase the number of generations of the insects (Spieth et al. 2011). If the phenology of food plants of multivoltine insects advances in response to higher temperatures, climate change may result in the starvation of multivoltine insects, or else they may evolve adaptations such as aestivation (Spieth et al. 2011).

Conclusions

- The multivoltine gregarious insect herbivore *P. brassicae* was only marginally affected by ontogenetic, seasonal and plant species-specific variation in food-plant quality. In addition, food-plant shifts had only minor effects (both positive and negative depending on plant species) on the performance of the herbivore.

- Survival and performance of *P. brassicae* appears to be constrained more by quantitative than qualitative aspects of the food plant.

- Development of the multivoltine gregarious endoparasitoid *C. glomerata* was also only marginally affected by seasonal and plant-specific variation in the food-plant quality and is confronted with similar quantitative constraints as its host.

- Oviposition preference of adult female *P. brassicae* butterflies declined with plant age (they preferred younger but smaller over older and larger plants). Female *P. brassicae* butterflies may thus be able to 'anticipate' future biomass or quality potential of the plants.

- Pre-adult experience had minor effects on oviposition preference in *P. brassicae* and had no effect on *C. glomerata* landing preference.

- Overall conclusion: insect-plant interactions are complex and dynamic. My research has shown that a better understanding of this complexity and of the mechanisms involved can be attained through exploring more intimate trait-mediated aspects of plant and insect life histories.

Future directions

Many studies on plant-insect interactions ignore temporal and spatial realism of these interactions in nature. Instead, many studies on tritrophic insect plant interactions focus on mechanisms underlying these interactions, such as how parasitoids respond to different HIPV blends in simple wind tunnels or Y-tube experiments or how (chemical) resistance of plants affects the behaviour and performance of herbivorous insects and their natural enemies. In these studies little attention is paid to the temporal and spatial dynamics of multitrophic interactions. These include food-plant species shifts (temporally dynamic interactions) as well as insect foraging behaviour in the field (spatially dynamic interactions), both of which have been little studied. Plant age-related differences in quality are also frequently ignored. Therefore more studies are required that pay attention to these areas in order to better understand how temporally dynamic changes in plant quality (inter- and intra-specifically) affect multitrophic interactions, and how herbivorous insects and their natural enemies respond to these changes. The life histories of both the plants and insects need to be examined in a more integrated framework. Furthermore, more fieldwork and long-term studies are needed to help to understand the effect of climate change on relationships between insects and their various food-plant species. In particular, few studies have examined trophic interactions integrating three levels of the food chain under conditions of a changing climate. This research will provide an important and hitherto missing link between controlled lab experiments and realistic but correlative field records. Moreover, predicting the direct effects of climate change on the development and survival of insects needs to take into account phenotypic and genotypic flexibility.

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Summary

As food resources for herbivorous insects, the quality and quantity of plants can directly affect the performance of herbivorous insects; it will also indirectly affect the performance of natural enemies of herbivorous insects such as parasitic wasps. Food-plant suitability for herbivores and potentially their natural enemies is determined by plant quality, such as the production of primary and secondary metabolites and morphological traits, but quantity can also be important. In nature, plant quality and quantity are dynamic and can change within individual plants over the course of a single growing season. When multivoltine insects associate with short-lived annual plants, each generation must move from the natal plant species/patch to a new food-plant species/patch. In this instance, food-plant quality and quantity may be highly variable for parents and their children and grandchildren. However, thus far, temporal realism is often missing in many studies of multitrophic interactions. Most studies are conducted in a single time frame or involve interactions between insects with a single plant species and tacitly assume that these interactions persist over a long period of time. This thesis explores constraints imposed by the seasonal phenology of several food-plant species on the development, survival and oviposition decisions in a gregarious insect herbivore and its parasitoid wasp both of which have 2-3 generations per year. As a model system, I used the large cabbage white butterfly, *Pieris brassicae* L, and its specialized gregarious endoparasitoid *Cotesia glomerata* L. *Pieris brassicae* is a specialist that feeds primarily on plants in with the Brassicaceae family. Three short-lived annual plants that are known to serve as food plants for *P. brassicae* were used in my thesis: *Brassica rapa* L., *Sinapis arvensis* L, and *Brassica nigra* L. These species grow rapidly and exhibit differing seasonal phenologies, each growing within a short period of time with little temporal overlap amongst them.

In central Europe, *B. rapa* typically grows in early spring, *S. arvensis* in late spring and early summer, and *B. nigra* in mid to late summer. *Pieris brassicae* generally has three generations per year, and *C. glomerata* two or three. This means that different generations of the insects must find and exploit different food-plant species that potentially differ in size, quality and in the habitats in which they grow. In Chapter 2, I explored whether growing on the same or different food-plant species in successive generations affects the performance of *P. brassicae* and its endoparasitoid, *C. glomerata*. Development time and biomass were measured in the herbivore *P. brassicae* and *C. glomerata* when they were either reared on each of the three plant species for three successive generations or shifted between generations from *B. rapa* to *S. arvensis* to *B. nigra* as the natural sequence of plant growing in the field across the season. The results show that when reared on the same food-plant species, development time of both the herbivore and the parasitoid decreased across different generations. The biomass of insects was larger when feeding on *S. arvensis* and *B. nigra* plants than on *B. rapa* plants. However, when insects were shifted to new food plants, insect performance was marginally affected depending on the identity of the new food plant. I conclude that plant species identity, seasonal phenology and food-plant shifts all may affect the performance of multivoltine herbivorous insects and their parasitoids, although both the herbivore and its parasitoid performed well on the three species, showing that they are well adapted to shifting food plants across a growing season.

One of the major challenges for each generation of multivoltine herbivorous insects searching for food plants is that female butterflies are likely to encounter individual food plants in various stages of size and hence developments, where plant quality is also different and could directly affect insect performance. In Chapter 3, I investigated whether female butterflies (*P. brassicae*) are able to distinguish between different developmental stages of two different food-plant species (*S. arvensis* and *B. nigra*) for oviposition. Oviposition preference was investigated in three-choice-experiments in tents placed outside in the garden of NIOO-KNAW on bare soil. One plant from each three different age classes was prepared. For each plant species, bioassays were performed separately. The results showed that oviposition preference order for food plants declined with plant age in both plant species; the butterflies preferred the smallest, youngest plants for oviposition. I also investigated whether female butterflies lay their eggs on plants in the developmental stage that is best for the growth and development of their offspring. I found that oviposition preference was positively correlated with larval performance when the food plant was *S. arvensis*, but not when the food plant was *B. nigra*. Actually, the overall effects of food-plant quality for *P. brassicae* on herbivore performance were relatively small. I also analyzed and quantified primary (amino acids and sugars) and secondary metabolites (glucosinolates) in tissues collected from plants in different developmental stages to link larval performance with qualitative characteristics of the plant tissues of both plant species. Differences in plant chemistry were more pronounced with respect to tissue type (leaves versus flowers) than among different developmental stages in both plant species. Correlations were found between insect performance and plant chemistry variables for both plants species. However, I did not detect any clear consistencies in phytochemistry characteristics and insect performance.

Gregariously-feeding insect herbivores can be constrained by the availability of sufficient food resources. Some multivoltine gregarious insects are known to associate with short-lived annual plants, which have limited biomass. In Chapter 4, I examined the effect of periodic starvation and density of natal plants on the development and survival of the herbivore and its endoparasitoid. In laboratory experiments, I tested how periodic food deprivation of healthy and parasitized final-instar caterpillars affected survival and fitness (body mass and development time) of *P. brassicae* and *C. glomerata*. I also investigated the ability of herbivores and their parasitoids to recover from variable periods of food deprivation when re-provided with plant diet after short-term starvation. The results of this investigation showed that depriving *P. brassicae* caterpillars of food plants for variable periods in the final instar significantly affected the survival and fitness of both the herbivore and its parasitoid. Moreover, any kind of interruption in the feeding behaviour in insects can have a significant effect on the fitness of survivors. In the herbivore, these effects are correlated with the age of the caterpillar in its final instar. I also studied how variation in plant biomass and natural vegetation barriers affected the survival of healthy and parasitized larvae in a semi-field experiment. In outdoor tents with natural vegetation, I monitored the survival of 25 healthy and 25 parasitized caterpillars that were placed on mustard plants in pots in groups of 1, 5 or 8 plants (as natal food-plant patch) in the center, and with 2 plants placed in all 4 corners of the tents 1.5 meters away. Emigration rates of both healthy and parasitized caterpillars from the natal food-plant patch to the other food-plant patches decreased with natal food-plant density. Survival of both the herbivore and the parasitoid increased with natal plant density and was higher for healthy than for parasitized caterpillars. I concluded that in *P. brassicae* and *C.glomerata* which are associated with short-lived annual plants, plant quantity is more vital for insect development and survival than plant quality.

How experience of insects in their natal habitat affects their later likelihood of accepting a similar food plant (herbivorous insect) or host (parasitoid) has also been examined. When multivoltine insects associate with short-lived annual plants, for each generation of insects, the food-plant individual/species on which the caterpillars (and by association, their parasitoids) had fed during their larval stage is not available anymore when they enclose as adults. If these insects are restricted by their natal experience, they may face the negative consequences of wasting time for searching for new food plants/hosts close to the natal patch. In Chapter 5, I tested whether rearing history, that is growing on the different food-plant species (*B. rapa*, *S. arvensis*, and *B. nigra*) in one generation affects oviposition site selection of adult *P. brassicae* and foraging behaviour of *C. glomerata*. The results show that rearing history only marginally affected oviposition preference of *P. brassicae* butterflies, and had no effect on *C. glomerata* landing preference. However, *P. brassicae* did not have a consistent preference for any plant species, whereas *C. glomerata* had a clear preference for *B. rapa*. Volatile compounds are known as important attractants for parasitoids. To explain the attractiveness of different food-plant species for the female wasps, the headspace of *B. rapa*, *S. arvensis*, and *B. nigra* that had been fed by *P. brassicae* larvae for 24 h was analyzed. Higher total amounts of volatile blends in *B. rapa* and of the glucosinolate breakdown product 3-butenyl isothiocyanate in the headspace of this plant species could explain the higher attractiveness of it to *C. glomerata*. My results show that being less choosy enables multivoltine insects to be flexible for different host species irrespective of their rearing history.

In conclusion, my PhD research shows that *P. brassicae* and *C. glomerata* are well-adapted to developmentally and behaviourally exploit three phenologically different plant species across consecutive generations in a single growing season. The performance and survival of both insects was marginally affected by season-related and plant species-specific variation in food-plant quality, whereas they were more constrained by quantitative aspects of one important food-plant species (*B. nigra*). Female *P. brassicae* butterflies may 'anticipate' future plant quantity or quality when choosing oviposition sites rather than select for plant traits at oviposition. Pre-adult experience had minor effects on oviposition preference in *P. brassicae* and had no effect on *C. glomerata* landing preference. The bottom line is that it is adaptive for these insects to be flexible in the cues they use to find new plants/hosts in different generations.

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Minghui, 24th of November, 2015

Curriculum vitae

Minghui Fei was born on the 5th of April 1985 in Siping, China. After finishing high school in 2004, she started the study at School of Life Sciences in Northwest A&G University, where she obtained her bachelor degree in Biotechnology in 2008. Then she continued her master studies at Northeast Forestry University, major in Cell Biology and she graduated with the honour of excellent graduate in 2011. During her master studies, she took part in research projects on effect of salt treatment on the glucosinolate-myrosinase system in *Thellungiella salsuginea* and proteomic investigation of glucosinolate systematically changes in Arabidopsis Rosette leaves to exogenous methyl jasmonate, supervised by Prof. dr. Xiufeng Yan. Both master works resulted in publications in scientific journals. After her master, Minghui got a national fellowship from China Scholarship Council for abroad PhD research in 2011. In November 2011, she started her PhD with Prof. dr. Louise Vet, Prof. dr. Jeffrey Harvey, and Dr. Rieta Gols at Department of Terrestrial Ecology, Netherland Institute of Ecology, The Netherlands. During her PhD, she conducted laboratory and semi-field experiments and presented her research at many national and international conferences. The results of her PhD research are presented in this thesis.

Publications

Pang Q, Guo J, Chen S, Chen Y, Zhang L, **Fei M**, Jing S, Li M, Wang Y, Yan X. Effect of salt treatment on the glucosinolate-myrosinase system in *Thellungiella salsuginea*. *Plant and Soil*, 355: 363-374 (2012).

Chen Y, **Fei M**, Wang Y, Chen S, Yan X. Proteomic investigation of glucosinolate systematically changes in Arabidopsis Rosette leaves to exogenous methyl jasmonate. *Plant Biosystems*, 1-8 (2013).

Fei M, Gols R, Harvey JA. Seasonal phenology of interactions involving short-lived annual plants, a multivoltine herbivore and its endoparasitoid wasp. *Journal of Animal Ecology*, 83, 234-244 (2014).

Fei M, Gols R, Zhu F, Harvey, JA. Plant quantity represents a greater constraint than quality for a gregarious insect herbivore and its endoparasitoid wasp. *PloS One*, Accepted.

In process

Fei M, Harvey JA. Yin Y, Gols R. Cabbage butterfly mothers are choosy when selecting plants that differ in age and size. Submitted.

Fei M, Gols R, Weldegergis B, Tzeyi H, Reijngoudt K, Vet LEM, Harvey JA. Integrating insect life history and plant phenology: offspring are not restricted by maternal choice. Submitted.

Harvey JA, **Fei M**, Lammers M, Kos M et al. Development of a solitary koinobiont hyperparasitoid in different instars of its primary and secondary hosts. Submitted.

Fei M, Harvey JA, Gols R, Huang Y et al. Comparing insect communities on native Dutch wild mustard plants over a growing season. In preparation.

Essens T, Gols R, **Fei M**, Heinen R, Harvey JA. Dietary sugars and host-feeding behaviour differentially affect reproductive success in parasitoid wasps. In preparation.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Spatial effects of plant identity and quality on the behaviour and development of multivoltine insect parasitoids and their hosts

Writing of project proposal (4.5 ECTS)

- Phenological differences in the seasonal growth of brassicaceous plants and effects on indirect plant defences

Post-graduate courses (4.7 ECTS)

- Consumer-Resource Interactions: Hunger, Health & Horror; PE&RC, SENSE, RSEE (2014)
- Introduction to R for Statistical Analysis; PE&RC (2015)
- Linear Models; PE&RC (2015)
- Generalized Linear Models; PE&RC (2015)
- Mixed Linear Models; PE&RC (2015)

Laboratory training and working visits (0.3 ECTS)

- Multitrophic Interactions: Plant-insect-parasitoids and biological control; College of Life Science, Nankai University, China (2014)

Invited review of (unpublished) journal manuscript (1 ECTS)

- Journal of Animal Ecology: Tertiary parasitism and trophic efficiency in aphid parasitoid food webs (2013)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic Statistics; PE&RC (2012)

Competence strengthening / skills courses (3.3 ECTS)

- Techniques for Writing and Presenting a Scientific Paper; Wageningen Graduate Schools (2012)
- Scientific Publishing; Wageningen Graduate Schools (2012)
- Reviewing a Scientific Paper; Wageningen Graduate Schools (2012)
- Scientific Writing; Wageningen in'to Language (2015)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)

- PE&RC Weekend first year (2012)
- PE&RC Day (2012)
- 8th Insect-Plant Interactions Workshop (2013)
- PE&RC Weekend last year (2014)

Discussion groups / local seminars / other scientific meetings (14.6 ECTS)

- Monthly NIOO Seminar (2012-2015)
- Department of Terrestrial Ecology PhD Monthly Journal Club Meeting (2012-2015)
- Netherlands Annual Ecology Meeting (NAEM) (2013-2015)
- NIOO Days (2015)

International symposia, workshops and conferences (7.5 ECTS)

- BES: British Ecology Society Annual Meeting; oral presentation (2012)
- IEIC3: International Entomophagous Insect Conference; oral presentation (2013)
- SIP: 15th International Symposium on Insect-Plant Relationships; poster presentation (2014)
-

Supervision of MSc Students

- Comparing insect communities on short-lived annual plants over a growing season

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