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Ecological fits, mis-fits and lotteries involving insect herbivores on the invasive plant, *Bunias orientalis*

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Abstract Exotic plants bring with them traits that evolved elsewhere into their new ranges. These traits may make them unattractive or even toxic to native herbivores, or vice versa. Here, interactions between two species of specialist (*Pieris rapae* and *P. brassicae*) and two species of generalist (*Spodoptera exigua* and *Mamestra brassicae*) insect herbivores were examined on two native crucifer species in the Netherlands, *Brassica nigra* and *Sinapis arvensis*, and an exotic, *Bunias orientalis*. *Bu. orientalis* originates in eastern Europe and western Asia but is now an invasive pest in many countries in central Europe. *P. rapae*, *P. brassicae* and *S. exigua* performed very poorly on *Bu. orientalis*, with close to 100% of larvae

failing to pupate, whereas survival was much higher on the native plants. In choice experiments, the pierid butterflies preferred to oviposit on the native plants. Alternatively, *M. brassicae* developed very poorly on the native plants but thrived on *Bu. orientalis*. Further assays with a German *Bu. orientalis* population also showed that several specialist and generalist herbivores performed very poorly on this plant, with the exception of *Spodoptera littoralis* and *M. brassicae*. *Bu. orientalis* produced higher levels of secondary plant compounds (glucosinolates) than *B. nigra* but not *S. arvensis* but these do not appear to be important factors for herbivore development. Our results suggest that *Bu. orientalis* is a potential demographic ‘trap’ for some herbivores, such as pierid butterflies. However, through the effects of an evolutionary ‘lottery’, *M. brassicae* has found its way through the plant’s chemical ‘minefield’.

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Introduction

The theory of co-evolution constitutes a long-standing foundation in understanding various aspects of plant-insect interactions (Ehrlich and Raven 1964; Fox and Morrow 1981; Jermy 1984; Bernays and

Graham 1988; Janz et al. 2001). In this scenario, plants evolve defences that repel or deter herbivore attack, whereas herbivores evolve counter adaptations to deal with plant defences. Ultimately this leads to an evolutionary ‘arms race’ whereas some measure of equilibrium is maintained between plant response to herbivory and insect-counter-response over time (Thompson 1988). Such an arms race is predicted to lead to specialization in herbivore diet, leading to intimate associations between plants with phylogenetically conserved chemical defences and certain herbivore lineages (Feeny 1976). The larvae of many species of butterflies in the family Pieridae, for example, feed only on plants in the order Brassicales that produce secondary compounds known as glucosinolates (Gols et al. 2008a; Hopkins et al. 2009). Adaptation to specific allelochemicals may explain why polyphagy is rare amongst insect herbivores, even amongst so-called ‘generalists’. This is because specific genotypes or populations of some apparent generalists tend to be found only on certain plants or else avoid plants in many families (Weis and Berenbaum 1989; Novotny and Basset 2005; Singer and Bernays 2008).

Many associations in nature, however, are not necessarily the result of long periods of co-evolution but are based on recent novel precedents. Novel associations amongst plants and herbivores may occur when an insect enters a community in which there are plants with which it is naïve, or when an exotic plant invades the range of an insect (Agosta and Klemens 2008). Described as ‘ecological fitting’ (Agosta 2006; Agosta and Klemens 2008), organisms are able to persist in novel environments if an evolved trait or suite of traits they bring from their original habitat enables them to enjoy realized fitness in their new habitat. Ecological fitting represents a different way of explaining the outcome of species interactions, and is notable in that evolution is not required between either party for the formation and/or persistence of the interactions between them.

Novel interactions can have either positive or negative effects on the behaviour and development of naïve herbivores (Keeler and Chew 2008). Interactions between herbivores and potential food plants involve several steps that, if successful from the herbivore’s perspective, lead to adult eclosion and reproduction. These steps include habitat location of the host plant by the herbivore, alighting on the plant

by the adult female, host plant acceptance (both oviposition by the adult female herbivore and the initiation of feeding by her progeny) and host plant suitability leading to successful adult eclosion (Scriber and Slansky 1981; Udayagiri and Mason 1995; Renwick and Lopez 1999; van Loon and Schoonhoven 1999). From the perspective of the herbivore, a failure to complete any one of these steps will lead to a ‘misfit’ (as opposed to fitting) with the novel plant. Novel plants may also become demographic traps if they are highly attractive to an insect on which their progeny perform very poorly (Renwick 2002; Keeler et al. 2006; Keeler and Chew 2008). On the other hand, they present evolutionary opportunities if herbivores can adapt to them.

Many studies involving novel interactions between plants and insect herbivores are based on exotic plants that have become established in new habitats (Agrawal et al. 1999; Louda et al. 2005; Siemann et al. 2006; Lankau and Strauss 2007). Plants that invade regions bordering their natural range, perhaps due to such processes as changes in land use or climatic warming may encounter novel herbivore species that exhibit differing degrees of adaptation to their secondary (= defensive) metabolites (Engelkes et al. 2008). The nutritional suitability of food plants is influenced by both levels of toxic secondary plant metabolites and nutrients found in plant tissues (Slansky 1992). In areas close to the natural range of the invasive plant, populations of many herbivores may be adapted to secondary metabolites in the exotic plant, particularly if the plant shares common secondary metabolites with other native food plants used by the herbivore. Closely related plants often possess chemical ‘fingerprints’ based on their secondary chemistry which are recognized by specialized insect herbivores (Feeny 1976; Müller-Schärer et al. 2004). However, novel secondary plant metabolites may lead to mis-fits between the plant and the herbivore, making these plants nutritionally unsuitable or even toxic to the herbivore (Renwick 2002; Verhoeven et al. 2009).

The warty cabbage, *Bunias orientalis* L. (Capparales: Brassicaceae) is a wild cruciferous plant that originates in western Asia and extreme eastern Europe but which has become an highly invasive species in various parts of northern and central Europe over the past 25 years (Dietz et al. 1996; Woitke and Dietz 2002). For instance, it is now

considered to be a serious pest in parts of Germany, Poland, Sweden, Norway, as well as in several other countries (Dietz et al. 1999). At the same time, the plant is less common in parts of western Europe, including the Netherlands, Britain, France, and Austria (Dietz et al. 1996). Where it is invasive, stands of *Bu. orientalis* may form dense monocultures in ruderal areas such as roadsides and waste ground, but the plant apparently can also out-compete mid-successional vegetation and agricultural crops (Dietz et al. 1996). Like all brassicaceous plant species, *Bu. orientalis* is able to biosynthesize secondary plant compounds called 'glucosinolates' (GS). These compounds play an important role against a range of attackers and are especially effective in reducing the performance of generalist herbivores (Chew 1988; Rask et al. 2000).

In this study we compare the development and survival of two specialist herbivores, *Pieris rapae* L. and *P. brassicae* L. (Lepidoptera: Pieridae) and two generalist herbivores, *Spodoptera exigua* Hübner and *Mamestra brassicae* L. (Lepidoptera: Noctuidae) on an accession of *Bu. orientalis* originating from a population in the Netherlands, as well as on two native Dutch crucifers, *Brassica nigra* L. and *Sinapis arvensis* L. (Capparales: Brassicaceae). These two crucifers were deliberately selected because they are abundant and ubiquitous during the growing season, produce large amounts of biomass and serve as the two most important naturally occurring food plant species for caterpillars of both *P. rapae* and *P. brassicae* over much of Eurasia (Feltwell 1982; Gols et al. 2008b). We also examine oviposition behaviour and preference in the two pierids on the three cruciferous plants. The cabbage moth *M. brassicae*, on the other hand, is commonly found on cultivated crucifers such as cabbage which have depressed levels of GS in shoots (Gols et al. 2008a).

Additional assays were conducted in which several species of generalist herbivores in the Noctuidae (including *M. brassicae* and *S. exigua*) and both pierids were reared from their 1st (L1) and 3rd (L3) instars on a German accession of *Bu. orientalis*. This enabled us to determine if the biological effects of this plant (if any) are similar in plants growing in different regions of central Europe.

The main aims of the study are (1) to determine if the performance of the specialist and generalist herbivores differs on three crucifers, (2) to determine

if oviposition preference of the two pierids differs on the three crucifers and is correlated with performance, and (3) to compare GS levels in healthy plants and plants damaged by larvae of *S. exigua*. Our main hypotheses are (1) that, irrespective of plant species origin, the development of the generalist herbivores will be negatively correlated with total glucosinolate levels as has been demonstrated in other studies with generalists developing on wild perennial crucifers such as wild cabbage (Gols et al. 2008a), and (2) that the development and oviposition preference of the specialist herbivores will be less affected on the different crucifers because they are adapted to the main secondary compounds (GS) contained in these plants (Hopkins et al. 2009).

Materials and methods

All experiments with plants and insects using Dutch accessions were maintained in climate rooms at $22 \pm 2^\circ\text{C}$ with a 16:8 h L:D photoperiod. This closely approximates the length of daylight in the Netherlands in the period between June and August.

Plants

Bu. orientalis is a perennial plant that may live up to 10 years or even longer. The seeds germinate in autumn with new rosettes produced the following year that consist of up to hundreds of leaves. Furthermore, the roots of parent plants may produce horizontal stems below ground, which produce spatially extensive networks of interconnected, yet functionally autonomous plants. Plants typically begin to flower in early May and produce fruits that are ripe by late July. Seeds of *S. arvensis* also germinate in late autumn and begin growing the following spring. Plants usually flower in May and produce seeds in late June, although occasionally there is a second generation later in the year. The life-cycle of *B. nigra* is similar, except that the plants generally do not flower until mid-July. *S. arvensis* and *B. nigra* are annuals with short life-cycles. However, because they grow quickly in ruderal sites, they are capable of producing significant above-ground plant biomass which is visible for about 8 weeks in the growing season. As stated earlier, both species serve as major food plants for spring and

summer generations of some specialist herbivores, most notably larvae of large and small cabbage butterflies (Gols et al. 2008a).

Seeds of *Bu. orientalis* were obtained from an established wild population growing along a railway line mid-way between Arnhem and Nijmegen, the Netherlands, within a few hundred meters of the River Rhine. The plants from this population originated from a small cluster of ~5 plants. Seeds of *B. nigra* and *S. arvensis* were obtained from wild plants growing naturally in large single populations in Wageningen, the Netherlands. For the German assays, plant leaves were collected during late summer from plants growing in fields adjacent to the Max Planck Institute, Jena, Germany.

The seeds of *Bu. orientalis* were extracted from dried fruits by gently cracking them open using a nutcracker. The seeds of the other crucifers were extracted from dried pods by hand. In all species the seeds were allowed to germinate in small plastic boxes (15 × 10 × 6 cm) containing glass beads immersed in tap water. Newly germinated seedlings were grown in 2 l plastic pots containing a soil mixture consisting of approximately 30% sand, 5% clay, and 65% peat. Plants were grown in a greenhouse at 22 ± 2°C, 50–70% r.h, with a photoperiod of at least 16 h. Natural daylight was supplemented by metal-halide lamps (225 μmol photons/m²/sec) during the 16 h photoperiod. Plants were watered daily, and after 2 weeks they were provided with nutrient-enhanced Hoagland solution to compensate for nutrient depletion in the soil. All the plants used in the herbivore development and oviposition assays were between 4 and 5 weeks old.

Distribution of *Bunias orientalis*

The Atlas Florae Europaeae (AFE) (Jalas and Suominen 1972–1994) has calculated the presence or absence of 2432 species of plants using grid cells that are approximately 50 km² but which vary according to latitude and longitude. These data have been combined with personal communication with other researchers in many countries throughout Europe where *Bu. orientalis* is either present or absent. An estimate of the current native, invasive and exotic (not yet invasive) range for *Bu. orientalis* is presented in Fig. 1.



Fig. 1 Distribution map of *Bunias orientalis* in Europe and western Asia. *Black circles* indicate area where *Bu. orientalis* is native; *open circles* where the species is highly invasive; *black stars* where the species is present but as of yet non-invasive. Data from Atlas Florae Europaeae (AFE) and in personal communication with various institutions throughout Europe

Insects

The small (*P. rapae*) and large (*P. brassicae*) cabbage white butterflies are native over much of Eurasia and are pests in collard crops. Adults of *P. rapae* lay single eggs on the underside of leaves, and the larvae, which are green in colour, feed solitarily. After completing 5 instars, the larvae pupate on plant stems or leaves. By contrast, female *P. brassicae* butterflies lay broods of up to 150 eggs on the underside of the leaves, and after hatching the dark green larvae feed gregariously until their third instar. They then disperse on the food plant and become yellow and black in colour, feeding on both leaves and flowers (Smallegange et al. 2007). After completing 5 instars, the larvae pupate on the food plants. Eggs of both pierid species were obtained from cultures maintained at the Laboratory of Entomology, Wageningen University (WUR) the Netherlands for many years. The insects were originally collected from agricultural fields in the vicinity of the University. Both species are reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv *Cyrus*). Although they readily oviposit and feed on a number of wild crucifers (Feltwell 1982), little is known about their natural association, if any, with exotic species such as *Bu. orientalis*.

The cabbage moth, *M. brassicae*, is a generalist herbivore that feeds on a wide variety of plants. It is native to Eurasia and is also a serious pest in collards and other cruciferous crops as well as in beet and onion fields. Large numbers of this species have been observed feeding on *Bu. orientalis* plants in Germany (J. Harvey, personal observations). The southern beet armyworm, *S. exigua* is a generalist herbivore whose larvae feed on a very wide range of plants in several different families. Its life cycle is similar to that of *M. brassicae* (Carter 1984). The species is a well-established pest of crop species over much of the world. Both noctuids lay their eggs directly onto the food plant and their larvae complete 5 instars before dropping to the soil to pupate. Eggs of *M. brassicae* and *S. exigua* were also obtained from cultures maintained at the Laboratory of Entomology at WUR. Larvae of *S. exigua* were reared on artificial diet as described by Vickerman and Trumble (1999). Adult moths were kept in groups of 5–10 in 1 l plastic beakers containing vermiculate, with water absorbed in cotton wool added in small plastic vials. Paper sheets were placed around the inside of the beakers as well as over the top, which was secured with an elastic band. Female moths oviposit directly onto the paper and newly hatched larvae were placed onto artificial diet in small plastic boxes. *M. brassicae* are reared on Brussels sprouts plants as described for both *Pieris* species (above). Adult moths were also kept in 1 l beakers and eggs were collected in the same fashion as with *S. exigua*.

For experiments with the German accession of *Bu. orientalis* experiments used the following insects: Cotton bollworm (*Helicoverpa armigera*, Too-woomba strain [Australia]), cabbage moth (*Mamestra brassicae*), beet armyworm (*Spodoptera exigua*), cotton leafworm (*Spodoptera littoralis*, both species obtained as lab strains from BAYER) and tobacco budworm (*Heliothis virescens*, YDK strain obtained from North Carolina; Gould et al. 1995). Larvae were reared on a laboratory-prepared pinto bean diet (Perkins et al. 1973) and held in a climatic-controlled room at 25°C, 16:8 (L:D) photoperiod, and 60% RH until used for experiments. Cultures of *P. rapae* and *P. brassicae* were originally obtained from Seritech (Warwick, UK) and reared on *Brassica oleracea gemmifera* cv. Rosella at 22°C and 70% humidity with a 16:8 (L:D) photoperiod. Both cultures were

supplemented with individuals from Jena, Germany, to reduce the risk of inbreeding.

Experimental protocol

Development of insect herbivores on Dutch populations of Brassica nigra, Sinapis arvensis and Bunias orientalis

Egg batches of *P. rapae* and *P. brassicae* were allowed to hatch on excised Brussels sprouts leaves and the neonate larvae were then carefully removed with a soft brush and transferred to experimental plants in large rearing cages (40 × 25 × 35 cm). Larvae were placed in groups of 20 on middle-aged leaves of single plants, and were then allowed to freely disperse on the plants. In addition to the plants containing larvae, an additional 2 plants were placed into the cages to ensure that the caterpillars had sufficient food. No more than 40 larvae were maintained in a single cage. As they grew and moulted, larvae were distributed to other cages containing fresh food plants. In total, larvae of *P. rapae* and *P. brassicae* were reared on no less than 16 plants of each species. Mature larvae crawl to the top of the cages where they initiate pupation. Fresh pupae of both pierids were carefully removed from the cages and weighed on a Mettler-Toledo MT5 Electrobalance (accuracy ±1 µg). In order to determine larval mortality, initial (L1) larval and terminal (pupal) counts were made for all herbivore-plant combinations. Mortality was determined as the percentage of neonate larvae that failed to pupate. Two replicates of the experiment were performed: the first between May and July of 2006 and the second between April and June of 2007. In the first replicate, 25 larvae were also randomly selected from each plant species and weighed on the microbalance when they were 10 days old (post hatching). Because many of the *P. rapae* and *P. brassicae* larvae had already died on *Bu. orientalis* plants, the sample sizes for these species on *Bu. orientalis* was smaller than on either *S. arvensis* or *B. nigra*.

Cohorts of newly hatched larvae of *S. exigua* and *M. brassicae* were allowed to feed on excised leaves of the three food plant species that were kept in separate in plastic boxes for the first 2 days. Approximately 60 larvae were then individually removed from the boxes and placed individually into small clip

cages (3 cm dia.) on mid leaves of their respective food plants. No more than 5 larvae were allowed to feed on a single plant. The clip cages were checked daily and were moved to another part of the same leaf if more than half of the leaf tissues under the cage had been consumed. On the 8th day, the larvae of both species were transferred to large clip cages (8 cm dia.) and these were placed onto new food plants. Because they are small, 3–5 larvae of *S. exigua* were maintained in a single clip cage, whereas the large *M. brassicae* were kept singly. The *S. exigua* experiment was based on a single replicate performed in April–May, 2007; two replicates were performed for *M. brassicae* (the same times as those performed for the two pierid species). Pupal mass and mortality were determined following the same procedure as for the pierids.

The growth curves (or trajectories) of *M. brassicae* larvae were also compared on the three crucifer species. In order to compare the larval growth trajectories of *M. brassicae*, 25 larvae per plant species were reared in a separate cohort during the second replicate as described above. Every 3 days from hatching the larvae were removed from clip cages and weighed on the Mettler-Toledo MT5 Electrobalance (accuracy $\pm 1 \mu\text{g}$). Once the larvae exceeded approximately 100 mg, they were then weighed on a Mettler-Toledo AG135 Electrobalance (accuracy $\pm 10 \mu\text{g}$). In the first replicate, 25 larvae were also randomly selected from clip cages on each plant species and weighed on the microbalance, but in this instance when they were 12 days old (post hatching). For *S. exigua*, many of the larvae had already died on *Bu. orientalis* by this time hence the sample size for this species on this plant was smaller than on either *S. arvensis* or *B. nigra*.

Survival of generalist and specialist herbivores on a German accession of *Bunias orientalis*

To determine if a different accession of *Bu. orientalis* has similar or different effects on the development of generalist (Noctuidae) and specialist (Pieridae) herbivores, assays were conducted at the Max Planck Institute. Eggs of the 5 generalist herbivores (*Heliothis virescens*, *Helicoverpa armigera*, *H. subflexa*, *S. exigua*, *S. littoralis* and *M. brassicae*) were collected from paper lids overlying plastic containers. Newly hatched L1 larvae were reared in groups of 40

in large plastic tubs (40 × 20 × 20 cm) that were covered with a fine nylon mesh. In these tubs they were provided with a constant supply of excised leaves of *Bu. orientalis* that had been collected from plants growing close to the Institute. A separate cohort of older caterpillars was taken from artificial diet as early L3 and placed in groups of 40 into separate containers. Leaves were also refreshed every 2 days as required. *Pieris rapae* and *P. brassicae* were reared in the same way as the noctuids, except that for the L3 treatment the larvae were removed from intact cabbage plants and reared on excised *Bu. orientalis* leaves. For all insects, survival was monitored daily and any dead larvae were immediately removed from the experiment and were counted. Survival was based on the number of larvae that were able to pupate successfully.

Oviposition behaviour in *Pieris rapae* and *P. brassicae* when provided with *Sinapis arvensis*, *Brassica nigra* and *Bunias orientalis* plants

In order to determine if adult female *P. rapae* and *P. brassicae* butterflies exhibit differences in preference for oviposition sites, an experiment was set up comparing the number of eggs laid on *S. arvensis*, *B. nigra* and *Bu. orientalis* plants. Because it is gregarious, total clutches of eggs laid were also counted in *P. brassicae*. Before the experiment was performed, it was necessary to show that both butterflies do not lay their eggs randomly on any available plants. In two small cages (30 × 25 × 25 cm) several female butterflies of each species were presented with either cultivated cabbage or black mustard plants, as well as with either lima bean (*Phaseolus lunatus*) or tomato (*Solanum lycopersicum*) plants. The latter two species are non-brassicaceous and do not produce GS. In both trials, butterflies laid eggs only on the cabbage or mustard plants, and thus completely ignored the non-crucifers (data not shown). This revealed that the insects sample the plants before ovipositing and thus do not lay their eggs randomly.

Two female and two male butterflies that were approximately 5 days old were placed into large indoor cages (1.1 m³) each containing 2 mature *S. arvensis*, *B. nigra* and *B. orientalis* plants that were 4–5 weeks old in a large greenhouse room (maintained at a constant 25°C with 16 h light). Because

Bu. orientalis plants are not as tall as plants of the other species, they were placed on 20 cm stands that made them of equivalent height. The butterflies were supplied with 20% sugar solution in small Eppendorf tubes that had been placed into the centre of plastic yellow and blue flowers attached to wooden sticks on stands. Both species were kept separately in cages with plants, and the butterflies were removed after 24 h and the eggs (as well as egg clutches for *P. brassicae*) were counted. There were 10 replicates for *P. rapae* and 12 for *P. brassicae*.

Glucosinolate analyses of leaf tissues of *Sinapis arvensis*, *Brassica nigra* and *Bunias orientalis*

To compare the quality and quantity of GS in leaf tissues of the three plant species, leaf tissues were collected from undamaged control plants and plants that had been damaged by *S. exigua* larvae for 5–7 days. This herbivore species was used because it was the one species which during early development fed about equally on the three plant species, meaning that damage was more uniform than in the case of the other herbivores. The distal parts (approximately 20 cm²) were cut from all fully developed leaves of a plant individual, pooled, and immediately stored in paper bags at –20°C. Eight samples were collected from each of the plant species for both treatments (damaged and control). Undamaged and damaged plants were of the same age and were sampled on the same date. Samples were freeze-dried and pulverized. GS were extracted from 40 mg of ground leaf material in boiling 70% methanol. The extract was purified and desulfated on a DEAE-Sephadex A26 column. The desulfated GS were separated on a reverse phase C-18 column (Alltima C-18, 3 µm, 150 × 4.6 mm, Alltech, Deerfield, IL, USA) on HPLC (Dionex, Sunnyvale, CA, USA) with an acetonitrile water gradient. Detection was performed with a Dionex PDA-100 Photodiode array detector set to scan from 200 to 350 nm. For quantification, sinigrin (Sigma, St. Louis, IL, USA) was used as an external standard. Peaks were integrated at 229 nm for which standard response factors have been defined. The different GS were identified based on their retention times and UV spectra compared to those of pure compounds, sinigrin (Sigma, St. Louis, IL, USA),

glucotropaeolin, sinalbin, and glucobrassicin, or compared with a certified oil seed reference (EC Community).

Statistical analyses

Development data: Effect of plant species, block, and their interactive effect on survival of the pierids and *M. brassicae* larvae to the pupae stage were tested using Generalized Linear Models (procedure GENMOD in SAS v. 9.1) with a type III binomial distribution and logit link function. For *S. exigua*, only one time block was performed and hence only the effect of plant species was tested. Effects of plant species on larval mass was analysed using one-way analysis of variance (ANOVA). Effects of plant species, time block, and their interactive effect on pupal mass and development data were analysed using 2-way ANOVA (procedure GLM, SAS v. 9.1, SAS Institute Inc., Cary, NC, USA). No data transformation was necessary to meet assumptions of normality and homogeneity of variances. Effects of cage, host plant species, and interactions between cage and host plant species on oviposition were analysed with Generalized Linear Models also using SAS v. 9.1 procedure GENMOD on ($x + 1$ transformed) count data with a type III Poisson error distribution and a log link function. Contrasts within the factor host plant species were used to test differences in oviposition between the three host plant species.

Results

Development and survival of generalist and specialist herbivores on *Brassica nigra*, *Sinapis arvensis*, and *Bunias orientalis* plants

Survival of *P. rapae* larvae to the pupal stage was strongly dependent on the identity of the plant species ($\chi^2 = 291.23$, $P < 0.001$). Effects of replicate and interactions with replicate were not significant. In both replicates, all larvae perished on *Bu. orientalis* plants, with most failing to survive during the first instar. By contrast, survival was over 70% in both of the native crucifers (Fig. 2a). Body mass in 10-day old larvae of *P. rapae* varied significantly across the different plant species ($F_{2, 56} = 68.66$, $P < 0.001$).

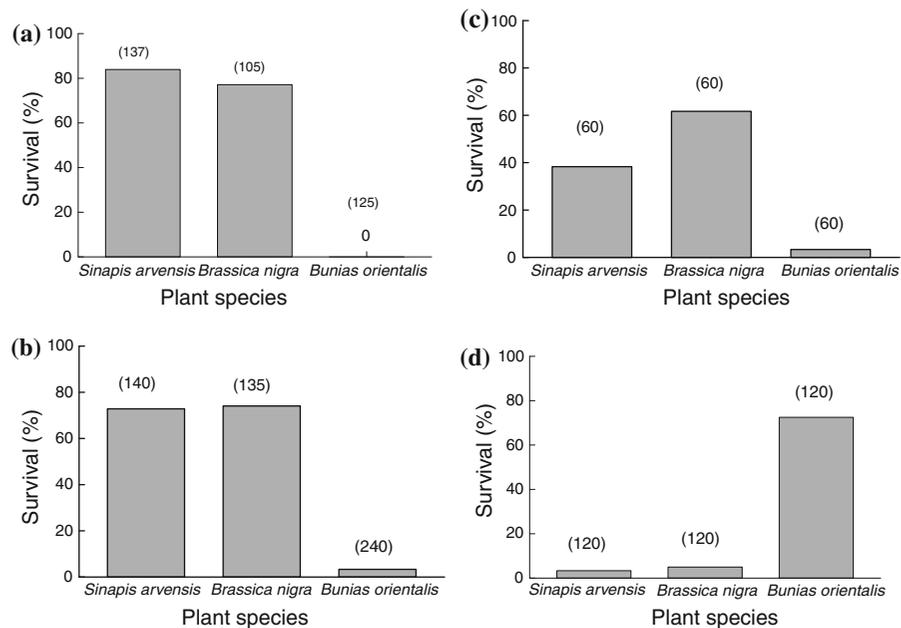


Fig. 2 Survival of different insects on *Sinapis arvensis*, *Brassica nigra* and *Bunias orientalis* plants. **a** *Pieris rapae*, **b** *P. brassicae*,

c *Spodoptera exigua*, **d** *Mamestra brassicae*. Sample sizes are indicated in parentheses over the bars

Only 9 larvae reared on *Bu. orientalis* survived this long, and they were a small fraction of the size of larvae developing on the native crucifers (Fig. 3a). Pupal mass in *P. rapae* varied significantly with replicate ($F_{1, 195} = 125.62$, $P < 0.001$) and between the two native plant species ($F_{1, 195} = 25.63$, $P < 0.001$) whereas the interactive effect of these parameters was not significant ($F_{1, 195} = 2.21$, $P = 0.138$). Insects developing in the second replicate were smaller than those developing in the first replicate (see “Materials and methods”) and *B. nigra* plants were of somewhat higher quality than *S. arvensis* plants for this herbivore (data not shown).

Survival of *P. brassicae* larvae also was significantly associated with the identity of the food plant ($\chi^2_2 = 356.34$, $P < 0.001$). As with its congener, *S. arvensis* and *B. nigra* were much more suitable plants than *Bu. orientalis*, with more than 70% of larvae pupating on the natives. In the second replicate, all larvae perished on *Bu. orientalis* plants, whereas approximately 6% managed to pupate on this plant in the first (Fig. 2b). Overall, survival was lower in replicate 2 ($\chi^2_1 = 37.88$, $P < 0.001$) and the magnitude of the effect of host species was slightly affected by replicate (host species \times replicate interaction, $\chi^2_1 = 6.11$, $P < 0.05$). Body mass in 10 day-old

larvae also varied significantly with plant species ($F_{2, 66} = 62.60$, $P < 0.001$). As with *P. rapae*, larval mass in 10-day old *P. brassicae* was much lower on *Bu. orientalis* plants (Fig. 3b). Pupal mass in *P. brassicae* also varied significantly between replicates ($F_{1, 205} = 9.92$, $P < 0.01$) but not between the two native crucifers ($F_{1, 205} = 0.561$, $P = 0.436$), and the interactive effect between these parameters was also not significant ($F_{1, 205} = 0.38$, $P = 0.541$). As with *P. rapae*, insects in the second replicate were smaller than in the first replicate, but the two crucifers were of similar quality. A one-way ANOVA, incorporating *Bu. orientalis* pupal data from the first replicate, revealed that pupal mass differed significantly with plant species ($F_{2, 124} = 9.99$, $P < 0.001$). Unlike *P. rapae*, many *P. brassicae* larvae readily fed on *Bu. orientalis* plants during early larval development, but most were unable to survive beyond their third instar. Those that did manage to pupate in the first replicate were significantly smaller as pupae on *Bu. orientalis* plants than on the natives.

The survival of *S. exigua* larvae was also found to be strongly associated with foodplant identity ($\chi^2_2 = 45.80$, $P < 0.0001$). Like the specialist herbivores, *S. exigua* survival was much higher on the

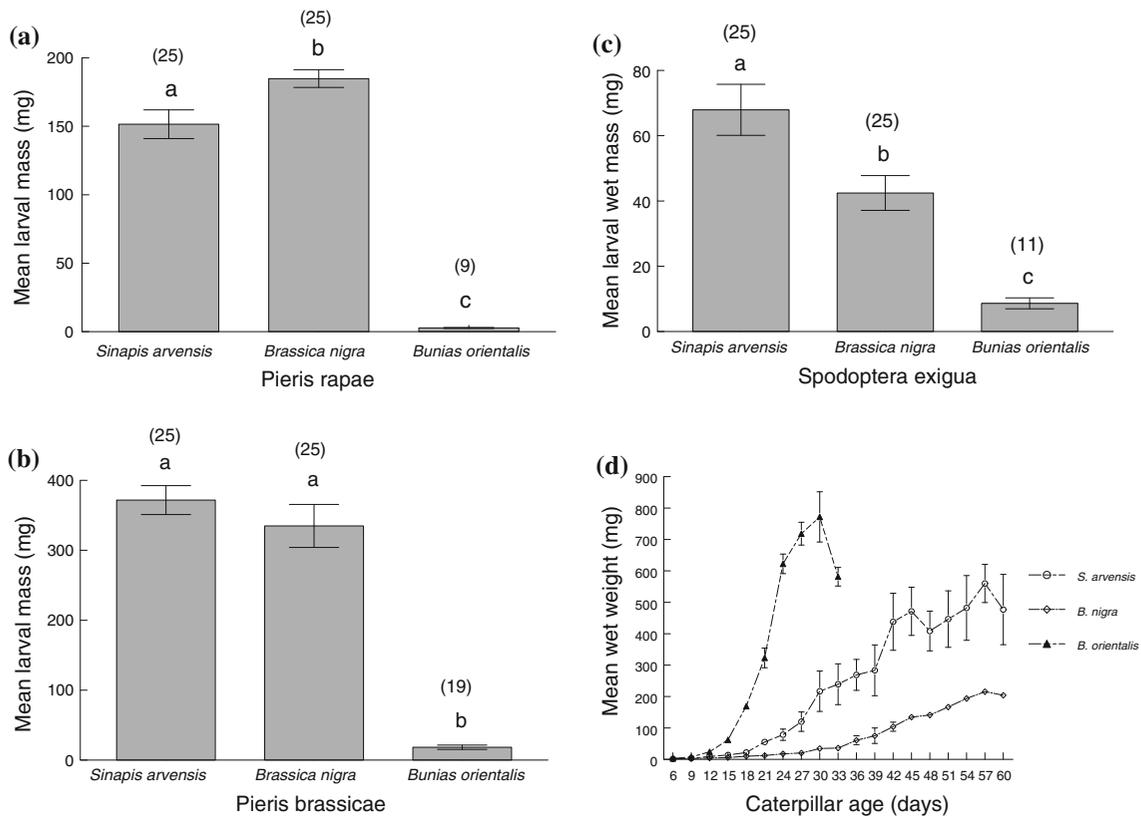


Fig. 3 **a, b, c** Larval wet mass at 10 days of age in different insect herbivores developing on *Sinapis arvensis*, *Brassica nigra* and *Bunias orientalis* plants. **d** Growth trajectories of *Mamestra brassicae* larvae from hatching until pupation or precocious death. Line bars represent standard error of the

mean. Bars with the same letter are not significantly different (Tukey–Kramer tests, $P > 0.05$). Sample sizes for **(a, b, c)** are as indicated in parentheses over the bars. **d** 20 larvae per plant from hatching

native crucifers than on the invasive (Fig. 2c). The mass of 12-day old *S. exigua* larvae also varied significantly with plant species ($F_{2, 58} = 14.83$, $P < 0.0001$). The pattern was similar to that in the pierids, with larval mass greatly depressed on *Bu. orientalis* plants. Larvae were largest at this time on *S. arvensis* plants (Fig. 3c).

In *M. brassicae*, larval to pupal survival was also found to vary amongst the different plants tested ($\chi^2_2 = 218.88$, $P < 0.001$). However, the pattern differed profoundly from that described in the other species, with survival in both replicates being considerably higher on *Bu. orientalis* plants than on the native crucifers (Fig. 2d). Overall survival did not differ between replicates, but the slightly higher survival of *S. arvensis* in replicate 2 and of *B. nigra* in replicate 1 resulted in a significant interaction between replicate and species ($\chi^2_2 = 12.88$, $P < 0.01$).

The mass of 12-day old *M. brassicae* larvae also varied significantly with plant species ($F_{2, 72} = 51.39$, $P < 0.0001$). The pattern was, however, completely opposite to that observed in the pierids and *S. exigua*, with larval mass greatly depressed on the native plants and highest on *Bu. orientalis* (data not shown). A comparison of the growth trajectories of *M. brassicae* caterpillars in the three plants revealed that larvae grew exponentially in *Bu. orientalis*, exhibiting a ‘J-curve’. By contrast, larval development was depressed in *S. arvensis* and especially on *B. nigra* plants (Fig. 3d).

Survival of generalist and specialist herbivores on a German accession of *Bunias orientalis*

Most of the insect herbivores reared on a German accession of *Bu. orientalis* were not able to pupate on

Table 1 Stage-specific and overall percentage survival (\pm SD) of generalist and specialist herbivores in the Lepidoptera on *Bunias orientalis* plants (German accession)

Insect species	n	Sp/Gen	1st instar	3rd instar
<i>Heliothis virescens</i>	120	Gen	0.0 (0)	0.0 (0)
<i>Helicoverpa armigera</i>	120	Gen	0.0 (0)	4.2 (1.6)
<i>Heliothis subflexa</i>	120	Gen	0.0(0)	0.0 (0)
<i>Spodoptera exigua</i>	120	Gen	0.0 (0)	0.0 (0)
<i>Spodoptera littoralis</i>	240	Gen	52.2 (3.5)	68.2 (1.8)
<i>Mamestra brassicae</i>	320	Gen	81.3 (2.4)	96.5 (1.2)
<i>Pieris rapae</i>	120	Sp	0.0 (0)	0.0 (0)
<i>Pieris brassicae</i>	160	Sp	0.0 (0)	0.0 (0)

Sp specialist herbivores, Gen generalist herbivores

this plant, irrespective as to whether they were provided with leaves as L1 or L3 instars. Both pierids suffered 100% mortality on *Bu. orientalis* as did three of the noctuids (*H. virescens*, *H. subflexa* and *S. exigua*). By contrast, *S. littoralis* and *M. brassicae* both survived well on *Bu. orientalis*, with >80% of *M. brassicae* pupating successfully (Table 1).

Oviposition behaviour in *Pieris rapae* and *P. brassicae* when provided with *Sinapis arvensis*, *Brassica nigra* and *Bunias orientalis* plants

Mean leaf area of the three plant species did not differ significantly when *P. rapae* butterflies were foraging ($F_{2, 27} = 3.20$, $P = 0.06$) but did so when *P. brassicae* butterflies were foraging ($F_{2, 27} = 10.175$, $P < 0.001$). With respect to *P. rapae*, leaf area of *Bu. orientalis* were marginally (though not significantly) greater than for the other two species. On the other hand, *B. nigra* plants had more leaf area than the other two species in cages with *P. brassicae*. However, in both experiments all of the plants had very large amounts of foliage and this was almost certainly not a limiting factor.

Pieris brassicae on average laid 3.8 egg batches (broods) per cage with an average brood size of 38.3 eggs per brood. Brood size did not differ between host plants ($F_{2, 35} = 0.06$, $P = 0.94$), but the number of oviposited broods was significantly lower on *Bu. orientalis* than on *B. nigra* (Table 2) and the total number of oviposited eggs was significantly lower on *Bu. orientalis* (8.2% of all eggs) than on *S. arvensis*

Table 2 Generalized Linear Model analysis of effects of cage and host plant species on oviposition (number of egg clusters or eggs deposited) by *Pieris brassicae* and *Pieris rapae* on three different host plant species, *Brassica nigra*, *Sinapis arvensis* and *Bunias orientalis* presented as three-choice in cages with three plants

	df	<i>P. brassicae</i>		df	<i>P. rapae</i>
		# Broods	# Eggs		
Cage (C)	9	2.4	45.4***	11	143.4***
Host species (H)	2	7.1*	46.9***	2	26.3***
Bo versus Bn	1	6.5*	40.2***	1	10.9**
Bo versus Sa	1	0.4	13.2***	1	23.2***
Bn versus Sa	1	3.8 ⁺	16.7***	1	0.5
C * H	18	12.4	1187.7***	22	140.9***

Degrees of freedom (df) and chi-square values are given

⁺ $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

(20.0%), both of which were lower than on *B. nigra* (71.8%) (Table 2, Fig 4a). The magnitude of the host plant-specific differences in oviposition varied among cages (cage*host plant interaction, Table 2, Fig. 4a).

Pieris rapae on average laid 37.9 eggs per cage. Oviposition was significantly lower on *Bu. orientalis* (13.9%) than on *S. arvensis* (38.2%) and *B. nigra* (47.9%), whereas the difference between the latter two species was not significant. Like for *P. brassicae*, the magnitude of the host plant-specific differences varied among cages (cage*host plant interaction, Table 2, Fig. 4b).

Glucosinolate analyses in leaf tissues of *Sinapis arvensis*, *Brassica nigra* and *Bunias orientalis*

Analysis of glucosinolates in leaf tissues revealed considerable quantitative and qualitative variation in concentrations of these secondary plant compounds among the three plant species (Table 3). *p*-Hydroxybenzyl glucosinolate (also called sinalbin) is the dominant compound in both *Bu. orientalis* and *S. arvensis* contributing 85–95% and 35–60%, respectively, to the total glucosinolate content. Different aliphatic gluosinolates (glucosinolates derived from methionine) further characterized *Bu. orientalis* and *S. arvensis*. In contrast, leaf tissues of *B. nigra* contained only one glucosinolate, i.e. allylglucosinolate (trivial name sinigrin), which was not found in foliar tissues of the other two plant species. In *S. arvensis*, concentrations of all the glucosinolate

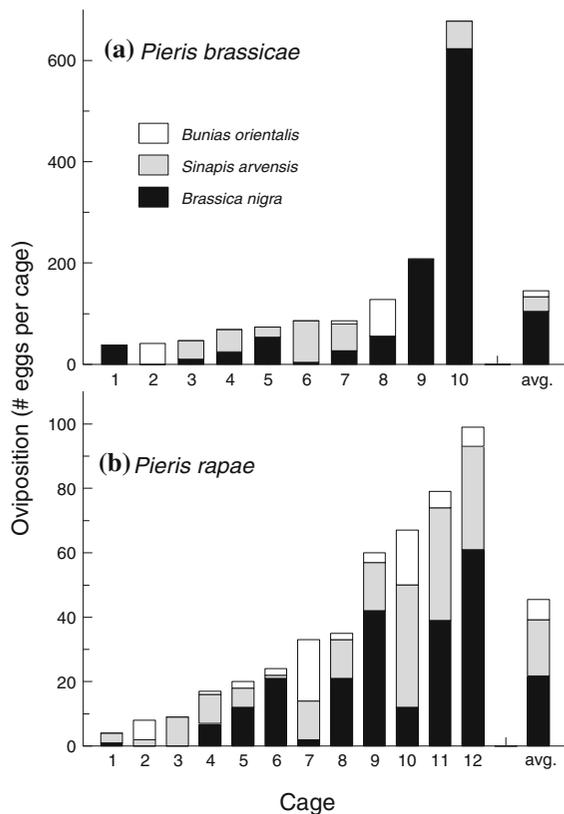


Fig. 4 Oviposition (number of eggs deposited) by **a** *Pieris brassicae* and **b** *Pieris rapae* on plants of three different host species offered in a three-choice experiment (three plants per cage). There were two female and 2 male butterflies per cage over the course of 24 h

compounds increased dramatically in response to *S. exigua* feeding, whereas in *Bu. orientalis* only sinalbin concentrations were significantly higher in damaged than in undamaged control plants. Contrastingly, sinigrin concentrations in *B. nigra* did not increase significantly in response to herbivory.

Discussion

Both species of *Pieris* studied here oviposit and successfully develop on a large number of wild species of plants in the Brassicaceae (Feltwell 1982; Robinson et al. 2009). This includes species with which they probably have no evolutionary history due to small plant size and/or differing seasonal phenologies in the life-cycles of the plants and the herbivores (Yano and Ohsaki 1993; Harvey et al.

2007; Pfalz et al. 2007). Moreover, larvae of both species readily feed and are able to pupate on several novel crucifer species whose distributions do not naturally overlap with local populations of *P. rapae* or *P. brassicae* (J. Harvey unpublished; Dethier 1947; Johansson 1951; Benrey et al. 1998; Robinson et al. 2009). By contrast, the results of this investigation show that *Bu. orientalis* plants were highly toxic to larvae of both pierids. Caterpillars grew much more slowly and mortality was at or close to 100% in both of the pierids when reared on *Bu. orientalis*, whereas both species developed very successfully on the native crucifers, *B. nigra* and *S. arvensis*, with pupal survival of more than 70% on both plants. Although adults of *P. rapae* and *P. brassicae* preferred the native crucifers on which to oviposit, both species laid some eggs on leaves of *Bu. orientalis* plants. Further assays also suggest that *Bu. orientalis* is toxic to other specialist crucivores, including *Plutella xylostella* (J. Harvey, unpublished data) and *Athalia rosae* (C. Müller, personal communication).

Development and survival of the generalist noctuids on the three crucifers was mixed. Like *P. rapae* and *P. brassicae*, survival of *S. exigua* larvae was much higher on the native Dutch crucifers than on *Bu. orientalis*. By contrast, larvae of *M. brassicae* readily fed on *Bu. orientalis* and the survival of this herbivore species was significantly higher on this plant than on either *S. arvensis* or *B. nigra*. Both pierids and several other noctuids also performed very poorly on a German accession of *Bu. orientalis*. In line with results on Dutch *Bu. orientalis*, larvae of *M. brassicae* developed very successfully on German plants, as did *S. littoralis*. This shows that the effects of *Bu. orientalis* on herbivore performance vary dramatically amongst closely related noctuids, even congeners, and that the ability to develop on this plant is not correlated with phylogeny but is species-specific. A cursory survey of *Bu. orientalis* plants in Jena, Germany, in September 2006 also revealed that many plants were infested with mature *M. brassicae* caterpillars, whereas few other insect herbivores were found (J. Harvey and R. Gols, personal observations). This supports data from the development assays performed here.

GS have been shown to play a role in the success of some invasive plants in the Brassicaceae (Müller 2009). Chemical analyses of leaf tissues in the three crucifers revealed that the main GS found in *Bu.*

Table 3 Glucosinolate concentrations (mean \pm SEM) in undamaged and herbivore-damaged leaf tissues ($n = 8$) of *Bunias orientalis*, *Sinapis arvensis* and *Brassica nigra*

Plants species	Glucosinolates	Undamaged control	Damaged by <i>S. exigua</i>
<i>Bu. orientalis</i>	4-Methylsulfinylbutyl Gls	0.73 \pm 0.07	1.07 \pm 0.21
	4-Methylsulfinylbut-3-enenyl Gls	2.72 \pm 0.40	3.80 \pm 0.68
	<i>p</i> -Hydroxybenzyl Gls	25.8 \pm 2.0	36.7 \pm 3.8*
	Total	29.2 \pm 2.3	41.6 \pm 4.0*
<i>S. arvensis</i>	8-Methylsulfonyloctyl Gls	0.54 \pm 0.13	2.53 \pm 0.44**
	9-Methylsulfinylnonyl Gls	0.42 \pm 0.05	1.87 \pm 0.28**
	9-Methylsulfonylnonyl Gls	4.25 \pm 0.81	12.5 \pm 1.9**
	10-Methylsulfinyldecyl Gls	0.74 \pm 0.10	2.03 \pm 0.29**
	10-Methylsulfonyldecyl Gls	1.73 \pm 0.32	3.57 \pm 0.53*
	<i>p</i> -Hydroxybenzyl Gls	9.03 \pm 1.02	21.9 \pm 3.7**
	Total	16.7 \pm 1.9	45.5 \pm 5.2**
<i>B. nigra</i>	Allyl GS	15.9 \pm 2.7	20.2 \pm 2.9
	Total	15.9 \pm 2.7	20.2 \pm 2.9

The compounds that are significantly induced by *S. exigua* feeding are indicated by * $P < 0.05$ or ** $P < 0.001$ based on two-sample *t*-tests on log₁₀-transformed concentrations

orientalis was sinalbin, which was also the dominant GS present in leaves of *S. arvensis*. Concentrations of GS were also higher in undamaged *Bu. orientalis* shoots than in undamaged *S. arvensis* or *B. nigra* shoots. However, both pierids are known to perform well on many species in the Brassicaceae that produce significantly higher total concentrations of GS in their shoots than were found in any of the plants used in this study (Gols et al. 2008b). At the same time, the development of *M. brassicae* is negatively correlated with total concentrations of GS found in shoots of wild cabbage plants (Gols et al. 2008a). Our results reveal that it is highly unlikely that GS, in particular sinalbin, play a major role in the defence of *Bu. orientalis* against either *M. brassicae* or the specialist herbivores studied here.

Many crucifers have developed a 'second line' of chemical defence that appears to be primarily directed against specialist herbivores (Feeny 1977; Renwick 2002). These defences often involve the production of non-GS that either act as oviposition or feeding deterrents, or else chemicals which are toxic to the feeding insects (Renwick 2002). *P. rapae* butterflies avoid ovipositing and feeding on *Iberis amara* plants because of the presence of cucurbitacin glycosides in leaf tissues of this plant (Sachdev-Gupta et al. 1993; Renwick and Huang 1996). *P. rapae* butterflies and their larvae have been found to reject *Erysimum cheiranthoides* plants because of the presence of cardenolides in plant shoots (Sachdev-Gupta et al. 1990). In this example, the most

active oviposition deterrents differ from the most active feeding deterrents, revealing that the plant employs two lines of defence against different stages of *P. rapae*. Very recently it has been found that, in addition to GS, *Bu. orientalis* produces an imidazol alkaloid, although it is yet to be determined if this secondary compound affects the development of specialist herbivores (H. Vogel and M. Riechelt, unpublished data). Characterizing the function of this chemical will be the focus of future research.

Recent studies by Chew and colleagues (Keeler et al. 2006; Keeler and Chew 2008) examined interactions between the Nearctic butterfly, *Pieris oleracea*, and an exotic weed, the garlic mustard *Alliaria petiolata* in the eastern United States. This plant originates in Eurasia but become highly invasive in parts of North America in recent decades, displacing native plants, including other species of crucifers that serve as important food-plants for larvae of *P. oleracea*. The authors found that although female *P. oleracea* butterflies lay their eggs on *A. petiolata*, their larvae develop very poorly on it because the plant produces cyanide, as well as the GS alliarinoside, which are apparently novel compounds to which *P. oleracea* has not co-evolved (Cipollini and Gruner 2007). However, some populations of this herbivore are apparently adapting to this plant where it is abundant (Keeler et al. 2006).

In much of Europe, the main foodplants of pierid butterflies are short-lived annuals, such as *S. arvensis* and *B. nigra*. The seasonal phenology of these two

species differs, with *S. arvensis* abundance peaking in May and June and *B. nigra* in July and August (Gols et al. 2008b). For multivoltine insects, this means that different generations of the same species may feed on different species of plants. In much of Europe, *P. rapae* is known to be trivoltine (three generations per year) and *P. brassicae* bivoltine (two generations per year; Feltwell 1982). This means that the first generations of both pierids may depend on *S. arvensis* as food plants, whereas second generations depend on *B. nigra* as food plants. By late August, when many butterflies are still active, most annual species have already seeded and lost their foliage, meaning that foraging adult females may find it difficult to locate suitable foodplants on which to oviposit. Unlike many annual crucifers, *Bu. orientalis* produces a rosette in spring that persists well until late in the growing season. Mature larvae of *P. brassicae* and *P. rapae* have been observed, albeit in small numbers, on wild *Bu. orientalis* plants in both Germany and The Netherlands (J. Harvey, personal observations). In line with these observations, in the oviposition choice experiments, *Pieris* females occasionally laid eggs on *Bu. orientalis*, indicating that there is opportunity for host plant switches. This suggests if frequency dependent selection to adapt to novel plants is strong, this may eventually enable these herbivores to incorporate these plants into their diet (Keeler et al. 2006; Keeler and Chew 2008).

In summary, this study has reported that invasive plants may be toxic for non-adapted insect herbivores, but that ecological ‘fits’ can also occur by chance without the need for any evolutionary precedent. Mal-adaptation in both generalist and specialist herbivores to *Bu. orientalis* supports the predictions of the ‘enemy-release hypothesis’ (Keane and Crawley 2002; Liu and Stiling 2006; Cincotta et al. 2009) and potentially the ‘novel-weapons hypothesis’ (Callaway and Ridenour 2004; Callaway et al. 2008) unless those few herbivores that can exploit this plant can numerically counter the absence of other attackers. Under reduced herbivore attack *Bu. orientalis* may be able to allocate more resources towards growth, allowing it to outcompete native competitors, a process shown in other plants and described as the ‘evolution of increased competitive ability’ (or EICA) hypothesis (Meyer and Hull-Sanders 2008; Eigenbrode et al. 2009). These areas require detailed studies in the field, a process

that is now underway in both the Netherlands and Germany.

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