Impacts of belowground herbivory on oviposition decisions in two congeneric butterfly species

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Abstract

Root-feeding insects can affect the performance of aboveground insect herbivores when they are forced to feed on the same host plant. Here we explored whether the oviposition behaviour of two closely related herbivorous species (cabbage butterflies; Lepidoptera: Pieridae) is influenced by root-feeding insects, when they are given the chance to choose between host plants with and without root herbivores. Considering that egg load is an important physiological factor influencing the foraging behaviour of insects, we also examined whether root-feeding insects differentially influence oviposition preference in butterflies with low and high egg loads. Oviposition preference in both butterfly species with low and high egg loads was monitored using host plants with and without root herbivores. To ascertain the status of butterfly age with low and high egg loads, the oviducts of a separate group of butterflies was dissected to record the number of immature and mature eggs in butterflies of various ages. *Pieris brassicae* L. butterflies with low egg loads preferred plants without root herbivores over plants with root herbivores, and laid more egg clutches on the leaves of plants that were not attacked by root herbivores. Butterflies with comparatively high egg loads also selected a larger proportion of plants without root herbivores, but laid a similar number of egg clutches on the plant shoots independent of the presence or absence of root herbivores belowground. Independent of the age and egg load, *Pieris rapae* L. butterflies selected a larger proportion of plants not attacked by root herbivores to lay eggs, but the number of eggs laid was similar in plants with and without root herbivores. This study shows that belowground insects can influence behavioural decisions of aboveground insect herbivores. Interestingly, the strength of these interactions depends on the physiological state of the insects which is probably correlated with their perception of environmental quality.

Introduction

The choices that female insect herbivores make for oviposition sites can greatly affect the performance of their offspring. The larvae of most holometabolous insect species possess limited dispersal capacity in their early developmental stages and consequently they depend on the quality

of the plant on which their mothers have chosen to lay their eggs (Schoonhoven et al., 2005). It has been shown that many species preferentially oviposit on host plant species where the performance of their progeny is optimized, i.e., through maximum size, survival, and/or reduced development time (Thompson & Pellmyr, 1991; Mayhew, 1997). By contrast, some studies have reported that female insects sometimes choose nutritionally suboptimal sites in which to oviposit (Thompson, 1988). However, in many of the cases where the chosen oviposition site is nutritionally suboptimal, the food plant was found to be less attractive to natural enemies such as parasitoid

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wasps (Stamp, 2001). In this way, the lower nutritional quality of the food plant selected by the female offered enemy-free space to her offspring, which reduces the risk of parasitism or predation and thus increases survival (Price et al., 1980).

Herbivore performance can be strongly affected by indirect interactions with other herbivore species that previously fed or are currently feeding on plant shoots (Rodriguez-Saona et al., 2005; Beckers & Spoel, 2006; Viswanathan et al., 2007; Zheng & Dicke, 2008; Dicke et al., 2009). The most common outcome of indirect interactions between herbivorous insects that feed from the same plant shoot appears to be competition: one herbivore species triggers plant defenses that impact not only on conspecifics but also on the other species of insect herbivores that co-occur in the plant (Denno et al., 1995; Kaplan & Denno, 2007). Interestingly, herbivore performance can also be influenced by insects feeding in spatially separate but physically connected parts of the plant, such as in roots and shoots (Masters et al., 1993; van der Putten et al., 2001; Wardle, 2002; Bezemer & van Dam, 2005; Kaplan et al., 2008; Soler et al., 2008). Damage by belowground insects can result in positive, neutral, or negative outcomes on the performance of the aboveground herbivores, depending on the species of the plant mediating the interactions and on the identity of the insect species interacting (Johnson et al., 2008). These effects are often mediated by changes in levels of primary (nutrients) and/or secondary compounds (phytotoxins) in plant shoots induced by root herbivory (Bezemer et al., 2002, 2003).

Considering that root-feeding insects can influence the performance of their aboveground counterparts via changes in the quality of the plant, it can be expected that belowground insects are capable of influencing oviposition decisions of foliar-feeding insects. Aboveground herbivores that benefit from damage incurred by herbivores feeding on root tissues should consequently prefer these plants over plants without root herbivory, whereas the opposite should be true if plant quality is reduced by root herbivory. The effects of belowground insects on host preference and behaviour of aboveground parasitoids has recently been explored, and there is clear evidence that belowground insects can affect oviposition decisions of female parasitoids (Masters et al., 2001; Poveda et al., 2005; Soler et al., 2007a; Rasmann & Turlings, 2007). Surprisingly, much less is known as to whether root-feeding insects can influence the oviposition behaviour of aboveground insect herbivores, with which they more directly share the plant, or compete for it.

The main aim of this study was to determine whether the presence or absence of a belowground (root-feeding) herbivore, the cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae), could influence oviposition decisions by females of two closely related aboveground insect herbivores, the cabbage butterflies *Pieris brassicae* L. and *Pieris rapae* L. (Lepidoptera: Pieridae). In a previous study (Soler et al., 2005) we found that in the presence of *D. radicum*, the larval development of *P. brassicae* was extended compared with caterpillars developing on plants not attacked by the root herbivore. An extended developmental window is often correlated with increased susceptibility to parasitism and/or predation [the 'slow-growth–high-mortality hypothesis', sensu Benrey & Denno (1997)]. Consequently, there are potential fitness costs for butterflies of this species which oviposit on plants that are under attack from root herbivores. We therefore hypothesized that the butterflies would prefer to lay eggs on plants that were not attacked by the root herbivore over plants that were previously colonized by the root herbivore.

Both pierids used in the experiments are synovigenic, and emerge with low egg loads, but continue to mature and accumulate eggs throughout the course of their adult life. Given that several studies have reported that variation in the number of mature eggs available for oviposition (= egg load) affects searching intensity and host site selection in butterflies (Pilson & Rausher, 1988; Odendaal & Rausher, 1989; Tatar, 1991; Minkenberg et al., 1992; Javois & Tammaru, 2004; Gibbs et al., 2005; but see Javois & Tammaru, 2006), we examined oviposition behaviour in butterflies with comparatively low and high egg loads. In this scenario, low egg loads in young butterflies may be correlated with a higher risk of egg limitation, thus making females choosier for higher-quality oviposition sites. We therefore hypothesize that female butterflies with low egg loads would more readily reject low-quality plants containing *D. radicum* larvae than butterflies with higher egg loads.

Materials and methods

Delia radicum is a common pest of many agricultural crucifers and develops very successfully on other wild crucifer species, including the model plant for this study, *Brassica nigra* L. (Brassicaceae) (Soler et al., 2005, 2007a,b). Both pierids are specialist herbivores that feed also on crucifers, including many wild and cultivated species. *Pieris brassicae*, the large cabbage white butterfly is a gregarious species that typically lays egg clutches on the leaves of its food plants. *Pieris rapae*, the small cabbage white butterfly, is a solitary species that lays single eggs on the leaves of the food plant. *Delia radicum* larvae were obtained from a culture maintained at the Netherlands Institute of Ecology, Heteren, The Netherlands. *Pieris brassicae* and *P. rapae* pupae were obtained from an insect culture maintained at

the Laboratory of Entomology of Wageningen University, The Netherlands. Pupae of the butterflies were allowed to develop in large rearing cages (30 × 30 × 40 cm) until adult emergence. Adults were maintained in these cages, where they were allowed to mate. Cotton balls soaked with water and 20% honey solution were constantly provided to the adult butterflies.

Brassica nigra seeds were collected from a single wild population growing in Wageningen, The Netherlands. Seeds were surface sterilized, and germinated for transplanting. One week after germination, seedlings were transplanted into 1.2-l pots. The plants were grown in a greenhouse for about 4 weeks, at L16(22 ± 1 °C):D8(16 ± 1 °C) photoperiod and 70% r.h. Natural daylight was supplemented by metal-halide lamps (225 µmol s^{-1} m^{-2} PAR). Plants were watered daily. Some plants began flowering after a few weeks; these were not used in the experiments.

Egg load measurements

To determine whether variation in the physiological state of the butterflies could influence their foraging behaviour in relation to the presence/absence of the root herbivore belowground, the choice experiments were carried out with females that had just mature eggs (relatively low egg loads) and females that had mature eggs for 2–3 days (relatively high egg loads). To determine what egg loads constitute 'low' and 'high' in both pierids, groups of approximately 30 *P. brassicae* and 30 *P. rapae* females of different ages were dissected under a microscope. Over the course of 7 days following eclosion, numbers of both immature (= germaria) and mature (= ovulated) eggs were counted. Ovulated eggs are ovoid, deep white, and hang below the ovaries; non-ovulated eggs are typically translucent and smaller than mature eggs. Under the conditions in which the experiments were performed, butterflies of both species have short life spans and live for about 10 days. It is important to stress that 7 days was determined as the upper limit for age-dissections in which to select females with high egg loads, because butterflies from 0 to 7–8 days of age showed similarly good general physical conditions and flying activity. Therefore, egg load can be expected to be a major difference among the experimental 'young' and 'old' females.

Dissections of the ovaries of *P. brassicae* showed that most butterflies emerged with no mature eggs, and only began to mature eggs in copious numbers on day 3 after emergence (see Results). Consequently, the category 'low egg load' consisted of 3-day-old females. The ovaries of *P. rapae* butterflies contained a few fully matured eggs on the day of eclosion. Three days later, they had matured less than half of their potential egg complements. Therefore,

3-day-old *P. rapae* females were also classified as 'butterflies with low egg loads'. In contrast, 6-day-old butterflies of both species were selected as 'butterflies with high egg loads' as they had about double amount of mature eggs than butterflies classified as with low egg loads and, importantly, as described above they were still actively flying.

Oviposition-preference experiment

To study whether belowground herbivory influences the oviposition preference of *P. brassicae* and *P. rapae* butterflies, choice experiments were performed in large netted and transparent tents (8 × 4 × 2.5 m) containing plants with and without *D. radicum* belowground. For root-herbivore-attacked *B. nigra* plants, six *D. radicum* third instars were introduced per plant, 6 days before each test, while control plants were kept without root herbivores. Six days after inoculation with the belowground herbivores, 10 plants with and 10 plants without root herbivores were placed alternated in the tents, and plants were approximately 1 m apart. The root-density and pre-infestation time period selected was based on previous studies, in which we found that an insect parasitoid species is able to discriminate between plants that were attacked by six larvae of *D. radicum* for 6 days and plants without root herbivore infestation (Soler et al., 2007a,b). Five *P. brassicae* females and five males or five *P. rapae* females and males were then released per tent, and were allowed to forage freely over the course of the next 72 h. The experiments were repeated eight times per species, thus the behavioural responses of 40 *P. brassicae* and 40 *P. rapae* females were observed. More precisely, old *P. brassicae* females were studied in five tents (i.e., 25 females, 100 plants); young *P. brassicae* females were studied in three tents (i.e., 15 females, 60 plants), and old and young *P. rapae* females were observed in four tents each (i.e., two times 20 females, 80 plants).

Cotton balls soaked with water and 20% honey solution were continuously supplied to the butterflies during the experiment. The experimental plants were checked for eggs twice daily, and the egg clutches (in the case of the gregarious *P. brassicae*) or single eggs (solitary *P. rapae*) were counted on each plant. All eggs were removed from the plants once a day at the end of the day. After the experiments were terminated, the root-attacked plants were inspected to ensure that *D. radicum* larvae were present and actively feeding on the plants. In all of the plants most of the larvae that had been introduced were recovered, confirming that all the root-attacked plants were indeed successfully infested during the course of the experiments.

Statistical analysis

The longitudinal structure of the data (repeated measures on the same plant over days) is modelled within a

generalized linear mixed model (GLMM) by an autoregressive process of order 1 (AR1). The model was fitted by the GLMM procedure of GenStat (Payne et al., 2009) that fits the model by the method of Schall (1991). For counts (number of egg clutches and number of eggs laid by the females) the Poisson distribution was used with the logarithm as link function. For the binary trait 'detected/undetected' (i.e., proportion of plants selected for oviposition by the females) a binomial distribution with the logit link function was used. The number of eggs per clutch on plants with and without root herbivores was tested using ANOVA (GenStat; Payne et al., 2009).

Results

Dissection of the ovaries of 1- to 7-day-old *P. brassicae* butterflies showed that this species began to produce significant numbers of mature eggs only on about the third day after emergence (Figure 1A). The number of mature eggs was strongly correlated with adult age, and increased almost linearly over the course of adult life, whereas the number of immature eggs did not significantly change

with age (Figure 1C). Dissections of the ovaries of *P. rapae* butterflies showed that the ovaries of this species contained a few mature eggs at eclosion (Figure 1B). The number of mature eggs for this species also increased with age, but the number of immature eggs decreased slightly but significantly as females aged (Figure 1D).

Both *P. brassicae* and *P. rapae* females with low and high egg loads selected a significantly higher proportion of plants without root herbivores over plants attacked by root herbivores on which to oviposit (Table 1A; Figures 2A and 2C). However, root herbivory also influenced the number of egg clutches laid by *P. brassicae* butterflies, but it did not significantly affect the number of eggs laid by *P. rapae* (Table 1B). *Pieris brassicae* females oviposited fewer egg clutches on plants with root herbivory, compared with control plants without root herbivores, but this was only significant for females with low egg loads, resulting in a significant root herbivory*egg load interaction (Table 1B; Figure 2B). The number of eggs per clutch was between 30 and 40 eggs on both plant types with and without root herbivores ($F_{1,127} = 1.29$, P = 0.3). Instead, *P. rapae* butterflies laid similar numbers of eggs on plants
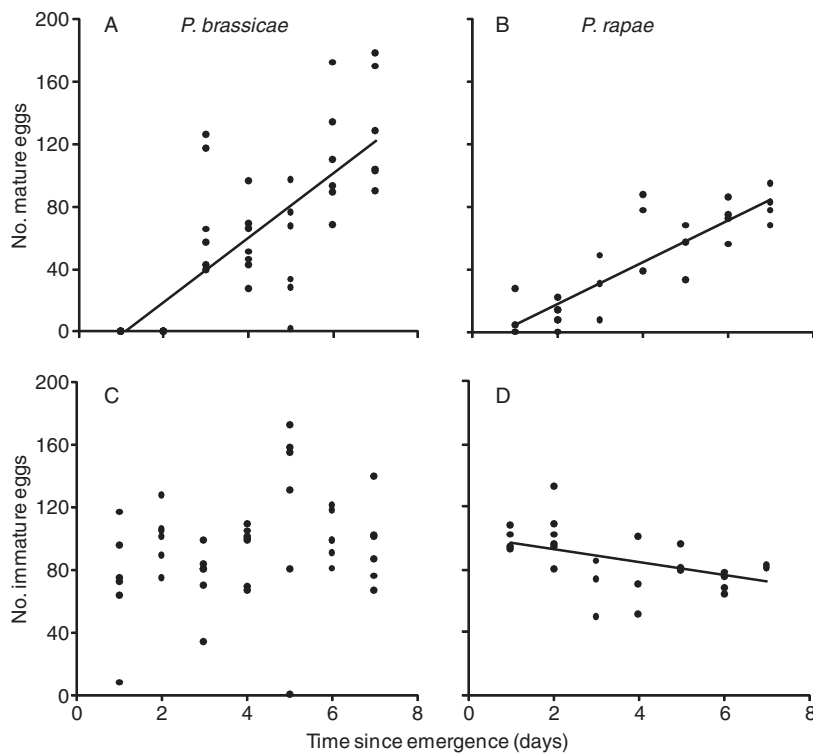

Figure 1 Average (± SE) number of (A,B) mature and (C,D) immature eggs present in the oviducts of *Pieris brassicae* and *P. rapae* butterflies, during the first 7 days after emergence. The regression equations are: *P. brassicae*: (A) number of mature eggs = 20.9 × day − 23.4, $R^2 = 0.61$, $F_{1,42} = 64.99$, P<0.001; (C) number of immature eggs: $F_{1,42} = 2.58$; P = 0.12; *P. rapae*: (B) number of mature eggs = 13.3 × day − 8.3, $R^2 = 0.76$, $F_{1,26} = 78.26$, P<0.001; (D) number of immature eggs = −4.1 × day + 101.3, $R^2 = 0.20$, $F_{1,42} = 7.53$, P = 0.011.

Table 1 Approximate F-test and P values for the fixed effect from GLMM of the effects of root herbivory by *Delia radicum* (yes vs. no) and egg load of the butterfly (low vs. high) on (A) the proportion of plants selected for oviposition, and (B) the number of egg clutches and eggs laid, by *Pieris brassicae* (total n = 40 females; per egg load category: n low = 15, n high = 25) and *P. rapae* (total n = 40 females; per egg load category: n low = 20, n high = 20)

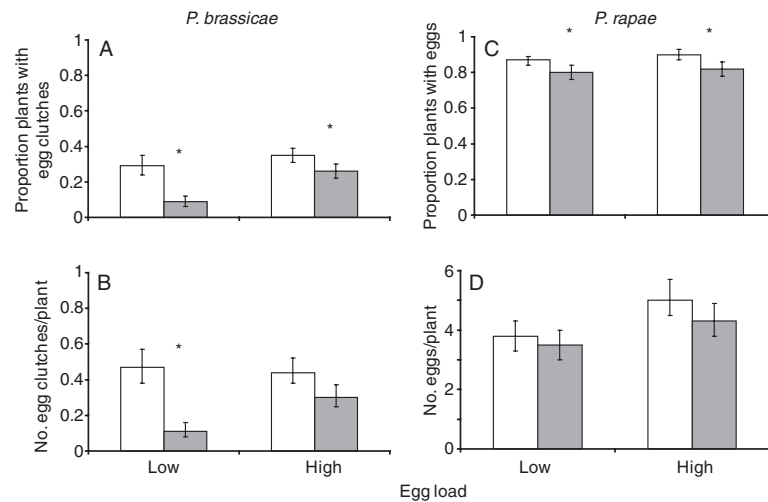| Factors	d.f.	*P. brassicae*		*P. rapae*	
		F	P	F	P
A					
Root herbivory	1	8.64	0.004	4.92	0.02
Egg load	1	3.85	0.06	0.56	0.45
Root herbivory*egg load	1	3.05	0.08	0.23	0.63
B					
Root herbivory	1	10.52	0.001	0.89	0.34
Egg load	1	0.53	0.46	3.36	0.07
Root herbivory*egg load	1	5.32	0.02	0.07	0.78

Figure 2 Back transformed means (± SE) of (A) the proportion of plants selected for oviposition and (B) number of egg clutches by *Pieris brassicae* females (total n = 40 females) with low (n = 15) or high (n = 25) egg load, and of (C) the proportion of plants selected for oviposition and (D) number of eggs by *Pieris rapae* females (total n = 40 females) with low (n = 20) and high (n = 20) egg load, on the foliage of *Brassica nigra* plants not attacked by the root herbivore (white bars) and on plants attacked by the root herbivore *Delia radicum* (grey bars). *Significant differences (Likelihood ratio test: P<0.05) between preference for plants with and without root herbivores within egg load category.

attacked by the root herbivore and control plants without root herbivores, independent of the egg load of the females (Figure 2D).

Discussion

Both butterfly species, *P. brassicae* and *P. rapae*, discriminated against plants with root herbivores by selecting a larger proportion of plants without root herbivores on which to oviposit. *Pieris brassicae* females also laid fewer clutches

on plants attacked by the root herbivore than on plants without herbivores feeding belowground. In a previous study we showed that *P. brassicae* larvae perform better on root-undamaged *B. nigra* plants than on plants exposed to root herbivory by *D. radicum* (Soler et al., 2005). Therefore, it is likely that the selection of plants without root herbivores over plants attacked by root herbivores as oviposition sites for *P. brassicae* is because of the lower quality of these plants as food for their progeny. We previously reported that the foliage of *B. nigra* plants attacked by

D. radicum belowground possessed two-fold higher concentrations of 2-propenyl glucosinolate (sinigrin) than the foliage of plants that were not exposed to the root herbivore (Soler et al., 2005, 2008). Although it is widely reported that specialist herbivores of crucifers, including both species of *Pieris* used in this study, have evolved physiological mechanisms to minimize the toxic effects of the glucosinolates (Wittstock et al., 2004), specialist feeders can also be negatively affected by phytotoxins of their host plants above certain concentrations.

We recently found that *Cotesia glomerata* (L.), an abundant and important parasitoid of *P. brassicae* in nature, is able to discriminate between volatile blends from *B. nigra* plants without root herbivores and *B. nigra* plants attacked by *D. radicum* belowground (Soler et al., 2007a,b). It is possible that both the herbivore and its parasitoids are similarly able to detect the distinct odor blend generated by the attack of root herbivores. Interestingly, plants attacked by the root herbivore *D. radicum* were less attractive for *C. glomerata*. *Cotesia glomerata* females preferred to search and parasitize hosts feeding on *B. nigra* plants without root herbivores, on which their offspring performed better, over plants attacked by the root herbivore (Soler et al., 2007a,b). Consequently, plants colonized by the root herbivore may offer enemy-free space to young caterpillars of *P. brassicae*. It is possible then that under certain conditions (e.g., environments with high presence of natural enemies) there is a trade-off for the herbivore between the higher nutritional quality of plants that had not been attacked by the root herbivore and an increased susceptibility of larvae feeding on these plants to their natural enemies (Ohsaki & Sato, 1994; Singer et al., 2004a,b).

As *P. brassicae* butterflies aged, discrimination between plants with and without root herbivores tended to break down. *Pieris brassicae* females are highly synovigenic, and thus eclose with only a proportion of their potential complement of eggs, but continue to mature eggs throughout their adult lives (Boggs, 1997). The experimental young butterflies began to mature large numbers of eggs only on the first day of the choice experiment, whereas older butterflies had already accumulated a large number of eggs in their ovaries when they were allowed to forage. Oviposition behaviour, including the 'motivation to oviposit', is strongly correlated with egg load in many species of insect herbivores and organisms of higher trophic levels such as parasitoid wasps (Hubbard et al., 1987; Mangel, 1987, 1989; Fletcher et al., 1994; Rosenheim, 1999; for a review see Minkenberg et al., 1992). Indeed, host searching and oviposition behaviour have been shown to be affected by maternal egg loads in several butterfly species (Jones, 1977; Courtney, 1981; Pilson & Rausher, 1988; Odendaal & Rausher, 1989; Tatar, 1991; Javois & Tammaru, 2004;

Gibbs et al., 2005). When there were many available resource patches of high quality such as plants not attacked by root herbivores, females with low egg loads were choosier than their counterparts with higher egg loads. Given that physiological condition generally worsens with age, it could be also possible that 6-day-old females were weaker fliers than their younger counterparts and also less capable of perceiving differences in chemical signals. However, as butterflies of different ages showed no apparent differences in their flying activity and general appearance, we assume that egg load was the most prominent dissimilarity between females of different age-classes. Nevertheless, it is possible that, in addition to egg load, the shorter perceived future life span of older females may account for a breakdown in their host-plant discrimination ability compared with younger females. It has been widely shown for insect parasitoids that the time spent by females in the foraging arena is influenced by experience, life expectancy/age, and egg load, and because the latter two are often correlated, host deprivation effects are sometimes confused with age effects (Rosenheim & Rosen, 1991).

Pieris rapae females discriminated less markedly than *P. brassicae* against plants attacked by the root herbivore. Although a larger proportion of plants without root herbivores were selected for oviposition by both older and younger *P. rapae* butterflies, the number of eggs laid on the shoots of the plants was independent of the presence or absence of insect herbivores belowground. In a field study where we observed the feeding and oviposition preference of aboveground insects on *B. nigra* plants with and without root herbivores, similar numbers of *P. rapae* eggs were also found on plants in both treatments (Soler et al., 2009). These results suggest two possible scenarios. First, the growth and development of the offspring of *P. rapae* butterflies, which has not been studied, may be less affected by the presence or absence of root herbivores than *P. brassicae*. Second, selection may be too diffuse in natural conditions for such discrimination to evolve.

Dissections of ovaries of female butterflies revealed that the number of mature eggs increased with age, indicating that both species are synovigenic (Boggs, 1986, 1997). In *P. brassicae*, the number of mature eggs never exceeded 200, even in older females, whereas in *P. rapae* maximum mature egg loads were always <100. However, these data may provide a misleading picture as to constraints on optimal oviposition behaviour in both species, because this relates to the risk of time- vs. egg-limitation in both species. It is known that *P. brassicae* can lay clutches of up to 100 eggs on a single plant in nature, although about 30 appears to be a more typical and average brood size for this species (Le Masurier, 1987). By contrast, *P. rapae* always lays a single egg during an oviposition sequence. In

P. brassicae, a female butterfly can become egg-limited after only a few oviposition events, whereas this is less of a constraint in *P rapae*. Under these conditions, the per capita effect of choosing a suboptimal plant on the fitness of a mother and her offspring is stronger in *P. brassicae* than in *P. rapae*.

In summary, this study has reported that both butterfly species discriminated and preferred to oviposit on plants without root herbivores over plants with root herbivores. *Pieris brassicae* females with low egg loads adjusted oviposition preferences to the absence of root-feeding insects belowground stronger than conspecific females with comparatively higher egg loads. Considering that changes in variables such as egg load and the physiological state of individual insects can play an important role in affecting foraging behaviour of butterflies regarding the presence or absence of insects belowground, these criteria should be considered in future above-belowground studies examining the foraging behaviour and oviposition strategies in insects. These results also provide compelling evidence that soil-dwelling insects affect not only the survival, growth, and development of their aboveground counterparts, as has been widely proven for a number of model systems in the last two decades, but also their behavioural decisions.

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