Endure and call for help: strategies of black mustard plants to deal with a specialized caterpillar

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Summary

1. Plants have evolved inducible resistance and tolerance mechanisms against insect herbivores. Resistance mechanisms that affect herbivorous insects directly can be effective against generalist herbivores, but will not deter specialist herbivores from attacking the plant. Tolerance mechanisms and indirect plant resistance are more likely effective strategies used by plants when dealing with specialist herbivores. However, inducible indirect resistance and tolerance mechanisms have rarely been investigated within the same study system.
2. We studied multiple tolerance mechanisms and the role of natural enemies in reducing fitness loss of *Brassica nigra* plants incurred by feeding by the specialist herbivore *Pieris brassicae*. For this, we measured the changes in carbon and nitrogen triggered by herbivore attack and quantified plant biomass and seed production under field conditions, in the presence or absence of natural enemies of the herbivores. We also assessed whether *B. nigra* increased selfing rates when exposed to herbivore infestation, and investigated whether infestation by *P. brassicae* rendered *B. nigra* plants more attractive to night-active pollinators than control plants.
3. We found that *B. nigra* flowers are rarely visited by insects during the night, and exposure to herbivores did not influence selfing rates. *Brassica nigra* plants compensated for herbivory in terms of vegetative biomass. Seed set was negatively affected by herbivory in the absence of natural enemies, but not in the presence of natural enemies. Plants responded to herbivory with drastic changes in nitrogen contents of leaves and flowers, whereas no changes in carbon concentrations were detected.
4. The investment in reproduction or re-growth of vegetative tissues is not sufficient to sustain plant fitness. Reproductive output of flowering mustard plants is only sustained when interactions with the natural enemies of the herbivores are preserved. We conclude that natural enemies of herbivorous insects play an important role as component of the plant's defence strategy. This study reveals that both tolerance and indirect resistance are strategies of this plant species to sustain fitness.

Key-words: carbon, flowering plants, indirect resistance, nitrogen, plant fitness, tolerance

Introduction

To maximize fitness, plants have evolved multiple resistance mechanisms against insect herbivory and tolerance mechanisms to compensate for the damage caused by herbivory (Dicke & Hilker 2003; Agrawal 2011; Fornoni 2011). Plants can directly resist herbivory through traits that deter herbivores from attacking the plant and/or negatively affect the performance and survival of the herbivores (Kessler & Baldwin 2002; Schoonhoven, van Loon & Dicke 2005; Hopkins, van Dam & van Loon 2009; Dicke & Baldwin 2010). This direct form of resistance is effective against generalist herbivorous insects, but generally does not affect specialist herbivores, as these insects have evolved ways of dealing with these resistance traits (Harborne & Grayer 1993; Schoonhoven, van Loon & Dicke 2005; Ferreres *et al.* 2009; Winde & Wittstock 2011). Plants then need to either endure the damage caused by a

specialist herbivore or promote the effectiveness of natural enemies of the herbivores (Dicke & Baldwin 2010; Fornoni 2011).

Tolerance and indirect resistance are likely effective strategies of plants to deal with specialist herbivores (Schoonhoven, van Loon & Dicke 2005). Insect herbivores can induce important physiological responses that allow plants to tolerate the damage caused by their feeding (Rosenthal & Kotanen 1994; Strauss & Agrawal 1999; Strauss & Murch 2004; Schwachtje & Baldwin 2008; Penet, Collin & Ashman 2009; Fornoni 2011; Lucas-Barbosa *et al.* 2013; Pashalidou *et al.* 2013). Plants can respond to herbivore attack by re-growing vegetative tissues or accelerate investment in reproduction (Strauss & Murch 2004; Schwachtje & Baldwin 2008; Penet, Collin & Ashman 2009; Fornoni 2011; Lucas-Barbosa *et al.* 2013; Pashalidou *et al.* 2013). In fact, herbivory is known to influence plant reproduction in a number of ways, for example by affecting pollinator behaviour, by altering the allocation of resources, by enhancing the speed of reproduction and by increasing selfing rates (Poveda *et al.* 2003, 2005; Kessler & Halitschke 2009; Penet, Collin & Ashman 2009; Lucas-Barbosa, van Loon & Dicke 2011; Lucas-Barbosa *et al.* 2013; Pashalidou *et al.* 2013).

Plants can benefit from investing in reproduction in response to herbivory. For instance, selfing is a reproduction mode through which plants can ensure reproduction in the absence of or under limiting visitation rates by pollinators, and selfing rates can be increased upon herbivory (Harder & Barrett 2006; Penet, Collin & Ashman 2009). Plants that can respond to attack by increasing selfing rates may benefit from producing seeds fast, especially when flowers are consumed by the herbivores, whereas the seeds are not consumed (Lucas-Barbosa *et al.* 2013). For plants, investment in reproduction can also mean increasing interactions with pollinators. Plant responses to herbivory can negatively affect pollinators, but can also increase their attraction (Poveda *et al.* 2003, 2005; Kessler & Halitschke 2009; Lucas-Barbosa, van Loon & Dicke 2011). By increasing interactions with pollinators, plants can potentially better tolerate herbivory. Interaction with pollinators is not the only mutualistic association that plants can invest in upon herbivore attack. Plants can also invest in indirect resistance; that is, plants can respond to herbivory with changes in odours emitted by leaves and flowers and in this way attract the natural enemies of their own enemies (Dannon *et al.* 2010; Mumm & Dicke 2010; Lucas-Barbosa, van Loon & Dicke 2011; Pareja *et al.* 2012; Schuman, Barthel & Baldwin 2012; Bruinsma *et al.* 2014). Thus, plants can employ multiple strategies to ensure reproduction after herbivore attack.

Many studies have identified mechanisms underlying indirect plant resistance (van Loon, de Boer & Dicke 2000; Fritzsche-Hoballah & Turlings 2001; Smallegange *et al.* 2008; Schuman, Barthel & Baldwin 2012; Gols *et al.* 2015) and tolerance to herbivores (Agrawal 2011; Fornoni 2011; Karban 2011), but these two strategies of plants to

maximize fitness have not been investigated within the same study system. The aim of this study was to investigate which strategies *Brassica nigra* plants use to sustain fitness, once attacked by a specialist herbivore. In the field, despite the extensive damage inflicted by *P. brassicae* to vegetative and reproductive tissues, *B. nigra* plants are able to tolerate herbivory and seed production was not affected (Lucas-Barbosa *et al.* 2013). We know that upon herbivore attack, *B. nigra* can speed up flowering and seed production (Lucas-Barbosa *et al.* 2013; Pashalidou *et al.* 2013) and maintain interactions with day-active pollinating insects (Lucas-Barbosa *et al.* 2013). Under natural conditions, carnivorous insects can significantly reduce herbivore abundance on *B. nigra* leading to mortality of *P. brassicae* that can get as high as 95% (Lucas-Barbosa *et al.* 2013, 2014). Therefore, here we investigated (i) the role of carnivores in *B. nigra* defence – we tested whether *B. nigra* can tolerate herbivory by investing in aboveground biomass and seed production when predators and parasitoids of *P. brassicae* are present or absent; (ii) mechanisms of tolerance to herbivory – we tested whether tolerance to herbivory results from reallocation of resources into different plant tissues in response to herbivory. Additionally, we have excluded possible alternative tolerance mechanisms that could lead to or aid *B. nigra* plants in sustaining fitness after herbivore attack; (iii) we investigated whether *B. nigra* increased selfing rates when exposed to herbivore infestation; and (iv) we investigated whether infestation by *P. brassicae* rendered *B. nigra* plants more attractive to night-active pollinators than control plants.

Materials and methods

STUDY SYSTEM

Brassica nigra L. (Brassicales: Brassicaceae) is an annual plant that is considered to be obligately outcrossing; in nature, it is pollinated by various insects including bees, syrphid flies and butterflies (Conner & Neumeier 1995; Lucas-Barbosa *et al.* 2013). In the Netherlands, this native wild species occurs as an early successional plant and commonly occurs in high densities.

Pieris brassicae L. (Lepidoptera: Pieridae) is a specialist herbivore of plants in the family Brassicaceae (Fig. 1). This lepidopteran species is gregarious and female butterflies lay clutches of up to 100 eggs, generally on the underside of leaves. Caterpillars of *P. brassicae* are voracious feeders. When larvae hatch from the eggs, caterpillars initially feed gregariously on leaves of a flowering *B. nigra* plant, but second-instar larvae soon move to and feed from the flowers (Smallegange *et al.* 2007), consuming flowers entirely and in large numbers (D. Lucas-Barbosa, personal observation). In the Netherlands, *P. brassicae* caterpillars are frequently attacked by various carnivorous insects: first- and second-instar larvae are attacked by the gregarious parasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae). Young larvae can also be preyed upon by ladybird beetles, and later instars can be intensively preyed upon by social wasps (Lucas-Barbosa *et al.* 2013, 2014).

Episyrphus balteatus (De Geer) (Diptera: Syrphidae) is one of the most common syrphid fly species world-wide (Jauker & Wolters 2008). Adult syrphid flies feed on nectar and pollen, but collect mainly pollen from *B. nigra* flowers (D. Lucas-Barbosa,

personal observation). *Episyrphus balteatus* adults serve as pollinators, increasing the fitness of *Brassica* plants (Jauker & Wolters 2008).

PLANTS AND INSECTS

Seeds of an early-flowering accession (CGN06619) of *B. nigra* were obtained from the Centre for Genetic Resources (CGN, Wageningen, the Netherlands) and multiplied by exposing plants to open pollination in the surroundings of Wageningen. The seeds collected from 25 plants were mixed to obtain seed batches for the experimental plants. For the glasshouse experiments, potted *B. nigra* plants were grown in a glasshouse compartment (23 ± 2 °C, 50–70% r.h., L16:D8). For the field experiments, potted *B. nigra* plants were reared outside on tables protected by insect screens, in a location close to the field site. Plants in the flowering stage, with several flowers open, and in the vegetative growth stage, that is stages 4·2 and 3·2, respectively, based on the classification for *Brassica napus* (Harper & Berkenkamp 1975), were used for the experiments. *Pieris brassicae* were obtained from a colony reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera*) plants in a climate room (22 ± 1 °C, 50–70% r.h., L16:D8). The adults were provided with a 10% sucrose solution as food. *Episyrphus balteatus* pupae were obtained from Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands. Adult flies were kept (22 ± 1 °C, 50–70% r.h., L16:D8) in a cage with a Brussels sprouts plant infested with the cabbage aphid *Brevicoryne brassicae*, known to promote the development of the female reproductive system (M. Kos, personal communication). Adult syrphid flies had access to sugar, pollen and water.

PLANT TREATMENT

Flowering plants with a few open flowers were infested with one egg clutch of *P. brassicae* by exposing plants to butterflies. For the glasshouse experiments, the plants were exposed to *P. brassicae* butterflies in an oviposition cage (100 × 70 × 82 cm). While the plant was inside the oviposition cage, the flowers were covered with a mesh bag to prevent being visited by the butterflies. For the field experiments, we exposed each plant to a mated female butterfly while the plant was covered with a tent. In field and glasshouse conditions, the number of eggs on a plant was reduced to 30 by gently removing surplus eggs shortly after egg deposition by the butterfly had been interrupted.

SEED PRODUCTION AND RE-GROWTH UPON HERBIVORE INFESTATION IN THE PRESENCE OR ABSENCE OF NATURAL ENEMIES

We investigated whether *B. nigra* plants can compensate for herbivory by *P. brassicae* in comparison with control plants. Plant biomass and seed production were quantified in two field conditions: (i) in open field conditions where plants and introduced herbivores were exposed to naturally occurring insects, including pollinators and carnivorous insects; (ii) in conditions where natural enemies (carnivorous insects) were excluded, and syrphid flies (*E. balteatus*) and male butterflies (*P. brassicae*) were used to ensure cross-pollination between plants of the same plot. For each of these two conditions, the field layout consisted of 16 plots of *B. nigra* plants infested with *P. brassicae* and 16 control plots. Each plot (50 × 50 cm) was composed of five plants. The central plant of the plot was either infested with 30 *P. brassicae* eggs or was not infested, and the other four plants were not infested with butterfly eggs. Once caterpillars hatched from eggs, they initially feed on the leaves; second-instar larvae move to flowers, and once caterpillars reach the flowers of the central plant of the plot, they

also colonize the other plants of the same plot (Lucas-Barbosa *et al.* 2013, 2014). Thus, plots with an infested central plant are termed 'infested plots' and plots with a non-infested central plant are termed 'control plots'. Equal numbers of control and infested plots were transplanted to the field on eight consecutive days. Plants that were infested on the same day were never planted in the same column or row in the field layout. Control and infested plots were planted alternately, and the distance between them was 1·5 m. When carnivorous insects were excluded, a tent was used to cover each plot of plants (95 × 95 × 190 cm, white fine polyester mesh) and two adult syrphid flies and two male *P. brassicae* butterflies were released in each of the tents as pollinators, within one week after eclosion from pupae. Adult syrphid flies and butterflies were replaced every week until the plants were harvested. Once harvested, above-ground dry plant biomass and seed biomass produced by infested and control plants in the two given conditions were determined as described above. We also measured the biomass of the herbivores reared in these two conditions. Caterpillars were recollected and weighed 28 days after egg deposition by the butterfly, for both the open field and exclusion conditions. We carried out two serially repeated trials between May and July 2013, at an experimental field site in Wageningen, the Netherlands. Data were analysed using a generalized linear model with either normal or Poisson probability distributions, and Wald chi-square test.

RESOURCE ALLOCATION BY *B. NIGRA* UPON HERBIVORE INFESTATION

We estimated how resources are allocated between leaves and reproductive tissues of *B. nigra* plants upon herbivory. At three time points, we measured total nitrogen and organic carbon concentrations in leaf and flower tissues of infested plants and non-infested control plants at the same development stages, and used this as a proxy of resource allocation. To do this, infested and control plants were kept in a glasshouse compartment (23 ± 2 °C, 50–70% r.h., L16:D8) until leaves and flowers were harvested. Carbon and nitrogen contents were determined at three time points: (i) egg-infested plants (4 days after oviposition), (ii) leaf-infested plants (folivory – 1 day after caterpillars had hatched from the eggs) and (iii) flower-infested plants (florivory – 5 days after caterpillars had hatched from the eggs).

At these three time points, all herbivores were removed from the infested plants and leaves and flowers of control and infested plants were harvested, frozen in liquid nitrogen and freeze-dried. Dried samples were ground, weighed and stored at −20 °C. Total nitrogen and organic carbon in leaf and flower tissues were determined by dry combustion using a CHN analyser. Data were analysed using a generalized linear model with normal probability distribution and Wald chi-square test, followed by pairwise comparisons with Bonferroni post hoc tests to determine the significant differences at the 0·05 level.

SELFING IN *B. NIGRA* PLANTS UPON HERBIVORE INFESTATION

We tested whether *B. nigra* increased selfing rates when exposed to herbivore infestation as selfing is a strategy through which plants can ensure reproduction, and several plant species are known to increase selfing rates upon herbivore attack (Harder & Barrett 2006; Penet, Collin & Ashman 2009). To do this, *B. nigra* plants were transplanted to the field at bud stage and individually isolated in tents (95 × 95 × 190 cm, white fine polyester mesh). Once the plants were flowering, 50% of the plants used for the experiments were infested with one egg clutch of *P. brassicae* and 50% were kept as control plants. After the caterpillars had

hatched, they were allowed to feed on the plant for 48 h and then removed. On the day when plants were infested with eggs, two adult syrphid flies and two male *P. brassicae* butterflies were released in each of the tents containing an individual *B. nigra* plant so that pollen could be carried among flowers of the same plant. Adult syrphid flies and butterflies were replaced every week until the plants were harvested. Plants remained in the field in tents isolated from other plants for 27 days. Once harvested, above-ground dry plant biomass and seed biomass produced by infested and control plants were determined. Plant shoots were dried overnight (105 °C), and dry biomass was measured. When the total number of seeds was smaller than one hundred for an individual plant, all seeds were counted. When the number of seeds was larger than one hundred seeds, the total number of seeds was estimated by dividing the total seed weight by the weight of one hundred seeds of that plant. In total, 60 plants were used for these experiments. Data were analysed with a generalized linear model using Poisson probability distribution and Wald chi-square test.

VISITATION OF INFESTED AND CONTROL *B. NIGRA* FLOWERS BY DAY- AND NIGHT-ACTIVE POLLINATORS AND PLANT VOLATILE EMISSION DURING DAY AND NIGHT

We investigated whether infestation by *P. brassicae* rendered *B. nigra* plants more attractive to night-active pollinators than control plants and whether this could explain how compensation in terms of seed production is achieved. Pollinator visitation to infested and control plants of *B. nigra* flowers was recorded during the day and the night, under field conditions. During the day, pollinator visitations were recorded by direct observation for 10 min using a handheld computer (Psion Workabout) programmed with the Observer XT software (version 10, Noldus Information Technology, Wageningen, the Netherlands). Observations were carried out whenever weather conditions were suitable (17–25 °C; wind speed ≤ 6 m s^{-1}), between 09.00 and 16.00 h (details outlined by Lucas-Barbosa *et al.* 2013). During the night, infrared cameras were used to record flower visitation by night-active insects. Each night, visitation to one or two plots of plants was recorded with an infrared camera and infrared light. During nine different nights, different plots, each with five plants in the flowering stage, were observed with the infrared camera for a total of 36 h. Images were captured using two analogue monochrome cameras (Ikegami, ICD-49E) at 5 frames per second. The near infrared cut filter was removed from the camera to make it sensitive to near infrared (see Allema *et al.* (2012) for more details). Radiation sources were placed next to each of the cameras. The cameras were connected to a laptop, and StreamPix software (4 × 64 edition, Norpix Inc., Montreal, Canada) was used for recording the video files (in AVI format). Observations were carried out whenever weather conditions were suitable (10–30 °C; wind speed ≤ 8 m s^{-1}; and no rain), between 08.00 and 20.00 h. Eight videos of 30 minutes were recorded per night. Field layout consisted of 16 plots of *B. nigra* plants, and each plot (50 × 50 cm) was composed of five plants (details have been previously described by Lucas-Barbosa *et al.* 2013). The observations were carried out between July and August 2012 in an experimental field site in Wageningen, the Netherlands. Data were analysed using the Mann–Whitney *U*-test.

Plant volatiles were collected from aerial parts of *B. nigra* plants during day and night. Experiments were performed in a glasshouse compartment (22 ± 2 °C, 60–70% r.h. L16:D8) and headspace volatiles were collected for 1·5 h. Diurnal collection was performed between 10.00 and 15.00 h and nocturnal collection was performed 1 h after the scotophase had started. Aerial parts of plants were enclosed in an oven bag (Toppits® Brat-Schlauch, polyester;

32 × 32 × 70 cm; Toppits, Minden, Germany). A strip of bag material wrapped around the stem and above the inflorescence was used to close the bag. Synthetic air was flushed through the bag at a flow rate of 300 mL min^{-1} by inserting Teflon tubing through an opening in the upper part of the bag. Air was sucked (224-PCMTX8, air-sampling pump Deluxe, Dorset, UK; equipped with an inlet protection filter) from the bag enclosing the aerial parts of the plants, and headspace volatiles were collected on Tenax (90 mg of Tenax-TA 25/30 mesh; Grace-Alltech, Breda, the Netherlands) in a glass tube at a flow rate of 250 mL min^{-1}. Bags were discarded after use. Volatiles from 11 individual plants were collected. Headspace samples were then analysed in a gas chromatograph with a thermodesorption unit (GC) (6890 series, Agilent, Santa Clara, USA) connected to a mass spectrometer (MS) (5973 series, Agilent, Santa Clara, UT, USA). We followed the methods outlined by Bruinsma *et al.* (2014) to analyse, identify and quantify volatiles emitted by *B. nigra* plants. Projection to latent structures discriminant analysis (PLS-DA) (SIMCA P + 12·0, Umetrics AB, Umeå, Sweden) was used to determine whether the samples subjected to the different treatments could be separated based on the composition of the volatile blend. To determine the significant differences between emission of volatile compounds by *B. nigra* plants during daytime and night-time, we used Wilcoxon matched-pairs tests as assumptions of normality were not met.

Results

SEED PRODUCTION AND RE-GROWTH OF ABOVE-GROUND TISSUES UPON HERBIVORE INFESTATION IN THE PRESENCE OR ABSENCE OF NATURAL ENEMIES

In open field conditions where carnivorous insects had access to the plants, infested and control plants produced similar numbers of seeds: both for the plants originally infested with butterfly eggs (Fig. 2a, A-plants, GLM with Wald chi-square test, $P = 0.988$) and for all plants within the plot (Fig. 2b, GLM with Wald chi-square test,

Fig. 1. Fifth-instar larva of *Pieris brassicae* feeding on flowers of *Brassica nigra* plants.

$P = 0.381$). In conditions where carnivorous insects were excluded, infested plants produced fewer seeds than control plants. Effects of herbivore treatment were observed for the plants originally infested with eggs (Fig. 2c, A-plants, GLM with Wald chi-square test, $P = 0.005$) and at plot level (Fig. 2d, GLM, $P = 0.006$).

To estimate re-growth capability in *B. nigra*, above-ground biomass was determined for infested and control plants. Shoot dry weights of infested and control plants were generally similar for experiments carried out in the two conditions, but there was an effect of trial. Irrespective of the treatment, plants of trial 2 produced lower biomass than plants of trial 1 (Fig. S1, Supporting Information).

In open field conditions, we recorded 46% survival of the caterpillars, whereas when carnivores were excluded, 80% of caterpillars were recollected at the end of trial 1. Under conditions in which natural enemies of the herbivores were excluded, caterpillars were heavier than under conditions where natural enemies were present (Fig. S2, Student's *t*-test, $P \leq 0.001$). No caterpillars were recovered in open field conditions during trial 2, whereas 80% of the herbivores were recollected from the exclusion experiment.

RESOURCE ALLOCATION BY *B. NIGRA* UPON HERBIVORE INFESTATION

Nitrogen content of infested plants was overall higher than that of control plants (Fig. 3, GLM with Wald chi-square test, $P = 0.001$). However, carbon content of above-ground tissues did not change in response to exposure to *P. brassicae* eggs and caterpillars (Fig. 3). Overall, C/N ratios in above-ground tissues decreased upon herbivore infestation (GLM with Wald chi-square test, $P = 0.035$).

SELFING IN *B. NIGRA* PLANTS UPON HERBIVORE INFESTATION

Plants induced by *P. brassicae* feeding produced as many selfed seeds as control plants (GLM, $P = 0.249$) and reached similar above-ground plant biomass (GLM with Wald chi-square test, $P = 0.913$). We observed an effect of trial on seed production (GLM with Wald chi-square test, $P \leq 0.001$) and plant biomass (GLM with Wald chi-square test, $P \leq 0.001$), but no overall effects of treatment, and no interaction between treatment and trial (GLM with Wald chi-square test, $P = 0.102$, $P = 0.166$, respectively).
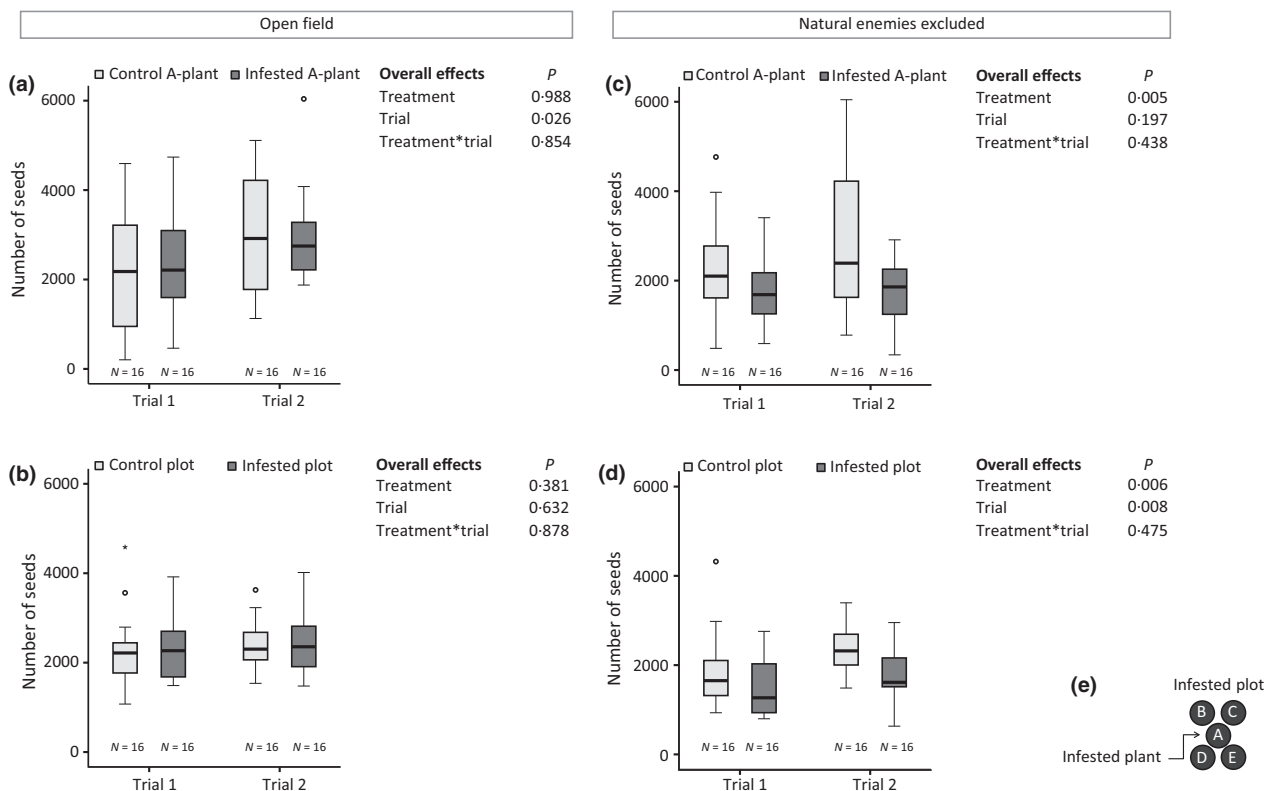

Fig. 2. The number of seeds produced (median, first and third quartiles, SD) by *Pieris brassicae*-infested and non-infested control *Brassica nigra* plants in the open field and in tents excluding natural enemies of *P. brassicae*. In the open field, plants and herbivores were exposed to naturally occurring pollinators and natural enemies. In the exclusion cages, syrphid flies (*Episyrphus balteatus*) and male butterflies (*P. brassicae*) were used to ensure cross-pollination between plants of the same plot. Data are shown for the central plant (A-plant) of the plot (a,c) and at plot levels (b,d) for trials 1 and 2. Schematic representation of a field plot (e); each plot is composed of five plants, and the central plant of the plot was either infested with 30 *P. brassicae* eggs or was not infested; second- to fifth-instar larvae colonized the other four plants of infested plots. A generalized linear model was used to determine significant differences at α = 0·05. The number of plants and plots is shown under the bars. Outliers are represented by " ° " (out) and " * " (far out).
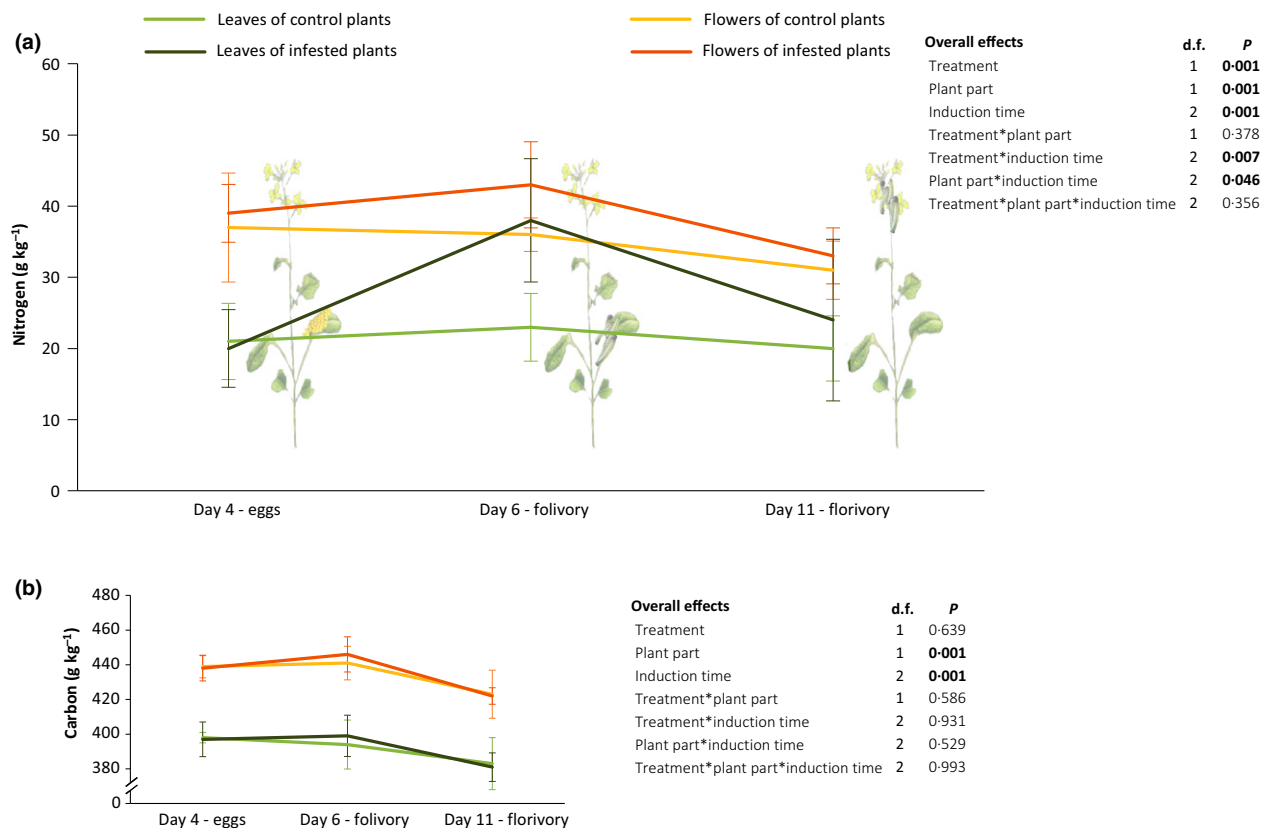
Fig. 3. Nitrogen (a) and carbon (b) contents of *Pieris brassicae*-infested and (non-infested) control *Brassica nigra* plants. Nitrogen (a) and carbon (b) contents (mean ± SD) are shown for leaf and flower tissues of plants infested with eggs and with caterpillars that feed on leaves and flowers, along with control plants of equivalent development stage. Plants were infested with *P. brassicae* eggs on the day the first flowers were open. Five plants per treatment and per development stage were used. A generalized linear model with Wald chi-square test, followed by pairwise comparisons with Bonferroni post hoc test, was used to determine significant differences at 0·05 level.

VISITATION OF INFESTED AND CONTROL *B. NIGRA* FLOWERS BY DAY- AND NIGHT-ACTIVE POLLINATORS AND PLANT VOLATILE EMISSION DURING DAY AND NIGHT

Brassica nigra flowers were rarely visited by insects during the night. Thus, visitation rates during the day were significantly higher than during the night (Fig. S3, Mann–Whitney *U*-test, $P \leq 0.001$). During daytime, the pollinator species most frequently observed was the honeybee *Apis mellifera* L. (Hymenoptera: Apidae). Many species of syrphid flies were also abundant flower visitors, including the syrphid fly *Eristalis tenax* (Diptera: Syrphidae). Solitary bees, butterflies and bumblebees were also observed visiting *B. nigra* flowers, but in lower numbers in comparison with honeybees and syrphid flies. During the night, a total of 11 moths were observed flying through the plots of *B. nigra* plants during the nine nights during which we recorded visitation. Only four moths were actually observed visiting the flowers. Of these four individual moths, three moths were identified as a member of the Noctuidae family and they visited eight flowers altogether. The fourth moth was a microlepidopteran which visited one flower. The total time spent by all moths on the flowers represented < 1% of the total recording time. Because

visitation rates by night-active pollinators were very low, they were not subjected to statistical analysis.

When regarding the effect of herbivory on visitation by day-active pollinators, herbivore-infested plants and control plants were visited at similar rates (Mann–Whitney *U*-test, $P = 0.780$).

Volatile emission rates by *B. nigra* plants were considerably lower during the night than during the day (Fig. S3, Wilcoxon matched-pairs tests, $P \leq 0.001$). A projection to latent structures discriminant analysis (PLS-DA) of volatile compounds collected from flowering *B. nigra* plants during the day and night resulted in a model with two principal components; the first principal component explained 55% and the second principal component 9% of the total variance (see Fig. S4). The PLS-DA completely separated the volatile profiles collected from plants during the day and those from night collections (Fig. S4). During daytime, several compounds were emitted in larger amounts than during the night (Table S1).

Discussion

Our data show that reproductive output of *B. nigra* plants exposed to the naturally occurring insect fauna was significantly higher than that of plants deprived of interactions

with natural enemies of the herbivores. The data collected in our field experiment support the importance of members of the third trophic level as a component of a plant's defence strategy. Thus, although *B. nigra* plants can, to some extent, compensate for the damage caused by herbivores, the tolerance mechanisms *per se* are not sufficient to sustain plant fitness. Moreover, our results show, for the first time for plants in the flowering stage, the effects of herbivore attack in terms of allocation of resources. Attack by a specialist herbivore induces significant changes in nitrogen content in the shoot of flowering mustard plants.

Tolerance to herbivory is a common phenomenon that is characterized by changes in primary metabolism that allow plants to make the necessary physiological adjustments to endure herbivory (Schwachtje & Baldwin 2008; Fornoni 2011). Here, we quantified the actual changes triggered by butterfly eggs and initial caterpillar feeding damage that can be largely associated with metabolic changes at primary metabolism level of flowering mustard plants. Overall C/N ratios declined upon herbivore infestation in both leaves and flower tissues. This resulted from increases in nitrogen concentration and was not related to the allocation of carbon to above-ground tissues. Thus, in the flowering stage, the short-lived *B. nigra* does not mobilize extra carbon for the re-growth of tissues after herbivory, but only invests in reallocation of nitrogen into above-ground tissues. Changes in N content can be potentially associated with plant defensive chemistry, and in the case of brassicaceous plants, with an increase in glucosinolate contents, for instance. However, it is important to realize that plant secondary metabolites are present in plant tissues in relatively low concentrations – often < 1% of the total C and N (Bourgaud *et al.* 2001; Li & Vederas 2009; Mithofer & Boland 2012). Thus, here, we mainly associate the observed changes in total N with re-allocation of resources into reproductive tissues. Interestingly, the overall effect on the N content suggests that *B nigra* responds to butterfly eggs with physiological changes that anticipate damage by caterpillars. However, initial damage by caterpillars triggers even stronger changes in the allocation of nitrogen to above-ground tissues: caterpillar damage to leaves (6 days since infestation with butterfly eggs or 24 h of caterpillar damage) induced 65% increase in nitrogen in leaves, and 20% increase in flowers, compared with levels measured in control plants. Thus, once caterpillars start to feed, plants increase the nitrogen uptake even more, either directly from the soil or by mobilizing nitrogen from the roots into above-ground tissues. Indeed, tolerance to herbivory can result from the reallocation of resources into different tissues (Schwachtje & Baldwin 2008). Plants in the vegetative stage can shift carbon and nitrogen resources from roots into above-ground tissues, when herbivores attack roots (Coughenour *et al.* 1990; Newingham, Callaway & BassiriRad 2007), and also allocate resources to roots when leaves are induced or attacked (Briske, Boutton & Wang 1996; Schwachtje & Baldwin 2008; Cooper & Rieske 2011; Robert *et al.* 2014). Biotic stresses

seem to induce the changes in C/N ratios that can result from uptake or allocation of either carbon or nitrogen, from roots to shoots or from shoots to roots, in different plant species (Briske, Boutton & Wang 1996; Schwachtje & Baldwin 2008; Robert *et al.* 2014). Mobilization of carbon for the regrowth of plant tissues after herbivory may be more likely when plants are still in the vegetative stage, at least for annual plant species (Schwachtje & Baldwin 2008). In our study, we focused on plants in the flowering stage, and the changes in C/N ratio resulted only from changes in the nitrogen contents, both for leaves and for flowers. Transition from the vegetative to the flowering stage has also been associated with an change in C/N ratio of leaves and flowers (Raper *et al.* 1988; Rideout, Raper & Gordon 1992) and with an increase in nitrogenous compounds (Corbesier *et al.* 2003). Optimal nitrogen levels in above-ground tissues are also important for maintenance of flowering, and plants may continue to depend upon reserves of nitrogen even after the deployment of carbon storage has declined (Rosenthal & Kotanen 1994; Strauss & Agrawal 1999). Our previous studies showed that *B. nigra* plants accelerated investment in flower (Pashalidou *et al.* 2013) and seed production (Lucas-Barbosa *et al.* 2013) in response to butterfly egg deposition. Thus, it seems that plant responses elicited by biotic stressors are very similar to those used to initiate flowering (Rosenthal & Kotanen 1994; Strauss & Agrawal 1999; Schwachtje & Baldwin 2008; Lucas-Barbosa *et al.* 2013; Pashalidou *et al.* 2013). This deserves more attention to further understand the mechanisms underlying tolerance to herbivory and consequences for plant fitness.

Tolerance mechanisms *per se* are not sufficient to sustain plant fitness. Whether plants manage to compensate for herbivore feeding damage depends on the degree of herbivore suppression by carnivorous insects. Although herbivore-infested *B. nigra* plants could still regrow and compensate for herbivory in terms of vegetative biomass, in the absence of natural enemies plants did not compensate for herbivory in terms of seed production. *Pieris brassicae* is a voracious herbivore, and second- through fifth-instar larvae feed exclusively on the flowers of *B. nigra* plants, entirely consuming large numbers of them (Smallegange *et al.* 2007; Lucas-Barbosa *et al.* 2013). When we excluded the natural enemies of the herbivores, seed set of *B. nigra* plants was negatively affected. Some studies have demonstrated that plants can benefit from the natural enemies of the herbivores (van Loon, de Boer & Dicke 2000; Fritzsche-Hoballah & Turlings 2001; Smallegange *et al.* 2008; Schuman, Barthel & Baldwin 2012; Gols *et al.* 2015). Most of these studies demonstrated that parasitized caterpillars feed less than healthy caterpillars, and the seed set of plants is, in view of this, positively affected when plants are exposed to parasitized larvae. In the present study, natural enemies that visited the plants in the open field included both parasitoids and predators. We have previously reported that hymenopteran predators cause high mortality of *P. brassicae* caterpillars (Lucas-Barbosa *et al.*

2014). Furthermore, herbivore-infested *Nicotiana attenuata* plants that emit green leaf volatiles produce more buds and flowers than plants that have been genetically engineered not to emit green leaf volatiles (Schuman, Barthel & Baldwin 2012). The production of green leaf volatiles was only correlated with higher reproductive output of tobacco plants when predatory bugs reduced herbivore numbers by a factor two. Collectively, these results emphasize the importance of carnivorous insects as part of plant defence strategy.

We have excluded possible alternative mechanisms that could lead to or aid *B. nigra* plants in sustaining fitness after herbivore attack by the specialist *P. brassicae*. We excluded that *B. nigra* plants increased self-fertilization upon herbivore attack (Penet, Collin & Ashman 2009; Wise & Hebert 2010) or that the plants attracted more night-active pollinators upon herbivore attack. Selfing and the accelerated reproduction in response to herbivory represent different strategies through which plants may ensure reproduction (Penet, Collin & Ashman 2009; Wise & Hebert 2010; Lucas-Barbosa *et al.* 2013). We observed that *B. nigra* can be self-fertilized. Selfing rates were, however, not influenced by prior infestation by *P. brassicae* and do not explain how *B. nigra* compensated for herbivory in terms of seed production. We also investigated whether flowers were visited during the night. This could possibly increase pollination success of herbivore-infested plants, if herbivore induction renders plants more attractive to night-active pollinators. Our data show that flowers of *B. nigra* are rarely visited by night-active insects, when compared with visitation rates during the day, and this correlated with reduced odour emission by plants. Because visitation rates during the night were very low, we did not statistically compare the differences between visitation rates to infested and control plants. We conclude that *B. nigra* is mainly pollinated by day-active insects, and exclude the possibility that responses of night-active insects to infested plants could explain how seed set of *B. nigra* is sustained after attack by *P. brassicae*.

We conclude that the interaction with natural enemies is vital in sustaining the reproductive output of black mustard plants. At the flowering stage, physiological changes triggered in *B. nigra* plants in response to butterfly egg deposition, that is in anticipation to caterpillar damage, will only translate into fitness benefit when combined with indirect plant resistance.

Authors' contributions

DLB, JJAvL and MD designed most experiments, and DLB collected and analysed most data. TK and YA contributed to the design, data collection and analysis of part of the experiments. MEH and TAvB contributed to the design and analysis of part of the experiments. DLB wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

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Data accessibility

Data are deposited in Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.n29s7 (Lucas-Barbosa *et al.* 2016).

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Plant biomass (median, first and third quartiles, whiskers) of *Pieris brassicae*-infested and non-infested control *Brassica nigra* plants in open field and when natural enemies of *P. brassicae* were excluded.

Fig. S2. Biomass (mean ± SD) of fifth instar *Pieris brassicae* caterpillars reared on flowering *Brassica nigra* plants in open field conditions and in conditions where natural enemies of the herbivores were excluded.

Fig. S3. Number of flower visitors of *Brassica nigra* (a) and total volatile emission (peak area) (b) by these plants during day and night.

Fig. S4. Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile compounds collected from flowering *Brassica nigra* plants during daytime and night-time.

Table S1. Volatile compounds from flowering *Brassica nigra* plants collected during the day and night.