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Synergistic effects of direct and indirect defences on herbivore egg survival in a wild crucifer

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Evolutionary theory of plant defences against herbivores predicts a trade-off between direct (anti-herbivore traits) and indirect defences (attraction of carnivores) when carnivore fitness is reduced. Such a trade-off is expected in plant species that kill herbivore eggs by exhibiting a hypersensitive response (HR)-like necrosis, which should then negatively affect carnivores. We used the black mustard (*Brassica nigra*) to investigate how this potentially lethal direct trait affects preferences and/or performances of specialist cabbage white butterflies (*Pieris* spp.), and their natural enemies, tiny egg parasitoid wasps (*Trichogramma* spp.). Both within and between black mustard populations, we observed variation in the expression of *Pieris* egg-induced HR. Butterfly eggs on plants with HR-like necrosis suffered lower hatching rates and higher parasitism than eggs that did not induce the trait. In addition, *Trichogramma* wasps were attracted to volatiles of egg-induced plants that also expressed HR, and this attraction depended on the *Trichogramma* strain used. Consequently, HR did not have a negative effect on egg parasitoid survival. We conclude that even within a system where plants deploy lethal direct defences, such defences may still act with indirect defences in a synergistic manner to reduce herbivore pressure.

1. Introduction

Plants deploy various direct and indirect defences against herbivore attackers. Direct defence traits induced by herbivory such as the production of secondary metabolites have an often systemic and broad effect on herbivores, whereas traits such as the abortion of plant organs and tissue necrosis are more locally targeted against specific attackers [1]. In addition, plant infochemicals may function as an indirect defence trait responsible for attracting natural enemies of herbivores [2,3]. However, a direct defence may not only have a major effect on the herbivore's survival, but also on the survival of the natural enemies of the herbivore, leading to a possible conflict between the plant and the natural enemy that is attracted to the herbivore on that plant [4–12]. Evolutionary theory thus suggests that defence strategies, such as direct and indirect defences, may negatively correlate within a multitrophic community resulting in possible trade-offs [13–15]. Among carnivores, such negative effects are especially relevant for parasitoids that require a single host to complete development. Also, many larval parasitoids have evolved an intimate physiological relationship with their host and, throughout their development, may be exposed to toxic plant defence chemicals ingested by their host [16–21].

Sub-lethal resistance traits, such as toxins or antinutritive agents, seem to be the most common form of plant resistance [22]. The claimed trade-offs between sub-lethal direct and indirect defences that result from deleterious effects on higher trophic levels are expected to be ubiquitous in plant–insect interactions and have been hypothesized to even shape consumer diversity of multitrophic systems [23]. However, sub-lethal plant defences that slow down herbivore growth may both compromise the herbivore's immune response against parasitism [17] and expand the temporal window of vulnerability to parasitoid attack, rather resulting in a higher than lower parasitism [22,24]. Also, plant defences may be shaped by a whole community of interacting herbivores than by single pairwise interactions between species [2,23,25,26]. Therefore, an alternative to the view of trade-offs between defences, is that they act additively or synergistically to combat different herbivore species simultaneously, and do not interfere with each other [22,27].

In contrast to sub-lethal plant defences, lethal traits are less common and mainly known to protect plants against sessile herbivore stages, i.e. egg deposition [28,29], or against fruit predators by fruit abortion [30]. A conflict with the attraction of natural enemies to plants expressing lethal defence traits is especially expected. Experimental evidence for this premise is scarce however, and there is a need for integrative approaches, where the effects of plant defences on fitness proxies across trophic levels are evaluated against the relevant genetic variation present in the plant–insect populations. In this study, we tested the prediction that a direct lethal defence trait killing herbivore eggs will conflict with the indirect defence trait of attracting parasitic wasps.

Egg deposition by herbivorous insects has been shown to trigger several plant responses lethal to herbivore eggs, such as (i) hypersensitive response (HR)—like necrosis and neoplasm formation both leading to egg desiccation and egg dropping [31–35], (ii) egg-crushing plant tissue [36] and (iii) ovicidal substances killing eggs [37,38]. At the same time, herbivore eggs may induce indirect defence traits, such as oviposition-induced plant volatiles (OIPVs) or plant cues perceived by contact, that attract or arrest parasitic wasps killing eggs before larvae hatch [29,39]. The black mustard *Brassica nigra* is commonly attacked by cabbage white butterflies (*Pieris* spp.) [40,41], the eggs of which sometimes induce HR-like necrosis [32,34] and serve as hosts for generalist parasitic wasps of the genus *Trichogramma* [42]. In a previous laboratory study with a *B. nigra* population originating from Greece, we showed that eggs of the gregarious large cabbage white butterfly (*P. brassicae*) induced HR-like necrosis [32]. Interestingly, this direct defensive trait against eggs has not been described so far in other crucifer species. One exception is *Arabidopsis thaliana* (Col-0), where *Pieris* eggs triggered a transcriptional response similar to HR, including an activation of the salicylic acid signalling and HR marker genes, i.e. pathogenesis-related genes (*PR*); but HR-like necrosis to eggs was absent [43]. In addition to HR, *B. nigra* emits volatiles in response to *P. brassicae* eggs attracting *T. brassicae* wasps [32]. However, how the direct egg-killing trait correlates with the attraction of natural enemies remains to be investigated.

We conducted a field survey and greenhouse and laboratory experiments to test (i) whether eggs of *Pieris* butterflies induce HR-like necrosis in different populations of *B. nigra* and if so, whether there is phenotypic variation therein, (ii) how the expression of HR affects the performance of *Pieris* eggs and

parasitism rates by *Trichogramma* species and to what extent, (iii) how expression of HR affects preference and performance of *Trichogramma* wasps and (iv) whether HR necrosis is associated with an activation of the marker gene *PR1*. We hypothesized that HR-like necrosis, and not oviposition itself, is associated with an induction of the *PR1* gene in the plant. Moreover, we expected that HR-like necrosis is a common trait for the black mustard against specialist cabbage whites and that herbivore and parasitoid survival under field conditions will be negatively influenced by the expression of HR. Consequently, *Trichogramma* wasps were expected to prefer volatiles of plants that did not express HR. This study provides evidence at several levels of biological organization of how insect eggs can shape plant–insect interactions.

2. Material and methods

(a) Plants and insects

Black mustard (*B. nigra* L.) plants were grown in a greenhouse ($18 \pm 5°C$, 50–70% RH, 16 L : 8 D). Seeds of at least 10 individual plants were collected from a *B. nigra* population in 2009 at the River Rhine in Wageningen (Steenfabriek), The Netherlands (coordinates: 51.96, 5.68) and pooled. To assess whether HR-like necrosis is common across populations, seeds were also collected from two other locations in The Netherlands namely north of Wageningen (Radix) (coordinates: 51.99, 5.68) and Rhenen (Blauwe Kamer) (coordinates: 51.95, 5.60). The distances between the populations were about 1–6 km. Moreover, seeds collected in West Sacramento, Yolo County, CA, USA (coordinates: 38.58, −121.57) and seeds from Peloponnesus, Greece (collected in 1975 and stored by the Centre for Genetic Resources, Wageningen, The Netherlands) were used. Plants of four weeks old were used in the experiments.

Pieris rapae L. and *P. brassicae* L. (Lepidoptera: Pieridae) were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv. Cyrus) in a climate room ($21 \pm 1°C$, 50–70% RH, 16 L : 8 D). Two native isofemale *Trichogramma evanescens* Westwood (Hymenoptera: Trichogrammatidae) strains (RU124 and RU263), originating from *P. rapae* eggs collected on *B. nigra* at the River Rhine in Wageningen in 2010, and one isofemale *T. brassicae* Bezdenko strain (Y175), originating from a *Mamestra brassicae* egg cluster collected in a cabbage field in Lierop, The Netherlands in 1999, were reared since then on *Ephestia kuehniella* eggs (Koppert, Berkel en Rodenrijs, The Netherlands) under standardized conditions in a climate chamber ($25 \pm 1°C$, 50–70% RH, 16 L : 8 D). Only mated, 2–3 days old, wasps were used in the preference experiments. All wasps used in the preference experiments lacked previous contact with any plant material or host residues and are referred to as inexperienced.

(b) Field survey

A survey was conducted to record survival and parasitism (by *Trichogramma* spp.) of *Pieris* eggs on individual *B. nigra* plants in a natural population. The survey was conducted at a *B. nigra* patch along the River Rhine in Wageningen (Steenfabriek), The Netherlands (coordinates: 51.96, 5.68) in three seasons/three butterfly generations: August—September 2010, May—July 2011 (1) and August—September 2011 (2). The total area monitored was approximately 100 m^2 consisting of about 1000 plants. In spring, plants were only 5–20 cm tall, whereas they could grow up to more than 1.5 m tall in late summer. Plants were monitored for eggs at the edges of a patch or on isolated growing plants. Eggs were collected on leaves and checked for the presence of a necrotic zone on the leaf and/or parasitism by *Trichogramma* wasps. In both seasons in 2011, each plant was

marked and revisited. Sometimes additional eggs were found on the same plant. After collection, eggs were kept in a climate chamber ($25 \pm 1^\circ$C, 50–70% RH, 16 L : 8 D) until caterpillars or wasps emerged. All hatched, dead and parasitized eggs were recorded. Hatched caterpillars were fed with black mustard leaves until fourth instar for species identification. The emerged wasps were preserved in 100% ethanol and kept at -25°C for standard molecular species identification using ITS-2 markers (for more details, see [44] and the electronic supplementary material).

(c) Greenhouse and laboratory experiments

(i) Plant treatments

To obtain egg-infested plants for monitoring HR-like necrosis induced by *Pieris* eggs in plants of different *B. nigra* populations, plants were placed into a cage with more than 100 *P. rapae* or *P. brassicae* adults to allow deposition of eggs onto the plants. Plants were exposed to the butterflies, to obtain about 20 single *P. rapae* eggs or one *P. brassicae* egg clutch per plant. After egg infestation, plants were kept in a greenhouse compartment ($21 \pm 2^\circ$C, 70% RH, 16 L : 8 D) and checked for HR-like necrosis 72–96 h after egg deposition (see below). For *P. rapae* egg performances and *T. evanescens* preferences and gene expression analysis, *B. nigra* plants from the local population (Steenfabriek) were used. Following *P. rapae* egg deposition, plants were placed in the same greenhouse compartment for either 24 or 72 h. Control plants, which had not been in contact with *P. rapae* or any other insects, were kept under the same conditions as treated plants.

(ii) Hypersensitive response-like necrosis

All egg-infested plants and leaves collected from plants in the field were checked for HR-like necrosis. The strength of HR was recorded and the plants were categorized into HR− (no necrotic zone observed) or HR+ (necrotic zone, eggs desiccating). Plants were kept under greenhouse conditions ($22 \pm 2^\circ$C, 70% RH, 16 L : 8 D) and the number of eggs was counted immediately and 5 days after oviposition.

(iii) Egg survival

The survival rate of eggs was calculated based on the total number of eggs and the number of caterpillars. The number of eggs was counted before and the number of caterpillars just after hatching by observation. HR intensity was noted just prior to larval hatching. HR necrosis was categorized into three severities: necrosis visible underneath the egg (HR+), necrosis also visible on other side of the leaf (HR++) and necrosis visible around the egg and underside of leaf (HR+++).

(iv) Two-choice bioassays

We tested the attraction of a laboratory strain of *T. brassicae* and two native strains of *T. evanescens* to volatiles of *P. rapae* egg-induced *B. nigra* plants. Bioassays were conducted by using a dynamic airflow Y-tube olfactometer. This olfactometer was adapted to small wasps like *Trichogramma* spp. to be released in groups [32]. We have previously established that group-release did not influence the behaviour of these *Trichogramma* wasps [32]. Ten adult females of one strain were released and their preference for one of the two odour sources was recorded. Thereafter, the position of the odour sources was exchanged and another group of 10 wasps from the other strain was released to test their preference for the same two odour sources. After 45 min, the wasps present in the collection flasks placed at the end of the arms section of the olfactometer were counted. When a wasp did not make a choice within 45 min, it was recorded as a 'no response'. The odour sources were plants infested with *P. rapae* eggs versus uninfested plants. In addition, we recorded the presence or absence of a hypersensitive-like response of the infested plants that were used in the bioassay. Per odour source combination, five to seven different plants with one replicate per experimental day were tested with 10–20 wasps of each strain released per replicate (70–100 wasps/strain/treatment). Each wasp was used only once.

(v) Quantitative RT-PCR analysis of hypersensitive response marker gene

For gene expression, two leaf discs (1.7 cm diameter) were taken from the third and fourth leaf from the top directly next to an egg. Discs of three to five individual plants per treatment were pooled. In total, three to five biological replicates were obtained per treatment. Leaf tissues were snap-frozen in liquid nitrogen and kept at -80°C until analysis. Total RNA was extracted from approximately 100 mg liquid nitrogen-ground leaf powder using the RNeasy Plant Mini kit (Qiagen). One microgram of total RNA was treated with DNaseI (Invitrogen) and subsequently transcribed into cDNA using the iScript cDNA synthesis kit (Bio-rad) following the manufacturer's protocol. The used sequence of *B. nigra* gene-specific *PATHOGENESIS-RELATED GENE1* primers were $PR1_F$ 5'-CTTGGCCATGGGTAGCGGCG-3' and $PR1_R$ 5'-ACACCTCGCTTTGCCACATCCA-3'. Quantitative RT-PCR was performed in a Rotor-Gene 6000 machine (Corbett Research) with a 72-well rotor. The amplification reactions were performed in 25 μl final volume containing 12.5 μl iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 300 nM of the gene-specific forward and reverse primer and 5 μl cDNA. All qRT-PCR experiments were performed in duplicate and average values were used in the analyses. The following PCR program was used for all PCR analyses: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 30 s at 60°C and 30 s at 72°C. Normalized gene expression was then calculated as $2^{-\Delta\Delta Ct}$ [45], using *GAPDH* as a reference gene (primer sequences: F 5'-GCTACGCAGAAGAC AGTTGATGG-3' and R 5'-TGGGCACACGGAAGGACATAC-3').

(d) Statistics

The effect of a plant showing an HR-like necrosis on *Pieris* eggs and parasitoid survival, as well as on *Trichogramma* parasitism was analysed using generalized linear models (GLM) with binomial distribution for errors and a logit link function. In these models, the dispersion parameter was estimated to correct for over-dispersion. The analysis of the data from 2010 could not include plant identity and therefore eggs (instead of plants) were considered as units. We tested whether HR affected *Trichogramma* clutch size by recording the number of wasps emerging from an egg as a categorical (multinomial) response variable using PROC CATMOD (SAS Institute, Cary, NC, USA). To test whether the number of eggs on a plant affects the induction of HR, a linear regression analysis between the number of eggs/plant/day and the number of eggs inducing HR/plant/day was conducted. Data from the olfactometer bioassays were analysed by expressing the number of animals that chose the test odour as the fraction of all responding wasps and arcsin ($\sqrt{x}$) transforming the variable. Subsequently, choices were tested against a 50 : 50 distribution with a one-sample *t*-test. The distributions in the choices that parasitoids made across the different treatment combinations were compared using a GLM with the arcsin ($\sqrt{x}$) fractions as dependent variables. 'Time after egg deposition', 'HR response' and 'parasitoid strain' were fixed factors. The model used for final inferences contained all main effects and significant interactions terms. The fit of the model was checked by the Kolmogorov–Smirnov test. The gene transcription data were square root-transformed to meet the normality assumption, and analysed with one-way ANOVA for the two time points independently. Then Tukey's test was used for post hoc comparisons.
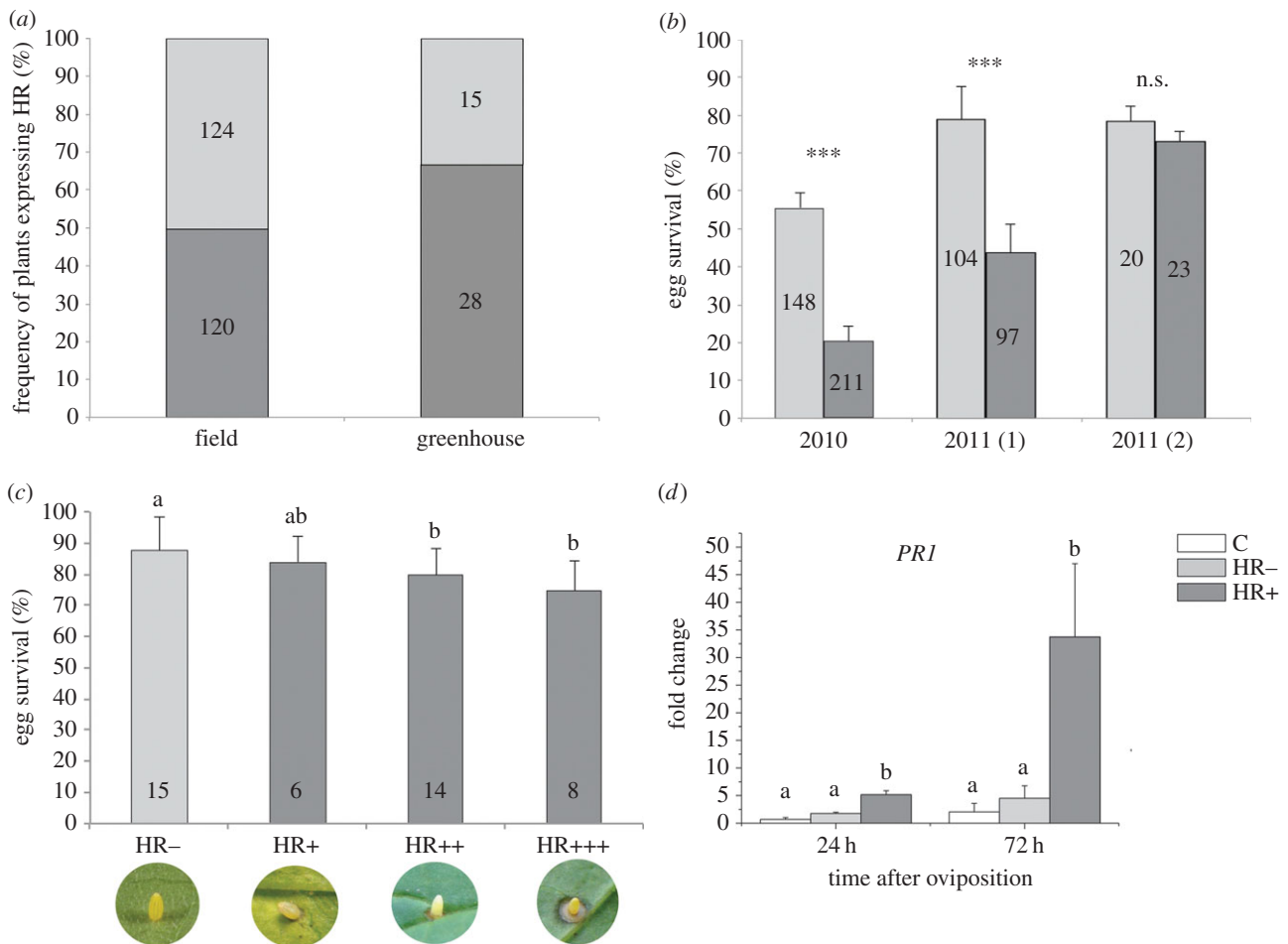
Figure 1. Frequency of *B. nigra* plants expressing HR-like necrosis induced by *Pieris* eggs and effects of HR-like necrosis on *Pieris* egg survival and *PR1* gene expression. (*a*) Percentage of HR-like necrosis on plants of a Dutch field population (Steenfarbiek) and in the greenhouse. (*b*) Percentage (estimated mean $\pm$ s.e.) of caterpillars emerging from *Pieris* eggs on plants of a Dutch field population (Steenfarbiek) (2010 and 2011, generation 1 and 2); ***$p < 0.001$; n.s., not significant (GLM, $p > 0.05$). (*c*) Variation in HR severity and effect on *P. rapae* egg survival (estimated mean $\pm$ s.e.) in the greenhouse (different severities were visually characterized as in the pictures); different letters indicate significant differences (GLM and LSD, $p < 0.05$). (*d*) Effect of HR-like necrosis induced by *P. rapae* eggs on expression changes (mean $\pm$ s.e.) of HR-marker gene *PR1* at 24 and 72 h after oviposition; different letters indicate significant differences (ANOVA, Tukey test $p < 0.05$, $n = 3 – 5$ biological replicates). Numbers inside the bars represent number of plants for data of 2011 and number of eggs for data of 2010. Hypersensitive response (HR), in light grey HR−: no necrotic zone observed, in dark grey HR+: necrotic zone. (Online version in colour.)

3. Results

(a) Field survey

(i) Monitored insects
The majority of the collected eggs were of *P. rapae* (96%, 191 out of 198 identified caterpillars), and a small fraction of *P. napi* (4%). Both species deposit single eggs. No egg batches of *P. brassicae* were found. All wasps that emerged from the collected *Pieris* eggs belonged to *Trichogramma* spp. (Hymenoptera: Trichogrammatidae). A group of 25 wasps identified through sequencing of the ITS-2 gene were *T. evanescens* Westwood.

(ii) Plant response to eggs
In the year 2010, 359 eggs were collected from approximately 50 plants. From all collected eggs in 2010, 211 had induced HR-like necrosis (HR+) (59%). On one exceptional plant, about 80 *Pieris* eggs were collected, however this plant was excluded from the analysis. In the year 2011 (both generations), 360 eggs were collected from 244 plants out of which 120 plants expressed HR (49%) (figure 1*a*). A maximum of nine eggs was found on a plant per sampling day (mean eggs/plant/day $\pm$ s.d.: 1.4 $\pm$ 1.1). There was no linear

relationship between egg density and induction of HR-like necrosis (electronic supplementary material, figure S1, $R^2 = 0.28$, $F_{1,48} = 0.06$, $p = 0.82$).

(iii) Effect of plant response on egg survival
In two cases out of the three censuses, we found a significant decrease in the egg survival of *P. rapae* when an HR-like reaction in response to egg deposition was present in the plant (figure 1*b*). In 2010, significantly more eggs survived on HR− than on HR+ plants (GLM, $F_{1,357} = 37.93$, $p < 0.001$). While a significant effect of HR on egg survival was present in the first generation in 2011 (GLM, $F_{1,199} = 16.84$, $p < 0.001$), there was no effect of HR on egg survival in the second generation (GLM, $F_{1,41} = 0.15$, $p = 0.70$).

(iv) Effect of plant response on egg parasitism
The highest *Trichogramma* egg parasitism rates were found in 2010: 104 eggs were parasitized (29%), and significantly more parasitized eggs were found with an HR-like necrosis (HR+) than with no necrosis (HR−) ($F_{1,357} = 18.19$, $p < 0.001$, figure 2*a*). In 2011, significantly more parasitized eggs were found on HR+ than on HR− plants in the first
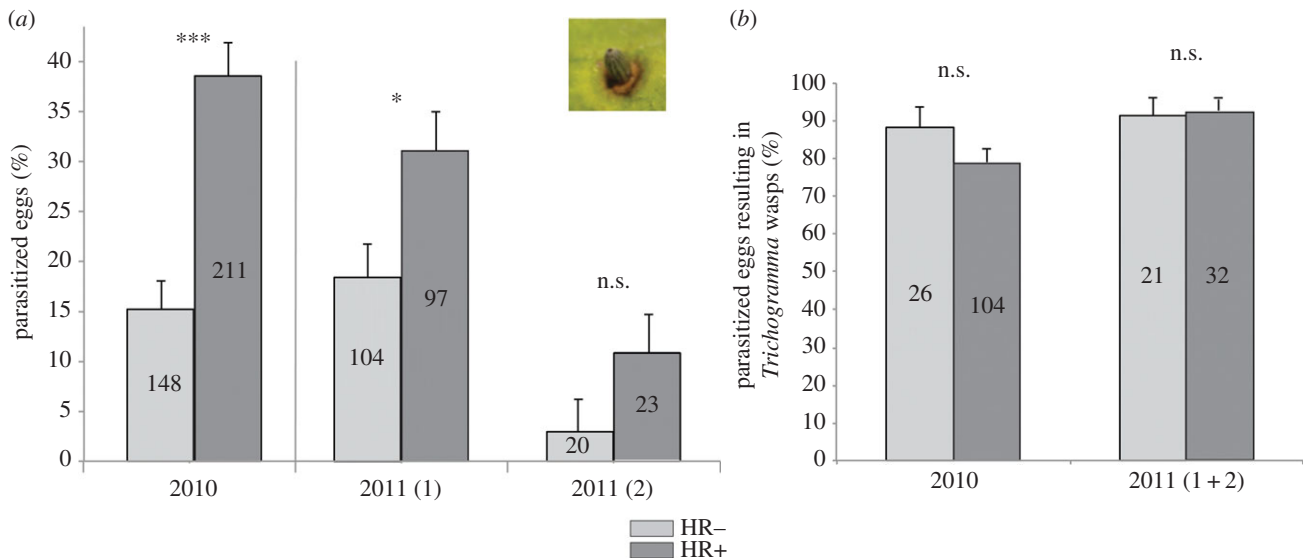
Figure 2. Effect of *Pieris* egg-induced HR on *Trichogramma* parasitism and performance in a natural *B. nigra* population collected in three different seasons in 2010 and 2011 (generations 1 and 2). (*a*) Percentage (estimated mean ± s.e.) of eggs that were parasitized by *Trichogramma* wasps. Picture shows a parasitized egg with HR-like necrosis. (*b*) Percentage of parasitized eggs (estimated mean ± s.e.) from which *Trichogramma* wasps emerged. Numbers inside the bars represent number of plants for data of 2011 and number of eggs for data of 2010. Different plant phenotypes: hypersensitive response (HR), HR−: no necrotic zone observed, HR+: with necrotic zone. ***$p < 0.001$, *$p < 0.05$; n.s., not significant (GLM, $p > 0.05$). (Online version in colour.)

generation ($F_{1,199} = 5.47$, $p = 0.02$), whereas no differences were found in the second generation ($F_{1,41} = 1.57$, $p = 0.22$).

(v) Effect of plant response on egg parasitoid survival and clutch size

Trichogramma spp. can successfully parasitize and complete their development inside single *Pieris* eggs that induced HR in leaves collected from *B. nigra* plants at the River Rhine. There was no effect of HR-like necrosis on the percentage of wasps emerging from the collected *Pieris* eggs in 2010 ($F_{1,128} = 1.25$, $p = 0.27$, figure 2*b*) and in 2011 ($F_{1,51} = 0.06$, $p = 0.81$, figure 2*b*). Moreover, we showed that up to four wasps emerged per *Pieris* egg but HR had no effect on clutch size ($\chi^2_4 = 4.92$, $p = 0.30$).

(b) Greenhouse and laboratory experiments
(i) Plant response to eggs
From all six tested *B. nigra* populations, a fraction of plants responded with HR-like necrosis induced by both singly deposited *P. rapae* eggs and/or gregariously laid *P. brassicae* eggs. Within each population expression of HR varied between 27 and 63% of the tested plants (table 1).

(ii) Effect of plant response on egg survival
Of the 42 *B. nigra* plants tested for *P. rapae* egg survival, 28 plants expressed HR (65%) (figure 1*a*) from which significantly less larvae hatched (22%) than from plants not expressing HR (GLM, $F_{1,41} = 9.51$; $p = 0.004$, figure 1*b*), probably due to egg desiccation. Only few eggs fell from the plant before hatching (HR−: 0.3%, HR+: 1%). Egg survival depended on the strength of the HR-like necrosis: the larger the area of necrosis, the lesser was the number of eggs that survived. Significantly more eggs survived on non-HR expressing plants (HR−) and on plants with little necrosis (HR+) than on plants expressing medium (HR++) or the strongest necrosis (HR+++) (+++) (GLM, $F_{3,39} = 4.0$; $p = 0.014$, figure 1*c*).

Table 1. Frequency of HR-like necrosis induced by *Pieris* eggs in different *B. nigra* populations.

B. nigra population	*Pieris* species	*N* plants	% HR
Peloponnese, Greece	*P. brassicae*	74	47.3
	P. rapae	38	31.6
South-Wageningen (Steenfabriek), The Netherlands	*P. brassicae*	76	51.3
	P. rapae	79	46.8
North-Wageningen (Radix), The Netherlands	*P. brassicae*	15	46.7
Rhenen (Blauwe Kamer), The Netherlands	*P. brassicae*	15	26.7
West Sacramento, CA, USA	*P. brassicae*	11	63.6
	P. rapae	15	26.7

(iii) Induction of hypersensitive response marker gene *PR1*
The deposition of single *P. rapae* eggs strongly induced the expression of the *PR1* both at 24 ($F_{2,9} = 18.35$, $p = 0.002$) and 72 h after oviposition ($F_{2,8} = 6.57$, $p = 0.03$) when compared with control plants, and only in HR+ plants, but not on HR− plants (Tukey, $p > 0.05$, figure 2*d*). Thus, phenotypic expression of necrosis was clearly correlated with genotypic expression of the HR marker gene *PR1*.

(iv) Effect of plant response on egg parasitoid-attracting plant volatiles
In olfactometer bioassays, two native strains of *T. evanescens* (RU124 and RU263), both collected from *P. rapae* eggs in 2010 and a laboratory strain of *T. brassicae* were tested for attraction to OIPVs (figure 3). Although in the overall
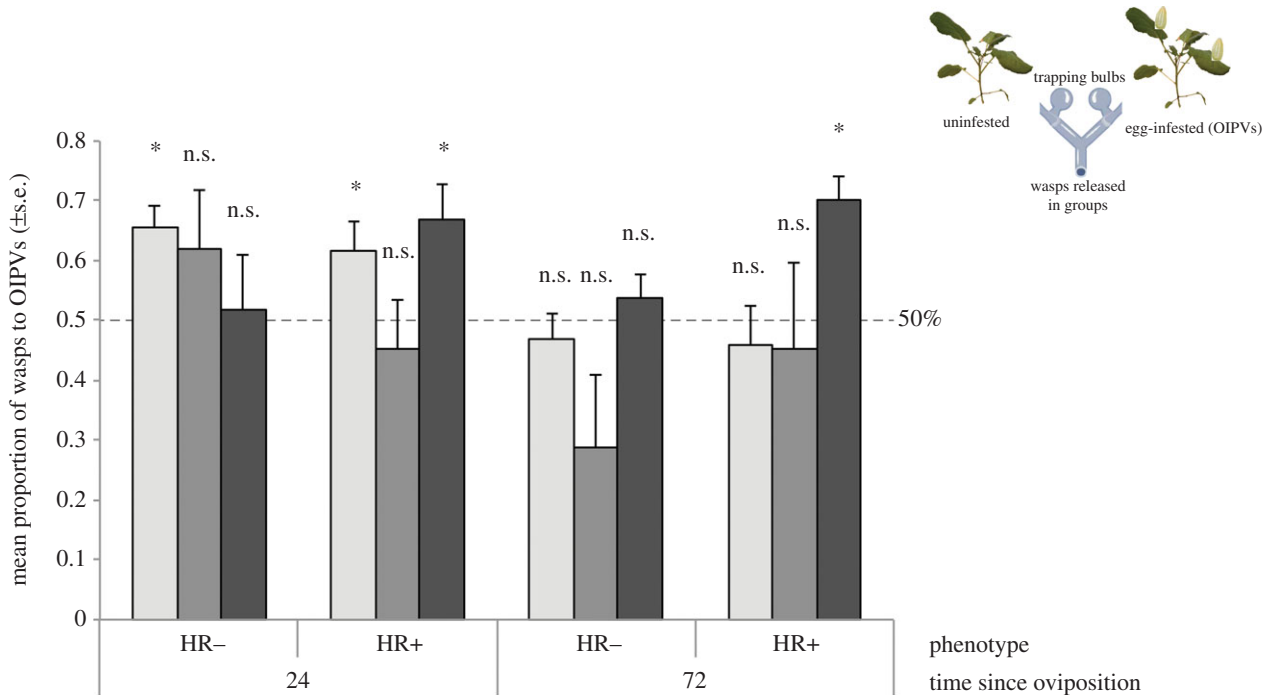
Figure 3. Proportion (mean $\pm$ s.e.) of female *Trichogramma* wasps choosing OIPVs of *B. nigra* plants. Plants were infested with single eggs of *P. rapae*. Columns represent the mean proportion of choice for OIPVs of isofemale lines from *Trichogramma brassicae* (lab strain Y175) (light grey) and *T. evanescens* (native strain RU124, medium grey and native strain RU 263, dark grey) tested in a Y-tube olfactometer. Numbers in the columns represent number of responding wasps. All experiments were conducted in a two-choice situation between plants infested with eggs of different ages (24 and 72 h) and uninfested control plants. *$p < 0.05$; n.s., not significant ($p > 0.05$); one-sample *t*-test. The dashed line indicates $0.5 =$ no preference. Each treatment combination was replicated with five to seven plant pairs and 10–20 wasps of each strain per plant pair ($n = 70$–100 wasps per treatment/strain). Different plant phenotypes: hypersensitive response (HR), HR$-$: no necrotic zone observed, HR$+$: necrotic zone. (Online version in colour.)

model, the presence/absence of HR response did not influence the distribution of the wasps' choices over the tested plant volatiles (GLM, $F_{1,93} = 0.68$, $p = 0.41$), when exposed to the same odour sources, the *Trichogramma* strains made different choices ($F_{2,93} = 3.63$, $p = 0.03$). Also, the time after oviposition significantly affected the distribution of the wasps' choices, and the differences between the tested odour blends were more pronounced 24 h after induction than after 72 h ($F_{1,93} = 6.58$, $p = 0.01$).

At 24 h after oviposition, only wasps of the *T. brassicae* laboratory line significantly preferred volatiles of egg-infested plants without a visible necrosis (HR$-$) (Y175: $t_4 = 4.55$, $p = 0.01$; RU124: $t_6 = 1.23$, $p = 0.27$; RU263: $t_6 = 0.23$, $p = 0.82$; one-sample *t*-test). However, when OIPVs of plants expressing HR (HR$+$) were tested 24 h after oviposition, wasps of the laboratory line and of one native line (RU263) were significantly attracted to them (Y175: $t_5 = 2.92$, $p = 0.03$; RU124: $t_7 = 1.8$, $p = 0.11$; RU263: $t_7 = 3.45$, $p = 0.01$). At 72 h after oviposition, none of the wasps were attracted to OIPVs of HR$-$ plants (Y175: $t_4 = -0.75$, $p = 0.5$; RU124: $t_6 = -1.67$, $p = 0.15$; RU263: $t_6 = 1.02$, $p = 0.35$), whereas one native line was significantly attracted to OIPVs of HR$+$ plants (Y175: $t_4 = -0.61$, $p = 0.58$; RU124: $t_6 = -0.29$, $p = 0.78$; RU263: $t_6 = 4.86$, $p = 0.003$).

4. Discussion

Our study finds a positive correlation between a potentially lethal direct and an information-mediated indirect defence. One tested *T. evanescens* strain was specifically attracted to volatiles emitted by *B. nigra* plants expressing HR-like

necrosis (HR$+$). This higher attractiveness to volatiles emitted by plants of the HR$+$ phenotype could explain the higher egg parasitism rates that were found on the latter compared with plants lacking HR (HR$-$) in the natural *B. nigra* population. A synergistic expression of both traits seems to lead to a more effective control of *Pieris* butterfly eggs leading to up to two times higher egg mortalities on HR$+$ plants.

We confirmed that HR-like necrosis against *P. rapae* eggs is a lethal resistance trait that leads to butterfly egg desiccation resulting in fewer caterpillars hatching especially then when necrosis was the strongest. We showed phenotypic variation within the same *B. nigra* population and between populations in the expression of HR induced by both tested *Pieris* species ranging from 27 to 64% (table 1). HR-like necrosis is not restricted to *B. nigra*. Similar necrotic responses have been observed in *Solanum* hybrids against egg masses of the Colorado potato beetle *Leptinotarsa decemlineata* or *Physalis* plants against eggs of the specialist moth *Heliothis subflexa* [31,33]. In the latter system, both HR-like necrosis sometimes in combination with neoplasm formation leads to 25% lower probability of larvae hatching and 28% lower probability of eggs remaining on the *Physalis* plants [33]. It is suggested that HR-like necrosis in both *Solanum* hybrids and *Physalis* spp. is genotypically determined and this might also be the case in the *B. nigra* population studied here. Previous studies in *Arabidopsis* (where several plants were pooled prior to analysis) have shown an upregulation of the classical HR marker gene *PR1* in response to eggs [43], but in our study only *B. nigra* plants that expressed the necrotic response showed an induction of *PR1* (figure 2*d*). This indicates that not all plants can detect the eggs and suggests a genotypic variation in the plants. A possible explanation for the

variation in strength of HR could be due to differences in the symbiotic microbial communities associated with the eggs (see also Petzold-Maxwell et al. [33]). The extent to which genotypic variation explains the observed phenotypic variation in HR between populations could be explained, e.g. by differences in herbivore pressure.

Abortion of herbivore-infected fruits is another known lethal defence trait used by plants whose pollinators larvae consume developing seeds and fruits [30]. The white campion (Silene latifolia) can selectively abort flowers that contain larvae of the nursery pollinating moth Hadena bicruris [46]. The plant benefits from this direct defence trait in terms of reduced secondary predation but pays costs in terms of residual fitness of non-aborted infested fruits. The latter is high also due to the presence of larval parasitoids killing larvae before completing fruit consumption showing a possible conflict between the two defence strategies in this nursery pollination system (A. Biere 2014, personal communication). In B. nigra, we expect that plant fitness should covary with presence/absence of the direct defence trait, as a conflict with the indirect defence trait was not shown.

Our study revealed that despite being a lethal trait, HR-like necrosis did neither render the eggs unsuitable for parasitoids nor did it negatively affect parasitoid fitness. Trichogramma wasps could successfully develop in desiccating eggs, without negative effects on their survival and clutch size. In addition, HR-like necrosis does not appear to reduce the window of opportunity during which the eggs are available for parasitism by another Trichogramma species [32]. Egg parasitoids are idiobionts that kill the embryo at the beginning of its development to feed on dead tissue and stored material in the egg. But the egg does not need to be alive for parasitization, as Trichogramma wasps can for example parasitize and develop in irradiated or even artificial eggs [42]. Apparently, Pieris eggs affected by HR-like necrosis provided sufficient conditions for successful development of Trichogramma wasps.

A direct defence trait like the observed necrosis may have evolved as an adaptation to specialist herbivores that are often feeding on reproductive plant tissue, with drastic fitness consequences [47,48]. Additionally, allelochemicals, e.g. glucosinolates, are not the most effective against specialists, because they can sequester or detoxify these compounds [49]. Therefore, HR-like necrosis seems to be targeted against glucosinolate specialists, i.e. Pieris spp., and not generalists such as M. brassicae, the larvae of which are strongly negatively affected by the plant toxins [12,50,51]. Such different lines of defences

would support the defensive synergism hypothesis, which states that different defence traits expressed in combination may provide a higher level of resistance against herbivory than when expressed separately [22]. Examples to support this hypothesis have been found in Nicotiana attenuata [52], in rainforest shrubs of Piper cenocladum [9] and in different Umbelliferae species [53]. Multiple defence traits may be adaptive in a complex plant–herbivore–carnivore community, because of the redundancy of defence lines; when one resistance trait fails, another acts as a safety net [54]. This was shown to be the case in a natural belowground tritrophic system involving common milkweed, where no negative correlation between direct (root cardenolides) and indirect defences (nematode attracting-root volatiles) was found [55].

We showed that parasitoid attraction towards volatiles of egg-induced plants was not influenced by HR-like necrosis, and OIPVs of HR+ plants were more attractive for a native strain of T. evanescens. We demonstrate that a synergism between direct and indirect defences may occur even when defence traits are evolved that are lethal for the herbivore and may potentially harm the natural enemies. This suggests that expressing HR is beneficial for B. nigra because it induces higher herbivore egg mortality. However, it remains unclear whether HR-like necrosis incurs fitness costs for the plant. If so, then expression of HR may provide plants with a higher fitness in some individuals over others, based on differences in e.g. herbivore pressure [56], that should result in within- and between population variation in HR responses. It still remains unclear why butterflies are abundant on such plants that kill their eggs by direct or indirect defences. Future studies should investigate how preferences of butterflies correlate with larval performances including the egg phase, to fully understand how directional selection of plant defence traits may shape multitrophic interactions.

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