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Original Article

Priming of anti-herbivore defence in *Nicotiana attenuata* by insect oviposition: herbivore-specific effects

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ABSTRACT

Oviposition by *Spodoptera exigua* on *Nicotiana attenuata* primes plant defence against its larvae that consequently suffer reduced performance. To reveal whether this is a general response of tobacco to insect oviposition or species-specific, we investigated whether also *Manduca sexta* oviposition primes *N. attenuata*'s anti-herbivore defence. The plant response to *M. sexta* and *S. exigua* oviposition overlapped in the egg-primed feeding-induced production of the phenylpropanoid caffeoylputrescine. While *M. sexta* larvae were unaffected in their performance, they showed a novel response to the oviposition-mediated plant changes: a reduced antimicrobial activity in their haemolymph. In a cross-resistance experiment, *S. exigua* larvae suffered reduced performance on *M. sexta*-oviposited plants like they did on *S. exigua*-oviposited plants. The *M. sexta* oviposition-mediated plant effects on the *S. exigua* larval performance and on *M. sexta* larval immunity required expression of the *NaMyb8* transcription factor that is governing biosynthesis of phenylpropanoids such as caffeoylputrescine. Thus, *NaMyb8*-dependent defence traits mediate the effects that oviposition by both lepidopteran species exerts on the plant's anti-herbivore defence. These results suggest that oviposition by lepidopteran species on *N. attenuata* leaves may generally prime the feeding-induced production of certain plant defence compounds but that different herbivore species show different susceptibility to egg-primed plant effects.

Key-words: herbivory; induced plant defence; *Manduca sexta*; phenolics; protease inhibitors; *Spodoptera exigua*.

INTRODUCTION

Plants are under attack by a plethora of different herbivores and defend themselves by multifarious traits that are constitutively expressed or inducible upon herbivore attack (Schoonhoven *et al.* 2005). The defensive effect of particular plant defence traits varies in different plant-herbivore interactions. Herbivores are phylogenetically diverse, exhibit numerous feeding modes and strongly differ in the degree of specialization to their host plants. Generalist herbivores use a wide range of host plants, whereas specialists attack single or few closely related host-plant species. Specialists are supposed to be less affected by the defence responses of their host plants as they often evolved mechanisms to detoxify plant metabolites or even sequester them for their own defence (Dyer & Bowers, 1996; Wink & Theile, 2002; Agrawal *et al.* 2012). Several plant species diverge in their induced responses to generalist and specialist herbivores (Voelckel & Baldwin, 2004; Vogel *et al.* 2007; Zong & Wang, 2007). On the one hand, this may be driven by the herbivore, for example, when specialist herbivores have evolved the ability to manipulate plant defence responses (Ali & Agrawal, 2012; Karban & Agrawal, 2002). On the other hand, distinct responses to different herbivores may enable plants to eventually mount the most effective combination of defence traits against a certain herbivore.

Plant responses to herbivores require perception of stimuli associated with the attacking herbivores. The feeding damage itself is one of the best investigated stimuli and is perceived by the plant based on the recognition of molecular patterns that are associated with the damage of plant tissue or the attacking herbivore (Heil, 2009). Molecular patterns of both origins shape the plant response to chewing herbivores. These herbivores typically elicit plant responses triggered by the phytohormone jasmonic acid (JA) that is also called the wound hormone. In many plants, JA mediates the production of defensive secondary metabolites, proteins or even morphological structures such as trichomes (Howe & Jander, 2008). The wounding response is modified by the perception of diverse herbivore-associated molecular patterns (Maffei *et al.* 2012) that often consist of modified plant components or are even products of plant enzymes that become active in the insect's midgut (Gaquerel *et al.* 2012). By integrating different stimuli associated with a feeding herbivore, plants convey responses that are specific to the attacking herbivore.

Yet plants do not only perceive the feeding damage by the herbivorous stage of an insect but also its eggs when they are deposited on the host plant (Hilker & Meiners, 2011; Reymond, 2013). The few oviposition-associated elicitors that are identified so far also differ between the insect species studied (Hilker & Fatouros, 2015). In addition to insect species-specific elicitors of plant responses to eggs, several studies show that the effects of oviposition on plant defence against insect eggs are specific to the insect species (Meiners *et al.* 2000;

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Mumm *et al.* 2005). Oviposition-induced responses that reduce egg survival include the formation of neoplasms, necrotic or egg-crushing tissues, the production of ovicidal substances and volatiles attracting egg predators and parasitoids (Hilker & Fatouros, 2015). Moreover, several plant species can increase their resistance to the herbivorous larvae upon insect oviposition (Beyaert *et al.* 2012; Geiselhardt *et al.* 2013; Pashalidou *et al.* 2013; Austel *et al.* 2015; Bandoly *et al.* 2015). Whereas these studies almost exclusively investigated specialist herbivores, one of them demonstrated that the generalist *Spodoptera exigua* oviposition on the desert tobacco *Nicotiana attenuata* primes the plant's feeding-induced defences against herbivory (Bandoly *et al.* 2015).

Priming of plant defence refers to a more efficient defence response to a biotic stress upon a preceding environmental stimulus (Gális *et al.* 2009; Conrath, 2011; Hilker *et al.* 2015). Whether the oviposition-mediated resistance to insect larvae is generally due to priming of feeding-induced plant defence or also to directly oviposition-induced plant traits remains to be determined. In *N. attenuata*, *S. exigua* oviposition primes the feeding-induced production of two defence traits, phenolic caffeoylputrescine (CP) and activity of trypsin protease inhibitors (TPIs). As a consequence, *S. exigua* larvae suffer increased mortality, reduced mass gain and a retarded development on oviposited plants, while the plant receives less feeding damage. The oviposition-mediated effects on *S. exigua* larvae require the expression of the transcription factor *NaMyb8* (Bandoly *et al.* 2015) that governs the production of phenylpropanoid-polyamine conjugates (PPCs) such as CP (Kaur *et al.* 2010).

Here, we investigate whether this oviposition-mediated priming of *N. attenuata*'s defence is specific to the herbivore with respect to (i) the plant response and (ii) the ecological effect on the feeding larvae. We compare the plant's response and resistance with larval feeding after oviposition by the specialist *Manduca sexta* to those after *S. exigua* oviposition. Both are leaf-chewing lepidopteran herbivores that frequently attack *N. attenuata* in native populations (Kessler & Baldwin, 2001; Steppuhn *et al.* 2004). Herbivory on *N. attenuata* induces several secondary metabolites and proteins that function as plant defence as has been shown by silencing genes mediating the biosynthesis of nicotine (Steppuhn *et al.* 2004), PPCs (Kaur *et al.* 2010), diterpene glycosides (DTGs) (Jassbi *et al.* 2008) and TPIs (Steppuhn & Baldwin, 2007; Zavala & Baldwin, 2004). *N. attenuata* responds differently to the feeding larvae of *S. exigua* and *M. sexta* (Voelckel & Baldwin, 2004; von Dahl *et al.* 2007; Winz & Baldwin, 2001), which likely results from a differential activation of phytohormone pathways in response to different ratios of herbivore-associated elicitors in the oral secretions (OS) of larvae of both species (Diezel *et al.* 2009). Yet it remains unknown whether this plant also discriminates between the oviposition by both species.

Specifically we asked (i) whether oviposition by *M. sexta* also primes or eventually induces *N. attenuata*'s defence traits; (ii) how it affects the larvae of *M. sexta*; (iii) whether the larval performance of both, *M. sexta* and *S. exigua*, is similarly affected by prior oviposition on *N. attenuata* independent of which moth species oviposited on the plant and (iv) whether the transcription factor *NaMyb8* is involved in the priming effects that *M. sexta* oviposition exerts on the plant's anti-herbivore defence. To address these questions, we analysed defence parameters of oviposited and egg-free plants as well as performance and immune parameters of larvae feeding on these plants. In addition to wild type (Wt) plants, we used transgenic plants to scrutinize the relevance of putative priming-involved factors.

MATERIAL AND METHODS

Plants and insects

We used inbred lines (15th, 17th and 18th generations) of *N. attenuata* Torr. ex Watson (Solanaceae) plants derived from seeds collected in the Great Basin Desert (Utah, USA). In addition to the Wt, we used plants stably transformed with an inverted repeat construct (ir) to post-transcriptionally silence gene expression of *NaMyb8* (GenBank: GU451752.1). We used T4-plants of line A07-810 that has been thoroughly characterized and described for *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure and vector (*pSOL8Myb8*) construction elsewhere (Kaur *et al.* 2010). Seeds germinated on agar plates (Gamborg 5, Duchefa, Haarlem, the Netherlands). After 7–10 d, seedlings were planted to individual cells (height: 6 cm; Ø: 4 cm) of propagation trays and after 4 weeks, potted in 1.5 L pots with potting soil Classic T (Einheitserde®, Uetersen, Germany; www.einheitserde.de). Plants grew continuously in a greenhouse [24 °C (±10):15 °C; 16:8 h L:D] under discharge lamps (Philips Master SON-T PIA PLUS 400 W E4, Amsterdam, the Netherlands). We used 5–6-week-old rosette plants in the experiments.

Larvae of *M. sexta* L. (Sphingidae) and *S. exigua* Hübner (Noctuidae) were reared in a climate chamber (24 °C; 16:8 L:D; metal halide lamp EYE CLEAN ACE MT250DL) on wheat germ-based artificial diets as described earlier (Bandoly *et al.* 2015; Trauer & Hilker, 2013).

Experimental overview

We analysed plant defence parameters in leaves of *M. sexta*-oviposited and egg-free plants in three experiments (experiments I–III) and *M. sexta* larval performance parameters in four experiments (experiments I–IV). Phytohormone induction kinetics were assessed in an experiment (experiment V) with *M. sexta*-oviposited and egg-free plants that were elicited with a standardized treatment to mimic herbivory. In two experiments (experiments III and VI), we examined the antimicrobial activity in the haemolymph of *M. sexta* larvae feeding on *M. sexta*-oviposited and egg-free plants. We then tested the effect of prior *M. sexta* oviposition on the performance of *S. exigua* (experiment VII). In another cross-resistance experiment with a full-factorial design (experiment VIII), we compared plants previously oviposited by either *M. sexta* or *S. exigua* moths and egg-free plants for the effects on mortality and mass of larvae of both species feeding on these plants. Finally, we tested the effect of prior *M. sexta* oviposition on the performance of *S. exigua* (experiment IX) and antimicrobial activity in the haemolymph of *M. sexta* larvae (experiment X) on *ir-Myb8* plants in comparison with that of Wt plants.

Plant exposure to oviposition

Prior to the experiments, we matched *N. attenuata* plants within the replicates for their ontogeny (by size and elongation state). To account for the known dependence of allocation of defensive compounds on leaf ontogeny, we always exposed the second youngest source leaf to *M. sexta* oviposition (van Dam *et al.* 2001). We positioned the plants around gauze cages (76 × 42 × 42 cm) and inserted this leaf through slots in the cage (Fig. 1i). Plants of the oviposition treatment were exposed in cages containing 10 3-day-old mated *M. sexta* females and males. Leaves of plants of the egg-free treatment were exposed in cages containing only male moths. This nocturnal moth lays single or two eggs on a plant under natural conditions, but many eggs in captivity. To avoid overloads of eggs on the leaves, we observed the moths from dusk onwards and removed a plant from the cage directly after an oviposition event. By this approach, we obtained plants with 2.3 ± 0.2 eggs (mean ± SEM). After the natural egg incubation time of 4 or 5 d, we removed eggs gently without damaging the leaf surface with featherweight forceps.

One cross-resistance experiment (experiment VII) involved oviposition by *S. exigua*. These moths lay egg clutches with about 5–95 eggs on *N. attenuata,* but not on leaves exposed through the slots of a cage as described previously. Therefore, we exposed whole rosette plants overnight in the gauze cages and obtained *S. exigua*-oviposited plants with 37.7 ± 5.6 (mean ± SEM) eggs per plant on one to four older leaves. After the natural egg incubation time of *S. exigua* (after 3 d), we removed the eggs gently with a soft brush.

Plant exposure to herbivory

We applied neonate larvae onto the plants within at maximum 12 h after egg removal. We placed either three (experiments I–II, VI and X) or two (experiments III–IV) *M. sexta* neonates on the same leaf that was exposed in the gauze cages (now third source leaf). In the cross-resistance experiments VII–IX, we applied 20 *S. exigua* or three *M. sexta* larvae (feeding comparable amounts) to a leaf that was systemic to the oviposition. We applied again neonates except in experiment IX, at which we used 1–2-day-old larvae because no neonates were available. The larvae started feeding one leaf position above the *M. sexta*-oviposited leaf (second source leaf), which was generally more distant to the old *S. exigua*-oviposited leaves.

Larvae were kept on the leaf using vented clip cages (ø 6.5 × 2.5 cm), and control plants received empty clip cages at the same leaf positions. To warrant sufficient leaf material for larval feeding, we positioned the cages one leaf position higher usually every second day.

Sampling for analysis of leaf defence parameters

In full factorial experiments, we sampled leaf material for analysis of plant defence parameters from plants oviposited by *M. sexta* and from egg-free plants with (experiments I/II/III: n_{Plant} = 10/6/10) and without (n_{Plant} = 10/6/5) larval feeding. The first two experiments involved an additional set of plants of which the egg-laden leaf or the corresponding leaf of egg-free plants was harvested after egg removal (n_{Plant} = 10/6 plants in experiments I/II). Of all other plants, we repeatedly sampled a part of the leaf that the larvae had fed upon for the first 2 d or the corresponding leaf of control plants. At every harvesting time point (2, 4 and 6 d after the larvae started feeding), about 100 mg (without midrib) were cut with a sharp scalpel from the tip onward. This leaf cutting was not sufficient to induce substantial increases of any of the defence parameter analysed in the control plants without larvae (Fig. 1).

In all these experiments, we analysed levels of nicotine, CP, chlorogenic acid (CA), rutin and DTGs by high-performance liquid chromatography and photodiode array detection (HPLC-DAD; Shimadzu SPD-M20A, Tokyo, Japan) as previously described (Keinänen *et al.* 2001) (n_{Plant} = 21–26). In the third experiment (experiment III), TPI activity was determined by a radial diffusion assay as described previously (van Dam *et al.* 2001). For more details on these assays, see Supporting Information (Appendix S1).

Manduca sexta performance and feeding damage

In four experiments (experiments I/II/III/IV: n_{Larvae} = 30/18/16–20/46, n_{Plant} = 10/6/10/23), we determined the accumulated feeding damage either 6 or 14 d after *M. sexta* larvae started feeding. We scanned the leaves, marked the fed areas in Photoshop© CS5 (Adobe Systems Incorporated, San Jose, CA, USA) and quantified the pixels of the fed areas. In three of these experiments (experiments I/II/IV), we determined larval mortality and mass every 2–3 d. In two experiments (experiments III/IV), we followed larval development daily by recording their instar and sub-instar (based on their head capsule to body diameter ratio, Supporting Information Fig. S1).

Treatment and sampling for phytohormone analysis

To measure the kinetics of the JA burst in *M. sexta*-oviposited and egg-free plants (experiment V), we used a standardized elicitation treatment by wounding and application of *M. sexta* OS, a well-established procedure to mimic the *M. sexta* herbivory (Diezel *et al.* 2009). For details on OS collection, plant treatments and analysis of JA and its conjugate to isoleucine (JA-Ile) by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC/ESS-MS/MS) according to Gaquerel *et al* (2012), see Supporting Information (Appendix S1).

Haemolymph collection and measurement of antimicrobial activity

Manduca sexta larvae that had been feeding for 6 d on *M. sexta*-oviposited/egg-free plants (experiment III: n_{Larvae} = 17/12; experiment VI: n_{Larvae} = 15/18; experiment X: n_{Larvae} = 20/23 on Wt plants and n_{Larvae} = 24/19 on *ir-Myb8* plants) were briefly chilled on ice and dabbed with a tissue soaked in 70% ethanol. Then we cut off the abdominal horn with sterilized scissors and collected the leaking haemolymph with a 10 μL pipette. The haemolymph samples were immediately flash-frozen in liquid

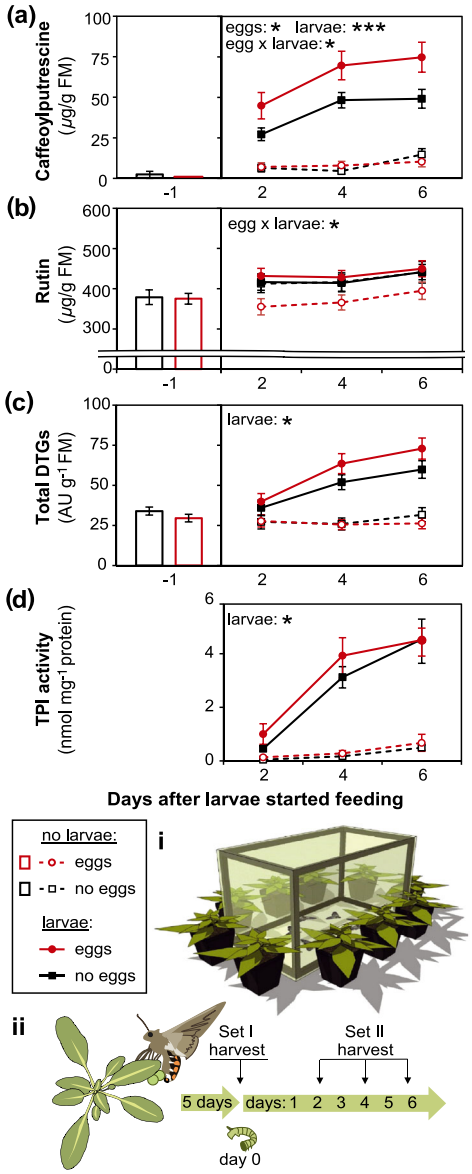

Figure 1. *Manduca sexta* oviposition affects feeding-induced defence parameters in *Nicotiana attenuata*. Mean ± SEM of levels of (a) caffeoylputrescine, (b) diterpene glycosides (DTGs), (c) rutin and of (d) trypsin protease inhibitor (TPI) activity in leaves of *N. attenuata* plants with or without prior oviposition by *M. sexta.* The eggs were deposited on standardized leaf positions using (i) flight cages with slots and were removed after 4 d. (ii) At this time (day-1), the formerly egg-laden leaf or a corresponding leaf of egg-free plants was analysed for defence parameters in a subset of plants (set I: $n = 16$) that was not further used. To the same leaves of plants of set II, either neonate larvae or empty clip cages were applied. The leaves were repeatedly sampled (2, 4, and 6 d after larvae started feeding) from the plants without larvae [dashed lines, $n = 21$ except for (d) $n = 5$] and from the plants exposed to larval herbivory [solid lines, $n = 24$–26, except for (d) $n = 8$–10]. The data in (a–c) are pooled from three experimental repetitions with 10, six and 10 plant replicates for all treatment groups (except for the last experiment in which the treatments without larvae were replicated with five plants). Although the experimental repetition did affect the absolute levels of metabolites, it did not affect the effects of the other factors (Table 1). Asterisks (*/***) indicate significant differences at $P < 0.05/0.001$ according to linear mixed models (Table 1, paired *t*-test revealed no differences at day 1).

nitrogen. We analysed the antimicrobial activity with a radial diffusion assay as previously described (du Toit & Rautenbach, 2000) and detailed in the Supporting Information (Appendix S1).

Cross-resistance experiments

Firstly, we recorded every other day larval mortality and mass of *S. exigua* feeding on *M. sexta*-oviposited and egg-free plants (experiment VII: $n_{Plant} = 8$). Additionally, we harvested a sample of the leaf that larvae had been feeding on for the first 3 d to determine TPI activity in a microwell plate assay as described earlier (Bode *et al.* 2013) and in the Supporting Information (Appendix S1).

Then, we examined in a full-factorial experiment, how oviposition by either species affects performance of larvae of the other species (experiment VIII). For 6 d, egg-free plants and plants oviposited by *M. sexta* or *S. exigua* were fed by *S. exigua* ($n_{Plant} = 11$) or *M. sexta* ($n_{Plant} = 12$) larvae. We recorded larval mortality daily and larval mass of 4-day-old *S. exigua* and 6-day-old *M. sexta* larvae.

To test whether the cross-resistance to *S. exigua* larvae of *M. sexta*-oviposited plants involves *NaMyb8*-mediated traits, we recorded every other day larval mortality and mass of *S. exigua* feeding on *M. sexta*-oviposited and egg-free Wt and *ir-Myb8* plants (experiment IX: $n_{Plant} = 11$).

Data analyses

Data were analysed using MS Excel (Microsoft, USA) and SPSS statistics 18-22 (IBM, Armonk, NY, USA) except for the Fisher's exact test (http://www.langsrud.com/stat/Fishertest.htm) on mortality data. We generally screened data graphically for normal distribution (Q-Q-plots) and by *F*-tests for variance homogeneity and used two-sided tests. Effects of oviposited plants on the antimicrobial activity of larval haemolymph were evaluated on log-transformed data to meet assumptions. We preferably used paired *t*-test for dual comparisons as we performed all experiments with matched pairs. However, we used unpaired *t*-test (Student's *t*-test for homoscedastic or Welch's *t*-test for heteroscedastic data) in cases of missing data, for example, due to larval mortality. We evaluated the effects of *M. sexta* oviposition and larval feeding on production of secondary plant metabolites and proteases inhibitors using linear mixed models (LMMs) with repeated measures over the days and plants as subjects with first-order autoregressive process [AR(1)] as covariance type. We started with the full-factorial model (factors: oviposition, larval feeding and experiment) including all interactions, reduced the model for non-significant interactions ($P \geq 0.2$) and evaluated the models for their goodness of fit based on the Akaike information criterion (AIC).

RESULTS

Oviposition by *Manduca sexta* primes a feeding-induced plant defence trait

Oviposition *per se* had no effect on the levels of CP, rutin, DTG, (Fig. 1a–c), nicotine and CA (Supporting Information

Table S1) 4 d after oviposition (i.e. just prior to larval hatching: day-1). Two, four and six days after empty clip cages had been applied to control plants, levels of most of the analysed secondary metabolites and TPI activity were still unaffected by prior oviposition (Fig. 1: dashed lines; Supporting Information Table S1). Because the levels of all defence metabolites in plants without larval feeding damage did not increase over time, the repeated sampling of the leaves did not relevantly induce biosynthesis of these metabolites. These results suggest that oviposition *per se* did not induce the analysed defence parameters.

However, prior oviposition significantly affected levels of CP and interacted with the effect of larval feeding on levels of CP and rutin (Table 1). While there was no consistent effect of oviposition on CP levels in undamaged control plants, the feeding-induced levels of CP were about 50% higher in previously oviposited plants than in egg-free plants at all time points analysed (Fig. 1a). Hence, *M. sexta* oviposition clearly primed the feeding-induced production of CP. In contrast, rutin levels were not feeding-induced and did not differ between oviposited and egg-free plants when fed by larvae. However, undamaged plants had always lower rutin levels when previously oviposited than when egg-free (Fig. 1b). DTG levels and TPI activity increased because of larval feeding activity, but they were not significantly affected by prior

Table 1. Model summaries on defence parameters of *Nicotiana attenuata*

Metabolite	Factor	d.f.	Denominator	*F* value	*P*-value
Caffeoylputrescine $\mu g\,g^{-1}$ FM					
	Intercept	1	90.96	138.602	<0.000
	Oviposition	1		4.393	**0.039**
	Larvae	1		69.969	**0.000**
	Experiment	2		4.864	**0.010**
	Oviposition * larvae	1		5.252	**0.024**
Rutin $\mu g\,g^{-1}$ FM					
	Intercept	1	91.08	3372.537	<0.000
	Oviposition	1		2.722	0.102
	Larvae	1		1.391	0.241
	Experiment	2		39.867	**0.000**
	Oviposition * larvae	1		3.964	**0.049**
Total DTGs AU per FM	Intercept	1	93.06	356.026	<0.000
	Oviposition	1		0.964	0.329
	Larvae	1		47.233	**0.000**
	Experiment	2		15.202	**0.000**
	Oviposition * larvae	1		2.307	0.132
TPI $nmol\,mg^{-1}$ FM	Intercept	1	30.87	51.80	<0.000
	Oviposition	1		0.44	0.513
	Larvae	1		33.83	**0.000**
	Oviposition * larvae	1		0.144	0.707
Nicotine $\mu g\,g^{-1}$ FM	Intercept	1	101.17	2821.960	<0.000
	Oviposition	1		0.006	0.940
	Larvae	1		0.275	0.601
	Experiment	2		11.235	**0.000**
	Oviposition * larvae	1		0.166	0.685
Chlorogenic acid $\mu g\,g^{-1}$ FM	Intercept	1	91.61	1511.947	<0.000
	Oviposition	1		1.687	0.197
	Larvae	1		3.548	0.063
	Experiment	2		36.610	**0.000**
	Oviposition * larvae	1		1.036	0.311
	Larvae * experiment	2		4.564	**0.013**

Linear mixed models (LMMs) were used to evaluate the effects of oviposition (present/absent) and feeding by the larvae (present/absent) by *Manduca sexta* on leaf metabolites surveyed as repeated measurement (2, 4 and 6 d after larvae started feeding) in all plant subjects [first order autoregressive process (AR1) was used as covariance type]. Additionally, the experimental repetition (experiment I/II/III) was included as fixed factor except for TPI activity that was only assessed in one experiment (experiment III). Full-factorial models were reduced for the insignificant interactions, and significant effects are indicated in bold.

FM, fresh mass; DTGs, diterpene glycosides; TPI, trypsin protease inhibitor activity; AU, area units

oviposition, although oviposited plants tended to produce more DTGs in response to feeding damage than egg-free plants (Fig. 1c and d; Table 1). Levels of nicotine and CA were neither affected by oviposition *per se* nor did oviposition prime the levels of these defence metabolites in feeding-damaged plants (Table 1; Supporting Information Table S1). The levels of defence metabolites differed between experiments, which did not influence the effect of prior oviposition or larval feeding except for CA, which was induced by larval feeding in only one experiment.

The *M. sexta* oviposition-mediated effect on CP levels induced by *M. sexta* larval feeding matches the effects by *S. exigua* oviposition on CP levels induced by *S. exigua* feeding, whereas the lack of an effect of *M. sexta* oviposition on the feeding-induced TPI activity contrasts the effects of *S. exigua* oviposition (Bandoly *et al.* 2015).

Because the analysed defence traits are inducible via JA signalling, we examined the induction kinetics of JA and JA-Ile in response to a standardized stimulus mimicking *M. sexta* feeding (wounding and application of *M. sexta* OS). This treatment induced levels of JA and JA-Ile similarly in oviposited and egg-free plants (Supporting Information Fig. S2). Therefore, the priming of the JA-mediated feeding-induced CP levels by prior oviposition is not facilitated by elevated JA or JA-Ile production.

Oviposition by *Manduca sexta* does not affect the performance, but the immune state of its larvae

None of the performance parameters of *M. sexta* larvae (mortality, mass and developmental time) nor the feeding damage they inferred were different between *N. attenuata* plants with and without prior *M. sexta* oviposition (Table 2; Supporting Information Table S2).

Interestingly, 6-day-old *M. sexta* larvae feeding on oviposited plants exhibited a lower antimicrobial activity than larvae feeding on egg-free plants (Fig. 2a). Thus, the oviposition-primed changes did not directly alter larval performance as known for *S. exigua* larvae on *N. attenuata* plants that were primed by *S. exigua* oviposition but clearly impacted negatively on an insect-immune parameter of the specialist *M. sexta* larvae.

Oviposition by *Manduca sexta* and *Spodoptera exigua* increases plant resistance to *Spodoptera exigua* larvae

In cross-resistance experiments with *M. sexta* and *S. exigua*, we aimed to disentangle whether (i) a species-specific oviposition-mediated effect on the plant or (ii) species-specific larval susceptibility to the oviposition-mediated changes can explain

Table 2. *Manduca sexta* oviposition on *Nicotiana attenuata* does not alter performance of conspecific larvae

Experiment	Parameter	Day	Eggs	No eggs	*P*-value
Experiment I					
	Mortality (%)	3	3 ± 3	3 ± 3	1.000
		6	20 ± 7	23 ± 7	1.000
		9	30 ± 8	40 ± 8	0.589
	Larval mass (mg)	3	7 ± 0	8 ± 0	0.366
		6	27 ± 3	26 ± 4	0.744
		9	103 ± 21	96 ± 19	0.746
	Feeding damage (cm^2)	6	36 ± 7	33 ± 6	0.691
Experiment II	Mortality (%)	3	0 ± 0	0 ± 0	1.000
		6	17 ± 11	6 ± 6	0.603
		9	28 ± 13	11 ± 7	0.402
	Larval mass (mg)	3	9 ± 0	8 ± 1	0.597
		6	33 ± 3	35 ± 6	0.729
		9	61 ± 11	57 ± 12	0.769
	Feeding damage (cm^2)	6	22 ± 4	24 ± 5	0.540
Experiment III	Feeding damage (cm^2)	6	11 ± 2	11 ± 2	0.826
Experiment IV	Mortality (%)	3	0 ± 0	4 ± 3	0.361
		5	2 ± 2	9 ± 4	0.145
		8	9 ± 4	22 ± 7	0.134
		10	9 ± 4	26 ± 7	0.495
		12	30 ± 8	48 ± 9	0.052
	Larval mass (mg)	3	8 ± 0	9 ± 0	0.305
		5	14 ± 1	14 ± 1	0.678
		8	46 ± 4	46 ± 4	0.825
		10	120 ± 15	97 ± 14	0.067
		12	278 ± 8	257 ± 12	0.285
	Feeding damage (cm^2)	14	60 ± 5	52 ± 5	0.223

Performance parameters of *Manduca sexta* larvae and the leaf damage they inflicted on oviposition-experienced (eggs) and unexperienced (no eggs) plants. Five days after oviposition, 2–3 neonate larvae started feeding on the previously oviposited or the corresponding leaf of egg-free plants. *P*-values refer to Fisher's exact test (mortality) or paired (larval mass) and unpaired *t*-test (feeding damage).

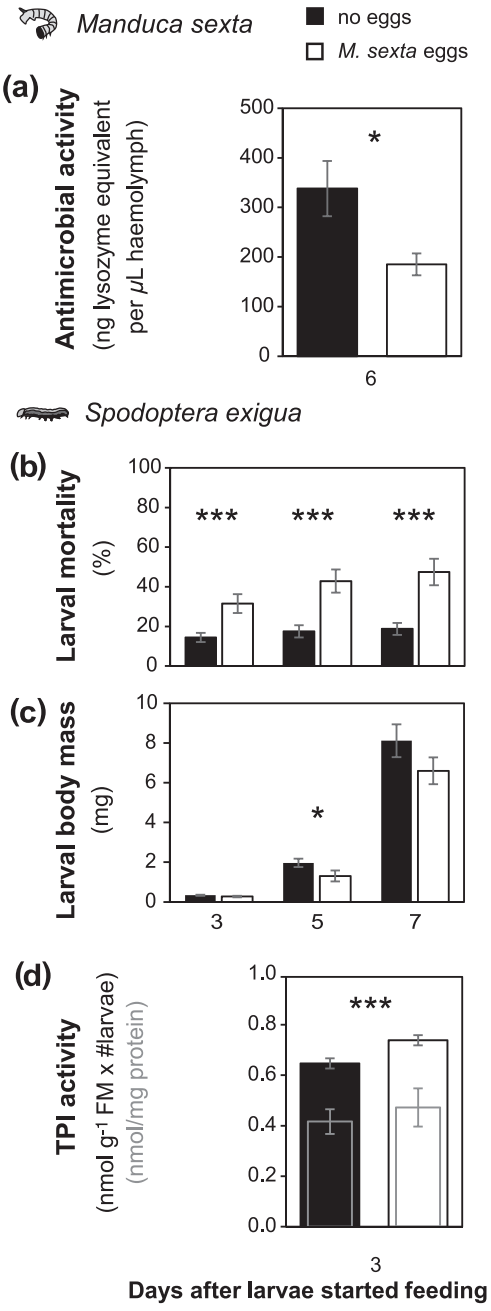

Figure 2. *Manduca sexta* oviposition on *Nicotiana attenuata* diminishes immunity of *M. sexta* and performance of *Spodoptera exigua* larvae. (a) Mean ± SEM of antimicrobial activity in the haemolymph of *M. sexta* feeding for 6 d on oviposited (*M. sexta* eggs) and egg-free (no eggs) *N. attenuata* plants (n_{Larvae} = 30 and 32; pooled data from two experiments in both of which the same trend was observed). Mean ± SEM (n_{Plant} = 8) of (b) *S. exigua* larval mortality after 3, 5 and 7 d of feeding on plants that were either oviposited or not on a leaf systemic to the larval feeding. (c) Trypsin protease inhibitor (TPI) activity in the leaves 3 d after larvae started feeding. The empty bars with grey margins depict TPI activity per mg total protein in leaves. Because mortality within the 3 d was twice as high on oviposited plants as on egg-free plants, TPI activity is expressed in nmol per leaf fresh mass (FM) normalized to the number of larvae feeding per plant (average of the 20 applied larvae and the number of larvae alive on day 3). Asterisks (*/***) indicate significant differences at $P < 0.05$/0.001 between oviposited and egg-free plants according to either (a) unpaired *t*-test, (b) Fisher's exact test or (c) paired *t*-tests.

the differential effect of oviposition by these moths on performance of their larvae.

When we exposed *M. sexta*-oviposited and egg-free *N. attenuata* plants to *S. exigua* neonates, we found a strongly increased larval mortality and reduced larval mass on oviposited plants (Fig. 2b and c). Mass of 5-day-old *S. exigua* larvae was 50% lower on *M. sexta*-oviposited than on egg-free plants. This result suggests the hypothesis that the discrepancy in the effect of prior oviposition on larval performance of both species may result from differences in the susceptibility of both insects.

However, the oviposition-primed plant response to feeding damage by both insect species did not match completely but differed with respect to the TPI activity. Egg-free *N. attenuata* responds also differentially to the larval feeding of both species (Voelckel & Baldwin, 2004; Diezel *et al.* 2009). Therefore, we asked whether *M. sexta* oviposition primes TPI activity induced by *S. exigua* feeding. We assessed TPI activity in the leaves that *S. exigua* was feeding on for the first 3 d. We normalized the TPI activity to the number of larvae per plant because *M. sexta*-oviposited plants received less feeding damage due to a twice as high larval mortality compared with egg-free plants (Fig. 2b) and because TPI activity positively correlates with feeding damage (Bandoly *et al.* 2015). This normalization revealed that *M. sexta*-oviposited plants showed a higher TPI activity per feeding *S. exigua* larvae than egg-free plants (Fig. 2d). This effect was not detected when ignoring the difference in the number of feeding larvae. Furthermore, the *S. exigua* oviposition-primed induction of TPI activity was only detected, when the extent of inflicted damage was kept equal.

We then examined the effect of oviposition by both moth species on the larvae of both species in a full-factorial experiment. Neither *M. sexta* nor *S. exigua* oviposition affected mortality and mass gain of *M. sexta* larvae (Fig. 3a and b). In contrast, 5-day-old *S. exigua* larvae had a 24 and 48% higher mortality when feeding on plants that experienced oviposition by *S. exigua* or *M. sexta* than on egg-free plants. In addition, *S. exigua* larval mass was 35% lower on plants oviposited by *M. sexta* than on egg-free plants, and by trend, 19% lower on prior *S. exigua*-oviposited plants (Fig. 3c and d). Thus, *M. sexta*-oviposited plants displayed even a more pronounced negative effect on *S. exigua* larval performance than plants oviposited by conspecific *S. exigua* moths. These data support the hypothesis suggested previously that generalist and specialist larvae show a differential susceptibility to the oviposition-primed plant response.

Effects of *Manduca sexta* oviposition on *Manduca sexta* and *Spodoptera exigua* larvae require *NaMyb8* expression

We tested whether priming of plant resistance against *S. exigua* larvae by *M. sexta* oviposition is – like that by *S. exigua* oviposition – functionally linked to the expression of the transcription factor *NaMyb8* that governs biosynthesis of PPCs including CP. We exposed *M. sexta*-oviposited and egg-free Wt and

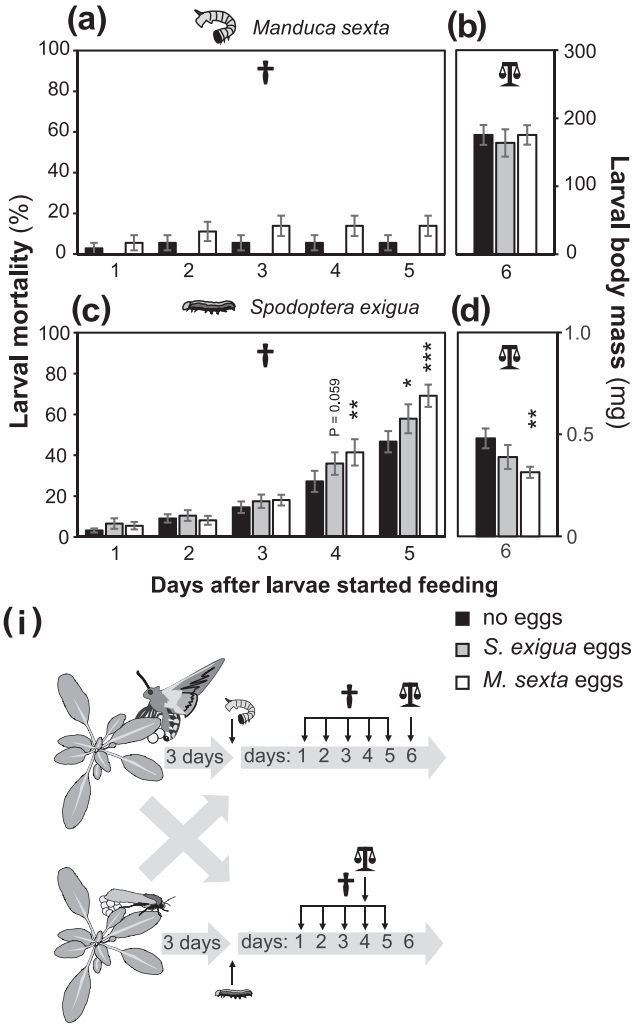

Figure 3. *Spodoptera exigua* and *Manduca sexta* oviposition on *Nicotiana attenuata* similarly affect plant resistance to larvae. Mean ± SEM of (a) mortality and (b) body mass (averaged per plant) of *M. sexta* ($n_{Plant} = 12$) and (c and d) *S. exigua* larvae ($n_{Plant} = 10$) feeding on *N. attenuata* plants that were oviposited by *S. exigua* (*M. sexta* mortality was 0) or *M. sexta* moths or received no eggs. The (i) experimental schedule involved egg deposition by *M. sexta* or *S. exigua* on plants; in the end of the egg incubation time (i.e. after 3 or 5 d), the eggs were removed, and larvae were applied. In a full-factorial design, either three neonates *M. sexta* or 20 neonates *S. exigua* were applied on a standardized leaf position, which was systemic to oviposited leaves. Asterisks (*/**/***) indicate significant differences between larval mortality (Fisher's exact test) and average mass per plant (paired *t*-tests) on oviposition-experienced and egg-free plants at $P < 0.05/0.01/0.001$.

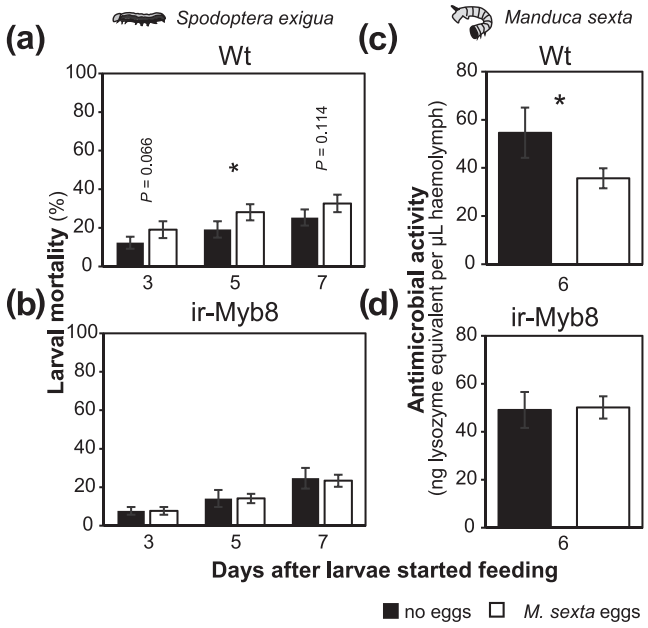

Figure 4. Silencing of *NaMyb8* abolishes the increased resistance of *Manduca sexta*-oviposited plants. Mean ± SEM ($n_{Plant} = 11$) of *Spodoptera exigua* larval mortality after 3, 5 and 7 d of feeding on either (a) wild-type (Wt) *Nicotiana attenuata* or (b) plants stably transformed to silence *NaMyb8* gene expression (*ir-Myb8*). The plants were oviposited (*M. sexta* eggs) or not (no eggs) on a leaf systemic to the larval feeding. (c and d) Antimicrobial activity in the haemolymph of *M. sexta* ($n_{Larvae} = 19–24$) feeding for 6 d on plants of the same treatments and genotypes in another experiment. Asterisks (*) indicate significant differences between larvae on oviposited and egg-free plants at $P < 0.05$ for either *S. exigua* mortality (Fisher's exact test) or antimicrobial activity in *M. sexta* haemolymph (unpaired *t*-tests).

NaMyb8-silenced (*ir-Myb8*) plants to *S. exigua* larvae. The larvae performed significantly better on *ir-Myb8* than on Wt plants, which was highly significant for oviposited plants and indicated by trend for egg-free plants (Fig. 4a and b; Supporting Information Table S3). Because only few neonates were available, up to 2-day-old first instar larvae were used, which probably resulted in the generally better performance of the larvae in that experiment. Despite less pronounced oviposition-mediated effects than in the experiments with neonate larvae (compare with Figs 2b and 3c), mortality of *S. exigua* larvae again increased when they fed on *M. sexta*-oviposited Wt plants (Fig. 4a). Yet mortality of *S. exigua* larvae did not increase on oviposited *ir-Myb8* plants (Fig. 4b). Thus, *Myb8*-mediated traits such as CP likely conferred the *M. sexta* oviposition-mediated increased resistance of plants against *S. exigua* larvae.

We also tested whether the effect of *M. sexta* oviposition on the antimicrobial activity in the larval haemolymph of *M. sexta* varies between *ir-Myb8* plants and Wt plants. Again, larvae feeding on *M. sexta*-oviposited Wt plants exhibited a lower antimicrobial activity than larvae feeding on egg-free Wt plants (Fig. 4c), but the antimicrobial activity of larvae on *ir-Myb8* plants remained equally high on oviposited and egg-free plants (Fig. 4d). Thus, oviposition-primed induction of CP or other PPCs regulated by *NaMyb8* is also involved in this oviposition-mediated effect.

DISCUSSION

Our study shows that oviposition by two phylogenetically divergent lepidopteran species that differ with respect to their host-plant specialization and egg-laying behaviour affects the production of *N. attenuata*'s defence metabolites in a very similar way. Both oviposition by the generalist *S. exigua* and the specialist *M. sexta* primed the feeding-induced production of

the defensive phenolic CP without affecting the feeding-induced levels of JA and JA-Ile. However, the larvae of both insect species showed different susceptibility towards the oviposition-mediated priming effects. While *S. exigua* larvae feeding on plants oviposited by *S. exigua* or *M. sexta* suffered increased mortality and gained less weight, *M. sexta* larvae were not affected in these parameters. Yet *M. sexta* larvae feeding on plants that had been oviposited by conspecifics exhibited a reduced antimicrobial activity in the haemolymph. This oviposition-mediated effect on *M. sexta* larval immunity required – similar to the plant resistance against *S. exigua* larvae – expression of the *NaMyb8* transcription factor governing biosynthesis of PPCs (e.g. CP). Studies on the interaction of plant secondary metabolites with insect-immune defences against pathogens and endoparasites are rare, but our finding suggests that plant defence traits that are primed by insect oviposition may impair the immune state of the feeding larvae.

Oviposition-mediated plant responses

Manduca sexta oviposition *per se* did not induce *N. attenuata*'s defence traits in the local leaf, neither one day before larval hatching nor after the larvae would have hatched (Fig. 1). However, the feeding-induced production of CP increased in leaves previously oviposited by *M. sexta*. This matches the findings on *N. attenuata*'s interaction with *S. exigua* (Bandoly *et al.* 2015). This priming of CP induction is likely also connected to the reduced levels of rutin in previously oviposited undamaged plants. The biosynthesis of the flavonoid rutin roots in the same metabolic pathway as the *NaMyb8*-regulated biosynthesis of PPCs (Onkokesung *et al.* 2012). Thus, altered rutin levels may reflect shifts in the metabolic flux of that pathway. However, priming by insect oviposition on *N. attenuata* consistently involved PPCs. Interestingly also, a recent study on the effects of oviposition by the elm leaf beetle on elm resistance to the beetle larvae revealed that a phenolic compound (robinin) plays a role in the oviposition-primed resistance against the larvae (Austel *et al.* 2015). Thus, it will be promising to investigate further whether priming of the feeding-induced biosynthesis of phenolic compounds may be a general plant response to insect oviposition.

Opposite to oviposition by *S. exigua*, *M. sexta* oviposition did not prime TPI activity induced by conspecific feeding larvae. Yet TPI activity in *M. sexta*-oviposited plants that were fed by *S. exigua* was more strongly induced than in egg-free plants when we normalized for the lower number of larvae feeding on these plants (Fig. 2). This suggests that TPI activity can also be primed by *M. sexta* oviposition. *N. attenuata* differentially responds to the feeding larvae of both species that exhibit different compositions of elicitors in their OS (Diezel *et al.* 2009; Voelckel & Baldwin, 2004), which may explain why oviposition primes TPI levels induced by *S. exigua* feeding, but not by *M. sexta* feeding. Alternatively, the induction of TPI activity is only primed in leaves that are systemic to the oviposited leaf, as *S. exigua* exclusively fed on systemic leaves, whereas *M. sexta* larvae started to feed on the egg-laden leaf of which the defence parameter was assessed. Furthermore, in tomato, oviposition by a generalist lepidopteran herbivore primes the wound-induced expression of a *PI* gene, which is accompanied by a primed JA burst (Kim *et al.* 2012). However, neither oviposition by *M. sexta* (Supporting Information Fig. S2) nor that by *S. exigua* (Bandoly *et al.* 2015) alters *N. attenuata*'s herbivory-induced levels of JA or JA-Ile.

Oviposition-mediated plant effects on larval performance

While the plant response to oviposition by *S. exigua* and *M. sexta* overlapped, the performance of their larvae on oviposited plants was differentially affected. Opposite to *S. exigua*, *M. sexta* larvae performed similarly on oviposited and egg-free plants in four experimental repetitions (Table 2), although its susceptibility to *N. attenuata*'s CP production has been previously shown (Kaur *et al.* 2010). However, in this previous study, the neonate larvae fed on systemically pre-induced leaves of Wt and *NaMyb8*-silenced plants. Opposite to this difference between a nearly complete CP deficiency and a strongly induced CP level, the 50% higher CP levels in oviposition-primed than in egg-free plants that are naturally induced by the feeding larvae was clearly not sufficient to significantly affect the classical larval performance parameters.

Specialist herbivores are often well adapted to secondary metabolites of their host plants, and as a consequence, the efficiency of defences may strongly vary between generalist and specialist species. Furthermore, *N. attenuata*'s nicotine production and JA-mediated defences of tomato impact on *S. exigua* much stronger than on *M. sexta* larvae (Steppuhn *et al.* 2004; Steppuhn & Baldwin, 2007; Bosch *et al.* 2014). The hypothesis that *M. sexta* larvae are less susceptible to the oviposition-primed defence induction than *S. exigua* larvae is corroborated by the fact that oviposition by neither of the two moth species altered the plant's resistance to *M. sexta* larvae, whereas oviposition by both moth species increased plant resistance against *S. exigua* larvae (Fig. 3). These findings in *N. attenuata* contrast the situation in *Brassica nigra*, in which oviposition by a specialist lepidopteran species increases plant resistance to the larvae of this specialist, but also to a generalist lepidopteran species, whereas oviposition by the generalist moth does not affect plant resistance to the larvae of both species (Pashalidou *et al.* 2013). On the other hand, our study shows parallels to a study in *Arabidopsis thaliana*, in which a common plant response to the oviposition by generalist and specialist moths differentially affects their larvae (Bruessow *et al.* 2010). However, in this case, the oviposition mediates a suppression of herbivore-induced defence likely because of the activation of pathogen-related signalling, which enhances the performance of the generalist's but not of the specialist's larvae.

The performance of *S. exigua* larvae was even worse when plants had been oviposited by *M. sexta* than by conspecific moths, which may have resulted on the one hand from a different localization of their egg depositions. The leaf that *M. sexta* oviposited on was always one leaf position below the leaf on which the larvae started feeding and which may have received

a stronger systemic signal than a similar leaf of plants oviposited by *S. exigua* on distant old leaves. On the other hand, the plant's response to the oviposition by the two species may differ ahead of the commonalities that we determined. The defence response primed by the oviposition by specialist *M. sexta* may have been even stronger than that primed by the generalist's eggs although only the latter is susceptible to this defence response. For example, DTGs may have played an additional role in plants oviposited by *M. sexta*. Priming of DTG production in the feeding-induced leaves was only indicated by trend, but we may have examined an unfavourable leaf to detect this effect because DTG biosynthesis is more strongly induced in systemic than in locally damaged leaves (Gulati *et al.* 2013).

Various studies show that plants can specifically respond to oviposition by different insect species with defensive responses targeting the eggs (Hilker & Meiners, 2011; Hilker & Fatouros, 2015). The notion that plants show overlapping and distinct responses to oviposition by different herbivores suggests that plants likely respond to general as well as specific cues associated with insect egg deposition. These plant responses may be adjusted for the particular herbivore species just as it is known for plant responses to insect feeding.

Effects of the primed plant defences on plant resistance

We tested whether the increased resistance against *S. exigua* larvae on *M. sexta*-oviposited plants is mediated by the primed feeding-induced PPC production. The detrimental effect of *M. sexta* oviposition on *S. exigua* survival was not detected on PPC-deficient *ir-Myb8* plants while still present on Wt *N. attenuata* (Fig. 4). The defensive effect of *NaMyb8*-dependent traits, such as CP, was evident by the better performance of *S. exigua* on *ir-Myb8* than on Wt plants, which was much stronger (and only significant) in oviposited plants. Thus, we conclude that priming of the production of PPCs by *M. sexta* oviposition is conferring the increased resistance of Wt plants against *S. exigua* larvae. Hence, *M. sexta* oviposition mediates a similar effect on the plant's anti-herbivore resistance as *S. exigua* oviposition on *N. attenuata* (Bandoly *et al.* 2015).

Oviposition-primed plant defence impairs the immune state of *Manduca sexta* larvae

While *M. sexta* larvae did not perform differently on oviposited and egg-free plants, they exhibited a lower antimicrobial activity in their haemolymph on oviposited Wt plants, but not when the plants were silenced for *NaMyb8* expression (Figs 2 and 4). Thus, the oviposition priming of plant defence, that is, the increase in levels of PPCs, may interact with *M. sexta* immunity.

Although scarcely investigated, effects of plant secondary metabolites and of herbivory-induced plants on insect immune responses have been described earlier. For example, lepidopteran larvae feeding on a catapol-rich diet (Laurentz *et al.* 2012) or on previously herbivory-induced host plants (Kapari *et al.* 2006) exhibit an increased encapsulation response to invading bodies. Gypsy moth larvae feeding on damaged-induced silver birches with distinct phenolic profiles show an enhanced antimicrobial activity in their haemolymph (Martemyanov *et al.* 2012a; Martemyanov *et al.* 2012b). Plant secondary metabolites can interact with insect-pathogen interactions at several levels, which may result in either increased or impaired resistance to pathogens (Cory & Hoover, 2006). Ingestion of milkweed cardenolides has been shown to reduce parasite growth and increase the lifespan of monarch butterflies infected with a neogregarine parasite (Gowler *et al.* 2015). On the other hand, induced plant responses can impair the insect's resistance to enemies, as exemplified in the reduced encapsulation of parasitoid eggs in the larvae of the small cabbage while feeding on plants that were induced by previous herbivory (Bukovinszky *et al.* 2009).

The lower antimicrobial activity in the haemolymph of *M. sexta* larvae on oviposited plants may result from effects of the primed *NaMyb8*-dependent plant defence either (i) on the microbial load of the larvae or (ii) directly on the larval innate immunity.

Biosynthesis of tobacco phenylpropanoids has been associated with antimicrobial activity (Maher *et al.* 1994), which may also apply for the oviposition-primed PPCs in *N. attenuata*. Accordingly, lower levels of insect-derived antimicrobials in the haemolymph of larvae feeding on oviposited plants could reflect a relaxed insect-immune state because of a lower microbial load. In addition, oviposition can also impact on a plant's resistance to pathogens as known for *A. thaliana*, which activates systemic acquired resistance (SAR) upon oviposition by *Pieris brassicae* (Hilfiker *et al.* 2014). Larvae of *P. brassicae* are negatively affected by a phytopathogen infection of the plant, which is attenuated on plants that express the egg-mediated SAR. Such a response may reduce the bacterial load on a plant and eventually of the larvae feeding on this plant. However, *N. attenuata* does not show parallels to the response of *A. thaliana* to larval feeding damage on previously egg-deposited plants. Leaves of *A. thaliana* show, for example, necrotic lesions at the site of egg deposition and suppress JA signalling (Bruessow *et al.* 2010), effects that were not observed in *N. attenuata*. Moreover, dietary CA that is metabolically related to CP does not affect the bacterial load of two pathogens infecting *M. sexta* (Del Campo *et al.* 2013).

Alternatively, the increased levels of oviposition-primed PCCs ingested by larvae feeding on oviposited plants may directly interfere with the insect's immune system and thus could harm *M. sexta* larvae when these are exposed to entomopathogens. This would resemble an indirect plant defence via entomopathogens, which has been often proposed (Elliot *et al.* 2000; Cory & Hoover, 2006). Further studies are needed to test whether and how the priming of *N. attenuata*'s direct defence traits by *M. sexta* oviposition may finally act as an indirect defence by suppressing this specialist's immunity.

CONCLUSIONS

Altogether, our data suggest that *N. attenuata* responds to oviposition by *S. exigua* and *M. sexta* in a very similar way with

respect to a priming effect on the feeding-induced PPC and eventually TPI production, despite some species-specific features. This similarity in the priming of a plant's anti-herbivore defence by oviposition raises the questions how the plant notices the egg depositions of the moths and whether the eggs of these two moth species share the same elicitors or whether the plant responds to different egg-associated elicitors in a similar way. However, the similar response of *N. attenuata* to oviposition by both moth species exerts different effects on their larvae, which clearly show different susceptibility to the oviposition-primed changes. Hence, this study shows both sides of a coin, one on the plant side with respect to a rather unspecific egg priming of feeding-induced plant defence metabolites and one on the insect side with respect to highly species-specific insect susceptibility.

CONFLICT OF INTERESTS STATEMENT

We have no conflict of interests.

AUTHOR CONTRIBUTIONS

A. S., M. B. and M. H. designed the study. M. B., R. G. and A. S. performed the experiments, chemical and data analysis. M. B. and A. S. wrote the first draft of the manuscript, and all authors revised it.

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