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Plant responses to insect eggs are not induced by egg-associated microbes, but by a secretion attached to the eggs

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Abstract

Plants can enhance their defence against herbivorous insects by responding to insect egg depositions preceding larval feeding. The similarity of plant responses to insect eggs with those to phytopathogens gave rise to the hypothesis that egg-associated microbes might act as elicitors. We tested this hypothesis by investigating first if elimination of microbes in the butterfly *Pieris brassicae* changes the responses of *Brassica nigra* and *Arabidopsis thaliana* to eggs and larvae of this insect species. An antibiotic treatment of butterflies mitigated the plant transcriptional response to the eggs and the egg-mediated enhancement of the plant's defence against larvae. However, application of cultivated microbial isolates from the eggs onto *Arabidopsis thaliana* did not enhance the plant's anti-herbivore defence. Instead, application of an egg-associated glandular secretion, which is attaching the eggs to the leaves, elicited the enhancing effect on the plant's defence against larvae. However, this effect was only achieved when the secretion was applied in similar quantities as released by control butterflies, but not when applied in the reduced quantity as released by antibiotic-treated butterflies. We conclude that glandular secretions rather than egg-associated microbes act in a dose-dependent manner as elicitor of the egg-mediated enhancement of the plant's defence against insect larvae.

KEYWORDS

Brassicaceae, herbivory, induction, lepidoptera, microbes, plant defence, priming

1 | INTRODUCTION

Plant infestation by herbivorous insects often starts with the insect's egg deposition onto a plant. Several plant species display early defence mechanisms targeting insect eggs (Hilker & Fatouros, 2015, 2016). Plants can also use the eggs as a cue to "get ready" (Frost, Mescher, Carlson, & De Moraes, 2008) for improved defence against the hatching larvae; their response to insect eggs can result in stronger, earlier or faster feeding-induced resistance to larvae (Hilker et al., 2016). It has been shown for several plant species that these egg-mediated responses to feeding larvae significantly impair larval performance (Altmann et al., 2018; Bandoly, Grichnik, Hilker, & Steppuhn, 2016; Geiselhardt et al., 2013; Geuss, Stelzer, Lortzing, & Steppuhn, 2017; Pashalidou et al., 2015). For instance, defence of brassicaceous plants against *Pieris brassicae* larvae is enhanced by the plant's response to prior egg deposition; larvae feeding on plants, on which eggs were

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previously deposited, gain less weight and inflict less leaf damage than larvae feeding on egg-free plants (Geiselhardt et al., 2013; Lortzing et al., 2018). Although a few elicitors of plant defences targeting the eggs are known (Hilker & Fatouros, 2015; Hilker & Fatouros, 2016; Reymond, 2013), the nature of the egg-associated cue that primes plant anti-herbivore defences is unknown.

Several plant species form necrotic or chlorotic tissue in response to eggs laid by *Pieris* butterflies (Fatouros et al., 2014; Gouhier-Darimont, Schmiesing, Bonnet, Lassueur, & Reymond, 2013; Griese, Dicke, Hilker, & Fatouros, 2017; Reymond, 2013; Shapiro & DeVay, 1987), thus mimicking the so-called hypersensitive response (HR) known to occur in response to infection by (hemi)biotrophic phytopathogens (Coll, Epple, & Dangl, 2011; Mur, Kenton, Lloyd, Ougham, & Prats, 2008). This reaction to the eggs is accompanied by accumulation of reactive oxygen species (ROS), enhanced levels of salicylic acid (SA) and expression of SA-responsive *PATHOGENESIS-RELATED* marker genes, such as *PR1* and *PR5* (Bruessow, Gouhier-Darimont, Buchala, Metraux, & Reymond, 2010; Gouhier-Darimont et al., 2013; Hilfiker et al., 2014; Little, Gouhier-Darimont, Bruessow, & Reymond, 2007; Lortzing et al., 2018).

Based on the similar nature of plant responses to *P. brassicae* eggs and (hemi)biotrophic pathogens, we addressed the question whether microbes are associated to the eggs and might elicit plant defence responses against the eggs and subsequently hatching larvae. In some insect taxa, mutualistic bacteria are transferred by the mother to the offspring via bacterial inocula adjacent to the eggs or directly on their surface (Engel & Moran, 2013; Kellner, 2002). When such egg-associated microbes are in contact with leaf tissue, they could potentially trigger the above-mentioned egg-mediated plant responses.

Furthermore, we addressed the question whether the secretion, by which egg clutches of *P. brassicae* are attached to leaves, could directly elicit the egg-mediated plant defence responses to hatching larvae independent from microbes. The sticky, exocrine secretion is produced by the butterfly female's accessory reproductive gland (ARG). This glandular secretion is in direct contact with the leaf epidermis and has been shown to elicit plant defences against *P. brassicae* eggs, both HR-like leaf necrosis at the site of egg deposition and egg parasitoid-attracting volatiles (Fatouros et al., 2015). Therefore, the ARG-secretion is another candidate to elicit egg-mediated plant defence responses. Its role in egg-mediated priming of plant defences against larvae has not been tested yet.

To analyse the role of *P. brassicae* egg-associated microbes and ARG secretion in elicitation of egg-mediated plant defences, we eliminated bacteria from *P. brassicae* adults by treating them with antibiotics (AB). We used *Arabidopsis thaliana* as a model plant species and *Brassica nigra* as a natural host plant. Our results showed that the AB-treatment mitigated the plant's transcriptional response to the eggs and the egg-mediated enhancing effect on plant defence against the larvae.

Based on these findings, we tested if the difference between plant responses to eggs laid by AB-treated and untreated butterflies is caused (a) by the AB-mediated elimination of egg-associated bacteria or (b) by changes in the ARG secretion due to the AB-treatment of the insects. To address the first question, we analysed *P. brassicae* eggs for the presence of microbes and tested whether plant exposure to cultivated microbial isolates from eggs can elicit plant responses against the feeding larvae. To approach the second question, we studied the effect of the AB-treatment on the released amount of ARG secretion during oviposition. We found that AB-treated butterflies released less secretion than control butterflies. To figure out whether the quantity of the egg secretion applied onto the plant is relevant for the occurrence and intensity of the egg-mediated enhanced defence against the larvae, we applied various amounts of ARG secretion onto the leaves.

Our study shows that the parallels of plant responses to insect eggs and (hemi)biotrophic phytopathogens are not due to egg-associated microbes. Instead, the sticky secretion, which attaches *P. brassicae* eggs to leaves, evokes plant defence responses in a dose-dependent manner.

2 | MATERIALS AND METHODS

2.1 | Plant species

Brassica nigra seeds (accession number: CGN06619; Centre of Genetic Resources, Wageningen, Netherlands) were sown on soil (Einheitserde Typ P; Kausek, Mittenwalde, Germany) mixed 3:1 with vermiculite (Kausek, Mittenwalde, Germany). They germinated and grew under long day conditions (16-hr/8-hr light/dark cycle, 85 μmol m^{-2} s^{-1} light intensity, 22°C, r. h. 50%). One-week-old seedlings were transplanted to individual pots. We used 4-week-old plants for all the experiments.

We used 7-week-old *A. thaliana* Col-0 plants for all the experiments. Plants were grown as described by Firtzlaff, Oberländer, Geiselhardt, Hilker, and Kunze (2016).

2.2 | Plant treatments

All treatments (eggs, glandular secretions or microbes) of both plant species were conducted at the same abiotic conditions in a climate chamber (18-hr/6-hr light/dark cycle, 220 μmol m^{-2} s^{-1} light intensity, 23°C, r. h. 70%).

For plant treatments with eggs, a plant was placed in a cage with a gravid female, which laid an egg clutch (~35 eggs) on the abaxial side of a leaf in position 14–17 within the *A. thaliana* rosette and on the third or fourth fully developed *B. nigra* leaf. Plants were laden with eggs deposited by either control (E treatment) or antibiotics-treated (AB) butterflies (E_{AB} treatment) (see below for treatment of the insects with antibiotics). *Arabidopsis thaliana* and *B. nigra* plants carried the eggs until larvae started hatching. The egg incubation time naturally varies dependent on the plant species; under our laboratory conditions, *A. thaliana* carries the eggs for 6 days until larvae hatch, *B. nigra* carries the eggs for 5 days until hatching of larvae.

Treatment of plants with secretion from the ARGs dissected from control (G treatment) and AB-treated (G_{AB} treatment) *P. brassicae* females were performed on *A. thaliana* on the same leaf positions and for the same duration as described for the treatment with eggs (for

the method of secretion collection see Data S1: collection of ARG secretion). For experiments regarding priming of anti-herbivore defences by treatment of plants with whole ARG content, we applied the secretion of an entire gland onto an *A. thaliana* leaf. We placed a freshly dissected glandular reservoir onto a leaf by using sterile tweezers. The glandular reservoir was punctured with a sterile needle (without puncturing the leaf) to release its content. For the experiments with specific amounts of ARG secretion, glands were pooled, punctured with a sterile needle and centrifuged for 15 s at 6,000 rpm to separate the secretion from the gland tissue. The supernatant was then taken with a pipette and used for the treatment.

Treatment of plants with bacterial and yeast isolates obtained from eggs freshly laid on a sterile surface (see below Section 2.5) were performed on *A. thaliana*. Overnight cultures of isolates of *Metschnikowia sp*., *Hanseniaspora sp*., *Lactococcus sp*., *Enterococcus sp*. and *Rahnella sp*. were diluted in 10 mM phosphate buffer (pH 7.0) to a final concentration of 10,000 colony forming units (CFUs) per 20 μl equally mixed and applied as a droplet (2 × 10 μl) on the same leaf position and for the same duration as in the treatments with eggs. A mock treatment with 2 × 10 μl sterile buffer was used as a control.

After the abovementioned treatments, *A. thaliana* plants were transferred into a climate chamber with short-day conditions (8-hr/16-hr light/dark cycle, 120 μmol m^{-2} s^{-1} light intensity, 20°C, r. h. 50%), whereas *Brassica nigra* plants were transferred into a climate chamber with long-day conditions (16-hr/8-hr light/dark cycle, 85 μmol m^{-2} s^{-1} light intensity, 22°C, r. h. 50%). The applied abiotic conditions mimic the natural conditions during the growing seasons of these plant species. Under these abiotic conditions, plants were kept for the duration of the abovementioned treatments (6 days for *A. thaliana*, 5 days for *B. nigra)*. When plants thereafter were treated with larvae, they were further kept under these conditions.

Treatments of plants with *P. brassicae* larvae were conducted after the abovementioned treatments. For treatment of *A. thaliana* plants, 10 neonate *P. brassicae* larvae were enclosed in a clip cage on the treated leaf or the respective leaf of a control plant, as previously described (Firtzlaff et al., 2016). On *B. nigra* plants, 10 neonates (reduced to three larvae after 7 days of feeding until pupation) moved freely per plant following a setup similar to the one of previous studies (Pashalidou, Fatouros, Van Loon, Dicke, & Gols, 2015; Pashalidou, Lucas-Barbosa, van Loon, Dicke, & Fatouros, 2013). We recorded the following performance parameters: larval biomass (2 days of feeding on *A. thaliana*, or 7 days of feeding on *B. nigra*), pupation time (on *B. nigra*), pupal biomass (on *B. nigra*).

2.3 | Plant gene expression analysis

We harvested *A. thaliana* leaves without petioles. The material was sampled 5 days after egg deposition or treatment with ARG secretion. Samples were frozen in liquid nitrogen and stored at −80°C until further use. Samples were bead-grinded for 40 s at 4,500 rpm in a tissue homogenizer (Precellys Evolution®). Total RNA was isolated as described by Oñate-Sánchez and Vicente-Carbajosa (2008) with minor modifications; we used twice the volume of buffers and isopropanol and added an additional centrifugation step after the DNA and protein precipitation. RNA was treated with DNA-free™ DNA Removal Kit (Thermo Fisher Scientific) following the manufacturer's protocol. RNA concentrations and quality were determined spectrophotometrically (Multiskan™ GO, Thermo Fisher Scientific), and by electrophoresis on a 1.2% agarose gel.

First strand cDNA was synthesised from 2 μg RNA with the RevertAID RT Reverse Transcription kit (Thermo Fisher Scientific) following the manufacture's protocol. For high efficiency of cDNA-synthesis we used 1 μl 100 μM random hexamer as well as 1 μl 100 μM oligo$(dT)_{18}$-primers (Table S1). All qPCRs were performed in duplicates in 10 μl volumes including 1 μl cDNA, 250 nM of each gene-specific primer and 5 μl Power SYBR® Green PCR master mix (Applied Biosystems). We used a Stratagene MX3005p Real-Time PCR System (StrataGene Systems, Washington) with the thermal profile: 1 × 10 min 95°C – 40 × (90 s 95°C–60 s 60°C). Normalised relative expression levels were calculated by the ΔΔC(T) method as described by Livak and Schmittgen (2001), using *AtACT2* (AT3G18780) and *AtUBQ10* (AT4G05320) as reference genes.

2.4 | Insects and antibiotics treatment

The Large White butterfly (*Pieris brassicae*) line we used for our experiments is derived from a colony at the Laboratory of Entomology, Wageningen University in the Netherlands. The F0 generation was reared during the larval stage on Brussels sprouts (*Brassica oleracea var. gemmifera*). Insects were kept in two treatment groups: control and antibiotics-treated (AB). Control and AB-treated caterpillars were reared in a climate chamber (18-hr/6-hr light/dark cycle, 160 μmol m^{-2} s^{-1} light intensity, 20°C and 70% r. h.) until pupation.

AB-treated larvae were reared on plants sprayed with a mixture of four antibiotics (rifampicin, chloramphenicol, streptomycin, ampicillin), each in a concentration of 0.5 mg/ml H_2O until the plant surface was uniformly covered with the solution. These antibiotics were selected based on previous studies showing no effect on larval mortality at such concentrations (Lin, Kang, Pan, & Liu, 2015). Furthermore, we observed no effects of the AB-treatment on larval biomass (Paniagua Voirol, Weinhold, Johnston, Fatouros, & Hilker, 2020). Pupae were transferred to a separate climate chamber (18-hr/6-hr light/dark cycle, 220 μmol m^{-2} s^{-1} light intensity, 23°C, r. h. 70%), where adult butterflies emerged.

Control butterflies were fed with a 15% wt/vol honey-water solution and AB-treated butterflies with 15% wt/vol honey dissolved in the above-mentioned combination of antibiotics. Honey solutions were provided in 1.5 ml tubes placed in the centre of artificial flowers. For all the experiments, butterflies were used 2 weeks after emergence from pupae. Eggs deposited by AB-treated butterflies were not treated with antibiotics.

2.5 | Analysis of microbes associated with freshly laid *P. brassicae* eggs on a sterile surface

To investigate the microbes associated with freshly laid eggs, we let the butterflies deposit their eggs on a sterile surface. Therefore, we covered the upper side of a sterile plastic petri dish with a Brussels sprouts leaf, while the lower side of the dish remained sterile. Petri dishes were placed for 4–6 hr in a cage with 10–15 butterflies (2 weeks after emergence). When butterflies landed on the leaf, their tarsi, but not the abdominal tip touched the leaf. This touch stimulated oviposition behaviour, and the butterflies immediately curved their abdomen directly to the (sterile) lower side of the dish for egg deposition. Eggs with their secretion were collected from the petri dishes using flame-sterilised tweezers and were processed in a laminar flow hood. We quantified the abundance of bacteria associated with eggs by determining (a) the 16S rRNA gene copy number using qPCR or (b) the number of CFUs.

For quantification of the bacteria by qPCR, we determined the bacterial 16S rRNA gene copy number from 2-week-old control and AB-treated butterflies and their freshly laid egg clutches (N = 8 for each type of sample). Genomic DNA was isolated from egg clutches and entire butterflies (after wing removal) using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions. Samples were homogenised by bead-grinding (15 s at 4,500 rpm in a tissue homogeniser, Precellys Evolution®) and treatment with lysozyme (25 U/μl, Epicentre) for 30 min at 25°C and RNaseA (0.1 μg μl^{-1}, Epicentre) 30 min 37°C. DNA yield was quantified using the Thermo Scientific Multiskan GO microplate spectrophotometer. Samples were stored at −20°C until further use.

We developed specific qPCR primers, which allowed us to amplify also minute bacterial numbers without interference from insect ribosomal sequences. Detailed information on the primers and qPCR conditions are given in the Data S1: Microbial analysis.

For quantification of egg-associated bacteria by CFUs, 46 egg clutches were collected (median 32.5 eggs per clutch), squeezed in 200 μl sterile LB medium using a pistil and then plated on three different media (LB-Lennox agar, Tryptic soy agar, and one-fifth-strength LB-agar). Plates were incubated at 27°C and colonies counted after 2, 4, and 7 days. Colonies were randomly picked, sub-cultured, and stocks were kept at −80°C in 10% DMSO. Isolates were identified by sequencing the full length 16S or 18S rRNA gene (for details see Data S1: Microbial analysis).

2.6 | Analysis of microbes associated with five-day-old eggs laid on *B. nigra*

To figure out which microbes are detectable in/on insect eggs that have been lying on a leaf for 5 days (i.e. almost for the entire egg incubation time), we sequenced the bacterial 16S rRNA genes detectable in such egg samples by the Illumina MiSeq system at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv). We excised egg clutches from *B. nigra* leaves 5 days after oviposition by either control or AB-treated butterflies. Plant tissue containing no eggs was also cut from the plant as a control for the autochthonous phyllosphere bacterial community (leaf sample size: 5 cm^2). After DNA extraction from homogenised samples, we performed PCR amplification of the bacterial 16S rRNA gene using the 341F and 805R primers, which are recommended by Illumina for the MiSeq platform. These primers target the bacterial V3-V4 16S rRNA gene regions. PCR products were purified with AMPure Beads (Beckmann Coulter, Brea) and ligated to barcoded Illumina-Adapters. Negative extraction control samples (NEC) containing no insect sample (but just the kit reagents and lysozyme addition) were processed at the same time to control for bacterial contamination (further details in Data S1: Microbial analysis).

2.7 | Statistical analysis

Statistical analysis was conducted with R Statistics (R Development Core Team 2017) by using car (Fox & Weisberg, 2011), gplots (Warnes et al., 2011), psych (Revelle, 2015), nlme (Pinheiro, Bates, DebRoy, & Sarkar, 2018), lme4 (Bates, Machler, Bolker, & Walker, 2015). We used the Shapiro–Wilk test to evaluate the data for normal distribution and the Levene's test for variance homogeneity. For normally distributed data we applied parametric test statistics, for non-normally distributed data non-parametric tests were used as described in the figure legends. p-values of multiple comparisons were adjusted according to the Benjamini and Hochberg procedure (BH).

The bacteria sequencing reads obtained from 5-day-old eggs laid on *B. nigra* leaves were trimmed, denoised and overlapped using a full-stack R pipeline (Callahan, Sankaran, Fukuyama, McMurdie, & Holmes, 2016) incorporating dada2 (Callahan et al., 2016) and phyloseq (McMurdie & Holmes, 2013). The data were further processed and evaluated as described in the Data S1: Microbial analysis. The resulting exact sequence variants were agglomerated at the genus level. Beta diversity distance matrices (including Bray-Curtis index) and ordinations were performed using phyloseq (McMurdie & Holmes, 2013). Multivariate analysis of variance (MANOVA) was tested using vegan (Oksanen et al., 2018).

3 | RESULTS

3.1 | Plant responses to insect eggs laid by AB-treated butterflies are attenuated

To elucidate whether the AB-treatment of butterflies changes a plant's response to their eggs, we compared (a) transcriptional responses of plants to eggs from AB-treated and control butterflies and (b) larval performance on plants previously laden with eggs from these types of butterflies.

The expression levels of the studied genes differed between *A. thaliana* plants with eggs from control butterflies compared to plants

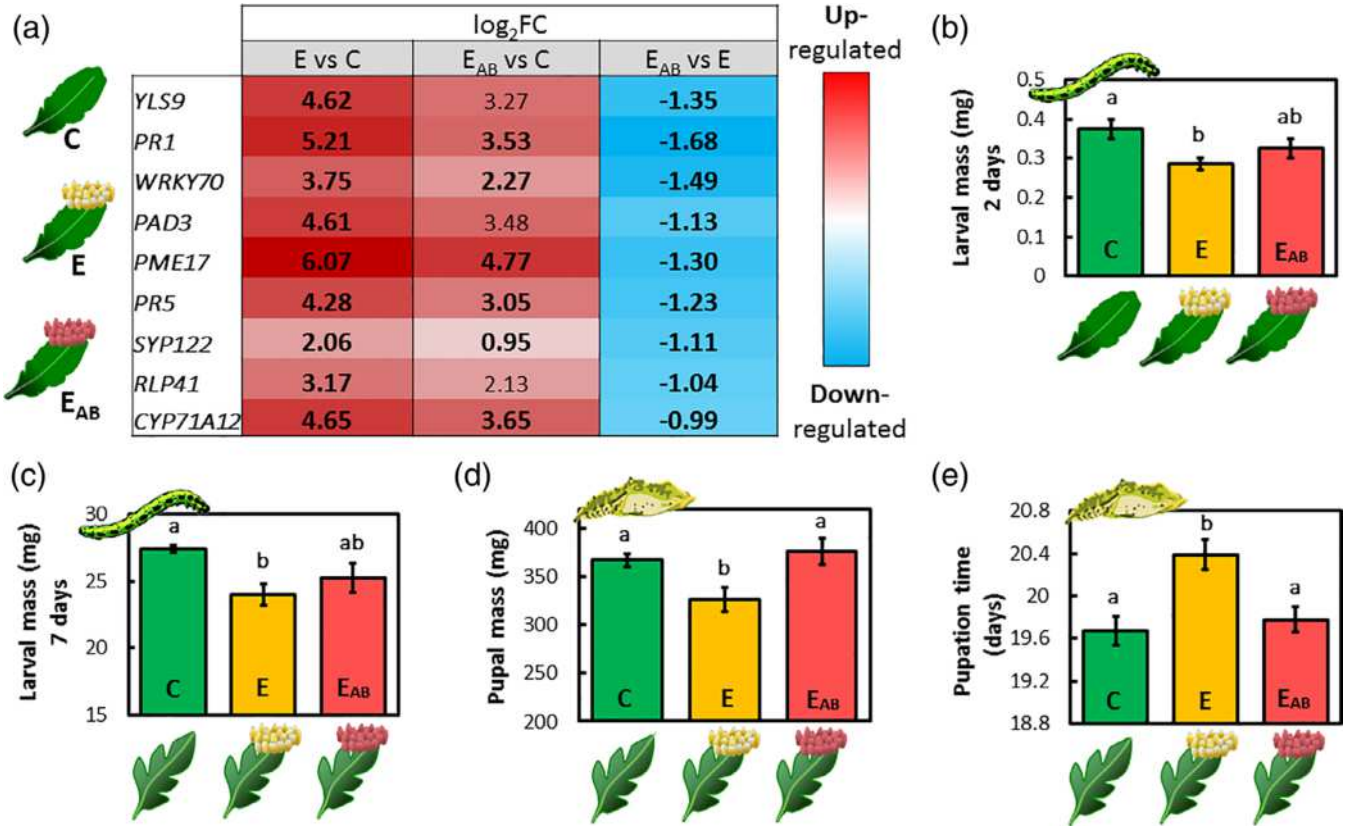

FIGURE 1 Plant transcriptional responses to eggs from control and antibiotic-(AB)-treated *Pieris brassicae* butterflies and insect performance on plants exposed to these types of eggs. (a) Mean relative expression (log_2FC) of genes in *Arabidopsis thaliana* leaves 5 days after egg deposition by control butterflies ("E") or AB-butterflies ("E_{AB}") compared to untreated control leaves ("C"). Bold numbers: significant differences between gene expression of the treatment groups ($p < .05$, multiple Student's *t* test, post hoc BH correction); $N = 5–8$ plants. (b–e) Performance of *P. brassicae* larvae on untreated control plants (green bars; "C") or on plants with eggs laid by control butterflies (yellow bars; "E") or on plants laden with eggs laid by AB-treated (red bars; "E_{AB}") butterflies. Performance was measured as fresh larval biomass (mg) after (b) 2 days of feeding on *A. thaliana* ($N = 9–10$ plants) or (c) 7 days of feeding on *Brassica nigra* ($N = 12–13$ plants), as (d) pupal mass and (e) time until pupation ($N = 8–9$ plants) when fed as larvae on *B. nigra*. All bars show means (± SE). Different letters above bars: significant differences between treatments ($p < .05$, multiple Mann–Whitney *U* tests, post hoc BH correction) [Colour figure can be viewed at wileyonlinelibrary.com]

with eggs from AB-butterflies. The transcript levels of *YLS9*, *CYP71A12*, *RLP41*, *PME17*, *PAD3*, *PR5* and *PR1* were significantly lower in plants exposed to eggs from AB-butterflies than in plants exposed to eggs from control butterflies. When comparing plants exposed to eggs from AB-butterflies and untreated plants, the expression of *PAD3*, *YLS9* and *RLP41* was not significantly induced, but their expression was significantly induced in plants exposed to control eggs (Figure 1a).

Performance of larvae on *A. thaliana* and *B. nigra* plants previously laden with eggs from control butterflies was worse than performance of larvae on egg-free plants. The larvae gained less weight, needed more time to pupate, and reached a lower pupal weight (Figure 1b–e). However, when the larvae fed on plants with eggs from AB-treated butterflies, these egg-mediated effects on larval performance were attenuated in the larval stage and abolished in the pupal stage (Figure 1b-e).

Thus, egg-mediated plant defence responses to larvae and expression of several egg–/phytopathogen-responsive *A. thaliana* genes were attenuated when eggs were from AB-butterflies.

3.2 | Microbes on *P. brassicae* eggs are scarce and do not enhance plant defence against larvae

The bacterial abundance determined by the number of 16S rRNA gene copies in eggs freshly laid on a sterile surface was marginally above the detection limit in control eggs and only slightly reduced in eggs laid by AB-treated butterflies (Figure 2a). In contrast, control butterflies harboured high numbers of 16S rRNA gene copies, whereas the AB-butterflies showed a strong reduction in the abundance of their microbiota (total median reduction of more than 21,000-fold compared to control butterflies) (Figure 2a).

The microbial abundance determined by the numbers of CFUs obtained from eggs (including glandular secretion) freshly laid on a sterile surface was very low and variable. Only about half of the analysed control egg clutches (52%) showed microbial growth, compared to an even lower percentage (13%) of the egg clutches from AB-treated butterflies (Figure 2b). No dominant isolate could be obtained or identified as a potential phytopathogen. The most frequent morphotypes observed

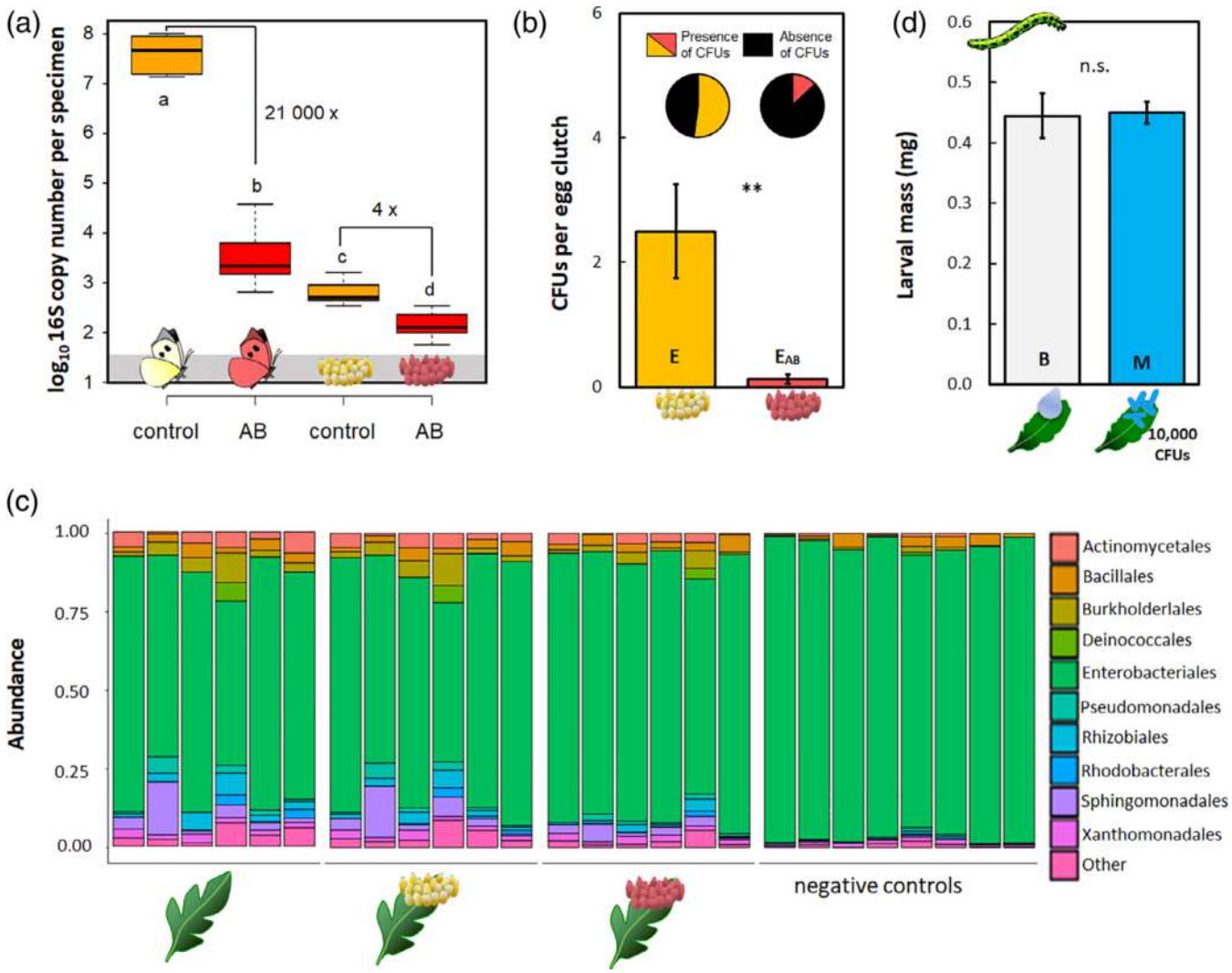

FIGURE 2 Analysis of microbes associated with eggs of control and antibiotic-(AB)-treated *Pieris brassicae* and bioassay testing the effect of the egg-associated microbiome on the plant's anti-herbivore defence. (a) Culture-independent quantification of bacterial 16S rRNA gene via qPCR for control (yellow) and antibiotic-(AB)-treated (red) butterflies and their eggs, freshly laid on sterile surface. Different letters above bars: significant differences ($p < .01$, Mann–Whitney *U* tests, post hoc BH correction). Grey horizontal bar: detection limit. $N = 8$ individual female butterflies or egg clutches, each of control and AB-butterflies. (b) Pie charts represent the presence and absence of colony forming units (CFUs) of microbes (bacteria and yeasts) isolated from egg clutches freshly laid on sterile surface by control (yellow) and AB-treated (red) butterflies. Bars represent the mean (±SE) number of microbial CFUs per egg clutch (∼30 eggs) on sterile surface, $N = 23$ egg clutches of each type. Asterisks (**): significant difference between treatments ($p < .01$, Mann–Whitney *U* test). (c) Bacteria taxa abundance at the order level after sequencing of 5-day-old egg clutches laid on *Brassica nigra* leaves by control females (yellow eggs) and by antibiotic-(AB)-treated butterflies (red eggs), only leaves, and in negative extraction controls. Each bar represents independent samples. (d) Mean (±SE) larval biomass 2 days after feeding on *Arabidopsis thaliana* plants pre-treated with egg-associated microbes (M, blue bar) or on plants exposed to the buffer control (B, grey bars). n.s. (not significant, Student's *t* test $p = .89$), $N = 8$ buffer-treated and 8 microbe-treated plants [Colour figure can be viewed at wileyonlinelibrary.com]

belonged to a nectar yeast (genus *Metschnikowia*, 5 of 23 egg clutches) or an enteric bacterium (genus *Rahnella*, 4 of 23 egg clutches), both in total with an average abundance of less than 0.9 CFUs per egg clutch. Other isolates were found in even lower frequencies. However, neither type of eggs showed a consistent colonisation by a certain microbe.

Because of the extremely low and inconsistent abundance of microbes detectable in/on eggs freshly laid on a sterile surface, we further asked whether egg deposition might change the bacterial communities on the leaf when eggs were lying on a plant for 5 days. The sequencing analysis of 5-day-old egg clutches excised from *B. nigra* leaves revealed no differences between the bacterial community composition associated with control eggs and with eggs from AB-butterflies. Neither did the egg-associated communities differ from the one on *B. nigra* leaves without eggs (Figure 2c). The sequencing analysis was dominated by organellar reads from the plant leaf. A permutational MANOVA of Bray–Curtis dissimilarity revealed that only the NECs

significantly differed from the other samples (F = 939.91, R^2 = 0.98, p < .001). This difference was due to the greater abundance of both *Brassica* mitochondrial 16S (53-fold differential abundance, p < .001) and chloroplast 16S (133-fold differential abundance, p < .001) in the leaf samples with eggs and in the plant tissue samples without eggs than in the NECs (Figure S1a). However, when organellar reads were removed, the samples of the eggs and plant tissues did not differ from the NECs anymore (F = 2.30, R^2 = 0.24, p = .095) (Figure S1b). All samples were mainly dominated by Enterobacteriaceae classified as *Escherichia/Shigella* (Figure S2).

These results support the previous findings of an extremely low abundance of bacteria associated with egg clutches of *P. brassicae*.

To elucidate whether the few egg-associated microbes could prime the plant anti-herbivore defences, we treated *A. thaliana* plants with a mixture of cultivated egg-associated microbes for 5 days, then placed larvae on the plant and recorded their biomass after a 2-day-feeding period. Despite the excess of microbes used (10,000 CFUs per leaf) - more than a 1000 times higher than the numbers obtained from *P. brassicae* eggs - larvae feeding on these plants did not differ in weight gain from those feeding on buffer-treated control plants (Figure 2d).

Thus, egg-associated microbes did not elicit the enhancement effect on plant defence against the larvae, which was detectable when the plant had received natural eggs.

3.3 | Application of glandular egg-associated secretion onto leaves has a similar enhancing effect on plant defence against larvae as natural egg deposition

Since egg-associated microbes did not prime the plant's defence against larvae, we investigated whether exposure of the plant to the glandular secretion, which attaches the eggs to the leaf surface, enhances the plant responses against larvae.

We applied the entire glandular secretion produced by control or AB-butterflies in their ARGs onto *A. thaliana*. Regardless of whether the secretion was from control or AB-butterflies, it mediated the same enhancing effect on the plant's defence against larvae as natural egg deposition by control butterflies. Irrespective of whether larvae fed on plants laden with eggs from control butterflies or on plants treated with secretion of control or AB-butterflies, they always gained less weight than larvae on egg-free plants (Figure 3).

These data show that application of secretion of an entire female ARG is sufficient to elicit the enhanced anti-herbivore plant defence. Our results also suggest that the glandular secretion of AB-butterflies has the same qualitative potential to amplify the plant's anti-herbivore defence as the secretion from untreated butterflies. This finding raised the question whether the attenuation in the plant's anti-herbivore defence mediated by eggs from AB-butterflies is due to a difference in the amount of released secretion. Therefore, we addressed the question whether AB-butterflies release a different amount of secretion than control butterflies.

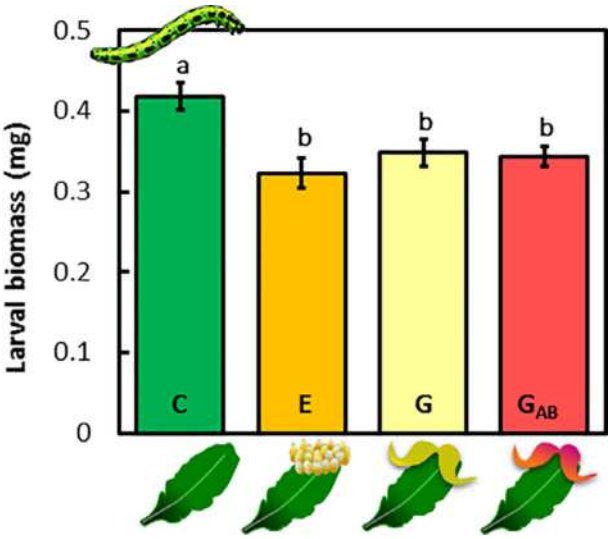

FIGURE 3 Effect of treatment of *Arabidopsis thaliana* with secretion of an entire Accessory Reproductive Gland (ARG) of a *Pieris brassicae* female on plant defence against larvae. Fresh biomass (mg) of *P. brassicae larvae* (mean ± SE) 2 days after feeding on pre-treated plants. Prior to larval feeding the plants were kept either untreated ("C," green bars) or had previously been exposed to *P. brassicae* eggs ("E," dark yellow bars), or had been treated with the entire secretion of an ARG of untreated females ("G," light yellow bars), entire secretion of an ARG of antibiotic-(AB)-treated females ("G_{AB}," red bars). Differences were tested using Linear Mixed Model (LMM), and GLHT post hoc with Tukey contrasts was used for pairwise comparisons. Different letters above bars indicate significant differences between treatments (p = .05). The number of biological replicates (plants) was N = 12 [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | The egg-mediated enhancer effect on plant defence against larvae depends on the amount of glandular egg-associated secretion

To investigate whether the AB-treatment of *P. brassicae* affects the amount of glandular secretion produced and released by the females during oviposition, we measured the gland biomass before and after oviposition. Before oviposition, the ARG biomass already differed significantly between control and AB-butterflies (Figure 4a). The analysis of ARG mass after oviposition revealed that the AB-butterflies released significantly less secretion when depositing an egg clutch (Figure 4a). Along with an egg clutch, control females released 2.1 mg ± 0.35 (mean ± SE) of secretion, whereas AB-butterflies released 0.8 mg ± 0.41 (mean ± SE) (Student's t test, p = .025).

We analysed whether the lower amount of secretion released by AB-butterflies was responsible for the attenuated plant responses to larval feeding. When the secretion was applied in the amount as released by control females (2.1 mg), larvae gained less biomass compared to larvae feeding on untreated plants (Figure 4b). These larvae gained similar biomass as those on plants exposed to eggs. In contrast, when secretion was applied in the same amounts as released by AB-

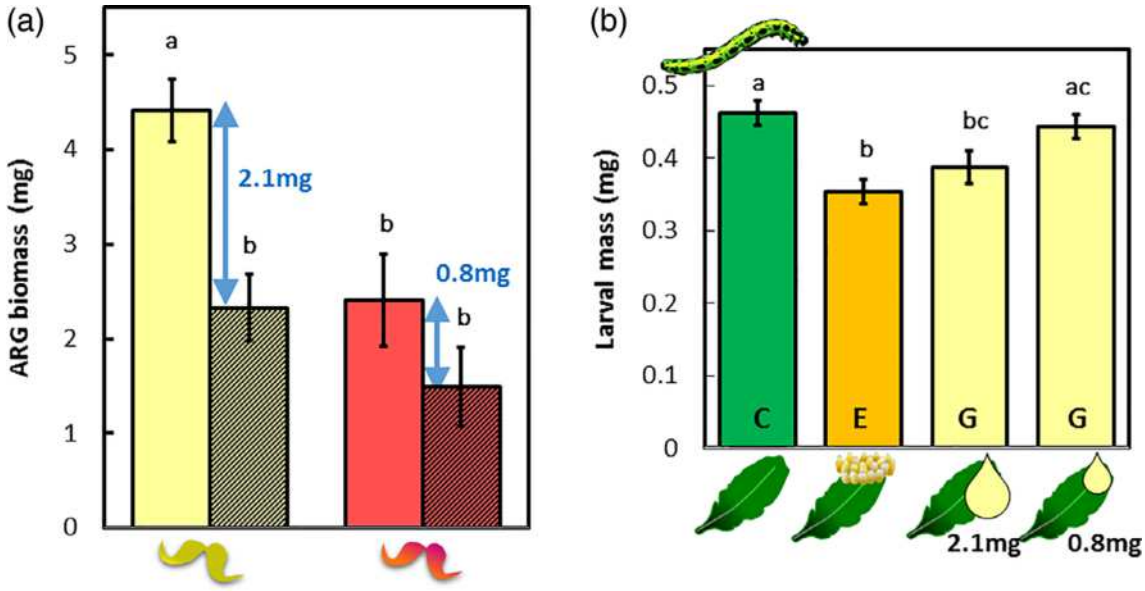

FIGURE 4 Biomass of accessory reproductive glands (ARGs) of AB-treated and control *Pieris brassicae* butterflies and the effect of the plant's response to various secretion quantities on larval performance. (a) Biomass (mean ± SE) of freshly dissected female *P. brassicae* ARGs before (solid filled bars) and after (dashed bars) deposition of an egg clutch with 35 eggs laid; untreated females (yellow bars) and antibiotic-(AB)-treated females (red bars). Different letters above bars indicate significant differences between treatment groups ($p < .05$, pairwise Student's *t* tests, post hoc BH correction). $N = 10$ glands per treatment. The blue arrows indicate the calculated amount of glandular secretion applied during egg deposition by control (i.e. 2.1 mg) and by AB-butterflies (i.e. 0.8 mg). (b) Biomass of *P. brassicae* larvae (mean ± SE) 7 days after feeding on *Arabidopsis thaliana* plants pre-treated with different doses of gland secretion (G, light yellow bars), eggs (E, dark yellow bars) and on untreated control plants (C, green bars). Different letters above bars indicate significant differences between treatment groups ($p < .05$, multiple Student's *t* test, post hoc BH correction), $N = 10$ [Colour figure can be viewed at wileyonlinelibrary.com]

butterflies (0.8 mg), larvae gained a similar biomass as larvae on untreated plants (Figure 4b). Hence, plant responses show a dosage dependency on the applied amount of glandular secretion.

To test whether different amounts of glandular secretion would explain a differential expression of egg- and phytopathogen-responsive genes, we analysed expression levels of the *PR1* and *PR5* marker genes on *A. thaliana* plants treated with various amounts of glandular secretion. When applying secretion from control females onto an *A. thaliana* plant in such an amount similar to the one released by control females (2.1 mg) during oviposition, this quantity upregulated the expression of *PR1* and *PR5* similarly as natural egg deposition does (Figure 5a,b). The expression of both genes did not significantly increase further when applying more secretion to a plant, that is, 4.5 mg, which is equivalent to the amount present in a filled gland of a control female prior to oviposition. However, when we applied the secretion of control butterflies in the amounts released by AB-butterflies (0.8 mg), the induction of *PR1* and *PR5* expression was significantly attenuated.

These results show a dose-dependent effect of the glandular secretion on plant responses, and this effect is independent of whether the secretion is produced by control or AB-butterflies.

4 | DISCUSSION

Based on studies showing that *A. thaliana* plants respond to *P. brassicae* eggs in a similar way as to (hemi)biotrophic pathogens (Little et al., 2007; Lortzing et al., 2018; Reymond, 2013), we tested the hypothesis that egg-associated microbes elicit the egg-mediated plant defence responses against *P. brassicae*. A detailed analysis of the microbiota associated with *P. brassicae* eggs revealed the presence of only few, environmentally ubiquitous microbes, which were only occasionally detected and probably carried over from the adult butterflies or the environment. Moreover, microbes isolated from *P. brassicae* eggs, cultured and then applied onto leaves did not elicit the egg-mediated enhancement of plant defence against larvae. Although not all bacterial species might have been cultured and applied onto the leaves, we showed with culture independent techniques (qPCR and MiSeq) that the amount of microbes associated with the eggs is negligible, thus making them unlikely to be involved in elicitation of the egg-mediated plant defence responses to larval feeding.

Instead, our findings show that the secretion associated with *P. brassicae* eggs elicits the enhancement effect on the plant's anti-herbivore defence against larvae. High amounts of secretion obtained from control or AB-butterflies elicited the same plant responses as natural egg deposition, whereas low amounts of secretion attenuated marker gene expression, regardless whether the secretion was obtained from control or AB-treated butterflies. The AB-treatment of butterflies led to a reduction in the ARG biomass and therefore in the amount of glandular secretion released during oviposition. Thus, our results showed (a) that the ARG secretion is sufficient to elicit plant defence responses similarly as the eggs do and (b) that the AB-

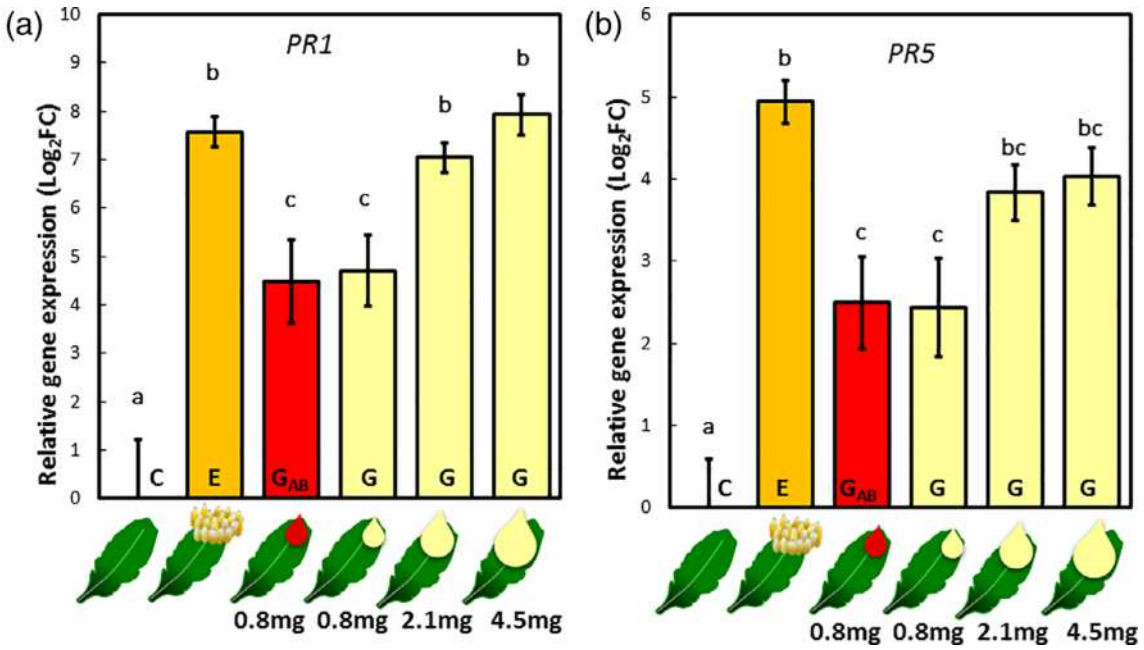

FIGURE 5 Effect of treatment of *Arabidopsis thaliana* with various quantities of Accessory Reproductive Gland (ARG) secretion of *Pieris brassicae* females on plant transcriptional responses. Relative expression (mean ± SE, log_2FC) of (a) *Pathogenesis-related gene 1* (*PR1*) and (b) *Pathogenesis-related gene 5* (*PR5*) in leaves laden with eggs by untreated *P. brassicae* female butterflies ("E," dark yellow bars), or in leaves treated with 0.8 mg of secretion obtained from glands of antibiotic-(AB)-treated female butterflies ("G_{AB}," red bars), or in leaves treated with different amounts of secretion (0.8, 2.1 and 4.5 mg) from glands of untreated control females (light yellow bars) or untreated control leaves ("C"). The amounts of secretion applied represent the secretion applied for attaching an egg clutch of 35 eggs by AB-fed butterflies (i.e. 0.8 mg) and by untreated control *P. brassicae* (i.e. 2.1 mg) and the total amount of secretion containing a single gland of untreated butterflies (i.e. 4.5 mg). Different letters above bars indicate significant differences between gene expression of the treatment groups ($p < .05$, LMM, post hoc glht with Tukey contrasts); $N = 7–8$ plants [Colour figure can be viewed at wileyonlinelibrary.com]

treatment of *P. brassicae* did not affect the eliciting quality of secretion, but the secretion quantity.

Application of control amounts of ARG secretion induced expression of *PR1* and *PR5*, which also respond to (hemi)biotrophic pathogens. The secretion of female lepidopteran ARGs is known to consist of numerous proteins and lipids (Beament & Lal, 1957; Hinton, 1981). So far, we do not know whether the ARG-immanent elicitor shares similarities with those known from phytopathogens, for example, proteins like flagellin, harpin and elicitins (Cordelier, de Ruffray, Fritig, & Kauffmann, 2003; Denoux et al., 2008; Peng et al., 2003), or whether the plant shows similar responses to different elicitors. However, our study revealed that the elicitor is not released by egg-associated microbes, but by the insect itself.

The cause for the reduced biomass of ARGs produced by AB-treated females remains unclear. The AB-treatment may have caused negative side effects hampering the production of the glandular secretion. However, the antibiotics we used have been reported to impair lepidopteran performance only at higher concentrations than the ones we used (Lin et al., 2015). On the other hand, the elimination of gut bacteria in the AB-butterflies might have exerted negative effects on the production of the glandular secretion. Insect-inhabiting bacteria providing essential amino acids (Douglas, 2015) might indirectly contribute to the production of proteinaceous ARG secretion. If so, the parental microbiome may be important for the production of sufficient secretion in the female ARGs, and thus, would play an indirect role with respect to the egg-mediated enhancement of plant responses against larvae. To disentangle whether the effect we see is caused by the antibiotic treatment *per se* or by the elimination of adult-associated bacteria, tests should ideally be carried out using an axenic insect line. Axenic insect larvae are usually obtained by first sterilising the eggs and then feeding the hatching larvae with sterilised artificial diets (e.g. Mason et al., 2019). However, no sterilisable artificial diet has been successfully developed so far for rearing *P. brassicae*, a specialist that feeds exclusively on fresh brassicaceous plant tissue.

From an evolutionary viewpoint, one might ask why *P. brassicae* is applying so much secretion to attach its eggs to a host plant. Among the common European pierid species that feed on Brassicaceae, *P. brassicae* is the only species which lays eggs gregariously in clutches (Rothschild & Schoonhoven, 1977). This oviposition behaviour requires high amounts of secretion to attach an egg clutch to the leaf surface, in contrast to closely related species like *P. rapae* and *P. napi*, which lay single eggs. Depositions of single eggs by *P. rapae* did not prime defence of *B. nigra* against larvae (Griese et al., 2020). However, *P. rapae* eggs induce an HR-like leaf necrosis in *B. nigra* that can kill singly laid eggs. In contrast, gregariously laid *P. brassicae* eggs are not killed by HR-like leaf necrosis (Griese et al., 2017). One can only speculate that avoidance of such a

lethal plant trait to singly laid eggs promoted the evolution of egg clustering in *P. brassicae*, an oviposition mode, which might be "paid" by eliciting enhancement of plant anti-herbivore defence against the larvae.

Because a large group of eggs needs a high amount of glandular secretion to be tightly glued to a leaf, our results suggest that the plant's response to *P. brassicae* eggs is dependent on the number of eggs laid. If so, this would contrast the response seen in tobacco plants to eggs of the generalist *Spodoptera exigua*; egg-mediated priming of defences against *S. exigua* larvae is independent of the number eggs (Bandoly & Steppuhn, 2016). Further studies are needed to elucidate correlations between the size of *P. brassicae* egg clutches, plant defensive responses and the effect on plant and insect fitness.

5 | CONCLUSION

Despite the similarities between plant responses to eggs and to phytopathogens, the results of our study do not support the hypothesis that egg-associated microbes in *P. brassicae* are involved in eliciting the enhancing effect that plant responses to insect eggs exert on subsequent defence against hatching larvae. Here, we elucidated for the first time that the elicitor of egg-mediated enhancement of feeding-induced plant defences is present in egg-associated secretion and is acting in a dose-dependent manner.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

Luis R. Paniagua Voirol, Georgios Valsamakis, Vivien Lortzing, Nina Fatouros, Reinhard Kunze and Monika Hilker conceptualised the study. Luis R. Paniagua Voirol, Georgios Valsamakis and Vivien Lortzing performed and evaluated the ecological and molecular experiments. Luis R. Paniagua Voirol, Paul R. Johnston and Arne Weinhold performed microbial assessments. Luis R. Paniagua Voirol, Georgios Valsamakis and Monika Hilker wrote a first draft of the manuscript. All authors contributed to later versions and agreed with the final version.

DATA AVAILABILITY STATEMENT

Illumina sequencing data are deposited in SRA database under the bioproject accession PRJNA544041. Ribosomal sequences of isolates are deposited in GenBank under the accession numbers: MK945762-MK945766.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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