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INVERTEBRATE MICROBIOLOGY

Legacy of a Butterfly's Parental Microbiome in Offspring Performance

Luis R. Paniagua Voirol,[a] Arne Weinhold,[a] Paul R. Johnston,[b,c,d] Nina E. Fatouros,[e] Monika Hilker[a]

[a]Institute of Biology, Applied Zoology/Animal Ecology, Freie Universität Berlin, Berlin, Germany
[b]Institute of Biology, Freie Universität Berlin, Berlin, Germany
[c]Berlin Center for Genomics in Biodiversity Research (BeGenDiv), Berlin, Germany
[d]Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany
[e]Department of Plant Sciences, Biosystematics Group, Wageningen University, Wageningen, The Netherlands

ABSTRACT An insect's phenotype can be influenced by the experiences of the parental generation. However, the effects of the parental symbiotic microbiome and host plant use on the offspring are unclear. We addressed this gap of knowledge by studying *Pieris brassicae*, a multivoltine butterfly species feeding on different brassicaceous plants across generations. We investigated how disturbance of the parental bacterial community by antibiotic treatment affects F_1 larval traits. We tested the effects depending on whether F_1 larvae are feeding on the same plant species as their parents or on a different one. The parental treatment alone had no impact on the biomass of F_1 larvae feeding on the parental plant species. However, the parental treatment had a detrimental effect on F_1 larval biomass when F_1 larvae had a different host plant than their parents. This effect was linked to higher larval prophenoloxidase activity and greater downregulation of the major allergen gene (*MA*), a glucosinolate detoxification gene of *P. brassicae*. Bacterial abundance in untreated adult parents was high, while it was very low in F_1 larvae from either parental type, and thus unlikely to directly influence larval traits. Our results suggest that transgenerational effects of the parental microbiome on the offspring's phenotype become evident when the offspring is exposed to a transgenerational host plant shift.

IMPORTANCE Resident bacterial communities are almost absent in larvae of butterflies and thus are unlikely to affect their host. In contrast, adult butterflies contain conspicuous amounts of bacteria. While the host plant and immune state of adult parental butterflies are known to affect offspring traits, it has been unclear whether also the parental microbiome imposes direct effects on the offspring. Here, we show that disturbance of the bacterial community in parental butterflies by an antibiotic treatment has a detrimental effect on those offspring larvae feeding on a different host plant than their parents. Hence, the study indicates that disturbance of an insect's parental microbiome by an antibiotic treatment shapes how the offspring individuals can adjust themselves to a novel host plant.

KEYWORDS Lepidoptera, symbionts, insect immune system, *Pieris brassicae*, transgenerational effects

The phenotype of an organism is determined not only by its genotype interacting with its environment (1–3) but also by environmental factors experienced by previous generations (4). The exposure of invertebrate and vertebrate animals to environmental conditions can exert transgenerational effects on the offspring phenotype, thus preparing the offspring for predictable environmental stressors (5).

In insects, especially in Lepidoptera, transgenerational effects are increasingly recognized as important factors influencing fitness across generations (4). Transgenera-

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Address correspondence to Monika Hilker, monika.hilker@fu-berlin.de.

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tional effects on the offspring phenotype may be caused by various environmental experiences in the parental generation (4, 6). For example, immune challenges imposed to the parental generation are well known to prime the immune status of the offspring generation in Lepidoptera (7, 8), as well as in other insect taxa, including Hymenoptera (9, 10) and Coleoptera (11–13). Furthermore, the parental food is known to affect offspring immunity and performance in several insect species (14–16). Moreover, the insect's immune status can have an impact on food detoxification, thus potentially affecting performance (17).

While the diet of an insect is known to contribute to shaping the gut microbiota community (18), the composition of the parental gut bacterial community can also influence the offspring performance, as has been shown for *Drosophila melanogaster* (19). In addition to such diet-mediated indirect effects, direct transgenerational effects of microbes are achieved by inoculation of the offspring with parental microbes. These vertically transmitted microbes can shape the metabolic, immunological, and behavioral traits of the offspring (20–22).

However, unlike many insect orders, lepidopteran larvae do not harbor abundant and resident bacterial communities and seem not to depend upon the transfer of bacterial symbionts across generations (23–25). In contrast, adult butterflies contain conspicuous amounts of bacteria compared to their larvae. While the parental diet and immune state are known to affect offspring traits in Lepidoptera, it is unclear whether also the parental microbiome imposes direct effects or diet-mediated indirect effects on the lepidopteran offspring.

Here, we addressed this knowledge gap by studying transgenerational effects of the large cabbage white butterfly (*Pieris brassicae* L.) on its offspring. We tested the hypothesis that disturbance of the parental *P. brassicae* microbiome by an antibiotic (AB) treatment has transgenerational effects on the offspring. We asked if the hypothesized transgenerational effect is dependent on whether the offspring feeds on the parental host plant species or on a different one. This question is based on the assumption that a host plant species which differs from the parental one is experienced as a challenge by the offspring. Adaptations to novel host plants have been intensively studied with respect to the evolution of insect species (26, 27). However, little is known about transgenerational effects on phenotypic adaptations of multivoltine insects to the various host plant species they use during a season.

Pieris brassicae is an oligophagous, multivoltine species specialized on Brassicaceae plants of different seasonal phenologies. Throughout a year, different generations usually feed on different host plant species with various compositions in secondary plant metabolites (28, 29). Brassicaceous plants are known to produce glucosinolates (GS) as defensive compounds against other herbivores (30–32). Within the Pieridae family, species feeding on Brassicaceae have evolved the ability to enzymatically detoxify GS. Among the GS-detoxifying enzymes are nitrile-specifier proteins (NSP) that promote the formation of nitriles instead of toxic isothiocyanates as glucosinolate breakdown products (33–35). *Pieris* sp. larvae can differentially regulate the expression of their genes encoding GS-detoxifying enzymes depending on the GS profiles of their host plants (36). This ability might facilitate shifts to other brassicaceous host plant species with different GS profiles. However, studies also suggest that the efficacy of the GS-detoxifying system varies depending on the host plant GS profile (37, 38).

Here, we studied the question whether disturbance of the parental *P. brassicae* microbiome and a transgenerational host plant shift affect offspring traits. We compared the traits of offspring from antibiotic-treated parents and control parents. Offspring larvae either could feed on the parental host plant or were subjected to a transgenerational host plant shift and fed on a different plant species than their parents. We studied shifts between the following three different host plants varying in their GS profiles: *Brassica rapa* L. (with glucobrassicin, gluconasturtiin, and gluconapin as the predominant GS) (29, 39), *Brassica oleracea* L. var. *gemmifera* (with sinigrin, gluconapin, and glucobrassicin as the predominant GS) (40), and *Brassica nigra* L. (containing mainly sinigrin) (39, 41). We assessed offspring (i) biomass, (ii) bacterial

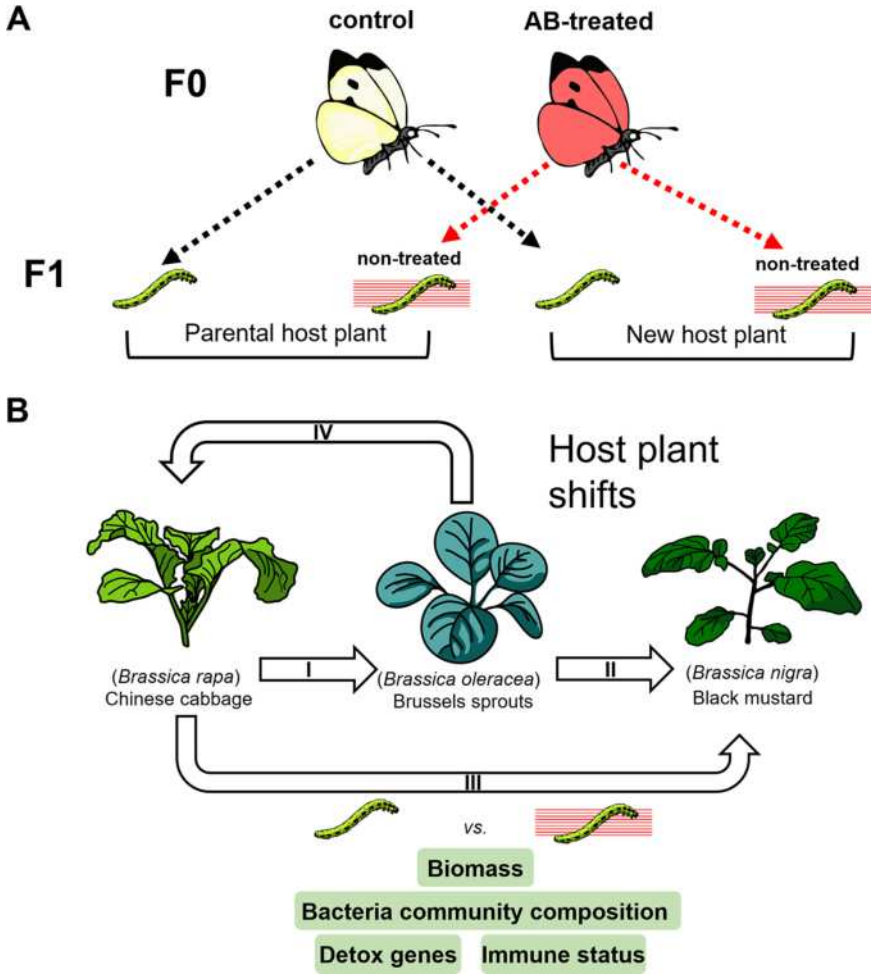

FIG 1 Schematic overview of the experimental design. (A) Effect of an antibiotic (AB) treatment of the parental generation on the F_1 offspring, feeding on either the parental or a new host plant species. The parental F_0 generation was fed on AB-treated plants (red butterfly) or untreated plants (yellow butterfly) during its whole life cycle. The offspring (F_1) generation was not treated with antibiotics. The F_1 generation from AB-treated parents (red stripes) or from control parents fed either on the same host plant species as their parents or on a different one. (B) Overview of host plant shifts performed in this study. *Pieris brassicae* larvae fed on three different host plant species with different glucosinolate profiles (*Brassica rapa* subsp. *pekinensis*, with glucobrassicin, gluconasturtiin, and gluconapin as predominant glucosinolates; *B. oleracea var. gemmifera,* with sinigrin, gluconapin, and glucobrassicin as predominant glucosinolates; and *B. nigra,* containing mainly sinigrin). We analyzed four parameters of F_1 larvae, (i) biomass, (ii) bacterial community composition, (iii) prophenoloxidase (PPO) activity, and (iv) expression of genes involved in detoxification of the host plant secondary metabolites. Offspring F_1 larvae from parents, which fed on *B. rapa*, were transferred to *B. oleracea* (I), *B. oleracea* was transferred to *B. nigra* (II), *B. rapa* was transferred to *B. nigra* (III), and *B. oleracea* was transferred to *B. rapa* (IV).

community composition, (iii) prophenoloxidase (PPO) activity as an immune parameter, and (iv) expression of putative detoxification genes (Fig. 1).

RESULTS

Efficacy of antibiotic treatment in the parental generation. To elucidate if the disturbance of the parental microbiota influences the offspring when coping with a host plant shift, we treated the parental generation (F_0) with antibiotics (AB). The antibiotic treatment itself showed no effect on larval biomass in the parental generation (see Fig. S1 in the supplemental material). To investigate effects of the AB treatment on the parental microbiome, we quantified the bacterial abundance in the F_0 generation by 16S rRNA gene quantitative PCR (qPCR) and counting of CFU.

The qPCR analyses revealed that the median relative abundance of bacteria in the F_0 adults was greatly reduced after the AB treatment. Overall, the relative bacterial

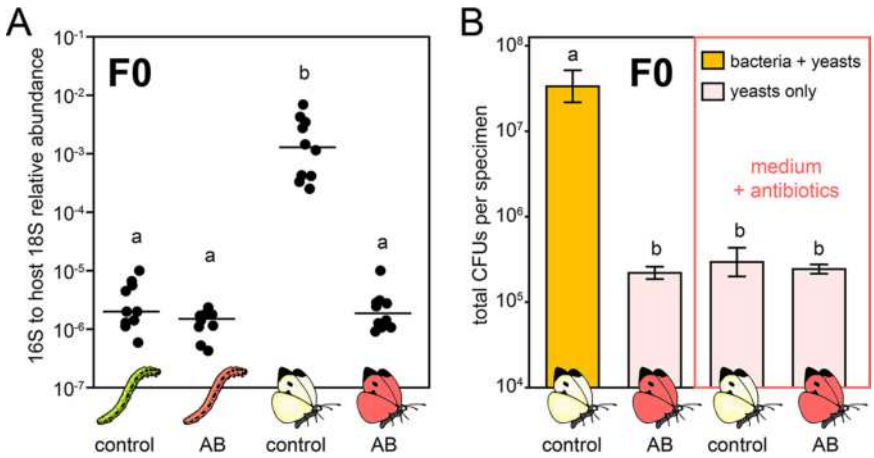

FIG 2 Efficacy of an antibiotic (AB) treatment in the parental generation (F_0) of *Pieris brassicae*. (A) Estimation of the bacterial abundance by qPCR from control and AB-treated F_0 larvae and F_0 adults shown as 16S rRNA gene copy numbers relative to insect 18S rDNA. The horizontal lines show the median from 10 insects per group. AB-treated F_0 larvae showed no difference from control F_0 larvae. Different letters indicate significant differences among groups ($P \leq 0.05$; Kruskal-Wallis test followed by Dunn's multiple-comparison test). Threshold cycle (C_T) values of AB-treated butterflies were 14-fold above the detection limit (water control samples). (B) Culture-dependent estimation of CFU on tryptic soy agar (TSA) obtained from control and AB-treated adult butterflies of the parental generation (± standard error of the mean [SEM] from 9 adults per group). Tryptic soy agar supplemented with antibiotics (same as fed to the butterflies) served as a control to reveal the background colonization by yeasts (see Fig. S2 for details). Different lowercase letters indicate significant differences among groups ($P \leq 0.05$).

abundance in untreated butterflies was much higher than that in F_0 larvae. Furthermore, the AB treatment had no effect on the low relative abundance of bacteria in the larvae (Fig. 2A).

A culture-dependent analysis of adult control butterflies showed a presence of 10^7 to 10^8 CFU per specimen. The AB treatment resulted in a median reduction of 99.2% of the total CFU (Fig. 2B). While most of the CFU from control butterflies originated from bacteria (with only a few yeast colonies), all of the CFU remaining in the AB-treated butterflies belonged only to yeasts. In the AB-treated adults, no bacteria were observable even in the lowest dilution (lower detection limit, 500 CFU; Fig. S2).

Hence, the AB treatment significantly reduced the bacterial load in F_0 adults, while F_0 larvae contained only a very low bacterial abundance regardless of the AB treatment.

Larval performance depends on both host plant species and parental AB treatment. To elucidate the impact of parental AB treatment on offspring (F_1) performance, we compared the biomasses of F_1 larvae from control and AB-treated parental lines when feeding on the same or a different host plant species as their parents. The F_1 generation was not treated with antibiotics.

The biomasses of the 7-day-old F_1 larvae from AB-treated parents and from control parents did not differ when feeding on the same host plant species as their parents (Fig. 3A to D, parental host plant). Thus, the antibiotic treatment of the parents had in general no detrimental effect on the performance of the offspring when larvae fed on the same plant species as their parents.

However, in three out of the four tested host plant shifts, F_1 larvae from AB-treated parents gained less biomass than did larvae from control parents when shifting to a new host plant (Fig. 3A to C, new host plant). For such shifts, we found a significant interaction effect of parental AB treatment and host plant shift (Fig. 3A and C; $P < 0.01$, generalized linear model [GLM]). Interestingly, this detrimental effect of the parental AB treatment was present only when the shift occurred from a plant with lower (or no) sinigrin content to a plant with higher sinigrin content (Fig. 3A to C). This effect was not detected when larvae shifted to a host plant with no sinigrin (Fig. 3D).

Additionally, the biomass of larvae from control parents was determined by the host plant species itself. Larvae gained generally more biomass when shifting to *B. nigra*

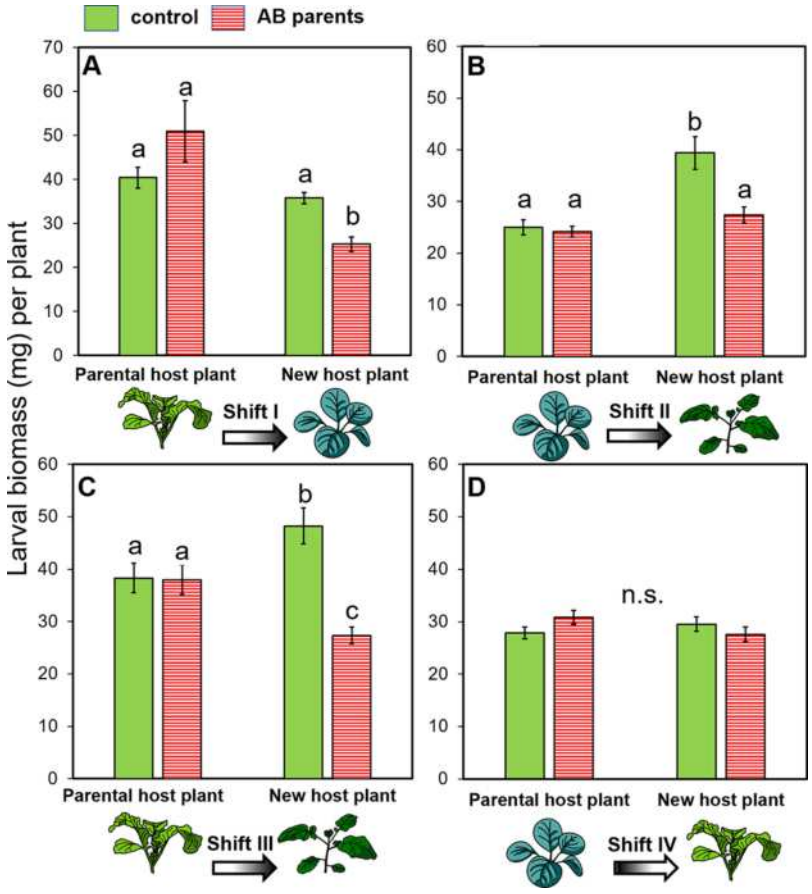

FIG 3 Transgenerational effect of a parental antibiotic (AB) treatment on the biomass of *Pieris brassicae* larvae (F_1 generation) experiencing different transgenerational host plant shifts. Mean (± standard error [SE]) F_1 larval biomass (in milligrams) per plant is shown 7 days after feeding on either the parental host plant species or on a new host plant species. Green, offspring from control parents; red stripes, AB-treated parents. (A) Shift I, *Brassica rapa* to *B. oleracea*. (B) Shift II, *B. oleracea* to *B. nigra*. (C) Shift III, *B. rapa* to *B. nigra*. (D) Shift IV, *B. oleracea* to *B. rapa*. For each shift, the shaded side of the arrow points at the plant with higher sinigrin proportion in its GS profile. Different letters above the bars indicate significant differences determined by *post hoc* tests among treatments, $P < 0.05$; n.s., not significant (Kruskal Wallis test, followed by Dunn's multiple-comparison test/multiple Student's *t* test, *post hoc* Benjamini-Hochberg [BH] correction). The different diet shifts were carried out independently with 10 to 12 plants (biological replicate), each with 5 to 10 larvae. For shifts I, II, and III, we found a significant interaction effect of parental AB treatment and host plant shift ($P < 0.01$, GLM).

(Fig. 3B and C), while shifting to the other host plants did not cause differences in the performance of larvae from untreated parents (Fig. 3A and D).

Thus, the parental AB treatment exerted a negative impact on F_1 larval biomass when F_1 larvae experienced a transgenerational host plant shift.

***Pieris brassicae* larvae harbor an inconspicuous bacterial community.** Since the parental AB treatment had an impact on the performance of those larvae feeding on a different host plant species than their parents, we hypothesized that the parental bacteria affect the ability of the offspring to cope with a host plant shift. Based on this hypothesis, we asked whether the parental AB treatment and/or host plant shift influence the bacterial community in the larval offspring.

Microbiome profiling by Illumina sequencing of F_1 larvae from AB-treated and control parents revealed that the bacterial amplicons in larval samples were indistinguishable from the negative controls. This result was independent of the parental treatment and of the experience of a host plant shift from the parental host plant *B. oleracea* to the larval host plant *B. nigra* (Fig. 4A). The larvae were found to harbor a negligible bacterial biomass, which did not exceed the background amplification of the

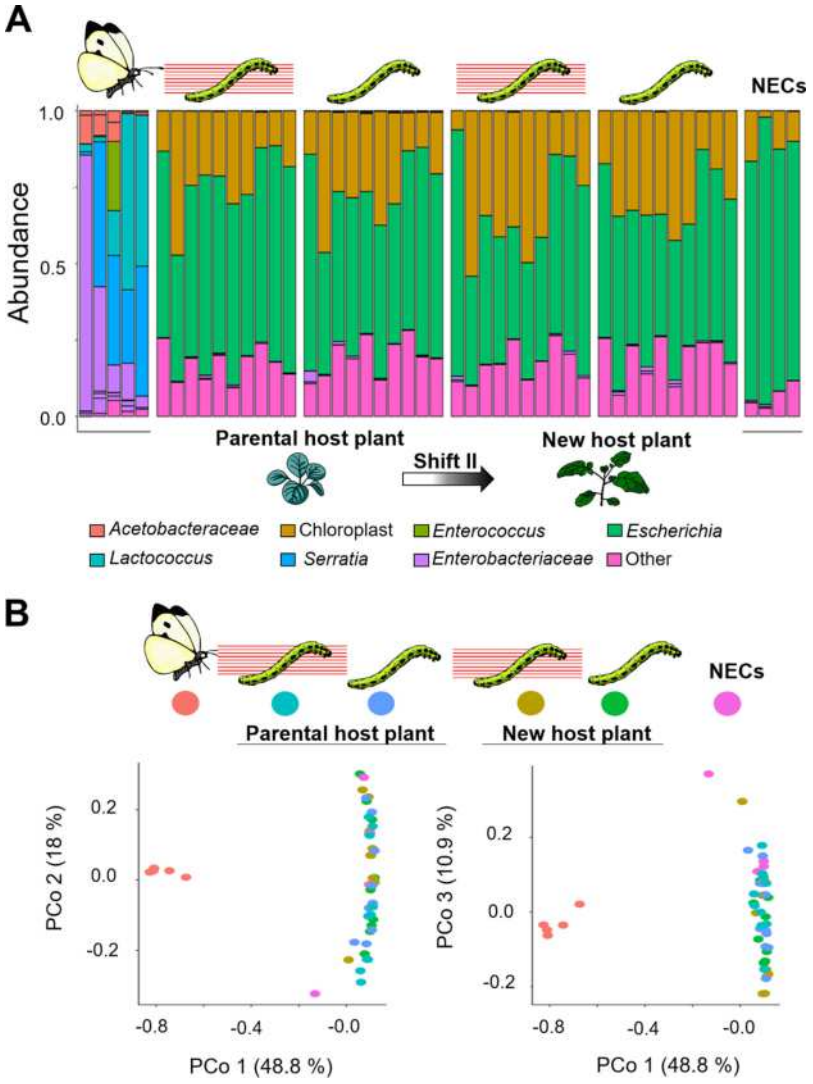

FIG 4 Bacterial community composition in F_0 adults and F_1 larvae of *Pieris brassicae*. (A) Bacterial taxon abundance in F_1 larvae from antibiotic-treated (red stripes) and untreated parents feeding on either the parental host (*Brassica oleracea*) or a new host plant (*B. nigra*). Abundances are shown in comparison to the taxon abundances found in butterflies (positive controls) and negative extraction controls (NECs). (B) Principal-coordinate (PCo) analysis of microbial beta diversity (based on Bray-Curtis dissimilarity) in F_1 larvae from AB-treated and control parents feeding on either the parental host plant (*B. oleracea*) or a new host plant (*B. nigra*), untreated F_0 butterflies, and NECs. The colors representing each type of sample are explained above the plot. A pairwise contrast between bacterial samples from F_1 larvae and F_0 adults shows a significant difference ($P = 0.001$, permutational multivariate analysis of variance [PERMANOVA]).

controls. These results were in line with the outcome of the qPCR analysis (Fig. 2A). In contrast, untreated *P. brassicae* adults contained a clearly defined bacterial community, with *Acetobacteraceae*, *Streptococcaceae,* and *Enterobacteriaceae* as the most abundant taxa.

Furthermore, we directly compared the operational taxonomic units (OTUs) obtained by Illumina sequencing from untreated adult F_0 butterflies to those sequences obtained from isolated bacteria from *P. brassicae*. All major groups found via Illumina sequencing matched to sequences from cultured bacterial isolates (Fig. S3). Hence, we consider the majority of the *P. brassicae* adult bacterial community (at least >98.6%) to be culturable. This comparison increased also the taxonomic resolution of the adult bacterial community, as members of the *Enterobacteriaceae* could be classified to the genus level (*Hafnia* and *Rahnella*).

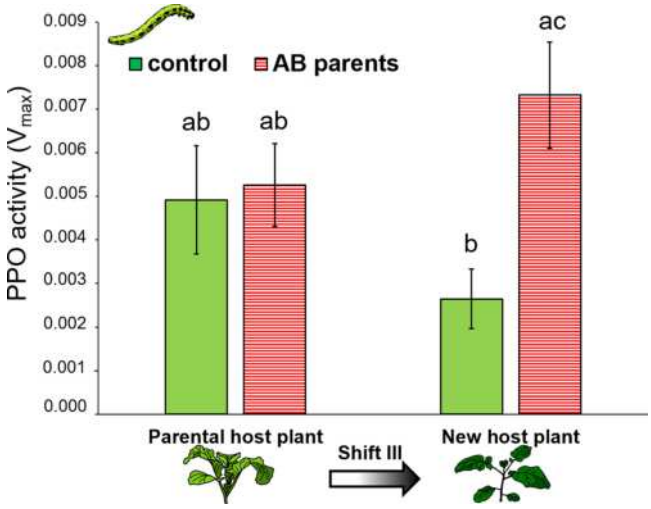

FIG 5 Prophenoloxidase (PPO) activity in the hemolymph of F_1 *Pieris brassicae* larvae. Shown is the PPO (mean ± SE) activity in the hemolymph of larvae from antibiotic (AB)-treated and control parents. Larvae were either feeding on the parental host (*Brassica rapa*) or shifted to a new host plant (*B. nigra*). Enzyme activity was measured during the linear phase of the reaction (V_{max}) (absorbance units/time). Different letters indicate significant differences among the treatments (Kruskal-Wallis test with Dunn's multiple-comparison test *post hoc* BH correction), $P < 0.05$. Each replicate consisted of hemolymph pooled from 2 or 3 larvae feeding on a plant. Data were collected from 10 independent replicates per treatment. We found a significant interaction effect of parental AB treatment and host plant shift ($P < 0.05$, GLM).

We further compared the bacterial communities identified by Illumina sequencing in F_1 larvae and F_0 adults. This comparison was done by principal-coordinate analysis based on Bray-Curtis dissimilarities (beta diversity) and showed a significant separation of samples from larvae and adults. Larval samples clustered together along with the negative controls regardless of parental treatment or host plant shift (Fig. 4B). Hence, F_1 larvae harbor a negligible amount of bacteria, regardless of the parental treatment and host plant.

PPO activity in larval hemolymph is determined by parental treatment and host plant shift. We asked whether the transgenerational effects of parental AB treatment and host plant shift on F_1 larval biomass were linked to differences in immune parameters of the F_1 larvae. As a relevant parameter of the insect's cellular and humoral immune responses, we measured prophenoloxidase (PPO) activity in the hemolymph of F_1 larvae from AB-treated and control parents feeding on either the parental host plant *B. rapa* or the new host plant *B. nigra*.

When F_1 larvae fed on the parental host plant, the PPO activity did not differ between larvae from control and AB-treated parents (Fig. 5). In contrast, when feeding on a new host plant, F_1 larvae of AB-treated parents had significantly higher PPO activity than did the larval offspring of untreated parents (Fig. 5). In line with the biomass results, we found a significant interaction effect of parental AB treatment and host plant shift ($P < 0.05$, GLM).

Hence, differences in PPO activities between larvae from AB-treated and control parents were only detected when F_1 larvae had experienced a transgenerational host plant shift.

Transcriptional changes of larval genes involved in detoxification. We tested whether a transgenerational shift (from *B. rapa* to *B. nigra*, Fig. 1B) elicits changes in the expression of larval genes involved in the detoxification of plant secondary compounds. Furthermore, we asked whether the detrimental effect of the parental AB treatment and host plant shift (on F_1 larval biomass) was linked to alterations in the expression of such genes. We tested expression of the *P. brassicae* genes coding for cytochromes P450 (*Drosophila* homologs *CYP304A1* and *CYP6AE12*) and glutathione-*S* transferase (*GSTD1*), known as general detoxifying enzymes in diverse organisms (42,

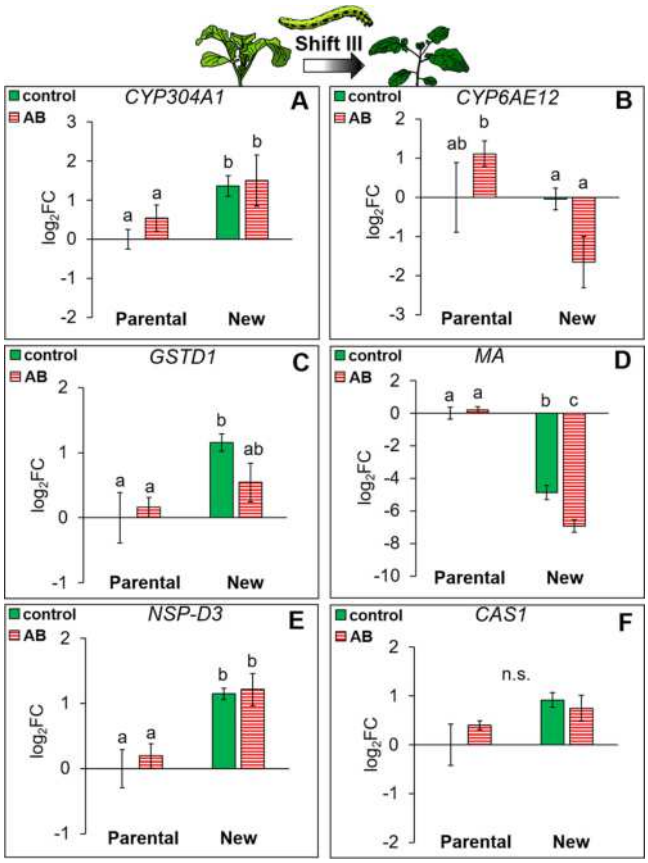

FIG 6 Expression of putative detoxification genes in 7-day-old (F_1) *Pieris brassicae* larvae. (A) *CYP304A1*. (B) *CYP6AE12*. (C) *GSTD1*. (D) *MA*. (E) *NSP-D3*. (F) *CAS1*. Quantitative real-time PCR analysis of putative host plant detoxification genes in 7-day-old untreated F_1 larvae from antibiotic-treated (red-striped bars) and control parents (green bars) when feeding on the parental host plant (*Brassica rapa*) or a new host plant (*B. nigra*). For each gene, expression is shown relative to the control group (the offspring of control parents feeding on the same host plant species as their parents). Data were further normalized to the expression of the housekeeping *EF-1α* gene. For each biological replicate of the 7-day-old larvae, two larvae were pooled per plant. The bars represent the average ± SE $\log_2$ fold change ($\log_2$FC) expression per plant. Different letters above the bars indicate significant differences among the treatment groups for each gene (multiple Student's *t* tests with BH correction, 10 independent replicates per treatment), $P < 0.05$. We found a significant interaction effect of parental AB treatment and host plant shift on *CYP6AE12* and *MA* gene expression ($P < 0.05$ for *CYP6AE12* and $P < 0.01$ for the *MA* gene, GLM).

43). In addition, we tested the expression of two nitrile-specifier protein genes, the major allergen gene (*MA*) and *NSP-D3*, and of another gene coding for cyanoalanine synthase (CAS1), all shown to be upregulated in *Pieris* spp. when feeding on a GS containing host plant (44).

When analyzing larvae from untreated parents, transcript levels of the *CYP304A1*, *GSTD1*, *MA*, and *NSP-D3* genes were different between larvae that fed on the parental host plant and larvae exposed to the host plant shift (Fig. 6). The expression of the *MA* gene was downregulated, whereas the *CYP304A1*, *GSTD1*, and *NSP-D3* genes were upregulated after the host plant shift. The expression levels of *CYP6AE12* and *CAS1* were not significantly affected by the host plant shift.

In a comparison of gene expression in larvae from control and AB-treated parents, we found that gene expression did not differ in larvae feeding on the parental host plant (Fig. 6). Only when experiencing a host plant shift was the *MA* gene significantly downregulated in the offspring of AB-treated parents compared to its expression in the control offspring (Fig. 6). When testing for an interaction effect between parental AB treatment and host plant shift, we found a significant effect on *CYP6AE12* and *MA* gene expression ($P < 0.05$ for *CYP6AE12* and $P < 0.01$ for the *MA* gene, GLM).

Thus, F_1 larvae exposed to a transgenerational host plant shift show a significantly differential expression of several genes putatively involved in GS detoxification. Furthermore, the parental AB treatment results in significantly stronger downregulation of the *MA* gene in host-plant-shifting F_1 larvae from AB parents than that from untreated parents.

DISCUSSION

We tested the hypothesis that a disturbance of the parental microbiota in *P. brassicae* butterflies by a treatment with antibiotics (AB) has effects on the ability of the offspring to cope with a transgenerational host plant shift. We found that a parental AB treatment changes the phenotype of (untreated) F_1 larvae only when they experience a different host plant species than their parents.

In three out of four different host plant shift experiments, larvae that derived from AB-treated parents gained less biomass than control larvae when experiencing a transgenerational host plant shift but not when they fed on the parental host plant. This effect of the parental treatment on host-shifting offspring was not seen in a transgenerational shift from a plant with sinigrin content to a plant without sinigrin, but the effect became obvious when larvae shifted from a parental plant with low (or no) sinigrin content to a plant with higher sinigrin content. These host-shifting larvae from AB-treated parents had also a significantly higher prophenoloxidase (PPO) activity in their hemolymph than did control larvae experiencing the shift. Moreover, they showed lower expression of *MA*, an NSP family gene, than did control larvae after the transgenerational host shift. We will discuss these results with respect to the suggestion that a disturbance of the parental bacterial community has transgenerational effects on the F1 larvae when feeding on a new plant species.

The host plant species determined the biomass of F_1 larvae in an expected manner when they were offspring of control parents. F_1 larvae from untreated parents could increase their biomass when shifting to *B. nigra*, a host plant species well known as an optimal host plant for *P. brassicae* (28, 45). However, when F_1 larvae from AB-treated parents shifted to *B. nigra*, they did not gain more biomass than did F_1 larvae from AB-treated parents feeding on the parental host (*B. oleracea* or *B. rapa*). Very little is known on how the chemical composition of the different brassicaceous species drives the performance of *P. brassicae*. The effect of specific glucosinolates (GS) or their profiles on *P. brassicae* performance is unexplored. Performance of the close relative *P. rapa* is differentially affected by indole and aliphatic GS (37, 38). Whether these GS exert similar effects on *P. brassicae* performance remains to be answered. Our data show that F_1 larvae from control parents gained the highest biomass on the host plant *B. nigra* containing mainly the aliphatic GS sinigrin. The presence or absence of GS *per se* does not necessarily impact *P. brassicae* larval performance, at least when comparing larvae feeding on wild-type and GS-free *Arabidopsis* mutant plants (44). However, *P. brassicae* larvae perform different depending on the host plant species (36, 45). Our study suggests that an antibiotic treatment, which disturbs the adult microbiome, significantly affects whether their larval offspring can optimally adapt themselves to a host plant chemical profile that has not yet been experienced in the parental generation.

In *P. brassicae* larvae, the bacterial presence was practically below the detection limit in both the AB-treated F_0 and the untreated F_1 generation. Bacterial amplicons in larval samples did not differ from those in negative extraction controls. These results suggest that no transgenerational transmission of microbes from the parental to the offspring generation takes place in *P. brassicae*. This supports recent findings showing a lack of resident gut bacteria in lepidopteran larvae as a result of a thorough screening of 124 species from 16 families (23). Furthermore, our data show that, under the laboratory conditions used here, the host plant species do not contribute to the bacterial colonization of *P. brassicae* larvae. Hence, it is highly unlikely that the effects of a parental AB treatment on the performance of transgenerationally host-shifting F_1 larvae are due to a direct disturbance of the bacterial community in the larvae.

In addition to the effect of the AB treatment on the parental microbiome, the AB

treatment could have other direct effects on the adults, which might be passed on to the next generation and affect the performance of the offspring. Treatment of insects with antibiotics is known to exert a wide range of direct effects, including nonspecific changes of the insect metabolic parameters, weight reduction, and increase in mortality (46, 47). To check whether the AB treatment affects the biomass of parental F_0 larvae, i.e., a performance parameter also tested in the F_1 generation, we weighed untreated and AB-treated F_0 larvae feeding upon *B. rapa* or on *B. oleracea*. The AB treatment used in our study did not have a direct effect on the biomass of F_0 larvae, regardless of the host plant upon which they were feeding (Fig. S1). We also did not observe an effect of the parental AB treatment on F_1 larvae, which do not experience a host shift. These findings suggest that direct effects of the parental AB treatment transgenerationally transferred to F_1 larvae seem unlikely, although they cannot fully be excluded. Our results rather suggest that the parental effects on the host-shifting F_1 generation are due to disturbance of the bacterial community of the parental generation or due to direct changes to the physiology of the adults.

Interestingly, the lower biomass of host-plant-shifting F_1 larvae from AB-treated parents was linked to a higher prophenoloxidase (PPO) activity than that with host-shifting F_1 larvae from control parents. In contrast, F_1 larvae feeding on the parental host plant did not differ in their PPO activity, regardless of whether they derived from control or AB-treated parents. Thus, a host plant shift seems to be a special challenge for the F_1 larvae of AB-treated parents because it might expose the larvae to a yet-unexperienced, unfamiliar phytochemistry. A wide range of previous studies have addressed effects of the plant's chemistry on the immunity and performance of herbivorous insects, and several of them found, as in our study, a trade-off between insect performance and immune activity (48).

In the wild, *P. brassicae* larvae might also experience the novel plant species as a challenge because of a yet-unexperienced plant microbiome (49). While the impact of the plant's microbiome on plant immunity against phytopathogens is well known (50), our knowledge on how the plant's microbiome affects the immunity of herbivorous insects is still limited (see, e.g., reference 51). It is tempting, although highly speculative, to ascribe the adult butterfly microbiota a similar effect on the offspring as postulated for the human microbiome by the microbial "old friends" hypothesis (52), which states that human health may depend on the (perinatal) preexposure to microbes, i.e., on "old friends" accompanying mammalian evolution.

The question of the mechanisms by which the parental AB treatment affects the offspring biomass, PPO activity, and gene expression remains open. In *D. melanogaster*, the parental microbiome has been shown to affect oogenesis and embryo development and, thus, offspring traits (53). A study of transgenerational immune priming in the moth *Galleria mellonella* suggests that bacterial fragments are transferred to the eggs from females fed on bacterium-containing food (11), thereby mediating immune priming in the offspring. In *Manduca sexta*, maternal transgenerational immune priming is facilitated by bacterial elements translocated from the female's gut to the eggs, resulting in transcriptional reprogramming of immune-related genes involved in histone acetylation and DNA methylation in the offspring (54).

We found an inverse relationship between expression of the NSP family gene *MA* and immune (PPO) activity. *MA* gene expression levels were significantly lower in host-shifting F_1 larvae from AB-treated parents than from control parents, while PPO activity was higher in host-shifting F_1 larvae from AB parents than from control parents. An inverse relationship between immune defense and detoxification ability was also found in other lepidopteran species (17, 55), suggesting that an efficient immune defense may act at the cost of detoxification of plant compounds. The strong down-regulation of the *MA* gene in host-shifting F_1 larvae from AB-treated parents parallels their low biomass gain, thus giving rise to the suggestion that elimination of bacteria in the parental generation might prevent the offspring from achieving the optimal expression of the *MA* gene to cope with a novel host plant species. However, differ-

ential expression levels of just one gene cannot be used as an explanation for the different larval biomass in host-shifting F_1 larvae from control and AB-treated parents.

The other five of the six studied detoxification genes (*CYP304A1*, *CYP6AE12*, *GSTD1*, *NSP-D3*, and *CAS1*) showed no differences in expression levels when directly comparing host-shifting F_1 larvae from control and AB-treated parents. However, three of these genes (*CYP304A1*, *GSTD1*, and *NSP-D3*) were significantly upregulated when larvae were exposed to a transgenerational host plant shift. This finding is in line with other studies which showed that a shift to a new host plant is likely to elicit a differential expression of detoxification genes in the herbivorous insect when the new host plant differs in its content of secondary metabolites (56–59). The contrasting expression patterns of the *MA* gene (downregulation upon host shift) and *NSP-D3* (upregulation upon host shift) are in line with a study on *Pieris melete* showing high expression levels of the *MA* gene but low ones of *NSP* on one plant species, and *vice versa*, high *NSP* expression levels and low *MA* gene expression on another one with a different GS profile (36). Genes of the NSP family have been described as the key innovation to enable pierid species feeding on Fabaceae to shift to Brassicaceae (35). Here, we show that the ability to differentially express such detoxification genes may be a key trait promoting adaptive phenotypic plasticity when facing different host plant species across generations.

Our findings show that a parental treatment of Lepidoptera adults with antibiotics significantly disturbs the adult microbiome but does not affect the microbial abundance in offspring larvae. Interestingly, the parental treatment influences the larval offspring only when this is exposed to a transgenerational host plant shift. When feeding on a host plant with a different phytochemical profile than their parents, larval offspring of AB-treated parents gained less biomass, had higher prophenoloxidase activity, and had lower expression of a potential key GS detoxification gene (*MA* gene) than did the offspring of untreated parents. Hence, our study suggests a complex interplay of the parental microbiome, host plant chemistry, and offspring performance and immunity. More detailed insights in how such a complex interplay works on the transcriptional level need to be provided by future studies using RNA sequencing (RNA-seq) analyses of larvae from control and AB-treated parents and feeding on the parental or novel host plants. While a high microbial abundance in insects has been shown to positively impact immune priming within a generation (see, e.g., reference 60), future studies need to further elucidate how the parental microbiome affects the immune responses of the offspring to entomopathogens or parasitoids (61). These future studies, as well as the current one, contribute to shedding some light on the adaptive phenotypic plasticity of oligophagous, multivoltine insects switching between various host plant species during a season.

MATERIALS AND METHODS

Insect rearing and parental antibiotic treatment. The *P. brassicae* F_0 generation was reared on either Brussels sprouts (*B. oleracea* var. *gemmifera*) or on Chinese cabbage (*B. rapa* subsp. *pekinensis*). For each experiment, we reared two F_0 lines, one being the control line and the other line treated with antibiotics (AB) (Fig. 1A). The F_1 generation did not receive any AB treatment. The untreated larvae of the F_0 generation (control line) were reared on untreated plants, while the antibiotic-treated larvae (AB-treated line) were reared on plants sprayed with a cocktail of four antibiotics (ampicillin, chloramphenicol, rifampin, and streptomycin [Sigma-Aldrich]), each in a concentration of 0.5 mg/ml H_2O, until the plant surface was uniformly covered with the solution. These concentrations had no effect on the larval weight in the F_0 generation (see Fig. S1). Similarly, these antibiotics did not affect larval mortality and pupal weight when tested in such concentrations on larvae of the diamondback moth, *Plutella xylostella* L. (46).

The adults of the untreated F_0 generation were fed with a 15% (wt/vol) honey-water solution and the AB-treated F_0 adults with 15% (wt/vol) honey dissolved in the above-mentioned combination of antibiotics (0.5 mg/ml H_2O). The honey solution was provided every second day in 1.5-ml Eppendorf tubes placed in the center of artificial flowers. Control and AB-treated larvae of the F_0 generation were reared in a climate chamber (18-h light/6-h dark cycle, 160 μmol m^{-2} s^{-1} light, 20°C, 50 to 70% rH) until pupation. F_0 pupae were then transferred to a separate climate chamber (18-h light/6-h dark, 220 μmol m^{-2} s^{-1} light intensity, 23°C, 70% rH), where adult butterflies emerged. F_1 larvae were never treated with antibiotics.

Plants. *Brassica rapa* subsp. *pekinensis* seeds were obtained from International Seeds Processing (ISP) GmbH in Quedlinburg, Germany. *Brassica oleracea* var. *gemmifera* seeds were purchased from Hermina-

Maier GmbH in Regensburg, Germany, and *B. nigra* seeds were obtained from the Centre for Genetic Resources, The Netherlands (CGN; accession number CGN06619). Seeds were sown on medium consisting of soil (Einheitserde Typ P; Kausek, Mittenwalde, Germany) and vermiculite (Kausek) in a ratio of 3:1 (wt/wt). They germinated in a climate chamber under long-day conditions (18-h light/6-h dark, 85 μmol $m^{-2}\,s^{-1}$ light, 22°C, 50% rH). One week after sowing, the seedlings were transplanted to individual pots and grown for 5 weeks prior to the host plant shift experiments.

Host plant shift experiments. A transgenerational shift from one host plant species to the other was performed as follows: in a climate chamber (18-h light/6-h dark, 220 μmol $m^{-2}\,s^{-1}$ light intensity, 23°C, 70% rH), a group of 30 to 35 gravid females (either untreated or AB treated) were placed in a cage and offered a host plant (parental or new host plant) for oviposition. Plants laden with approximately 20 to 25 egg batches originating from different females were transferred to a different climate chamber (18-h light/6-h dark, 85 μmol $m^{-2}\,s^{-1}$ light intensity, 22°C, and 50% rH), where larvae hatched 5 days later. Neonate offspring (F_1) from AB-treated and control parents (F_0) were divided by random selection into two treatment groups, as follows: (i) feeding on the same host plant species as the F_0 generation (parental host plant) or (ii) feeding on a different host plant species with a different glucosinolate (GS) profile from that experienced by the F_0 generation (new host plant), as summarized in Fig. 1.

Larval biomass. Larvae were collected 7 days after feeding, and their biomass was measured using a microbalance (Sartorius Lab Instruments GmbH & Co.). The average larval weight per plant was then calculated. Each host plant shift experiment comprised 10 to 12 biological replicates (plants) per treatment.

Insect DNA isolation. Insects were individually frozen in liquid nitrogen and bead homogenized for 15 s at 4,500 rpm in a tissue homogenizer (Precellys Evolution). DNA was extracted with the Qiagen DNeasy blood and tissue kit, following the manufacturer's instructions, with an extra lysozyme digestion step for 30 min (Epicentre Ready-Lyse) after the addition of the lysis buffer to maximize the lysis of Gram-positive bacteria.

Determination of bacterial abundance in parental F_0 generation by CFU counts and qPCR. We investigated how the AB treatment affected bacterial presence in the parental (F_0) insect generation by counting microbial CFU (in adults) and rRNA gene copy numbers (in adults and larvae).

For the quantification of CFU, 4-week-old female butterflies (without head and wings, $n = 9$ for control and AB groups) were ground in sterile phosphate-buffered saline (PBS) solution (pH 7.4). Samples were serially diluted (10^{-2} to 10^{-7}) in PBS solution, and droplets of 20 μl were spotted on tryptic soy agar (TSA) with and without supplementation of the antibiotic cocktail at 50 μg/ml (ampicillin, chloramphenicol, rifampin, and streptomycin). CFU were counted after overnight incubation at 30°C.

For assigning the colonies to OTUs, we subcultured those with different morphologies, amplified their full-length rRNA genes, and sequenced them as previously described (62). Isolates were identified to the genus level using EZBioCloud (https://www.ezbiocloud.net/).

To determine the rRNA gene copy numbers, we isolated genomic DNA from 12-day-old control and AB-treated larvae (F_0), as well as from 4-week-old control and AB-treated female butterflies. We performed qPCRs and determined bacterial abundances by using relative quantification of the rRNA genes against host 18S ribosomal DNA (rDNA). The qPCR assay was performed on a Stratagene Mx3005P system with the reaction mixture containing 5 μl PowerUp SYBR green master mix (Thermo Fisher Scientific), 300 nM forward and reverse primers, and 50 to 100 ng template DNA. The cycling conditions included activation for 2 min at 50°C, 2 min at 95°C, 45 cycles at 95°C for 15 s and 60°C for 1 min, followed by a dissociation curve as the final step. We used the custom primer pair 558FAW (5′-GGAWTKAYTGG GCGTAAAGNGMDC-3′) and 783RAW (5′-RTGGACTACCAGGGTATCTAATCCTG-3′) for 16S quantification, which excludes the amplification of insect host DNA and minimizes coamplification of fungal and plant chloroplast sequences (62). Bacterial abundance was normalized against the abundance of insect 18S rDNA sequences using the *P. brassicae*-specific primers 18SF2 (5′-TCAAAGCGGGCTCAAAATGC-3′) and 18SR3 (5′-TTCGCTGATGTTCGTCTTGC-3′). The relative abundance of 16S rRNA gene copy numbers to 18S copy numbers was calculated using the ΔC_T method. Amplification of AB-treated adults was 3.8 cycles above the no-template control (NTC) water samples.

Sequencing of the bacterial community of *P. brassicae* using the Illumina platform. We explored the effects of parental treatment and host plant shift on the composition of the microbiome of 7-day-old larvae from AB-treated and control parents when they fed on either the parental host (*B. oleracea*) or a different host (*B. nigra*) plant (Fig. 1B, shift II).

Following DNA extraction, we performed PCR amplification of the bacterial 16S rRNA gene using the 341F and 805R primers, which target the bacterial V3-V4 16S rRNA gene regions. The following primers were designed with an Illumina overhang adapter sequence for compatibility with the Illumina index and sequencing adapters: Illumina_16S_341F-5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGN GGCWGCAG-3′ and Illumina_16S_805R-5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVG GGTATCTAATCC-3′. PCR was done using the JumpStart *Taq* ReadyMix from Sigma-Aldrich. Fifty nanograms of DNA template was used in a total reaction volume of 50 μl. Cycling parameters consisted of an initial denaturation cycle at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, and a final extension cycle at 72°C for 5 min. PCR products were separated on a 1.2% agarose gel stained with ethidium bromide and visualized under UV light. PCR products were purified with AMPure beads (Beckman Coulter, Brea, CA, USA) and ligated to barcoded Illumina adapters. Each library contained a specific combination of index adapters (dual indexed) to allow later discrimination of samples after pooling. A total of 10 biological replicates per treatment were processed.

Negative extraction controls (NEC) (containing no insect sample) were processed in parallel to control for bacterial DNA contamination. Homogenates (5 mg) of samples from untreated F_0 adults (without head and wings) were included as positive controls.

The pooled, barcoded samples were sequenced at the Berlin Centre for Genomics in Biodiversity Research (BeGenDiv) on a MiSeq platform v3 (Illumina, San Diego, CA, USA) using 600 cycles of (paired-end) sequencing. Sequencing reads were trimmed, denoised, and overlapped using a full-stack R pipeline (63) incorporating dada2 (64) and phyloseq (65). Forward and reverse reads were trimmed to 275 bp and 175 bp, respectively, truncated at the first instance of a quality score less than 2, and filtered on a maximum expected error rate of 2 errors per truncated read. The remaining forward and reverse reads were dereplicated and denoised using a parameterized model of substitution errors. The resulting denoised read pairs were merged and subjected to *de novo* chimera removal. Taxonomy was assigned using the Ribosomal Database Project training set version 16.

Supplementing OTU resolution obtained by Illumina sequencing with information from culturable bacterial isolates. To improve the phylogenetic resolution of the data set obtained by the Illumina sequencing, we compared the major groups of the bacterial community obtained from control adults (OTUs with in sum >98.6% relative abundance) with 16S rRNA gene sequence information from the culturable isolates obtained from *P. brassicae*. Sequences were trimmed to the same length matching the OTU fragment size (~180 bp) and aligned using ClustalW, with a gap opening penalty of 15 and gap extension penalty of 6.66. The alignment was used to construct a maximum likelihood tree using the Tamura-Nei model and the maximum composite likelihood approach including bootstrapping as implemented in MEGA X (66).

Collection of larval hemolymph and PPO activity assay. We analyzed how the AB treatment of *P. brassicae* in the F_0 generation and/or a transgenerational host plant shift affect the prophenoloxidase (PPO) activity of F_1 larvae. We used F_1 larvae (7 days old) of the AB-treated F_0 generation and, for a control, larvae of the same age of the untreated F_0 generation. We analyzed both offspring larvae feeding on another host plant species than their parents (*B. nigra*) and, for a control, larvae feeding on the parental host (*B. rapa*).

For hemolymph collection, the larvae were immobilized by placing them at –20°C for 10 min and then briefly washing them in PBS solution (pH 7.4) followed by 70% ethanol. Thereafter, they were placed on a clean filter paper to dry. An incision was made at the ventral side between the medial prolegs to collect hemolymph with a 10-μl pipette. The collected hemolymph sample was flash frozen in liquid nitrogen and stored at −80°C until further analysis. Hemolymph samples were centrifuged for 10 min (9,000 × *g*, 4°C) and diluted 1:20 by mixing 5 μl of the supernatant with 95 μl of 10 mM sodium phosphate buffer (pH 6.6) containing 0.2 mg ml^{-1} chymotrypsin (Sigma) solution.

To determine the PPO activity, 50 μl of the hemolymph sample was placed into a 96-well plate, and 100 μl of a 3 mM L-DOPA (3,4-dihydroxyphenylalanine [Sigma]) solution dissolved in 10 mM sodium phosphate buffer [pH 6.6] was added to each well using a multichannel pipette. The samples were incubated at 30°C in a microplate reader (Tecan Infinite 200Pro) with a total of 400 readings every 15 s at 490 nm for 100 min. The output was analyzed using the program PO-CALC (67), and the slope of this reaction curve in its linear phase (V_{max}) was used as readout for the enzyme activity, similarly to what was done in references 68 and 69. Values below 0.02 were not considered for the statistical analysis, as this was the highest value obtained from the hemolymph sample without the addition of L-DOPA.

Isolation of larval RNA and gene expression analysis by qPCR. We measured changes in the expression of six putative detoxification genes of *P. brassicae* F_1 larvae. We analyzed 7-day-old larvae used in the host plant shift experiments (shift from *B. rapa* to *B. nigra*). Two individual larvae per plant were pooled for each biological replicate.

Total RNA was extracted as described by Oñate-Sánchez and Vicente-Carbajosa (70). Genomic DNA was removed using RNase-free DNase I (Thermo Fisher Scientific), according to the manufacturer's instructions, for 30 min at 37°C. Strand cDNA was synthesized from 2 μg total RNA in 10-μl reaction mixtures with RevertAid RT reverse transcription kit (Thermo Fisher Scientific) and oligo$(dT)_{18}$, following the manufacturer's protocol.

Quantitative real-time PCR (qPCR) analyses were performed in 10-μl reaction mixtures containing 1 μl cDNA, 250 nM each gene-specific primer, and 5 μl Power SYBR green PCR master mix (Applied Biosystems) on a MX3005p real-time PCR system (Stratagene Systems, WA, USA). The thermal profile was 10 min at 95°C, followed by 45 cycles each of 90 s at 95°C and 60 s at 60°C. The primers for the tested genes of *P. brassicae* were designed using the Primer3 software based on the transcriptomic data from reference 44. The primer sequences used for transcript analysis are shown in Table 1. We calculated relative expression levels as specified by Livak and Schmittgen (71) using elongation factor 1 alpha (*EF-1αF1*, 5′-TGAAATGCACCACGAAGCTC-3′; and *EF-1αR1*, 5′-AACCACGACGCAATTCCTTG-3′) as the reference gene.

Statistical analysis. Statistical analyses of the data on bacterial quantification, larval performance, PPO activity, and gene expression were conducted with R (version 3.5.1). Data were tested for normal distribution (Shapiro-Wilk test) and variance homogeneity (Levene's test). According to the distribution of the data, we used either multiple Student's *t* test with Benjamini-Hochberg *post hoc* correction or the Kruskal-Wallis test followed by Dunn's multiple-comparison test with Benjamini-Hochberg *post hoc* correction. To test for an interaction effect of parental treatment and host plant shift, data on larval biomass, PPO activity, and gene expression were additionally analyzed using a generalized linear model (GLM; Gaussian distribution), with parental antibiotic treatment and host plant shift (scored as "yes" or "no") as fixed factors and biomass, PPO activity, gene expression as response variables.

TABLE 1 Primers used for gene expression analysis of putative detoxification genes known to be upregulated in *Pieris brassicae* after uptake of glucosinolates[a]

Primer by target	Sequence (5′→3′)	Description	References
Cytochrome P450			
CYP304A1 F	AGACTGTGTGCTGGTGAAAC	Methanol detoxification in *Drosophila* spp.	44, 74
CYP304A1 R	TGGAGACGTGGTAAGAGTGCAT		
CYP6AE12 F	AACTTGACGATGGCAGCAAC	Gossypol-induced in *Helicoverpa armigera*	44, 75
CYP6AE12 R	GGCGGCTGACAACTTTAATACC		
Glutathione-*S* transferase			
GSTD1 F	AACGTCTGCACTTTGACAGC	GS detoxification in *Scaptomyza* spp.	44, 76
GSTD1 R	TGAACTCGTACGCAGATGTG		
Nitrile-specifier proteins			
MA F	TGAAGCTTTGGAAGGTGCTC	GS detoxification in pierids (*NSP*-like genes)	35, 44, 77
MA R	TTGGAGCGTCAATAGCGTTG		
NSP-D3 F	AAAGGAGCCTGAAGTGCAAG		
NSP-D3 R	CGTTTGCGTGCAACTTTTGG		
Cyanoalanine synthase			
CAS1 F	TGCAGCGAGCTATTCACATG	CAS homolog	44, 78
CAS1 R	TCTTCAGCTGCTTTGACGTC		

[a]Cytochromes are named after their *Drosophila melanogaster* homologs. Genes were selected based on a study comparing the transcripts of *P. brassicae* larvae feeding on glucosinolate-deficient mutants or wild-type (Col-0) plants (44).

For the statistical analysis on bacterial community composition, 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 (65). Multivariate analysis of variance (MANOVA) was tested using vegan (72). Differential abundance testing was performed using DESeq2 (73) in conjunction with phyloseq.

Data availability. The rRNA gene sequences of *Pieris* isolates were deposited in GenBank under the accession numbers MN833383 to MN833385. Illumina sequencing data were deposited in the SRA database under the BioProject accession number PRJNA555186.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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L.R.P.V., A.W., N.E.F., and M.H. conceptualized the study. L.R.P.V. and A.W. performed the experiments. P.R.J. performed the sequencing analysis. L.R.P.V. and M.H. wrote a first draft of the manuscript. All authors contributed to later versions of the manuscript and agreed with the final version.

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