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Research

Interspecific Differences in the Larval Performance of *Pieris* Butterflies (Lepidoptera: Pieridae) Are Associated with Differences in the Glucosinolate Profiles of Host Plants

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Abstract

The tremendous diversity of plants and herbivores has arisen from a coevolutionary relationship characterized by plant defense and herbivore counter adaptation. Pierid butterfly species feed on Brassicales plants that produce glucosinolates as a chemical deterrent against herbivory. In turn, the larvae of pierids have nitrile specifier proteins (NSPs) that are expressed in their gut and disarm glucosinolates. Pierid butterflies are known to have diversified in response to glucosinolate diversification in Brassicales. Therefore, each pierid species is expected to have a spectrum of host plants characterized by specific glucosinolate profiles. In this study, we tested whether the larval performance of different *Pieris* species, a genus in Pieridae (Lepidoptera: Pieridae), was associated with plant defense traits of putative host plants. We conducted feeding assays using larvae of three *Pieris* species and 10 species of the Brassicaceae family possessing different leaf physical traits and glucosinolate profile measurements. The larvae of *Pieris rapae* responded differently in the feeding assays compared with the other two *Pieris* species. This difference was associated with differences in glucosinolate profiles but not with variations in physical traits of the host plants. This result suggests that individual *Pieris* species are adapted to a subset of glucosinolate profiles within the Brassicaceae. Our results support the idea that the host ranges of *Pieris* species depend on larval responses to glucosinolate diversification in the host species, supporting the hypothesis of coevolution between butterflies and host plants mediated by the chemical arms race.

Key words: *Pieris* butterfly, Brassicaceae plant, coevolution, host preference, larval performance

Ehrlich and Raven (1964) introduced the 'plant–herbivore coevolutionary theory' to explain the remarkable diversity of plants and herbivorous insects. Plants evolved novel chemicals to defend against herbivores, and in turn herbivores evolved adaptive traits to counter these defenses. This 'arms race' has contributed to the diversification of both plants and herbivores. Chemical defense profiles in plants tend to be specific to species or higher taxonomic groups (Futuyma and Agrawal 2009). Additionally, the variety in plant defenses is thought to affect herbivore host spectra, as herbivores can only detoxify a limited range of plant chemicals (Janz 2010).

Plants are known to produce an enormous variety of secondary metabolites that function as chemical defenses against herbivores (Fraenkel 1959, Ehrlich and Raven 1964, Futuyma and Agrawal 2009). Accordingly, numerous chemically mediated interactions between plants and herbivore groups have also been reported (Futuyma and Agrawal 2009). For example, interactions between plants of the Brassicales order and pierid butterflies have been well studied (Edger et al. 2015). Plants of the Brassicales order produce secondary metabolites known as glucosinolates (GLSs) as a chemical defense against herbivory (Hopkins et al. 2009). Upon damage to plant tissues, GLSs are hydrolyzed by myrosinase enzymes stored in specialized plant cells, producing compounds such as epithionitriles, nitriles, or toxic isothiocyanates (Wittstock and Halkier 2002). GLSs are composed of three building blocks: a β-thioglucose moiety, a sulphonated oxime moiety, and a variable side chain (Mithen 2001). More than 140 GLSs have been identified to date and variations in GLS structure are mainly due to differences in the side chains (Fahey et al. 2001, Olsen et al. 2016). In general, GLSs are sorted into aliphatic-, benzylic-, and indolic GLSs, depending on which amino acids were used in their biosynthesis (Wittstock and Halkier 2002). Side chain elongation or modification also occurs in the biosynthetic process, leading to the high diversity of GLSs observed in Brassicales.

As a result of this side chain diversity, the products of GLS breakdown are also highly diverse, with a variety of functions and effects on herbivores (Beekwilder et al. 2008, Müller et al. 2010, Winde and Wittstock 2011).

Butterfly larvae of the Pieridae family can overcome this GLS defense system by expressing nitrile specifier proteins (NSPs) in their guts. The NSPs redirect the GLS hydrolysis reaction to produce nontoxic nitriles instead of toxic isothiocyanates (Wittstock et al. 2004). It has been hypothesized that the high diversification rate of pierid butterflies is a consequence of acquiring the ability to produce NSPs when the pierid lineage first evolved (Wheat et al. 2007). Thus, the evolution of NSP production was a significant event in pierid evolutionary history (Wheat et al. 2007, Heidel-Fischer et al. 2010). A recent phylogenetic study revealed that the speciation rate of pierid butterflies increased in tandem with GLS diversification events in Brassicales, implying that coevolution between the two groups is mediated by GLS diversification (Edger et al. 2015).

Host plant shifts may represent the first step towards reproductive isolation and ultimately speciation of phytophagous insects. These speciation events subsequent to ecological niche differentiation are known as ecological speciation (Rundle and Nosil 2005, Matsubayashi et al. 2010, Ohshima 2010). If codiversification in both Pieridae and Brassicales were mediated by GLS diversification, the host preference and larval performance of each pierid species may be expected to correspond to specific plant GLS profiles. Although pierid butterflies use the same general mechanisms, namely NSPs, to overcome the GLS-based defenses of their host plants, they also exhibit interspecific host preference differences (Chew 1980). The host preferences of pierid butterflies have been examined in previous studies, but the associations of specific host preference, larval performance, and GLS profiles in host plant species have not been tested in detail (Renwick and Lopez 1999).

The objective of this study was to identify plant defense traits that explain differences in larval performance among *Pieris* spp. that feed on plants of the Brassicaceae family. We investigated the responses of three closely related butterfly species, *Pieris melete* Ménétriès, *Pieris napi* Linnaeus, and *Pieris rapae* Linnaeus (Lepidoptera, Pieridae) to potential host plants comprising 10 species of the Brassicaceae family. In several field observations, these species are known to have different host plant preferences as *P. melete* and *P. napi* tend to use wild brassicaceous plants, whereas *P. rapae* more frequently uses *Brassica* crops as host plants (Ohsaki 1979, Ohsaki and Sato 1994, Ohsaki and Sato 1999). We conducted comprehensive feeding experiments, measured five plant physical defense traits, and analyzed the GLS profile of each plant species. Larval performance differed among the three *Pieris* spp., and was affected by both physical and GLS defenses. Additionally, differences in larval performance among *Pieris* spp. were associated with differences in GLS profiles among host plants, but not with differences in the physical traits of the host plants.

Material and Methods

Feeding Experiments

Female butterflies of the three *Pieris* spp. used in this study were collected in the field in Hokkaido (*P. napi* and *P. rapae*) and Chiba prefecture (*P. melete*), Japan. In the laboratory, the female butterflies laid eggs on leaves of *Brassica oleracea* var. *capitata* or *Cardamine occulta* under high light intensity. The parts of the leaves where eggs were laid were cut out and partially dried to dissuade newly hatched larvae from feeding before the feeding experiments started. Eggs were incubated at 25°C until they hatched.

The seeds of the plant species *Arabidopsis kamchatica* (DC.) K.Shimizu et Kudoh, *Arabidopsis thaliana* (L.) Heynh., *Arabis hirsuta* (L.) Scop., *Brassica napus* L., *Cardamine hirsuta* L., *Cardamine occulta* Hornem., *Cardamine regeliana* Miq., *Draba nemorosa* L., *Nasturtium officinale* R.Br., *Rorippa indica* (L.) Hiern were collected from wild populations (Supplementary Table S1). Plants were grown at 25°C and 60% relative humidity, and with a light/dark cycle of 16:8 L:D. Plants were watered and fertilized weekly with a Hyponex solution that was diluted 2,000×, and had an N:P:K ratio of 6:10:5 (Hyponex, Osaka, Japan). Plants were used for feeding experiments and measurement of leaf traits after 2 mo of cultivation.

We collected neonate larvae within 12 h after hatching and placed them onto study plants with a soft-haired brush. Each plant hosted three larvae from one of the *Pieris* spp., and two plants from each plant species were used per *Pieris* sp. in each feeding experiment. To minimize changes of plant condition across the feeding experiments of each *Pieris* species, we started all the feeding experiments as simultaneously as possible (within 5 d). After placement, larvae were allowed to feed for 5 d and then weighed (to 0.1 mg). Feeding experiments were replicated twice for all three *Pieris* spp. (n = 12 larvae per *Pieris* sp. for each plant species). However, the host plant *C. regeliana* was only used in the first feeding experiment (n = 6 larvae). Regarding *P. napi*, we placed two larvae on each plant per host species in the second feeding experiment (n = 10 larvae).

Measurement of Plant Defense Traits

We measured six leaf traits associated with plant defense: leaf toughness, trichome density, water content, specific leaf area (SLA), C:N ratio, and individual concentrations of 22 GLS types (if detectable). Physical leaf traits were measured using five plant replicates. Leaf toughness was measured using a force gauge penetrometer (1 mm radius) on undamaged leaves (Feeny and Jul 2007). A CN coder (NC-220F; Sumika, Tokyo, Japan) was used to measure C:N ratios. For trichome density, water content, and SLA, we prepared 10 leaf discs of area 7.8 mm^2 each for each plant species (two discs from each plant replicate). Trichomes were counted on both sides of each disc under 10–40× magnification using a stereomicroscope, and mean trichome density (trichomes/mm^2) for each plant species was calculated. The SLA and water content were quantified by comparing wet and dry weights of leaf discs for each species.

To measure the typical GLS profile of each plant species, we sampled three plant individuals from each species from wild populations as representatives. We froze the fresh plant materials at –20°C immediately after sampling. Undamaged leaves from the samples were then selected and freeze-dried. Freeze-dried leaves were ground using Stainless Steel Beads (QIAGEN), and 2 mg of ground leaves were analyzed for each plant individual. We conducted a widely targeted metabolome analysis using tandem quadrupole mass spectrometry coupled with ultra-performance liquid chromatography (UPLC; n = 3; Sawada et al. 2009). The freeze-dried samples were weighed 2 mg each in 2 ml tube, and 5 mm zirconia bead and 500 µl of extraction solution (0.1% formic acid in 80% MeOH) were added in the 2 ml tube. The samples were extracted by beads shocker for 2 min at 1,000 rpm (Shake Master NEO, Biomedical Science). The extracted solution was transferred to 2 ml tube and dried up by nitrogen gas. The samples were dissolved by ultrapure water of LC-MS grade. One microliter of sample solution (final concentration 40 µg samples/µl) was injected to LC-QqQ-MS system (UPLC-TQS, Waters). The LC separation was carried out by the reverse phase column (ACUITY HSS T3 1.0 × 50 mm, particle size 1.8 µm) with the separation conditions: solvent A, 0.1% formic acid in ultrapure water; solvent B,

0.1% formic acid in MeCN, gradient conditions: 0 min, B solution 0.1%; 0.25 min, B solution 0.1%; 0.40 min, B solution 9%; 0.8 min, B solution 17%; 1.9 min, B solution 99.9%; 2.1 min, B solution 99.9%; 2.11 min, B solution 0.1%; 2.7 min, B solution 0.1%. MS detection condition was followed: capillary voltages, positive mode 0.5 kV and negative mode –0.8 kv; source temperature, 150°C; desolvation gas flow, 1,000 (liter/hour). Negative controls that only included an internal standard (10-camphorsulfonic acid) were run three times to characterize background noise. Peaks with signal/noise ratios > 30 represented GLS detection. GLSs were identified based on standard libraries of 22 types of GLSs listed in Supplementary Table S2 (Sawada et al. 2009). The relative concentration of each GLS detected was calculated by comparing the peak area with the peak area of the internal standard (10-camphorsulfonic acid). The GLSs detected were categorized according to their chemical classes, i.e., aliphatic, benzylic, and indolic (Supplementary Table S2).

Statistical Analyses

Comparisons of larval performance

For each *Pieris* sp., we standardized larval growth data for each feeding experiment by 'scale()' function in R Studio for interspecific comparisons (R Studio Team 2016). Mean standardized larval growth, representing larval performance, was then calculated for each host plant species. We performed Tukey HSD test to see performance differences among larvae that fed with different host plants in each species. We conducted hierarchical clustering and the Pearson's correlation test to determine if there were any similarities in larval performance across butterfly species.

Multivariate analyses to investigate plant defense and larval performance

We used multivariable regression analysis to see which plant defense potentially affect the larval performance of the three *Pieris* spp. Since plants use various defense traits as multiple defenses, we summarized measured plant defense traits by principal component analysis (PCA) and used each PC axes as defense profiles. PCA was conducted separately for physical traits and GLSs detected with scaling the variables. We retained the PCs with a cumulative proportion of at least 80% for each PCA.

As the larval weights of each *Pieris* sp. were not normally distributed ($P < 0.01$, Kolmogorov–Smirnov test), we conducted a generalized linear mixed model (GLMM) analysis with Gamma distribution (log link), setting larval weight as the response variable, the PCs representing plant traits as the explanatory variables, and the two feeding experiments as a random effect. The GLMM analyses using the PCs representing physical traits and GLS defenses were conducted separately to avoid multicollinearity. For each GLMM analysis, we selected the best model based on the Akaike information criterion for small sample sizes (AICc). The model with the lowest AICc was selected as the best model if it was > 2 points lower than the AICc value of the null model (ΔAICc = AICc *null model* – AICc *best model*). GLMM analyses were conducted in R Studio with the packages 'lme4' and 'MuMIn' (Bates et al. 2015, R Studio Team 2016, Bartoń 2018).

Multiple regression analysis to compare interspecific larval performance

The larval performance of *P. rapae* was different from that of *P. melete* and *P. napi*. Thus, we conducted a multiple regression analysis to identify the plant defense profiles associated with this difference. First, we conducted linear regressions using the standardized growth of *P. rapae* as the explanatory variable and the standardized growth of *P. melete* or *P. napi* as the response variable. The residuals (observed minus estimated values) of the regression for each pair of species were calculated and then pooled into a statistic representing the difference in larval performance between *P. rapae* and the other two species. As the values for differences in larval performance followed a normal distribution ($n = 20$, $P = 0.952$, Kolmogorov–Smirnov test), we conducted multiple linear regression with the PCs as explanatory variables. Model selection was based on AICc as described above.

Regression Tree analysis

Since PCA handled defense traits as combined defense profiles, we also performed regression tree analysis to find the most important defense trait that affect differently to the larval growth of different *Pieris* spp. (Breiman et al. 1984). In this analysis, we used standardized larval growth as response variable and all the measured defense traits were used as explanation variables. We used the 'rpart' packages in R studio for this analysis and selected the best tree with lowest cross-validate relative error (Therneau and Atkinson 2018).

Results

Larval Performance of *Pieris* spp.

The Tukey's Honest Significant Difference (Tukey HSD) tests showed that larvae of *P. melete* and *P. napi* significantly grew better on *R. indica* than on some of the other plant species (*P. melete*, *A. thaliana*, *P. napi*; *D. nemorosa*, and *C. hirsuta*) ($P \leq 0.05$; Fig. 1). *Pieris rapae* did not show this trend on *R. indica*, however, showed significantly higher larval growth on *Cardamine* spp. ($P \leq 0.05$; Fig. 1). The larval performances of *P. melete* and *P. napi* were significantly and positively correlated across all plant species (Fig. 2a, $P = 0.007$, $r^2 = 0.615$, Pearson's correlation test), but the larval performance of *P. rapae* was not significantly correlated with either *P. melete* ($P = 0.070$, $r^2 = 0.354$) or *P. napi* ($P = 0.400$, $r^2 = 0.090$; Fig. 2a). Cluster analysis also indicated that the larval performance of *P. melete* and *P. napi* was closer than that of *P. rapae* (Fig. 2b).

Chemical and Physical Plant Defense Traits

We detected 22 types of GLS from the 10 host plant species (Supplementary Table S2): 16 aliphatic-, 3 benzylic-, and 3 indolic GLSs. GLS profiles varied among plant species (Fig. 3a). GLS profiles in the host plants can be summarized into four PCs (GLSPC1-GLSPC4, cumulative proportion = 84.3%; Fig. 3a and Supplementary Fig. S1a). GLSPC1 (38.35%) was negatively correlated with the total amount of GLS in the plants (Fig. 3a and Supplementary Fig. S1a). GLSPC2 (25.81%) was mainly related to the concentrations of benzylic- and indolic GLSs (Fig. 3a and Supplementary Fig. S1a). GLSPC3 (11.71%) was characterized by high concentrations of 1-methoxyindol-3-ylmethyl GLS (1MOI3M) and low level of benzylic GLS, and GLSPC4 (8.43%) was negatively correlated with the concentrations of benzylic- and indolic GLSs associating with some types of aliphatic GLSs (Supplementary Fig. S1a).

Physical defense traits were summarized into two PCs (phyPC1 and phyPC2, cumulative proportion = 82.5%; Fig. 3b). phyPC1 was positively associated with the C:N ratio and negatively associated with water content and SLA (Fig. 3b and Supplementary Fig. S1b). A larger phyPC1 value is thus associated with lower nutrition and water content, and thicker leaves. phyPC2 was positively associated with leaf toughness and trichome density (Fig. 3b and Supplementary Fig. S1b). Thus, larger phyPC2 values indicated tougher leaves with higher trichome densities.

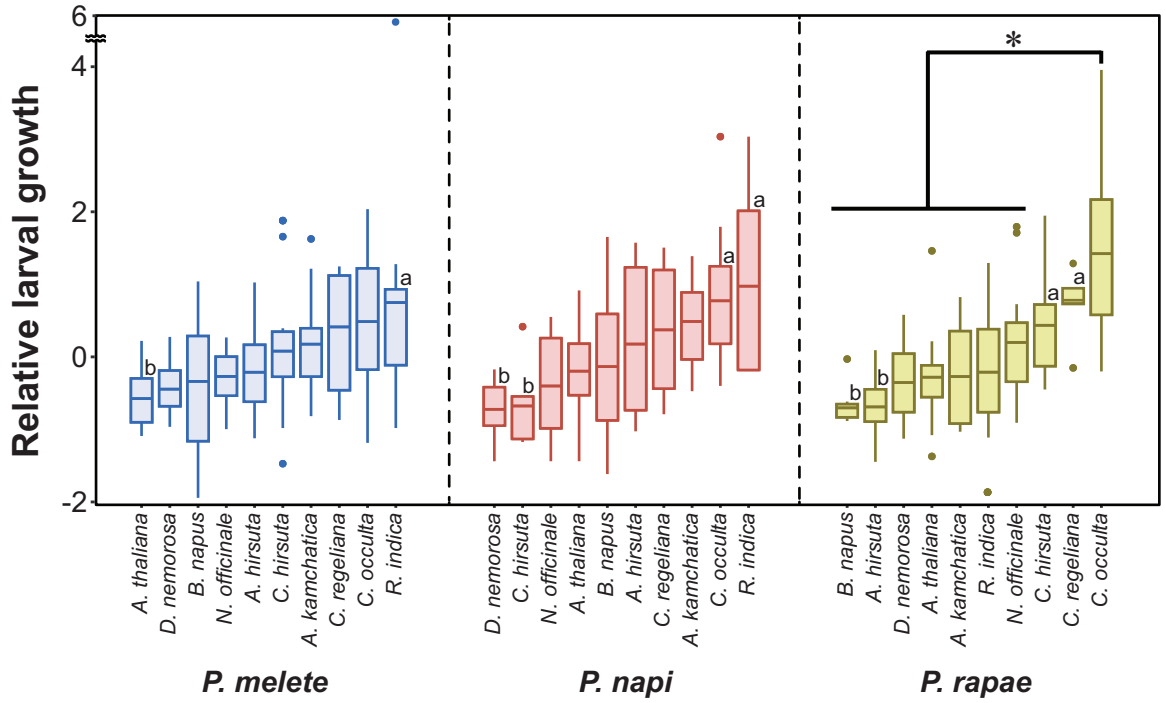

Fig. 1. Standardized larval growth of three *Pieris* spp. on 10 species of brassicaceous plants. The plant species are aligned based on the mean larval growths. The bottom and top limits of each box show the lower and upper quartiles; the horizontal line within each box is the mean; error bars show ± 1.5 times the interquartile range; dots represent outliers (n = 10–12; *C. regeliana*: n = 6). Significant larval growth differences are indicated with different letter on each box or '*' (Tukey HSD, adjust $P \leq 0.05$).

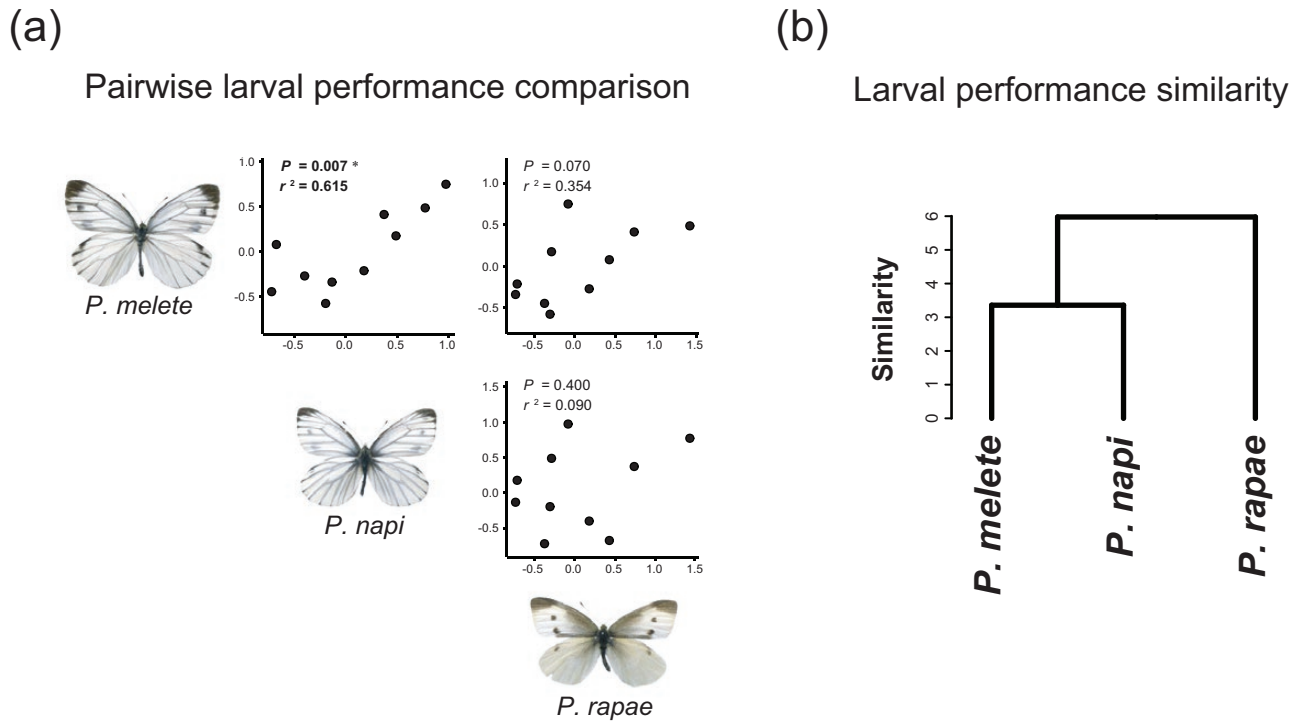

Fig. 2. (a) Scatterplots comparing larval performance between pairs of *Pieris* spp. The larval performances of *P. napi* and *P. melete* were significantly and positively correlated (Pearson's correlation test, $P \leq 0.05$). By contrast, the larval performance of *P. rapae* was not significantly correlated with those of *P. napi* and *P. melete*. (b) Hierarchical clustering analysis of the larval performances of the three *Pieris* spp. The larval performances of *P. melete* and *P. napi* were similar and clustered in the same group.

Plant Defense Profiles That Affected Larval Performance

From the GLMM analyses involving GLS defenses, the best models explaining the larval performance of *P. melete* and *P. napi* did not include GLSPC axes whereas that of *P. rapae* included GLSPC2 and GLSPC3 as negative explanatory variables (ΔAICc = 24.6; Table 1). The first three models with lower AICc of *P. melete* and *P. napi* were also tested and we found that they did not include

GLSPC3 as negative coefficient, which was included as a significant negative coefficient in *P. rapae* model (Supplementary Table S3a). These showed that *P. rapae* showed larval performance trend responding to some GLS profiles, however, *P. melete* and *P. napi* seemed not to respond detected GLS profiles.

With regard to physical defense, the best model of the larval performance of *P. melete* and *P. napi* included phyPC1 (ΔAICc = 2.17 and 2.34, respectively), whereas the best model of the larval performance of *P. rapae* included phyPC2 (ΔAICc = 9.82; Table 2). This result indicated that the larvae of *P. melete* and *P. napi* exhibited higher growth on plants with less nutrition and thicker leaves, whereas the larvae of *P. rapae* exhibited higher growth on plants with lower leaf toughness and trichome densities. We also found in the second-best models that phyPC1 was positively associated, and phyPC2 negatively associated, with larval growth for all three *Pieris* spp., indicating that the larvae of all three species exhibited higher growth on plants with thicker leaves, lower leaf toughness and trichome density, and less nutrition (Supplementary Table S3b). However, these models did not have strong explanatory power.

GLS Profiles Associated with Differences in Larval Performance Among *Pieris* spp.

The best model for explaining the differences between the larval performance of *P. rapae* and the larval performances of *P. melete* and *P. napi* included GLSPC1 and GLSPC3 as positive coefficients (ΔAICc = 5.47; Table 1). Therefore, this showed that the larvae of *P. melete* and *P. napi* exhibited higher growth than the larvae of *P. rapae* on plants with higher values of GLSPC1, i.e., lower total GLS concentrations, and higher values of GLSPC3, i.e., higher concentrations of 1MOI3M GLS and lower concentrations of benzylic GLSs (Supplementary Fig. S1a).

For physical defense, the model with the lowest AICc value included phyPC1. However, the null model was selected as the best model (ΔAICc = 0.22; Table 2). This showed that the differences in physical defense among plant species did not have a strong effect on the larval performance of the three *Pieris* spp.

Important Defense Traits That Affect *Pieris* Larval Performances

Based on regression tree analysis, GLSs but not plant physical traits, were selected as the most important determinant to the larval growth (the first node in the trees), and the selected GLSs were different among *Pieris* spp. (Fig. 4). Indol-3-ylmethyl GLS (I3M) was selected as the most important determinant to the larval growth of *P. melete* and *P. napi*, whereas, Benzyl GLS was selected to *P. rapae* (Fig. 4). In both cases, larvae grew better on plants with higher concentrations of selected GLSs. Furthermore, these selected GLSs (I3M and Benzyl GLS) were not shared as determinants in other *Pieris* spp. which have different larval performance (I3M was not selected in *P. rapae* tree and Benzyl was not selected in *P. melete* and *P. napi* trees).

Discussion

In this study, we conducted feeding assays using 10 species of the Brassicaceae family and larvae of three *Pieris* spp., which have been reported to have interspecific differences in host range from field observations (Ohsaki 1979, Ohsaki and Sato 1999). We aimed to determine if host plant traits can affect interspecific differences in larval performance across the three *Pieris* spp., and identify the traits contributing to these differences. Our results revealed that the larval performance of *P. rapae* differed from that of the other two species. The plant species used in the feeding assays varied in their GLS profiles and physical defenses. The larvae of *Pieris* spp. exhibited interspecific differences in responses to GLS profiles but responded similarly to physical defense traits. Although *Pieris* spp. are specialists of brassicaceous plants, members of the *Pieris* genus have not

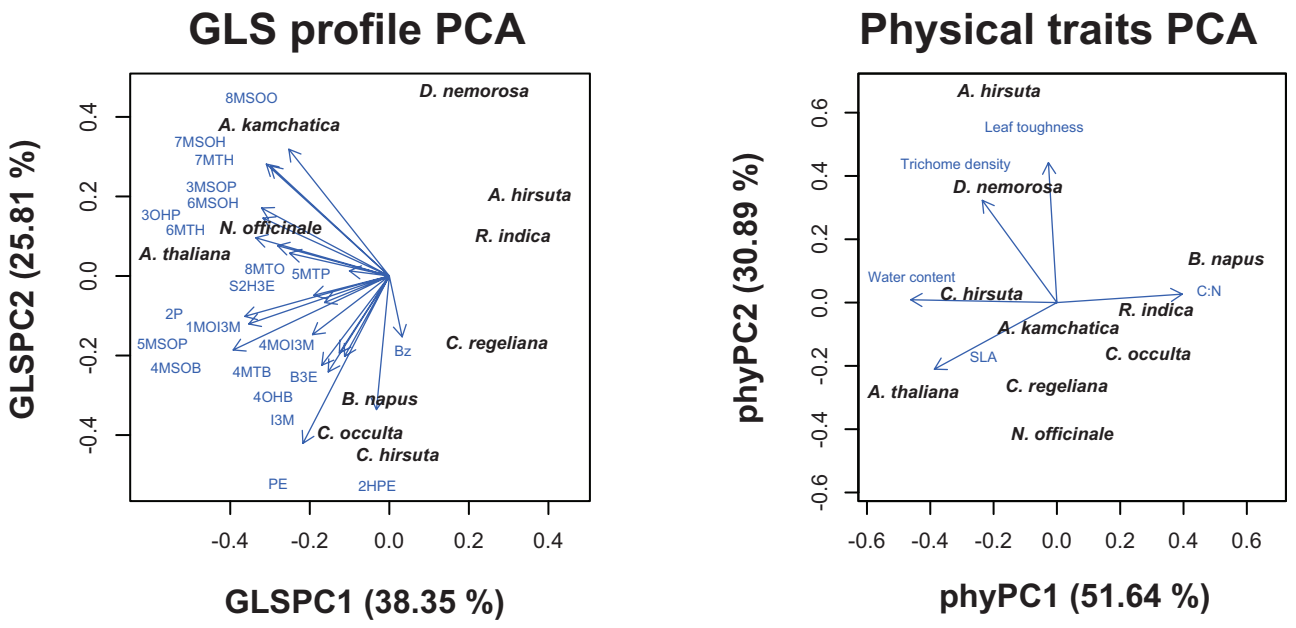

Fig. 3. Plots of PCAs of glucosinolate and physical defense traits. (a) PCA biplot with the GLS profiles of the 10 species of brassicaceous plants used in the feeding experiments. The first two principal component (PC) axes are labelled as GLSPC1 and GLSPC2, with the corresponding proportion of total variance in parentheses. Blue arrows indicate GLS type, and the placement of each plant species name represents the PC scores of GLSPC1 and GLSPC2. (b) PCA biplot with the physical defense traits of the 10 species of brassicaceous plants used in the feeding experiments. The first two PC axes are labelled as phyPC1 and phyPC2, with the corresponding proportion of total variance in parentheses. Blue arrows indicate physical defense traits, and the placement of each plant species name represents the PC scores of phyPC1 and phyPC2.

Table 1. The best models explaining the larval performance of each *Pieris* sp. and the difference in larval performance between *P. rapae* and the other two *Pieris* spp. in relation to GLS profiles

Species	Coefficients				Intercept	AICc	ΔAICc
	GLSPC1	GLSPC2	GLSPC3	GLSPC4			
P. melete					−5.289	−964.8	0
P. napi					−5.086	−810.8	0
P. rapae		−0.0079	−0.0159		−5.313	−1031.5	24.6
Interspecific differences in larval performance	0.0064		0.0210		4.9E−17	20.5	5.47

The first four principal components of the analysis with GLS profiles are labeled as GLSPC1–GLSPC4.

AICc = Akaike information criterion for small sample sizes; ΔAICc = AICc *null model* - AICc *best model*. The difference in larval performance is positive for plants where larvae of *P. melete* and *P. napi* exhibited higher growth than larvae of *P. rapae*, and negative when the situation is reversed.

Table 2. The best models explaining the larval performance of each *Pieris* sp. and the difference in larval performance between *P. rapae* and the other two *Pieris* spp. in relation to physical defense traits

Species	Coefficients		Intercept	AICc	ΔAICc
	phyPC1	phyPC2			
P. melete	0.063		−5.297	−967.0	2.17
P. napi	0.051		−5.085	−813.1	2.34
P. rapae		−0.135	−5.304	−1016.7	9.82
Interspecific differences in larval performance	0.101		3.49E−18	25.7	0.22

The first two principal components of the analysis with physical defense traits are labeled as phyPC1 and phyPC2.

AICc = Akaike information criterion for small sample sizes; ΔAICc = AICc *null model* - AICc *best model*. The difference in larval performance is positive for plants where larvae of *P. melete* and *P. napi* exhibited higher growth than larvae of *P. rapae*, and negative when the situation is reversed.

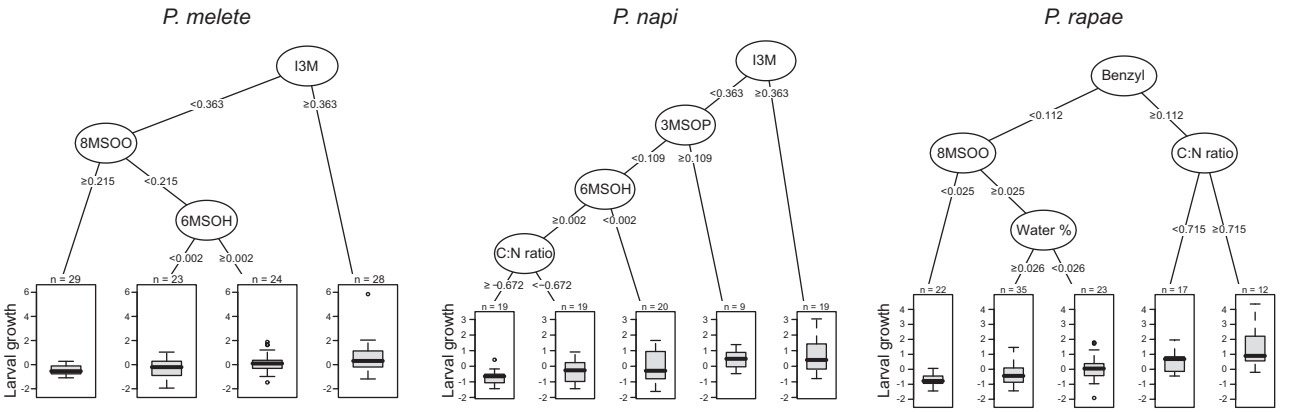

Fig. 4. Regression tree of each defense trait coefficient to the larval growth of *Pieris* butterflies. Each node shows the potential defense trait that affect larval performance. Each split is labeled with the value that determines the split. Lower boxplots show standardized larval performances. The bottom and top limits of each box are the lower and upper quartiles; the horizontal line within each box is the median; error bars show ± 1.5 times the interquartile range; dots represent outliers. I3M: indol-3-ylmethyl GLS, Benzyl: Benzyl GLS, 3MSOP: 3-(methylsulfinyl)propyl GLS, 6MSOH: 6-(methylsulfinyl)hexyl GLS, 8MSOO: 8-(methylsulfinyl) octyl GLS, Water %: water content.

evolved the capability of countering all GLS types present in the Brassicaceae. It is likely that each *Pieris* sp. has evolved adaptations to specific GLS profiles within a subset of Brassicaceae, and preferentially uses plant species within this subset as its hosts.

The feeding experiment showed that the three *Pieris* spp. tended to have similar larval performance in general (Fig. 2), however, they also performed differently on some host plants. *P. melete* and *P. napi* exhibited higher growth on *R. indica* and *P. rapae* performed better on *Cardamine* spp. (Fig. 1). On *C. hirsuta*, *P. rapae* relatively performed better, however, *P. napi* showed reduced larval weight gain (Fig. 1). Both the correlation test and cluster analysis revealed that the larval performances of *P. melete* and *P. napi* were similar (Pearson's correlation, $P = 0.007$), whereas the larval performance of *P. rapae* was not significantly correlated with those of the other two species (Fig. 2). Since these three species are closely related, they can have similar larval performance trends. However, our feeding experiments also showed that larval performance trend of *P. rapae* was slightly different from that of the other two species. Host plant utilization pattern of *P. rapae* in the field is known to be different from that of *P. melete* or *P. napi* (Ohsaki and Sato 1994, Benson et al. 2003, Gols et al. 2008, Kitahara 2016). The larval performances of herbivores in the laboratory may not match their host preferences in the field, as a number of ecological factors, such as oviposition preference, competition, and parasitism, can affect host preferences (Ohsaki and Sato

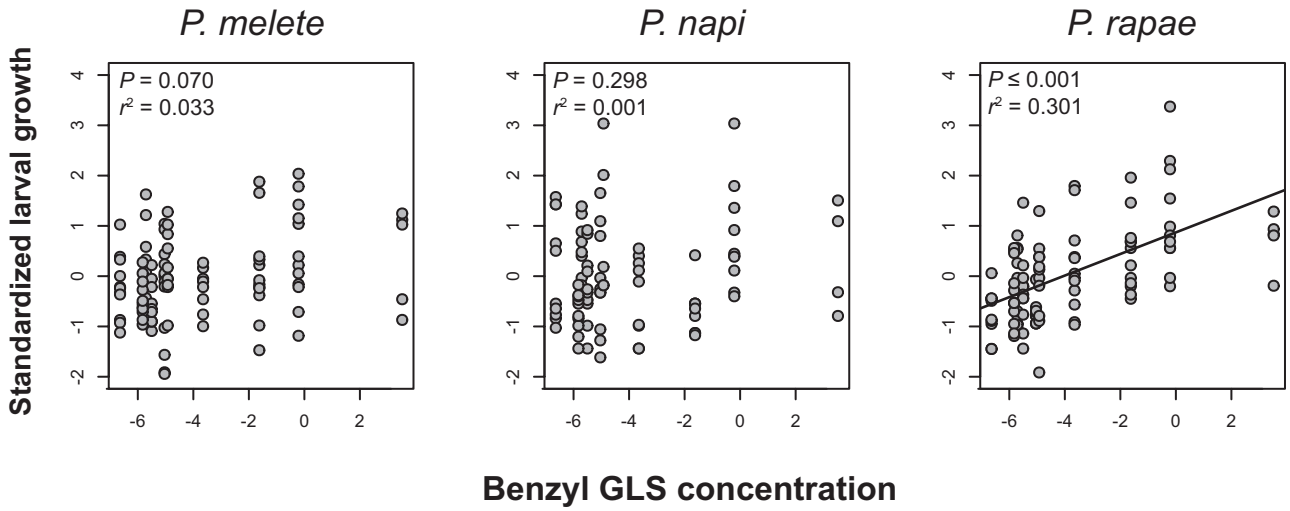

Fig. 5. Scatterplots comparing Benzyl GLS concentration (log-transformed) in plants and larval performance of *Pieris* spp. Solid line shows regression line. One outlier point (*P. melete*) is not shown for displaying the plots in scale. The larval performance of *P. rapae* showed significant positive correlation with Benzyl GLS concentration ($P \leq 0.001$, regression analysis), whereas, those of *P. melete* and *P. napi* did not show significant correlation.

1999). However, the observed interspecific differences in the larval performance of *Pieris* spp. from our feeding experiments are not in contradiction with the patterns of host use in the field.

From the 10 wild brassicaceous plant species used in this study, our widely targeted GLS analysis detected 22 GLSs that had been profiled in previous studies (Supplementary Table S2). In the samples of wild *A. thaliana*, we detected high concentrations of 4-(methylsulfinyl) butyl GLS, which is abundant in the Col-0 wild type of *A. thaliana*. We confirmed that our samples of *B. napus* contained high concentrations of but-3-enyl GLS, and samples of *N. officinale* contained high concentrations of phenethyl GLS, as was previously reported as typical for these plant species (Velasco et al. 2008, Jeon et al. 2017). GLS profiles within a plant species can vary depending on the developmental stage, part of the plant sampled, and among populations (Brown et al. 2003, Windsor et al. 2005, van Leur et al. 2006, Bidart-Bouzat and Kliebenstein 2008). However, each plant species also tends to have a typical, species-specific GLS profile as well (Fahey et al. 2001). Our plant samples contained high concentrations of the typical GLSs reported for the same species in previous studies and useful to see differences of GLS profiles among plant species.

From the PCA and GLMM analyses, the larval performances of *P. rapae* was associated with GLSPC2, and GLSPC3, however, the other two species did not show significant relationship with GLS profile of their host plants (Tables 1 and 2). GLSPC2 and GLSPC3 were both mainly negatively characterized by the amount of benzylic GLSs (Supplementary Fig. S1a). *Pieris rapae* exhibited higher growth, on plants with low GLSPC2, and GLSPC3 values (Table 1), i.e., those characterized by higher concentrations of benzylic GLS. We also compared the second and third best models of all the three species and found that GLSPC3, which were significant in *P. rapae*, was not selected in these models of *P. napi* and *P. melete* as a negative explanatory variable. This suggests that the responses of *P. rapae* to benzylic types of GLS might be different from that of other two *Pieris* species (*P. melete* and *P. napi*). However, as other GLSs are also represented by these GLSPCs in this PCA analysis, we are unable to specify which GLS accounted for the difference in larval growth among the *Pieris* spp. (Supplementary Table S3a). Overall, our results indicate that *Pieris* spp. may react in a species-specific manner to some GLS profiles.

Larval performance was also significantly affected by plant physical defenses. The larvae of *P. melete* and *P. napi* exhibited higher growth on higher phyPC1 associated with higher C:N ratio, lower water content, and lower SLA (Table 2; Supplementary Fig. S1b). The larvae of *P. melete* and *P. napi* thus performed better on plants with less nutrition and thicker leaves. Larval growth for *P. rapae* was negatively associated with phyPC2, which represents higher leaf toughness and trichome density. In general, the larvae of all three species had higher growth on plants with less nutrition, and lower leaf toughness and trichome density (Supplementary Table S3b; Supplementary Fig. S1b). Although herbivores should grow better on plants with higher nutrition values, such plants are also hypothesized to produce high levels of chemical defenses, according to the 'plant defense syndrome' (Agrawal and Fishbein 2006). This phenomenon may explain the apparently contradictory relationship between the high larval growth rates of *Pieris* spp. and the low nutrition values of their host plants. In general, the larval performance of all three *Pieris* spp. was affected similarly by the plant physical traits measured in this study.

As such, differences in larval performance between *P. rapae* and the other two *Pieris* spp. were only associated with differences in GLS profiles among host plant species, and not with plant physical defense. The larvae of *P. melete* and *P. napi* exhibited higher growth than the larvae of *P. rapae* on plants associated with high values of GLSPC1 and GLSPC3 (Table 1, Supplementary Table S3a). This indicates that the difference in larval performance possibly resulted from species-specific adaptations to selected GLS profiles, and not from adaptations to plant physical traits. Although *Pieris* spp. specialize on feeding on brassicaceous plants, individual species evolved adaptations to a subset of the Brassicaceae only, and may not be fully adapted to the entire range of GLSs found in the Brassicaceae.

In the regression tree analysis, we were able to focus on the effect of each defense trait to larval performance. Interestingly, different GLSs were selected as the most important determinant to *Pieris* spp. which have different larval performance trends (I3M to *P. melete* and *P. napi* and Benzyl GLS to *P. rapae*; Fig. 4). This again highlight that the sensitivity of *Pieris* larvae to a certain type of GLS can be different among *Pieris* spp. In addition, the trend we found in this regression tree analysis was not in contradiction with the results of

our PCA and regression analyses. GLSPC3 which showed significant correlation only with *P. rapae* was highlighted with Benzyl GLSs, and this axis also explained the differences in larval performance between *P. rapae* and the other two *Pieris* spp. (Table 1, Supplementary Fig. S1a). These suggest that the Benzyl GLS can be an important candidate that potentially interacts differently with different *Pieris* spp. (Fig. 5).

Still, we note that there are a number of undetectable GLSs and non-GLS chemical defenses in plants of the Brassicaceae family that may affect the larval performance of *Pieris* spp. but were not assayed in our study. To date, more than 140 GLS types have been identified (Fahey et al. 2001, Olsen et al. 2016), however, it was difficult to target all known GLS types at once in this study. For example, *A. hirsuta* has at least four types of chain-elongated aromatic GLSs that are not in our standard library (Agerbirk et al. 2010). Furthermore, some plants of the Brassicaceae family are also known to produce other secondary metabolites, such as saponins or cardenolides (Sachdev-Gupta et al. 1993, Badenes-Perez et al. 2014). Therefore, the plant species used in this study may also have these non-GLS secondary metabolites. Further analyses of GLS types and a better understanding of the plant chemistry of the Brassicaceae family would help to elucidate the major mechanisms that shape host selection in *Pieris* spp.

Differences in host plant ranges among *Pieris* spp. are well known (Ohsaki 1979, Chew 1980), but the plant traits that drive these differences had not been previously well studied. Our results indicate that *Pieris* spp. we tested have species-specific adaptations in response to the GLS profiles, but not the physical defense traits, of their host plants. These species-specific adaptations may influence the host plant ranges of individual *Pieris* spp., an expected consequence of a chemical arms race mediated by GLS production in host plants (Wheat et al. 2007, Edger et al. 2015). In this study, we also found potential effect of individual GLS types on larval performance (Figs. 4 and 5). However, this should be interpreted in the context of multiple defense strategies of plants as a variety of GLSs tend to be present within the same plant species and may interact in a synergistic manner (Travers-Martin and Müller 2008, Okamura et al. 2016, Dyer et al. 2018). More focused individual GLS effect can be also tested by using intraspecies GLS variations which were found in some Brassicaceae species (Müller et al. 2010, Prasad et al. 2012, Müller et al. 2018). Additional experiments, such as those using the intraspecies GLS variants or mutants of *A. thaliana* with different GLS profiles, are needed to confirm which GLS types affect differently the larval performance of each *Pieris* sp. Nevertheless, our results support an idea that the host plant ranges of *Pieris* spp. are influenced by a chemical arms race with diversifying GLS profiles in their host plants, but not by an arms race with physical defense traits. Further research on the molecular mechanisms that underpin the responses of *Pieris* spp. to GLS types would shed light on how *Pieris* spp. adapt to the constantly evolving chemical defenses of their host plants.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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Competing Interests

The authors declare no competing interests.

Contribution of Authors

Y.O., N.T., S.K., A.S., Y.S., and M.Y.H. carried out the laboratory work. Y.O. and M.M. conceived, designed and coordinated the study. All authors gave approval for publication and agree to be accountable for the content.

References Cited

Agerbirk, N., C. E. Olsen, E. Poulsen, N. Jacobsen, and P. R. Hansen. 2010. Complex metabolism of aromatic glucosinolates in *Pieris rapae* caterpillars involving nitrile formation, hydroxylation, demethylation, sulfation, and host plant dependent carboxylic acid formation. Insect Biochem. Mol. Biol. 40: 126–137.

Agrawal, A. A., and M. Fishbein. 2006. Plant defense syndromes. Ecology 87: 132–149.

Badenes-Perez, F. R., J. Gershenzon, and D. G. Heckel. 2014. Insect attraction versus plant defense: young leaves high in glucosinolates stimulate oviposition by a specialist herbivore despite poor larval survival due to high saponin content. PLoS One 9: 39–42.

Bartoń, K. 2018. MuMIn: multi-model inference. R package version 1.40.4. https://CRAN.R-project.org/package=MuMIn.

Bates, D., M. Mächler, B. Bolker, M., and S. Walker, C. **2015.** Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67: 1–48.

Beekwilder, J., W. van Leeuwen, N. M. van Dam, M. Bertossi, V. Grandi, L. Mizzi, M. Soloviev, L. Szabados, J. W. Molthoff, B. Schipper, et al. **2008.** The impact of the absence of aliphatic glucosinolates on insect herbivory in Arabidopsis. PLoS One 3: e2068.

Benson, J., A. Pasquale, R. Van Driesche, and J. Elkinton. 2003. Assessment of risk posed by introduced braconid wasps to *Pieris virginiensis*, a native woodland butterfly in New England. Biol. Control 26: 83–93.

Bidart-Bouzat, M. G., and D. J. Kliebenstein. 2008. Differential levels of insect herbivory in the field associated with genotypic variation in glucosinolates in *Arabidopsis thaliana*. J. Chem. Ecol. 34: 1026–1037.

Breiman, L., J. H. Friedman, R. A. Olshen, and C. G. Stone. 1984. Classification and regression trees. Wadsworth International Group, Belmont, CA.

Brown, P. D., J. G. Tokuhisa, M. Reichelt, and J. Gershenzon. 2003. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochem. 62: 471–481.

Chew, F. S. 1980. Foodplant preferences of *Pieris* caterpillars (Lepidoptera). Oecologia. 46: 347–353.

Dyer, L. A., C. S. Philbin, K. M. Ochsenrider, L. A. Richards, T. J. Massad, A. M. Smilanich, M. L. Forister, T. L. Parchman, L. M. Galland, P. J. Hurtado, et al. **2018.** Modern approaches to study plant–insect interactions in chemical ecology. Nat. Rev. Chem. 2: 50–64.

Edger, P. P., H. M. Heidel-Fischer, M. Bekaert, J. Rota, G. Glöckner, A. E. Platts, D. G. Heckel, J. P. Der, E. K. Wafula, M. Tang, et al. **2015.** The butterfly plant arms-race escalated by gene and genome duplications. Proc. Natl. Acad. Sci. USA 112: 8362–8366.

Ehrlich, P., and P. Raven. 1964. Butterflies and plants: a study in coevolution. Evolution. 18: 586–608.

Fahey, J. W., A. T. Zalcmann, and P. Talalay. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochem. 56: 5–51.

Feeny, P., and N. July 2007. Seasonal changes in Oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. Ecology 51: 565–581.

Fraenkel, G. S. 1959. The raison d'etre of secondary plant substances. Science. 129: 1466–1470.

Futuyma, D. J., and A. A. Agrawal. 2009. Macroevolution and the biological diversity of plants and herbivores. Proc. Natl. Acad. Sci. USA 106: 18054–18061.

Gols, R., L. M. A. Witjes, J. J. A. van Loon, M. A. Posthumus, M. Dicke, and J. A. Harvey. 2008. The effect of direct and indirect defenses in two wild brassicaceous plant species on a specialist herbivore and its gregarious endoparasitoid. Entomol. Exp. Appl. 128: 99–108.

Heidel-Fischer, H. M., H. Vogel, D. G. Heckel, and C. W. Wheat. 2010. Microevolutionary dynamics of a macroevolutionary key innovation in a Lepidopteran herbivore. BMC Evol. Biol. 10: 60.

Hopkins, R. J., N. M. van Dam, and J. J. van Loon. 2009. Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annu. Rev. Entomol. 54: 57–83.

Janz, N. 2010. Ehrlich and Raven revisited: mechanisms underlying codiversification of plants and enemies. Annu. Rev. Ecol. Evol. Syst. 42: 71–89.

Jeon, J., S. J. Bong, J. S. Park, Y. K. Park, M. V. Arasu, N. A. Al-Dhabi, and S. U. Park. 2017. De novo transcriptome analysis and glucosinolate profiling in watercress (*Nasturtium officinale* R. Br.). BMC Genomics 18: 401.

Kitahara, H. 2016. Oviposition plants and seasonal migratory movements of sympatric *Pieris melete* and *P. napi japonica* (Lepidoptera, Pieridae). Lepid. Sci. 67: 32–40.

van Leur, H., C. E. Raaijmakers, and N. M. van Dam. 2006. A heritable glucosinolate polymorphism within natural populations of *Barbarea vulgaris*. Phytochem. 67: 1214–1223.

Matsubayashi, K. W., I. Ohshima, and P. Nosil. 2010. Ecological speciation in phytophagous insects. Entomol. Exp. Appl. 134: 1–27.

Mithen, R. 2001. Glucosinolates – biochemistry, genetics and biological activity. Plant Growth Regul. 34: 91–103.

Müller, R., M. de Vos, J. Y. Sun, I. E. Sønderby, B. A. Halkier, U. Wittstock, and G. Jander. 2010. Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. J. Chem. Ecol. 36: 905–913.

Müller, C., M. Schulz, E. Pagnotta, L. Ugolini, T. Yang, A. Matthes, L. Lazzeri, and N. Agerbirk. 2018. The role of the glucosinolate-myrosinase system in mediating greater resistance of barbarea verna than *B. vulgaris* to *Mamestra brassicae* Larvae. J. Chem. Ecol. 44: 1190–1205.

Ohsaki, N. 1979. Comparative population studies of three *Pieris* butterflies, *P. rapae*, *P. melete* and *P. napi*, living in the same area I. ecological requirements for habitat resources in the adults. Res. Popul. Ecol. (Kyoto) 20: 278–296.

Ohsaki, N., and Y. Sato. 1994. Food plant choice of *Pieris* butterflies as a trade-off between parasitoid avoidance and quality of plants. Ecology. 75: 59–68.

Ohsaki, N., and Y. Sato. 1999. The role of parasitoids in evolution of habitat and larval food plant preference by three *Pieris* butterflies. Res. Popul. Ecol. (Kyoto) 41: 107–119.

Ohshima, I. 2010. Host-associated pre-mating reproductive isolation between host races of *Acrocercops transecta*: mating site preferences and effect of host presence on mating. Ecol. Entomol. 35: 253–257.

Okamura, Y., Y. Sawada, M. Y. Hirai, and M. Murakami. 2016. Effects of different secondary metabolite profiles in plant defense syndromes on specialist and generalist herbivores. Entomol. Sci. 19: 97–103.

Olsen, C. E., X. C. Huang, C. I. C. Hansen, D. Cipollini, M. Ørgaard, A. Matthes, F. Geu-Flores, M. A. Koch, and N. Agerbirk. 2016. Glucosinolate diversity within a phylogenetic framework of the tribe Cardamineae (Brassicaceae) unraveled with HPLC-MS/MS and NMR-based analytical distinction of 70 desulfoglucosinolates. Phytochem. 132: 33–56.

Prasad, K. V., B. H. Song, C. Olson-Manning, J. T. Anderson, C. R. Lee, M. E. Schranz, A. J. Windsor, M. J. Clauss, A. J. Manzaneda, I. Naqvi, et al. **2012**. A gain-of-function polymorphism controlling complex traits and fitness in nature. Science. 337: 1081–1084.

Renwick, J. A. A., and K. Lopez. 1999. Experience-based food consumption by larvae of *Pieris rapae*: addiction to glucosinolates? Entomol. Exp. Appl. 91: 51–58.

R Studio Team. 2016. RStudio: Integrated development for R. RStudio, Inc., Boston, MA. http://www.rstudio.com/.

Rundle, H. D., and P. Nosil. 2005. Ecological speciation. Ecol. Lett. 8: 336–352.

Sachdev-Gupta, K., C. Radke, J. A. Renwick, and M. B. Dimock. 1993. Cardenolides from *Erysimum cheiranthoides*: feeding deterrents to *Pieris rapae* larvae. J. Chem. Ecol. 19: 1355–1369.

Sawada, Y., K. Toyooka, A. Kuwahara, A. Sakata, M. Nagano, K. Saito, and M. Y. Hirai. 2009. *Arabidopsis* bile acid:sodium symporter family protein 5 is involved in methionine-derived glucosinolate biosynthesis. Plant Cell Physiol. 50: 1579–1586.

Therneau, T., and B. Atkinson. 2018. rpart: recursive Partitioning and Regression Trees. R package version 4.1–13. https://CRAN.R-project.org/package=rpart.

Travers-Martin, N., and C. Müller. 2008. Matching plant defence syndromes with performance and preference of a specialist herbivore. Funct. Ecol. 22: 1033–1043.

Velasco, P., P. Soengas, M. Vilar, M. E. Cartea, and M. Del Rio. 2008. Comparison of glucosinolate profiles in leaf and seed tissues of different *Brassica napus* crops. J. Am. Soc. Hortic. Sci. 133: 551–558.

Wheat, C. W., H. Vogel, U. Wittstock, M. F. Braby, D. Underwood, and T. Mitchell-Olds. 2007. The genetic basis of a plant-insect coevolutionary key innovation. Proc. Natl. Acad. Sci. USA 104: 20427–20431.

Winde, I., and U. Wittstock. 2011. Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. Phytochem. 72: 1566–1575.

Windsor, A. J., M. Reichelt, A. Figuth, A. Svatos, J. Kroymann, D. J. Kliebenstein, J. Gershenzon, and T. Mitchell-Olds. 2005. Geographic and evolutionary diversification of glucosinolates among near relatives of *Arabidopsis thaliana* (Brassicaceae). Phytochem. 66: 1321–1333.

Wittstock, U., and B. A. Halkier. 2002. Glucosinolate research in the Arabidopsis era. Trends Plant Sci. 7: 263–270.

Wittstock, U., N. Agerbirk, E. J. Stauber, C. E. Olsen, M. Hippler, T. Mitchell-Olds, J. Gershenzon, and H. Vogel. 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proc. Natl. Acad. Sci. USA 101: 4859–4864.