Host plant chemistry enhances herbivore cellular immunity with differential effectiveness against two parasitoid species

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Abstract

Insect herbivores must simultaneously balance bottom-up effects of plant defensive chemistry and the top-down effects of natural enemies. At the intersection of these effects are herbivore immune systems, an herbivore trait that has largely been overlooked in studies of plant-insect interactions. Counter to the majority of studies showing that herbivores feeding on plants containing higher levels of toxins are immunocompromised, we demonstrate that Pieris rapae caterpillars feeding on more toxic host plants have enhanced cellular immunity at the cost of reduced growth rates and body size. However, whether enhanced immune systems are effective defense against parasitoids depends on parasitoid identity. Whereas enhanced immunity provided increased protection against the parasitoid Cotesia glomerata, it did not provide protection against C. rubecula that suppressed and evaded the host's immune system. Our study demonstrates that both herbivore immunity and species identity of trophic participants are crucial in determining the structure of multitrophic interactions.

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Host plant chemistry enhances herbivore cellular immunity with differential effectiveness against two parasitoid species

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ABSTRACT

Insect herbivores must simultaneously balance bottom-up effects of plant defensive chemistry and the top-down effects of natural enemies. At the intersection of these effects are herbivore immune systems, an herbivore trait that has largely been overlooked in studies of plant-insect interactions. Counter to the majority of studies showing that herbivores feeding on plants containing higher levels of toxins are immunocompromised, we demonstrate that *Pieris rapae* caterpillars feeding on more toxic host plants have enhanced cellular immunity at the cost of reduced growth rates and body size. However, whether enhanced immune systems are effective defense against parasitoids depends on parasitoid identity. Whereas enhanced immunity provided increased protection against the parasitoid *Cotesia glomerata* , it did not provide protection against *C. rubecula* that suppressed and evaded the host's immune system. Our study demonstrates that both herbivore immunity and species identity of trophic participants are crucial in determining the structure of multitrophic interactions.

INTRODUCTION

Whether populations are primarily regulated by resource limitation (including traits that reduce palatability) or the activities of consumers (e.g., predators, parasites, pathogens) is a long-standing debate in ecology (Hunter & Price 1992; Lill et al. 2002; Farkas & Singer 2013). Traditionally, bottom-up selective forces (i.e., plant traits such as nutritional quality, plant chemical defenses) have been thought to have primacy (Schoonhoven et al. 2005). However, more recent studies suggest that top-down forces can be equally, if not more, important than bottom-up selective forces on herbivores (e.g., Vidal & Murphy 2018). In part, this controversy arises because plant-herbivore interactions have often been studied in isolation from herbivore-natural enemy interactions. Bi-trophic studies of plant-herbivore interactions in the absence of natural enemies, as well as studies of herbivore-natural enemy interactions in the absence of plants, ignore the potential for indirect effects of plants on natural enemies and vice versa (Kaplan et al. 2016; Ode 2019). Furthermore, studies of how plant traits influence herbivore interactions with multiple natural enemies are even rarer. Yet virtually all plants are attacked by insect herbivores and all herbivores are in turn attacked by one or more species of natural enemies like parasitoids (parasitic wasps).

As consumers that develop inside their hosts, endoparasitoid larvae are exposed to the effects of plant toxins in three potential ways: 1) parasitoid larvae directly encounter unmetabolized (including sequestered) plant toxins or their toxic metabolites, 2) plant toxins reduce host size and thus resource availability for the growing parasitoid thereby indirectly affecting parasitoid success, and 3) in another form of indirect effects, plant toxins may modulate the expression of host immune defenses, altering the ability of the herbivore to mount a successful encapsulation and melanization response (Kaplan et al. 2016; Ode 2019). In this latter context, how herbivore immune responses are affected by host plant quality and trade off with herbivore growth and survivorship as well as defense against their natural enemies are important, but understudied, factors that determine the outcome of tri-trophic interactions.

Several studies have shown a correlation between plant defensive chemistry and herbivore immunity (Diamond & Kingsolver 2010; Smilanich et al. 2009; Smilanich et al. 2011; Garvey et al. 2021; Smilanich & Muchoney 2022 and references therein). Caterpillar species that sequester plant toxins as defenses against their own natural enemies generally suffer reduced immunocompetence when feeding on more toxic diets (e.g., Smilanich et al. 2009; Lampert & Bowers 2015). Such studies support the 'vulnerable host' or 'immunocompromised host' hypothesis (Reudler et al. 2011). However, a few studies have found that elevated levels of plant defense chemistry may enhance immune responses (e.g., Garvey et al. 2021; Ghosh et al. 2022). The majority of eco-immunological studies of plant defense chemistry effects on caterpillar immune

responses have been bi-trophic in that immunity has been measured in terms of responses against artificial implants (see review by Smilanich & Muchoney 2022). Our understanding of how these relationships interact with higher trophic levels is largely lacking with rare exception (e.g., Bukovinzsky et al. 2009).

We investigated the effects of two species of *Brassica* plants on the immune response of a specialist herbivore against two parasitoids. We use two host plants, collards (*Brassica oleracea*) and field mustard (*Brassica rapa*), known to differ in their production of glucosinolates, metabolites that provide defense against a wide range of herbivores (Hopkins et al. 2009). Larvae of the small cabbage white butterfly *Pieris rapae* (Lepidoptera: Pieridae) are able to feed on several species of *Brassica* , including *B. oleracea* and*B. rapa* , in part because of their ability to metabolize glucosinolates into less toxic nitriles rather than the more toxic isothiocyanates that are typically produced upon feeding damage (Wittstock et al. 2004). We compared the immune responses and the performance of larvae reared on either *B. oleracea* or the more toxic *B. rapa* . We then compared the ability of two larval parasitoids of *P. rapae* , *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae), to successfully develop within*P. rapae* when this host fed on *B. oleracea* or *B. rapa* . *Cotesia glomerata* is a gregarious (multiple offspring can develop per host) endoparasitoid of many pierid butterfly larvae, whereas *C. rubecula* is solitary (only one offspring can successfully develop per host) and strict specialist endoparasitoid of*P. rapae* (Laing & Levin 1982).

METHODS

Study Organisms

Seeds of collards (*Brassica oleracea* var*. viridis*) and field mustard (*Brassica rapa* subsp. *dichotoma*) were obtained from the USDA-GRIN seed bank. All plants were grown under greenhouse conditions (26 ± 8°C, 40-60% RH, and a L:D 16:8 photoperiod) in 7-inch diameter pots filled with potting soil (Lambert LM-HP) and 5g of Osmocote fertilizer (N: P: K- 1: 1: 1). After 4 weeks, plants with four to six expanded leaves were used for all experiments. Adult*P. rapae* were collected from an organic crucifer farm near Fort Collins, CO. *Cotesia glomerata* cocoons were collected from the same crucifer fields near Fort Collins, CO and *C. rubecula*colonies were started from cocoons collected on crucifers on the University of Minnesota campus in St. Paul, MN. Both parasitoid colonies were maintained by parasitizing second instar *P. rapae* larvae. These colonies were periodically supplemented with field-collected parasitoids. Adult parasitoids were maintained in humidity-controlled incubator (15°C, 50% RH, and L:D 16:8h photoperiod).

Glucosinolate profiles of *Brassica oleracea* and *B. rapa*

Ten undamaged plants (five *B. oleracea* , five *B. rapa*) were flash frozen in liquid nitrogen followed by lyophilization for 24h. Plant leaves were separated from rest of the plant only after drying to avoid induction of glucosinolates due to mechanical damage. Dried leaf tissue was ground into a fine powder using a tissuelyser. For each sample, approximately 50 mg of leaf powder was added to a 1-mL Eppendorf tube. To each tube, 1 mL of 70% methanol and 50 µL of 2.5mM internal standard (sinigrin for *B. rapa* samples; sinalbin for *B. oleracea* samples) was added. The internal standards were selected based on our preliminary glucosinolate analysis that showed the absence of sinigrin in *B. rapa* and the absence of sinalbin in *B. oleracea* . Each Eppendorf tube was vortexed for 30 seconds and sonicated for six minutes in a water bath set at 23°C. Post sonication, each tube was centrifuged at 13,200 rpm for 10 minutes and the supernatant was added onto an anion exchange column. This process was repeated twice to increase the extraction efficiency. The columns were prepared in 1mL pipette tips plugged with glass wool. To each column, 0.5mL of DEAE-Sephadex bead solution (A25 chloride form, Millipore Sigma; 5g Sephadex in 75 mL of water) was added and allowed to drain before adding the sample supernatants. The columns were washed twice with both 1 mL 70% methanol and 1 mL HPLC grade water. Next, the bottoms of the columns were capped and 50 µL of sulfatase enzyme solution was added to each column. All the columns were incubated overnight after covering the top with parafilm. The next day, desulfated glucosinolates were collected in an Eppendorf tube by passing 2 mL of HPLC-grade water through the column. The collected flow-through was dried on a vacufuge at 45°C for 8h and then reconstituted in 100 µL of water for sample analysis.

Quantification and analysis were performed with a Shimadzu LC-30AD with SIL-30AC autosampler and

SPD-M30A photodiode array (PDA) detector. A Shimadzu LCMS-8040 mass spectrometer was used to confirm identities of glucosinolates. Glucosinolate separation was performed on Phenomenex NX-C18 column (100mm, 2.0mm I.D., 3µm pore size) with a pure water-acetonitrile gradient with a flow rate of 0.4mL/min. Quantities were measured by PDA using UV absorbance at 229nm. Compound identities were deduced from established literature and confirmed by retention time and coupled MS/MS analysis using the H+ -desulfoglucosinolate ion for each compound with 35eV ionization energy.

Host plant effects on caterpillar weight and relative growth rate (RGR)

To assess the effect of glucosinolate concentration on *P. rapae*larval performance, we recorded the larval weight ($n = 20$) and development duration ($n = 6$) of *P. rapae* grown on either*B. oleracea* or *B. rapa* . Development duration was calculated as the total number of days from egg to adult emergence. Following Lariviere et al. (2015), relative growth rate (RGR) was estimated as: RGR = (ln(weight at day 13) – ln(weight at day5))/8 days.

Host plant and parasitism status effects on caterpillar immune status

Four major classes of hemocytes form the basis of cellular immunity in larval lepidopterans: plasmatocytes, granulocytes, oenocytoids, and pro-hemocytes (Strand 2008). Upon recognition of a parasitoid egg, plasmatocytes, granulocytes, and oenocytoids form a capsule surrounding the parasitoid egg. Oenocytoids in the capsule release phenol oxidase, converting tyrosine to melanin, resulting in melanization of the capsule. The combination of encapsulation by the hemocytes and the oxidative reactions involved with melanization act to asphyxiate and kill the parasitoid egg (Carton et al. 2008; Strand 2008). Finally, pro-hemocytes are stem-cell like in that they can rapidly differentiate into other cell types. Hence, hemocyte number and diversity are important indicators of caterpillar immune status.

Both *Cotesia* species will attack a range of host larval stages. To quantify the ontogenetic effect of host plant species on the constitutive cellular immune status of unparasitized *P. rapae*larvae, first through fifth instar larvae were collected from each host plant species. A lateral incision was made across the abdomen of each larva using a sterile needle. Hemolymph that leaked from the incision was collected and added to a pre-chilled Eppendorf tube. To quantify the total hemocytes, the collected hemolymph was diluted with phosphate buffer saline (PBS) in a 1:2 ratio (hemolymph: PBS). Approximately 8µL of diluted hemolymph was added onto a Neubauer hemocytometer and left to settle for 15 minutes, after which total hemocyte counts (THC) were recorded. The individual hemocytes were identified morphometrically by performing a phalloidin-DAPI staining following established protocols (Ghosh & Venkatesan 2019). Briefly, approximately 10µL of diluted hemolymph was added onto a teflon-coated, pre-chilled slide and incubated for 30 minutes at 4°C. Hemocytes were fixed with 4% formaldehyde, followed by PBS washes, and stained using alexafluor 488 (1:2000, Thermo Fisher Scientific) for 6h. Images were recorded using Zeiss LSM-800 confocal microscope from slides mounted with the DAPI-glycerol solution. Morphological identification for differential hemocyte count (DHC) was done as described by Gupta (1979) (THC:$n = 20$, DHC: $n = 10$ per instar).

To measure phenol oxidase (PO) activity in unparasitized caterpillars, hemolymph samples from larvae were individually mixed with ice-cold PBS in a 1:2 ratio in an Eppendorf tube and centrifuged at 4000g for 15 min at 4°C. The supernatant was added to a microplate well containing 20 µL of PBS and 50 µL of 2mM L-Dopa as a substrate for PO. The reaction was allowed to proceed for 30 min. The degree of melanization was recorded spectro-photometrically at 490 nm (Versamax multimode reader) (n= 10 for each instar from second through fifth). As first instar larvae were too small to collect enough hemolymph to measure PO activity, only second to fifth instar larvae were measured.

Host plant effects on caterpillar immune responses against parasitoids and parasitoid success

To examine host plant-mediated effects of caterpillar immune status on parasitoid performance, we individually exposed second instar *P. rapae* caterpillars that had been reared on either *B. oleracea* or*B. rapa* to either a single *C. glomerata* or *C. rubecula* female in a 60x15mm petri dish. Both parasitoid species readily oviposit in first and second instar *P. rapae* (Vyas et al. 2019). For this study, second instar larvae were

chosen for two reasons: 1) to allow the caterpillar to feed on the host plant for long enough to show the effect of glucosinolates on their cellular immune system and 2) to be able to collect sufficient quantities of hemolymph to measure PO activity. Parasitism events were monitored closely to avoid superparasitism. Larvae were transferred to their respective host plants immediately after parasitism and reared until the formation of parasitoid cocoons (*C. glomerata* reared on *B. oleracea* -fed caterpillars: $n = 75$; *C. glomerata* reared on *B. rapa* -fed caterpillars: $n = 75$; *C. rubecula* reared on*B. oleracea* -fed caterpillars: $n = 17$; *C. rubecula*reared on *B. rapa* -fed caterpillars: $n = 17$). These larvae were maintained on their respective host plants under greenhouse conditions. Successful parasitoid development was defined as the presence of at least one cocoon on each caterpillar. The size of each*C. glomerata* brood was estimated by counting the number of parasitoid larvae that emerged out of each host caterpillar to form cocoons ($n = 10$ host caterpillars).

To examine host plant effects on the likelihood of encapsulation of the two parasitoids, a separate group of 124 second instar *P. rapae*larvae that had fed on either *B. oleracea* or *B. rapa* were parasitized by either *C. glomerata* ($n = 31$ on each plant) or *C. rubecula* ($n = 31$ on each plant). After parasitism,*P. rapae* larvae were placed back on their respective host plants for another 24-36h, a period sufficient for parasitoid eggs to hatch if they were not encapsulated. After this time, all caterpillars were dissected under a stereomicroscope with fine forceps to assess whether parasitoid eggs were encapsulated. We did not estimate the proportion of the brood that was encapsulated because it was impossible to accurately count eggs once they were encapsulated. Encapsulation was treated as a binary variable with 'yes' if any parasitoid eggs were encapsulated and 'no' if no evidence of encapsulation was found.

Statistical analysis

Total, aliphatic, and indole glucosinolate concentrations were each compared between the two host plants by using Welch's t-test, which adjusts for unequal variance in glucosinolate concentrations between the two host plants. The effect of host plant species on caterpillar weight, development duration, relative growth rate, and THC were analyzed with separate pooled t -tests when variances between the two host plants were similar. To test the effect of host plant species on individual hemocyte counts (i.e., plasmatocytes, granulocytes, oenocytoids, prohemocytes) and phenol oxidase activity (a measure of melanization capability), we used pooled t -tests when variance in the response variable was similar between *B. rapa* -fed and*B. oleracea* -fed caterpillars, otherwise we used Welch's t-test. We focus our analyses of host plant effects on caterpillar immune response on second instar caterpillars as younger instars are preferred by both ovipositing *C. glomerata* and *C. rubecula* females (Vyas et al. 2019).

To compare the effect of host plant species and larval instar on THC, plasmatocytes, granulocytes, oenocytoids, pro-hemocytes, and melanization data, we used a series of two-way ANOVAs followed by Tukey HSD multiple-comparisons tests where host plant, larval instar, and their interaction terms were considered as fixed effects. Granulocytes could not be detected from first instar larvae; hence, they were not included in the analysis. Oenocytoids and prohemocytes counts were square root transformed to normalize their skewed distributions.

The proportions of caterpillars where at least one parasitoid egg was encapsulated when fed either *B. oleracea* or *B. rapa* were compared with separate Fisher's exact tests for each parasitoid species. Likewise, the proportion of caterpillars where at least one *C. glomerata* egg was encapsulated was compared to the proportion of caterpillars where a *C. rubecula* egg was encapsulated using separate Fisher's Exact tests for caterpillars that fed on *B. oleracea* or *B. rapa* . Fisher's Exact tests, separate for each parasitoid species, were also used to compare the proportion of caterpillars where at least one parasitoid successfully formed a cocoon when host caterpillars fed on either *B. oleracea* or *B. rapa* . Finally, the brood size of *C. glomerata* emerging from caterpillars fed with either *B. rapa* or *B. oleracea* were compared with an independent two-tailed t -test.

Data were analyzed using IBM SPSS statistics version 26.0 and JMP Pro 15.2.1 (© 2019 SAS Institute Inc.) and figures were made using Origin 2021b and Biorender software.

RESULTS

Host plant differences in glucosinolate content

Foliar levels of glucosinolates were significantly higher in *B. rapa* than in *B. oleracea* (Figure 1a). *Brassica rapa*contained 52-fold higher total glucosinolate concentrations than*B. oleracea* (*B. rapa* : 24.58μmol/g d.w., *B. oleracea* : 0.47μmol/g d.w; Welch's $t_{4.0013} = 15.59$, $P < 0.001$). Similar patterns were observed for both aliphatic and indole glucosinolates. *Brassica rapa* had 68 times the average concentration of aliphatic glucosinolates than *B. oleracea*(*B. rapa* : 22.31μmol/g d.w, *B. oleracea* : 0.33μmol/g d.w; Welch's $t_{4.0022} = 20.86$, $P<0.001$) and 5.7 times the average concentration of indole glucosinolates than *B. oleracea* (*B. rapa* : 0.80μmol/g d.w., *B. oleracea* : 0.14μmol/g d.w.; Welch's $t_{4.08} = 8.35$, $P<0.001$). The two host plants also varied qualitatively in their glucosinolate profiles. The aliphatic glucobrassicanapin and the indole 4-methoxyglucobrassicin were only recorded from *B. rapa* , whereas the aliphatic gluconapoleiferin, an unidentified aliphatic glucosinolate, and an unidentified indole glucosinolate were only found in *B. oleracea* . The aliphatic gluconapin and the indole glucosinolates glucobrassicin and neoglucobrassicin were found in the leaves of both plant species. The major glucosinolate found in *B. rapa* was gluconapin which measured 348 times higher than the average concentration found in *B. oleracea* (*B. rapa* : 21.81μmol/g d.w., *B. oleracea* : 0.062μmol/g d.w.; Welch's $t_4 = 19.9881$, $P<0.001$). The average concentration of glucobrassicin and neoglucobrassicin measured at-least five times higher in *B. rapa* in comparison to *B. oleracea* (Welch's $t_4 = 7.729$, $P= 0.001$; Welch's $t_4 = 10.943$, $P= 0.0001$).

Host plant effects on caterpillar weight and relative growth rate (RGR)

Pieris rapae larval performance was poorer on *B. rapa* than it was on *B. oleracea* suggesting that feeding on plants with higher glucosinolate concentrations reduced larval weight and increased development time (Figure 1b-c). After 13 days of feeding, caterpillars that fed on *B. rapa* were 25% lighter than caterpillars that fed on *B. oleracea* (*B. rapa* -fed caterpillars: 46.41mg,*B. oleracea* -fed caterpillars: 61.22mg; $t_{38} =$ -12.27, $P<0.001$). However, the relative growth rate between day 5 and day 13 did not differ between caterpillars that fed on*B. oleracea* (0.2398 g/g/d) and *B. rapa* (0.2460 g/g/d) ($t_{38} = 1.06$, $P=0.29$) indicating that glucosinolate differences between these two host plants does not affect relative growth rate. On the other hand, the development duration of these larvae was prolonged on *B. rapa* (Figure 1c). Larvae took approximately seven additional days to develop into adults when they were fed with*B. rapa* ($t_{10} = 7.74$; $P < 0.0001$).

Host plant effects on caterpillar immune status

Total hemocyte counts (THC) increased 32 times from the first to the fifth instars for *B. oleracea* -fed caterpillars ($F_{4,95} = 571.1$, $P<0.001$) and nearly 60 times for *B. rapa* -fed caterpillars ($F_{4,95} = 445.7$, $P<0.001$) (Figure 2a). Beginning with the second instar, caterpillars that fed on *B. rapa* exhibited higher levels of THC production than those that fed on*B. oleracea* at each instar (*B. rapa* -fed vs. *B. oleracea* -fed: 1st instar: $t_{38} = 0.80$, $P=0.43$; 2nd instar: Welch's $t_{26.5} = 10.93$, $P<0.001$; 3rd instar: $t_{38} = 8.50$, $P<0.001$; 4thinstar: $t_{38} = 15.69$, $P<0.001$; 5th instar: $t_{38} = 10.68$, $P<0.001$) suggesting that the higher glucosinolate content of*B. rapa* elicits a greater cellular immune response in caterpillars that fed on this plant. Similar increases across caterpillar instar and differences between the two host plants were observed for individual hemocyte counts of plasmatocytes, granulocytes, and oenocytoids (Figures 2b-e; see figure legend for analyses). In particular, second instar *P. rapae* caterpillars, the stages readily used for oviposition by both *C. glomerata* and *C. rubecula* , produce significantly higher counts of plamatocytes (2.6x), granulocytes (23x), and oenocytoids (5.6x) when they feed on *B. rapa* compared to *B. oleracea* (plasmatocytes: $t_{18}= 12.75$, $P<0.001$; granulocytes: Welch's $t_{9.16}= 9.60$, $P<0.001$; oenocytoids: Welch's $t_{9.57} = 4.29$, $P<0.01$; Figures 2b-d). Phenol oxidase activity was 3.4 times higher in second instar *P. rapae* that fed on *B. rapa*compared to those that fed on *B. oleracea* (Welch's $t_{10.69} = 7.55$, $P < 0.001$; Figure 2f).

Host plant effects on caterpillar immune responses against parasitoids and parasitoid success

The odds that any eggs within a clutch of *C. glomerata* would be encapsulated were 6.3 times more likely when laid in a *P. rapae*caterpillar that fed on *B. rapa* than in a caterpillar that fed on*B. oleracea* (Fisher's Exact Test, $P = 0.005$, $N = 62$; Figure 3b1-2; 4a). Furthermore, *C. glomerata* oviposition attempts in

caterpillars that fed on *B. oleracea* were 3.2 times more likely to result in the formation of at least one cocoon than oviposition attempts in caterpillars feeding on *B. rapa* (Fisher's Exact Test, $P < 0.001$, $N = 150$; Figure 4b). *Cotesia glomerata*broods yielded 1.8 times as many cocoons when they developed in caterpillars that fed on *B. oleracea* compared to those that fed on *B. rapa* ($t_{16} = 8.67$, $P < 0.001$; Figure 4c). On the other hand, the odds of a *C. rubecula* egg being encapsulated did not differ whether host caterpillars fed upon*B. rapa* or *B. oleracea* (Fisher's Exact Test, $P = 0.49$, $N = 62$; 4a); no eggs were encapsulated by caterpillars feeding on *B. oleracea* and only 2 out of 31 eggs were encapsulated by caterpillars feeding on *B. rapa* . Likewise, the likelihood that oviposition by*C. rubecula* results in the formation of a cocoon did not differ between caterpillars that fed on *B. oleracea* and *B. rapa*(Fisher's Exact Test, $P = 0.84$, $N = 32$; Figure 4b). Whereas the odds of being encapsulated did not differ between *C. glomerata* and*C. rubecula* when their hosts fed on *B. oleracea* (Fisher's Exact Test, $P = 0.11$, $N = 62$, Figure 4a), the odds of at least one egg from *C. glomerata* clutch being encapsulated were 13.6 times higher than for a *C. rubecula* egg when laid in a caterpillar that fed upon *B. rapa* (Fisher's Exact Test, $P < 0.001$, $N = 62$, Figure 4a).

DISCUSSION

Insect herbivores must balance the nutritional quality of their host plants with their ability to defend against natural enemies. An increasing number of studies have focused on the interaction between plant secondary chemistry and herbivore immune responses when explaining the relationship between larval performance on host plants and defense against parasitoids, predators, and pathogens. Nearly all these studies have shown that herbivore immune systems are weakened when they feed on more toxic host plants (Reudler et al. 2011; Smilanich et al. 2009a; Smilanich & Muchoney 2022; but see Garvey et al. 2021, Ghosh et al. 2022).

Our study shows the opposite pattern whereby *P. rapae*caterpillars that feed on the higher glucosinolate-containing *B. rapa* have enhanced immunity against parasitoids. Hemocytes involved in encapsulation (plasmatocytes, granulocytes, and oenocytoids) as well as the processes involved in melanization (namely PO activity) are all elevated in caterpillars that feed on *B. rapa* during the second instar, when caterpillars are vulnerable to attack by *C. glomerata* and *C. rubecula* , resulting in increased encapsulation and melanization of *C. glomerata* eggs. However, enhanced immunity comes at the cost of reduced larval weight and increased development time, which may increase exposure to parasitism (Benrey & Denno 1997). As a result, an elevated immune response when feeding on plants containing higher levels of glucosinolates may provide an effective counter to the increased exposure duration to parasitoids. At present, the mechanism responsible for the positive association between glucosinolate concentration and encapsulation response is unknown, but clearly deserves further attention.

Furthermore, our study shows that the effectiveness of the immune system of *P. rapae* is not only dependent on the host plant species on which they feed, but also the identity of the parasitoid species. Whereas *C. glomerata* suffered high encapsulation rates and reduced brood sizes when their hosts fed on *B. rapa* , encapsulation of *C. rubecula* eggs was very rare in our study and nearly all oviposition attempts resulted in the production of adult offspring regardless of the plant species on which the host caterpillar fed. These observations strongly suggest that *C. rubecula* can successfully evade or suppress the immune response of *P. rapae* .*Cotesia rubecula* and *C. glomerata* inject venom and calyx fluid containing the symbiotic *Bracovirus* which suppress the encapsulation and melanization responses of *P. rapae* (Asgari et al. 2003; Zhang et al. 2004a; Zhang et al. 2004b). Yet, how *C. rubecula* can evade encapsulation whereas *C. glomerata* is more susceptible, especially when their hosts feed on glucosinolate-rich diets, remains an open question. *Cotesia rubecula* calyx fluid contains a Cr*Bracovirus* that interferes with adhesion among*P. rapae* hemocytes and a Crp32 protein that adheres to the egg's surface, both of which prevent successful encapsulation by *P. rapae* (Asgari et al. 1996, 1998). Whether *C. glomerata* has similar means of evading *P. rapae* 's immune system is unknown, but if these are absent or less effective, it may explain the difference in susceptibility between these two parasitoids. In an ongoing study we have found that total hemocyte counts in *B. oleracea* -fed caterpillars are induced three-fold when parasitized by *C. glomerata* , whereas parasitism by *C. rubecula* appears to suppress induction of total hemocyte counts (Ghosh et al. unpubl. data). In a separate study, we show that injection of *C. rubecula* calyx fluid into caterpillars that had already been parasitized by *C.*

glomerata results in decreased encapsulation rates of *C. glomerata* eggs, suggesting that important differences exist in the*Brachovirus* -containing calyx fluids of these two parasitoids (Paul et al. unpublished).

Caterpillar species that sequester plant toxins as well as species where plant toxins accumulate in the hemolymph generally have weak immune systems (e.g., Freitak et al. 2003; Smilanich et al. 2009; Lampert & Bowers 2015; Tan et al. 2019; Smilanich & Muchoney 2022), suggesting that the costs of sequestration trade off with the ability to invest in a robust immune system. Sequestration may reduce the need to invest in costly immune responses. Yet in some cases, while sequestration may provide protection against predators, reduced immunocompetence may expose herbivores to higher levels of parasitism (e.g., Kelly & Bowers 2018) indicating that a single herbivore defense mechanism is not equally effective against all natural enemies. Specialist caterpillars that can effectively detoxify plant toxins may reduce the negative impacts of toxins on the production of hemocytes involved in cellular immunity. The larvae of *P. rapae* do not sequester glucosinolates (Müller et al. 2003). Rather, *P. rapae* is thought to specialize on brassicaceous plants that contain glucosinolates because of its ability to efficiently metabolize these compounds into relatively benign compounds. *Pieris rapae* larvae possess a nitrile specifier protein in the midgut that converts ingested glucosinolates into relatively non-toxic nitriles, which are excreted, rather than the more toxic isothiocyanates (Wittstock et al. 2004). Caterpillars feeding on plants containing higher glucosinolate levels may induce increased production of the nitrile specifier protein (NSP) or a very high concentration of glucosinolates could overwhelm the enzymatic efficiency of NSP, either of which could result in decreased growth and larval survivorship (Agrawal & Kurashige 2003).

Interestingly, our results contradict those of a previous study (Bukovinszky et al. 2009) that showed that glucosinolates had an immunosuppressive, rather than an immune-enhancing, effect on the immune response of *P. rapae* against *C. glomerata* . At least two explanations may account for this discrepancy. First, the Bukovinszky et al. (2009) study compared the effects of different wild and cultivated populations of *B. oleracea* whereas our study compared the effects of two different *Brassica* species. Comparisons of two different plant species results in greater quantitative and qualitative differences in glucosinolate profiles as well as the potential for significant differences in other aspects of nutritional quality that may affect the expression of immune responses in herbivores. Second, the European populations of *C. glomerata* used in the Bukovinszky et al. (2009) study primarily attack *P. brassicae* , not *P. rapae* (Geervliet et al. 2000) and are therefore not typical of the caterpillar – parasitoid association in Europe. The populations of*C. glomerata* used in our study were collected in North America where they have had an association with *P. rapae* for nearly 150 years (Herlihy et al. 2012). The difference in host-parasitoid associations between European and North American populations of *C. glomerata* has been maintained over a long enough period that there may be important selective differences in the immune responses of *P. rapae* to *C. glomerata* and how they respond to variable levels of glucosinolates.

This is one of the few studies that have shown an immuno-enhanced effect of plant toxins on herbivore immunity that also translates into reduced parasitoid fitness. And, to our knowledge, this is the first study to demonstrate that parasitoid species identity can determine the outcome of plant toxin-mediated effects on herbivore immunity against their natural enemies. Clearly, a meaningful understanding of the significance of bottom-up effects of plant quality on herbivore immunity cannot occur in the absence of understanding the importance of the species-specific nature of top-down effects of natural enemies on herbivores. More studies are needed across different plant systems with a broader range of defensive chemistries to better understand the generality of patterns already elucidated. Equally important are studies that include multiple and diverse natural enemies to understand how plant toxin – herbivore immune interactions are shaped by the food-webs in which they reside.

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Competing interests: The authors declare no competing interests.

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Figure legends:

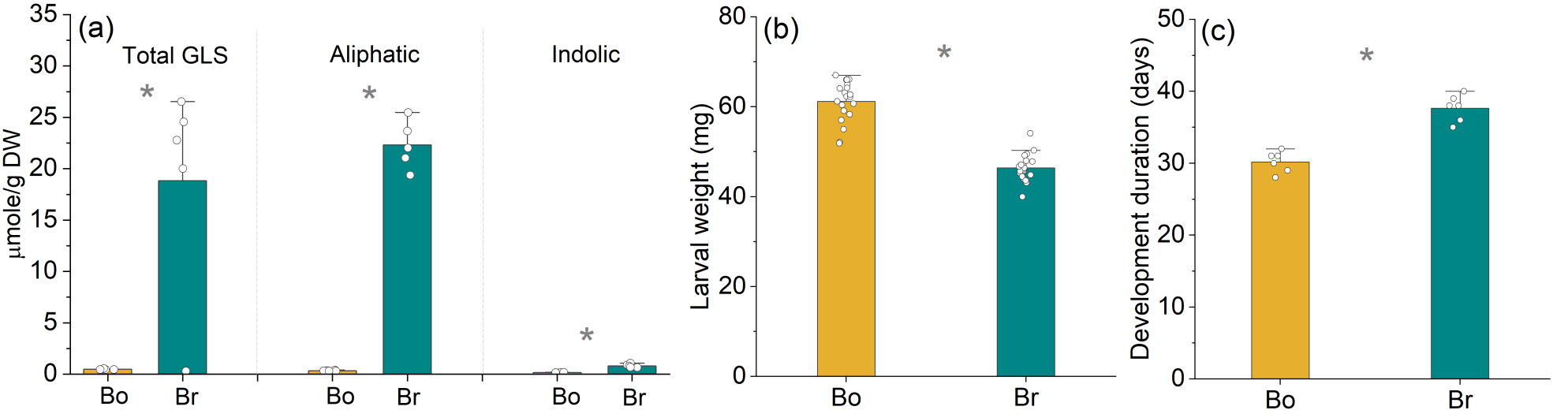
Figure1. Host plant differences in glucosinolate (GSL) content and impact on larval development: (a) profile of total, aliphatic, and indole GSL in the leaves of host plants ($n = 5$), (b) weight ($n = 20$) and (c) development duration (days) ($n = 6$) of larvae fed with experimental host plants. Values are means ± SE, significant differences are based on t - tests, '*' above bars mean significantly different means at alpha level 0.05, see text for details. Bo = *Brassica oleracea* var *viridis* , Br =*Brassica rapa* .

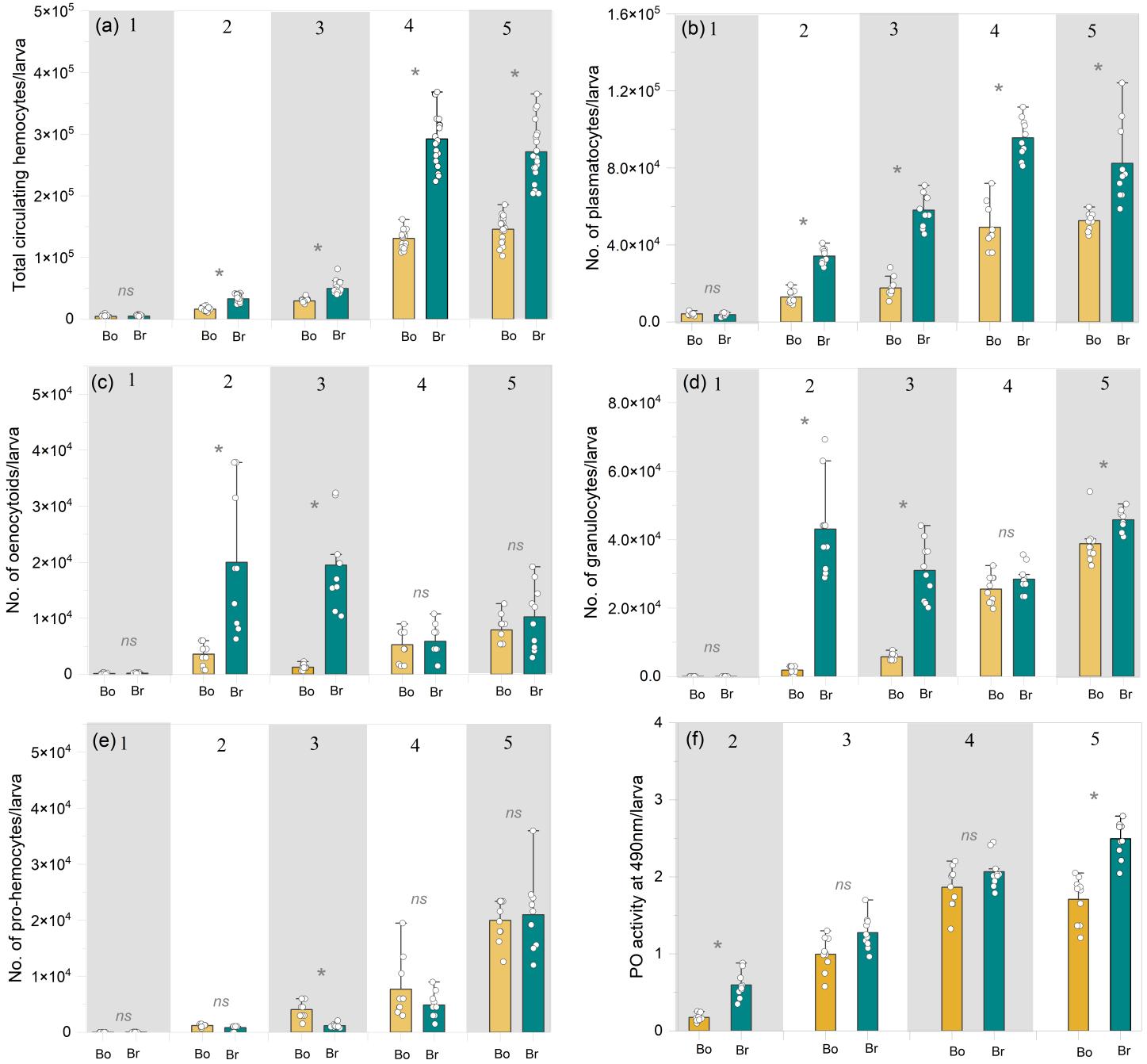
Figure 2. Effect of host plant on the ontogenetic profile of larval cellular and humoral (phenol oxidase activity) immunity: (a) Total circulating hemocytes/larva ($n = 20$ per instar/host plant combination), (b) Number of plasmatocytes/larva ($n = 10$ per instar/host plant combination), (c) Number of oenocytoids/larva ($n = 10$ per instar/host plant combination), (d) Number of granulocytes/larva ($n = 10$ per instar/host plant combination), (e) Number of pro-hemocytes ($n = 10$ per instar/host plant combination), and (f) PO activity at 490nm/larva ($n = 10$ per instar/host plant combination). Bo- *Brassica oleracea* var*viridis* , Br- *Brassica rapa* ; 1, 2, 3, 4, 5- first, second, third, fourth, and fifth instar, respectively. Values are means ± SE, significant differences are based on t -tests comparing the effect of host plant species on hemocyte counts (Figs. 2a-e) or phenol oxidase activity within each caterpillar instar (Fig. 2f). '*' above bars mean significantly different means at alpha level 0.05, 'ns' indicate comparisons are not significantly different; see text for analyses of second instar hemocyte counts and phenol oxidase activity; comparisons of the effects of host plant species on first, third, fourth, and fifth instar immune measures are as follows: total circulating hemocytes: first instar ($t_{38} = 0.80$; $P = 0.43$), third instar ($t_{38} = -8.50$, $P < 0.001$), fourth instar ($t_{38} = -15.69$, $P < 0.001$), fifth instar ($t_{38} = -10.68$, $P < 0.001$); plasmatocytes: first instar ($t_{18} = 0.91$, $P = 0.38$), third instar ($t_{18} = -7.60$, $P < 0.001$), fourth instar ($t_{18} = -9.44$, $P < 0001$), fifth instar ($t_{18} = -4.43$, $P < 0.001$); oenocytoids: first instar ($t_{18} = 1.17$, $P = 0.26$), third instar ($t_{18} = -12.80$, $P < 0.001$), fourth instar ($t_{18} = 0.48$, $P = 0.64$), fifth instar ($t_{18} = 1.18$, $P = 0.25$); granulocytes: first instar (*no granulocytes recorded*), third instar ($t_{18} =$

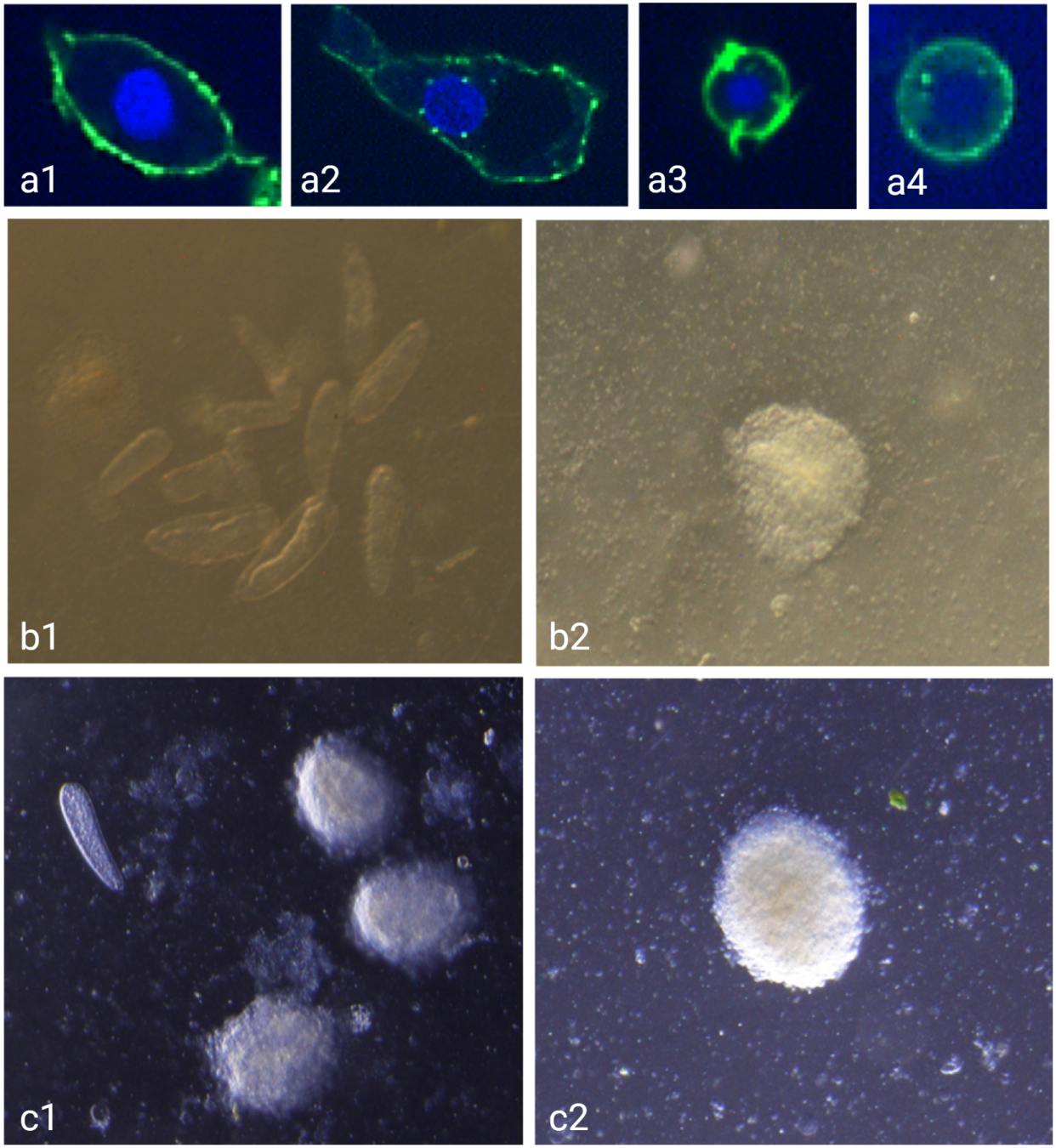
-9.34, P < 0.001), fourth instar (t_{18} = -1.59, P = 0.13), fifth instar (t_{18} = -3.34, P = 0.004); pro-hemocytes: first instar (t_{18} = -0.30, P = 0.77), third instar (t_{18} = 5.59, P < 0.001), fourth instar (t_{18} = 1.56, P = 0.16), fifth instar (t_{18} = -0.42, P = 0.68); phenol oxidase activity: third instar (t_{18} = -2.90, P < 0.001), fourth instar (t_{18} = -1.84, P = 0.08), fifth instar (t_{18} = -6.57, P < 0.001).

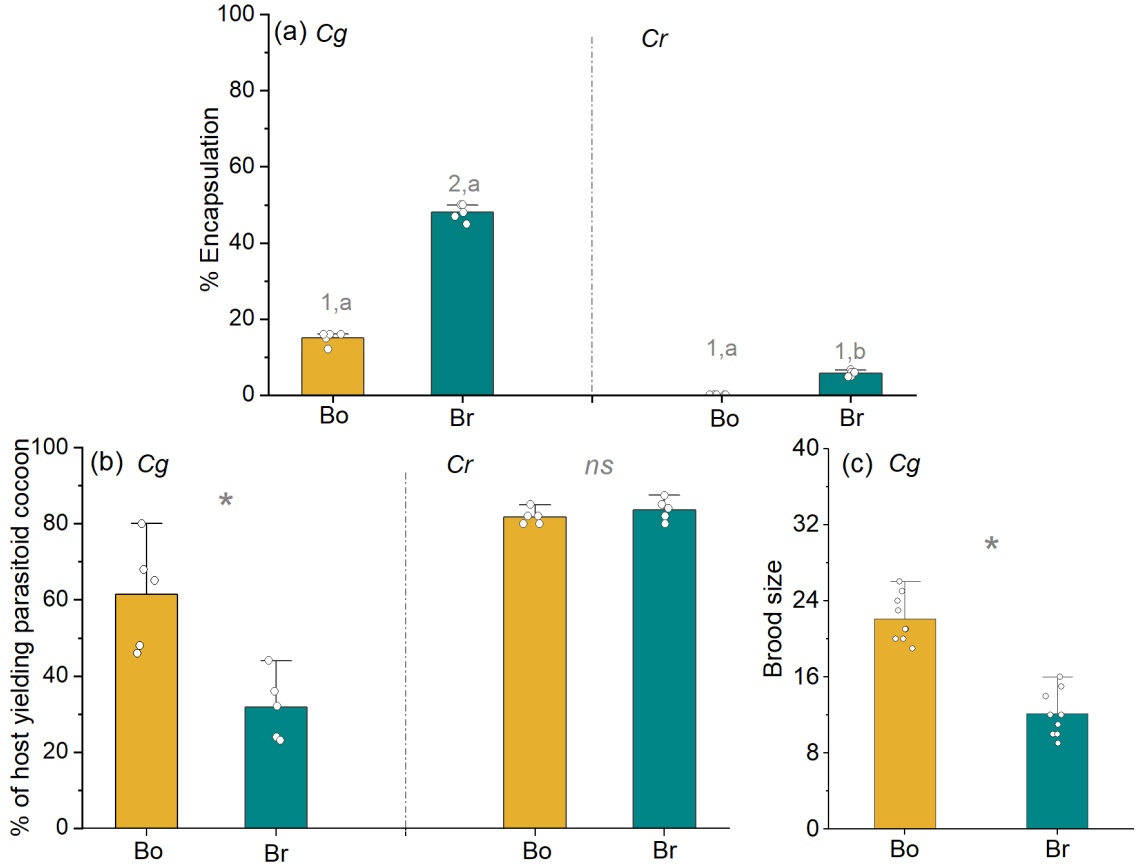
Figure 3. Hemocytes involved in encapsulation *Pieris rapae* larvae: (a1-2) Plasmatocytes, (a3) granulocytes and (a4) oenocytoids. Encapsulation of *Cotesia glomerata* eggs by*Brassica oleracea* var *viridis* , and *Brassica rapa*fed *P. rapae* (b, c).

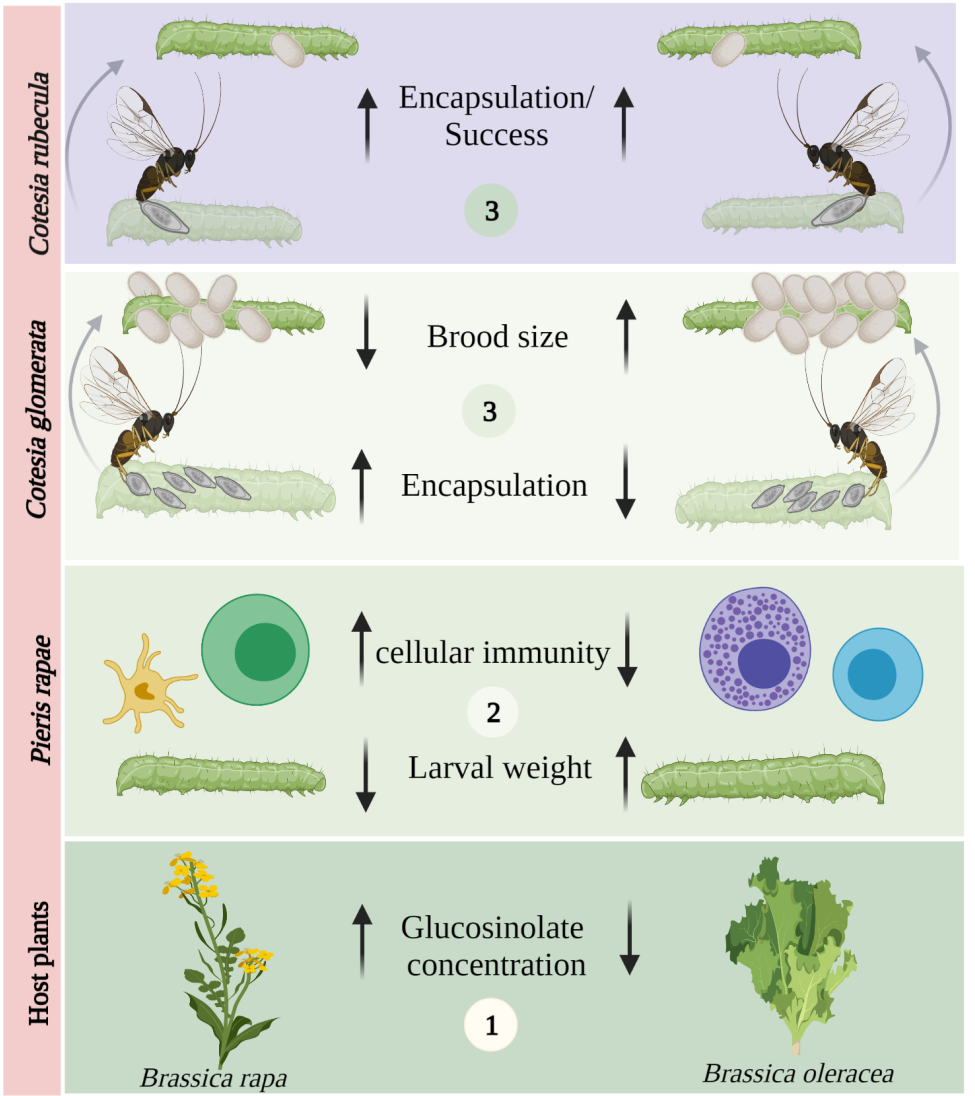
Figure 4. Host plant mediated larval immunity and parasitoid success: (a) % of caterpillars where at least one *Cotesia glomerata* (Cg) egg or the *C. rubecula* (Cr) egg has been encapsulated (n = 31), (b) % of caterpillars where at least one*C. glomerata* (n =75) or *C. rubecula* (Cr, n = 17) cocoon has successfully formed, (c) brood size of *C. glomerata* (n = 10). Values are means ± SE, significant differences are based on t -tests (c) or Fisher's exact test (a, b). Bars with different symbols (host plants are indicated with numbers and parasitoid species with letters)/'*' are significantly different from each other, see text for details. Bo-*Brassica oleracea* var viridis, Br- *Brassica rapa* , Cg- *C. glomerata* , Cr- *C. rubecula* .


(a)
Total GLS
Aliphatic
Indolic
µmole/g DW
35
30
25
20
15
10
5
0
Bo
Br
*
(b)
Larval weight (mg)
80
60
40
20
0
(c)
Development duration (days)
50
40
30
20
10
0


(a)
Total circulating hemocytes/larva
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
(b)
No. of plasmatocytes/larva
1.6×10^5
1.2×10^5
8.0×10^4
4.0×10^4
0.0
(c)
No. of oenocytoids/larva
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
(d)
No. of granulocytes/larva
8.0×10^4
6.0×10^4
4.0×10^4
2.0×10^4
0.0
(e)
No. of pro-hemocytes/larva
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
(f)
PO activity at 490nm/larva
4
3
2
1
0
1
2
3
4
5
Bo
Br
ns
*


a1
a2
a3
a4
b1
b2
c1
c2


(a) Cg
Cr
% Encapsulation
100
80
60
40
20
0
1,a
2,a
1,a
1,b
Bo
Br
Bo
Br
(b) Cg
Cr
ns
*
% of host yielding parasitoid cocoon
100
80
60
40
20
0
Bo
Br
Bo
Br
(c) Cg
*
Brood size
40
32
24
16
8
0
Bo
Br


Cotesia rubecula
Encapsulation/
Success
3
Cotesia glomerata
Brood size
3
Encapsulation
Pieris rapae
cellular immunity
2
Larval weight
Host plants
Glucosinolate
concentration
1
Brassica rapa
Brassica oleracea