Ontogenetic differences in the chemical defence of flea beetles influence their predation risk

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Author contributions

TS and FB designed experiments, TS, JK and FB performed experiments and analysed data, TS and FB wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Conflict of interest

The authors declare no conflict of interest.

Data availability

Data are available from the Edmond repository under https://dx.doi.org/10.17617/3.1e.
ABSTRACT

1. Several insect species have evolved two-component chemical defences that enable the rapid release of deterrent or toxic metabolites upon predator attack. However, whether these chemical defences vary across insect ontogeny and how this affects the predation risk of different life stages has rarely been addressed.

2. The horseradish flea beetle *Phyllotreta armoraciae* possesses a two-component chemical defence that consists of sequestered glucosinolates and an insect myrosinase capable of converting the non-toxic glucosinolates to deterrent isothiocyanates. Here, we show that the levels of sequestered glucosinolates only varied 2-fold across beetle ontogeny, but that insect myrosinase activity differed up to 43-fold among ontogenetic stages.

3. Specifically, glucosinolate levels were 1.5-fold lower in larvae of *P. armoraciae* than in pupae, but they showed 43.4-fold higher levels of myrosinase activity. Consistent with the distinct levels of myrosinase activity in larvae and pupae, only larvae released high amounts of toxic isothiocyanates when they were attacked by the generalist predator *Harmonia axyridis*. *P. armoraciae* larvae deterred the predator and survived one attack, whereas pupae were killed.

4. Feeding of *P. armoraciae* larvae on plants that differed in glucosinolates and plant myrosinase activity influenced the accumulation of glucosinolates in larvae and their subsequent interaction with *H. axyridis*. Larvae with low levels of sequestered
glucosinolates were much more susceptible to predation than larvae containing high glucosinolate levels.

5. Our results demonstrate that sequestered plant defence metabolites selectively protect specific ontogenetic stages of *P. armoraciae* from predation. The strong influence of plant defensive chemistry on sequestration indicates that predators have played an important role in the evolution of host use in this specialist herbivore. The distinct life styles of flea beetle life stages and their strategies to prevent predation by biologically relevant predator communities deserve further investigations.

**KEY WORDS**
chemical defence, *Harmonia axyridis*, myrosinase, ontogeny, *Phyllotreta armoraciae*, predation, sequestration, tritrophic interaction

1. INTRODUCTION

Insects evolved numerous strategies to escape predation including behavioural, structural, and chemical defences (Rettenmeyer 1970; Pasteels, Grégoire & Rowell-Rahier 1983; Gross 1993; Humphreys & Ruxton 2019). Which defence strategies an insect uses depends on the community of natural enemies it encounters, and this can change across insect ontogeny depending on the respective life style and habitat (Pasteels, Grégoire & Rowell-Rahier 1983; Boege, Agrawal & Thaler 2019; Lindstedt, Murphy & Mappes 2019). Some insects that feed on chemically defended plants utilize plant defence compounds to deter predators by sequestering them in their bodies (Opitz & Müller 2009; Erb & Robert 2016; Petschenka & Agrawal 2016). Sequestering insects are frequently specialist feeders, which led to the hypothesis that higher trophic levels played an important role in the evolution of herbivore diet breadth (Dyer 1995; Opitz & Müller 2009; Petschenka & Agrawal 2016). The sequestration of plant defensive chemicals appears to be widespread in herbivorous insects of the orders Coleoptera, Lepidoptera and Hemiptera (Opitz & Müller 2009). However, we know comparatively little about how sequestration patterns vary across insect ontogeny and how this affects predators.

Several plant defence compounds are stored as glucosylated pro-toxins. Upon herbivory, the pro-toxins come into contact with β-glucosidases and are converted to deterrent and toxic compounds (Morant *et al.* 2008). Such two-component defence systems also evolved in insects and other arthropods, which either sequester or de novo synthesize the glucosylated pro-toxins and produce
the corresponding β-glucosidases themselves (Beran et al. 2019). In contrast to defence strategies that deter the predator before it attacks, two-component defences are usually activated upon injury and thus expose the insect to toxic metabolites. To be beneficial to the individual, the defence should deter the predator before it kills the prey. However, chemical defences frequently do not protect the individual but are still beneficial because predators learn to avoid conspecifics (Zvereva & Kozlov 2016).

The horseradish flea beetle *Phyllotreta armoraciae* is a highly specialized herbivore that is monophagous on horseradish *Armoracia rusticana* in nature, but also feeds on other brassicaceous plants under laboratory conditions (Nielsen 1978; Vig & Verdyck 2001). Most species of the genus *Phyllotreta* are closely associated with Brassicaceae plants (Gikonyo, Biondi & Beran 2019). The most obvious and characteristic antipredator strategy of adult flea beetles is their ability to jump in order to escape (Furth 1988). There are anecdotal reports on predation of *Phyllotreta* adults from observations in agricultural settings (e.g. Burgess 1977; Burgess 1980; Burgess 1982), but how much influence predators and other natural enemies have on flea beetle populations in natural or agricultural ecosystems is unknown.

Studies with *Phyllotreta striolata* and *P. armoraciae* revealed that adults possess a potent chemical defence that consists of sequestered glucosinolates (GLS) and a beetle-derived β-thioglucosidase enzyme (myrosinase) that catalyses the conversion of GLS to highly reactive isothiocyanates (Beran et al. 2014; Körnig 2015). However, *Phyllotreta* beetles are apparently able to control GLS hydrolysis because high levels of sequestered GLS and myrosinase activity are simultaneously present in adults (Beran et al. 2014; Körnig 2015).

GLS are a group of about 130 structurally different amino acid-derived thioglucosides produced by Brassicales plants (Agerbirk & Olsen 2012; Blažević et al. 2020). Plant GLS levels and compositions vary within and between species and are also influenced by biotic and abiotic factors (Burow 2016). In plant tissue, GLS and myrosinases are separately stored until tissue damage leads to the formation of biologically active breakdown products, e.g. isothiocyanates (Wittstock et al. 2016). Isothiocyanates protect plants from non-adapted herbivores and pathogens due to their broad reactivity towards biological nucleophiles (Avato et al. 2013; Jeschke, Gershenzon & Vassão 2016; Pastorczyk & Bednarek 2016). Adapted herbivores and pathogens use different strategies such as sequestration, metabolic detoxification, and excretion
to overcome this plant defence (Jeschke, Gershenzon & Vassão 2016; van den Bosch, Niemi & Welte 2019; Vela-Corcía et al. 2019). The ability to sequester intact GLS evolved independently in specialized insects belonging to the orders Hemiptera, Hymenoptera and Coleoptera, but not all GLS-sequestering species evolved endogenous myrosinase activity (Müller & Wittstock 2005; Opitz & Müller 2009; Beran et al. 2018). One of these is the turnip sawfly Athalia rosae where rapid GLS sequestration has been suggested to function as a detoxification mechanism by preventing hydrolysis by plant myrosinases (Abdalsamee et al. 2014; van Geem, Harvey & Gols 2014).

Previous studies with P. armoraciae revealed the presence of high GLS levels in newly emerged adults, which indicates that sequestered GLS are transferred from the larval to the adult stage (Yang et al. 2020). Compared to P. armoraciae adults, we know much less about the chemical defence of the less mobile immature life stages, which have a different life style than adults (Vig 1999). P. armoraciae females prefer to oviposit on leaf petioles. Neonates penetrate into the plant and mine in the petioles or leaf midribs until the final (third) instar. Mature larvae leave the plant and search for a place to dig into the soil where they build an earthen chamber for pupation. There appears to be some variation in the life style of Phyllotreta spp. larvae, which either mine in plant petioles or roots, or feed externally on roots (Vig 2004). There is scarce information on predation of the immature life stages of Phyllotreta spp., but several laboratory and field studies investigated the efficacy of entomopathogenic fungi and nematodes to control the soil-dwelling life stages of P. striolata and Phyllotreta cruciferae, showing variable success (Xu et al. 2010; Yan et al. 2013; Reddy et al. 2014; Yan et al. 2018).

Here, we investigated how the GLS-myrosinase defence system of P. armoraciae shapes stage-specific predation by a generalist predator. We selected the Asian ladybird Harmonia axyridis as a model predator because this generalist is highly abundant in typical habitats of P. armoraciae and preys on different groups of insects including beetles (Koch 2003). Analyses of the levels of sequestered GLS and myrosinase activity across all life stages revealed rather similar GLS levels, but large differences in myrosinase activity between the life stages, with notably higher levels in larvae than in pupae. We tested the predation risk (predator-induced mortality rate) of P. armoraciae larvae and pupae in experiments with H. axyridis larvae. H. axyridis showed distinct responses to these life stages, which resulted in high mortality of P. armoraciae pupae but not of larvae. Based on these results we investigated how P. armoraciae larvae can deter
H. axyridis and survive the predator attack. Therefore, we manipulated the levels of sequestered GLS in larvae by feeding them with different food plants, and subsequently exposed these larvae to predation by H. axyridis. Finally, we investigated the mechanism of GLS hydrolysis upon predation by determining the localization of sequestered GLS and myrosinase activity in P. armoraciae larvae.

2. MATERIALS AND METHODS

2.1. Insect rearing and plant cultivation

P. armoraciae adults were collected from A. rusticana plants around Jena (Thuringia, Germany) and reared on potted Brassica juncea cv. Bau Sin (Known-You Seed Co., Ltd., Kaohsiung, Taiwan), because horseradish plants grow too slowly for a continuous supply at a suitable plant stage. However, B. juncea and horseradish contain the same major GLS (Li & Kushad 2004). Beetles were reared on three- to four-week old B. juncea plants in a controlled-environment chamber at 24 °C, 60% relative humidity and a 14/10-h light/dark period. New plants were supplied every week and plants with eggs were kept separately for larval development. After three weeks, any remaining above ground plant material was removed and the soil containing pupae was transferred to plastic containers (9 L volume, Lock&Lock, Seoul, South Korea). Emerging adults were collected every two to three days and were supplied with plants until used in experiments.

Asian ladybird beetles H. axyridis collected in Ober-Mörlen (Hesse, Germany), Jena, and Ottendorf (Thuringia, Germany) were reared in a controlled-environment chamber at 23 °C, 60% relative humidity, and a 16/8-h light/dark period on pea aphids Acyrthosiphon pisum. Egg clutches were transferred to Petri dishes, and hatched larvae were reared separately on pea aphids.

Arabidopsis thaliana plants were cultivated at 21 °C, 55% relative humidity, and a 10/14-h light/dark period. We used the A. thaliana Col-0 wild type and three mutant lines in the Col-0 background that differ from the wild type in their GLS accumulation patterns and foliar levels of myrosinase activity. The myb28×myb29 double knockout mutant (myb) is devoid of aliphatic GLS (Sønderby et al. 2007), the myb28×myb29×cyp79B2×cyp79B3 quadruple knockout mutant (mybcyp) has no detectable levels of GLS (Sun et al. 2009), and leaves of the tgg1×tgg2 double knockout mutant (tgg) are lacking myrosinase activity (Barth & Jander 2006).

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2.2. GLS levels and myrosinase activity in different life stages of *P. armoraciae*

To compare GLS levels and myrosinase activity across all life stages of *P. armoraciae*, we collected eggs, larvae, pupae, and adults (newly emerged and after 14 days of feeding) from the rearing colony. Samples were weighed, frozen in liquid nitrogen and stored at -20 °C until GLS extraction (n = 7-8) or at -80 °C until protein extraction (n = 5-9). Before sampling, eggs were washed three times in 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5). Fed adults were starved for one day before sampling but larvae were not starved because they are more sensitive than adults. GLS were extracted, converted to desulfo-GLS and analysed by HPLC-UV as described in Beran et al. (2014). Myrosinase activity was determined in crude protein extracts prepared from different *P. armoraciae* life stages using 0.5 mM allyl GLS (Carl Roth, Karlsruhe, Germany) as a substrate as described in Beran et al. (2018).

The levels of GLS and myrosinase activity were compared across *P. armoraciae* life stages using the generalized least squares method (gls from the nlme library (Pinheiro et al. 2018) in R3.3.1 (R Core Team 2018)). We applied a constant variance function structure (varIdent) implemented in the nlme library in R, which allows each life stage to have a different variance. The *P*-value was obtained by removing the explanatory variable and comparing both models using a likelihood ratio (LR) test. Significant differences between life stages were determined by post hoc multiple comparison of estimated means using Tukey contrasts (emmeans from the emmeans library (Lenth 2019)).

2.2.1. Feeding of *P. armoraciae* larvae with different food plants

To investigate whether sequestered GLS protect *P. armoraciae* larvae from *H. axyridis*, we manipulated their GLS levels by feeding early second instar larvae for 11 to 13 days with rosette leaves of six-week old *A. thaliana* wild type plants, *mybcyp* (devoid of GLS), *tgg* (no myrosinase activity), or *B. juncea* leaves without the midvein. Afterwards, we determined the levels of GLS and myrosinase activity in fed *P. armoraciae* larvae (GLS: n = 12-20, myrosinase activity: n = 6) as described above.

2.3. Predation experiments

2.3.1. Observations
Individual *P. armoraciae* larvae, pupae, and adults were exposed to a *H. axyridis* third instar larva for 10 min in a custom-made polyoxymethylene arena (25 mm length × 12.5 mm width × 3 mm height) closed with a Plexiglas plate. Sixteen independent observations were recorded for each life stage using an EOS 600D (Canon, Tokyo, Japan) camera mounted on a Stemi 2000-C microscope (Zeiss, Jena, Germany). For each observation, the arena was cleaned and new predator and prey individuals were used. The number of attacks and the feeding time per attack were recorded.

### 2.3.2. Predator feeding preference

To analyse the feeding preference of third instar *H. axyridis* larvae, we simultaneously offered one *P. armoraciae* larva and pupa as prey in a Petri dish (60 mm diameter, Greiner Bio-One, Frickenhausen, Germany), and recorded their survival after 24 hours (n = 55).

### 2.3.3. Predator-induced mortality

To determine the consequence of a single predator attack, we compared the proportions of injured larvae and pupae that developed into adults with that of uninjured individuals, respectively (larvae: n = 51; pupae: n = 15-16). Larvae were kept in Petri dishes with cut *B. juncea* petioles until pupation. Pupae were kept on moistened soil until adult eclosion. Mortality and adult eclosion were recorded every day.

### 2.3.4. Survival rate of flea beetle larvae with low and high GLS levels in the presence of predators

The survival of *P. armoraciae* larvae that were reared on different food plants (described in section 2.2.1) and thus differed in their levels of sequestered GLS was recorded over 6 h exposure to third instar *H. axyridis* larvae in 30 min intervals. Each replicate consisted of one *P. armoraciae* larva that was exposed to one predator larva (n = 60-61). One replicate was excluded from the analysis because the *H. axyridis* larva had moulted to the fourth larval instar. Survival data were analysed using a parametric survival regression model with a log-logistic hazard distribution in R (Therneau 2015). Log-rank tests with Benjamini and Hochberg correction were performed using the R package survminer (Kassambara & Kosinski 2018). Factor level reduction was used to determine which treatments differ from each other (Crawley 2013).

### 2.3.5. Flea beetle chemical defence and predator survival

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We compared the survival of early third instar *H. axyridis* larvae fed with *P. armoraciae* larvae that were either reared on *A. thaliana* wild type or *tgg* plants and thus contained low or high levels of sequestered GLS, respectively. Each *H. axyridis* larva was provided with one new *P. armoraciae* larva every day (*n* = 20). Predator feeding, weight, and survival were recorded every day, except that predators were not weighed on the first day. Survival data were analysed by a log-rank test as described above.

### 2.4. Hydrolysis of sequestered GLS upon predator attack

To determine whether sequestered allyl GLS is converted to allyl isothiocyanate (AITC) upon predator attack, we collected the headspace of five larvae or pupae that were exposed to one *H. axyridis* third instar larva in a 50 mL glass bottle (DURAN®, DWK Life Science, Mainz, Germany) for four hours (*n* = 8). Afterwards, the numbers of injured or dead *P. armoraciae* individuals were counted. Volatile collections performed with larvae or pupae not exposed to predators served as controls (*n* = 3-4). A constant flow of humidified and active charcoal-filtered compressed air (< 100 mL/min) was led through the bottle and the headspace was collected on Porapak-Q™ volatile collection traps (25 mg; ARS, Inc., Gainsville, FL, USA). Volatile traps were eluted twice with 100 µL of hexane (98% purity; Carl Roth) and samples were stored at -20 °C until analysis by gas chromatography-mass spectrometry. Headspace samples were analysed using a 6890N gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a Zebron ZB-5MSi capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex, Aschaffenburg, Germany) coupled to a 5973 quadrupole mass spectrometer (Agilent Technologies). The carrier gas was helium at a constant flow rate of 1 mL/min. One microliter per sample was injected in splitless mode. The front inlet temperature was set to 220 °C. The oven program started at 40 °C for 2 min, increased at 10 °C/min to 100 °C, and then with 50 °C/min to 300 °C, which was held for 1 min. Mass spectrometry conditions were electron impact mode (70 eV), and scan mode *m/z* 33-250. AITC was quantified using an external standard curve prepared from an authentic AITC standard (95% purity; Sigma-Aldrich, Steinheim, Germany).

To determine where sequestered GLS and beetle myrosinase are stored in *P. armoraciae* third instar larvae, we collected the haemolymph, gut, and the remaining body parts. One day before dissection, we shifted larvae from *B. juncea* to *A. thaliana myb* or *tgg* plants to ensure that larval
guts were devoid of aliphatic GLS or myrosinase activity. Larvae were dissected in phosphate-buffered saline (PBS) pH 7.4 (Bio-Rad, Munich, Germany), and haemolymph was collected in the dissection buffer. Samples for GLS extraction were collected on ice in 80% (v/v) methanol (> 99.9% purity, Carl Roth), and stored at -20 °C until GLS extraction. Samples for protein extraction were collected in PBS buffer containing proteinase inhibitors (cOmplete EDTA-free, Roche, Mannheim, Germany). Protein extraction and myrosinase activity assays were performed immediately after dissection as described above. For each sample, tissues and haemolymph of ten individuals were pooled (GLS: n = 5, myrosinase activity: n = 6).

To test whether GLS are hydrolysed in the gut of *H. axyridis* third instar larvae, we measured myrosinase activity in crude gut protein extracts. Larvae were dissected in PBS buffer (pH 7.4) supplemented with protease inhibitors (cOmplete EDTA-free). For each replicate, five guts were pooled in 130 µL of dissection buffer, frozen in liquid nitrogen and stored at -80 °C. Protein extraction and myrosinase activity assays were performed as described in section 2.2 (n = 4).

3 RESULTS

3.1 The levels of GLS and myrosinase activity differ between *P. armoraciae* life stages

To determine which *P. armoraciae* life stages are capable of producing toxic isothiocyanates for their defence, we compared the levels of GLS and myrosinase activity among eggs, larvae, pupae and adults. The average total GLS concentrations detected in *P. armoraciae* ranged from 20 to 44 nmol/mg FW and were thus generally higher than those detected in leaves of the rearing plant *B. juncea* (Fig. 1a and Table S1, refer to Supplementary Methods and Results 1 for details on *B. juncea*). Allyl GLS, the major GLS in *B. juncea*, accounted for more than 95% of the GLS detected in *P. armoraciae* (Table S1). The GLS levels differed significantly between different *P. armoraciae* life stages (generalized least squares method, LR = 57.077, *P* < 0.001) and were significantly lower in larvae (L3 and prepupae) than in eggs, pupae and adults. All *P. armoraciae* life stages contained myrosinase activity but the levels differed drastically (generalized least squares method, LR = 57.077, *P* < 0.001; Fig. 1b, Table S2). The lowest levels of myrosinase activity were detected in eggs and pupae, which corresponded to about 2% of the highest activity detected in *P. armoraciae* larvae (Fig. 1b).

3.2 *P. armoraciae* larvae deter the generalist predator *H. axyridis*

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We analysed the interaction of different *P. armoraciae* life stages (larvae, pupae, and adults) with a generalist predator by exposing these individually to *H. axyridis* third instar larvae. Predator larvae attacked *P. armoraciae* larvae and pupae significantly more frequently than adults (Kruskal-Wallis test, $H = 23.613$, $P < 0.001$, Table S3). In fact, we observed only one unsuccessful attempt of *H. axyridis* to injure a *P. armoraciae* adult and thus excluded this life stage from follow-up experiments. Examples of interactions between *H. axyridis* and *P. armoraciae* larvae, pupae, and adults are shown in video files 1, 2 and 3, respectively. After attack, the predator fed for a much shorter time on larvae compared to pupae (median feeding time 3 s and 354 s, respectively; Mann-Whitney U test, $U = 42.000$, $P < 0.001$, Table S3), and even regurgitated the ingested larval haemolymph in six out of sixteen independent observations (examples are shown in video 1). When *P. armoraciae* larvae and pupae were offered simultaneously to *H. axyridis* in choice assays, the predator clearly preferred to feed on pupae (paired Wilcoxon rank sum test, $W = -462.0$, $P \leq 0.001$; Table S4). Next, we determined the consequences of a single predator attack by comparing the mortality rates of attacked and non-attacked larvae and pupae. While we observed similar mortality rates of attacked and non-attacked larvae (25% and 22%, respectively; Chi-square-test, $\chi^2 = 0.0545$, $P = 0.815$), the mortality of attacked pupae was significantly higher than that of non-attacked pupae (93% and 6%, respectively; Chi-square-test, $\chi^2 = 12.25$, $P < 0.001$).

### 3.3 The GLS levels of *P. armoraciae* larvae influence their predation risk

Because only *P. armoraciae* larvae survived the predator attack, we asked whether the levels of sequestered GLS influence their survivorship. To answer this question, we manipulated the GLS levels in *P. armoraciae* larvae by feeding early second instar larvae with leaves of three different *A. thaliana* lines or *B. juncea* (generalized least squares method, $LR = 74.500$, $P < 0.001$; Fig. 2a, Table S5). Larvae that were reared on leaves of *A. thaliana* wild type or *mybcyp* plants contained only traces of GLS, whereas larvae reared on the myrosinase-deficient *A. thaliana tgg* mutant accumulated GLS (Table S5). *Tgg*-fed larvae contained high GLS concentrations and differed in GLS composition from *B. juncea*-fed larvae (Table S5). In contrast, the food plant did not affect the levels of insect myrosinase activity in larvae (ANOVA, $F = 0.383$, $P = 0.766$; Fig. S1).
We then exposed these larvae to *H. axyridis* and found that the survival rate strongly depended on the food plant (log-rank test, *P* < 0.05; Fig. 2b, Table S5). The predator killed about 90% of the wild type- and *mybcyp*-fed larvae with low GLS levels, but less than 30% of the *tgg*- and *B. juncea*-fed larvae, which contained higher GLS levels. Moreover, the survival rate of *tgg*-fed larvae was significantly higher than that of *B. juncea*-fed larvae.

To test whether GLS-containing larvae are toxic for *H. axyridis*, we fed the predator with *P. armoraciae* larvae that had been reared on *A. thaliana* wild type (low-GLS larvae) or *tgg* leaves (high-GLS larvae). Predators fed with low-GLS larvae survived significantly better than predators fed with high-GLS larvae, which frequently refused to feed (log-rank test, *P* < 0.001; Fig. 2c). In agreement with this observation, only predator larvae fed with low-GLS larvae gained weight (Table S6).

### 3.4 Predator attack induces hydrolysis of sequestered GLS

To determine whether sequestered GLS are hydrolysed upon predator attack, we measured the formation of AITC during exposure of *P. armoraciae* larvae and pupae to *H. axyridis*. In agreement with the different levels of myrosinase activity, attacked larvae released much more AITC than attacked pupae (9.5 ± 3.5 and 0.1 ± 0.1 nmol AITC per injured individual, respectively, mean ± SD). In the absence of *H. axyridis*, there was no detectable emission of AITC from larvae or pupae (Fig. 3a).

To better understand how sequestered GLS are hydrolysed upon predation, we analysed the distribution of GLS and myrosinase activity in dissected *P. armoraciae* larvae. The larval haemolymph contained significantly higher proportions of both sequestered GLS and myrosinase activity than the remaining tissues (paired *t*-test, GLS: *t* = -15.242, *P* ≤ 0.001, myrosinase activity: *t* = -9.442, *P* ≤ 0.001; Fig. 3b, Table S7).

To establish whether ingested GLS might also be hydrolysed in the gut of *H. axyridis* independently of *P. armoraciae* myrosinases, we measured the levels of myrosinase activity in crude protein extracts prepared from dissected larval guts. However, our enzyme assays revealed only minimal myrosinase activity in *H. axyridis* (1.09 ± 0.17 nmol × min⁻¹ × mg protein⁻¹; mean ± SD), which corresponded to 1.6% of the myrosinase activity detected in *P. armoraciae* larvae (Table S2).
4 DISCUSSION

The results of this study show the defensive function of GLS sequestration against predation, similarly as has been shown for specialist aphids that sequester GLS and possess endogenous myrosinase activity (Bridges et al. 2002; Francis et al. 2002; Kazana et al. 2007). In *P. armoraciae*, distinct levels of myrosinase activity in larvae and pupae correlated with their predation risk in experiments with the generalist predator *H. axyridis*. One predator attack had no influence on the survival rate of *P. armoraciae* larvae, whereas pupae suffered high mortality. Pupae contained only 2% of the myrosinase activity detected in larvae and thus released only traces of toxic isothiocyanates upon predator attack. Predation experiments with myrosinase-deficient *P. armoraciae* larvae may be a promising approach to determine whether low myrosinase activity is associated with a higher susceptibility of *P. armoraciae* to *H. axyridis*.

Qualitative and quantitative differences in chemical defences between insect life stages have been suggested to reflect the exposure to different communities of natural enemies, trade-offs between different predator defence or avoidance mechanisms (Pasteels, Grégoire & Rowell-Rahier 1983; Lindstedt, Murphy & Mappes 2019). During development from egg to adult, *P. armoraciae* occupies different habitats and thus encounters different above and belowground communities of natural enemies (Vig 2004). However, since isothiocyanates are broadly active against non-adapted fungal and bacterial pathogens, nematodes, and insect herbivores (Avato et al. 2013; Jeschke, Gershenzon & Vassão 2016; Pastorczyk & Bednarek 2016), their production should be beneficial to all life stages, in particular those that are less mobile than adult flea beetles. It was thus surprising to find that the immobile pupae have the lowest capacity to form deterrent isothiocyanates. Minor myrosinase activity in pupae might protect them from uncontrolled GLS hydrolysis during larval-adult metamorphosis. Alternatively, the resource allocation in larvae and pupae might differ, resulting in differential investment in chemical defence in both life stages. Behavioural observations by Vig (2004) suggest that *P. armoraciae* pupae might use a different strategy to escape from natural enemies. The mature larva buries between 5 and 10 cm deep into the soil and builds a pupal chamber by using an anal secretion. This chamber might represent a physical and/or chemical barrier against natural enemies.

To understand the causes and consequences of the stage-specific chemical defence in *P. armoraciae*, we need more knowledge about the distinct communities of natural enemies this...
insect encounters throughout ontogeny. Entomopathogenic nematodes might represent a group of relevant natural enemies because specific strains caused mortality of soil-dwelling stages of *P. striolata* and *P. cruciferae* in laboratory and field studies (Xu et al. 2010; Yan et al. 2013; Reddy et al. 2014; Yan et al. 2018). Similarly, the root-feeding larvae of the western corn rootworm *Diabrotica virgifera virgifera* use sequestered maize benzoxazinoid glucosides for protection from non-adapted entomopathogenic nematodes (Robert et al. 2017). However, adapted nematodes caused higher mortality rates because they developed resistance against this insect two-component chemical defence (Zhang et al. 2019). At this background, it would be interesting to analyse the role of the GLS-myrosinase system in *Phyllotreta* spp. in the interaction between soil-dwelling life stages and below-ground predators such as entomopathogenic nematodes, and how adapted predators deal with this defence system.

In our laboratory experiments, the predation risk of *P. armoraciae* larvae was significantly lower than that of pupae. However, when we manipulated the GLS levels in larvae using different food plants, we found that only larvae with GLS deterred the predator, whereas larvae with low GLS levels were killed by *H. axyridis*. These findings demonstrate that GLS sequestration can protect *P. armoraciae* larvae from a generalist predator, but is dependent on the food plant. Larvae were not able to accumulate ingested GLS from *A. thaliana* wild type plants, but sequestered GLS from the myrosinase-deficient *tgg* mutant and from *B. juncea*. This result was unexpected, because *P. armoraciae* adults accumulated all GLS types from *A. thaliana* wild type plants (Yang et al. 2020). To find out why larvae can sequester GLS from *B. juncea*, but not from *A. thaliana*, we compared the levels of myrosinase activity in leaves. However, myrosinase activity levels were similar in both plants under our assay conditions (refer to Supplementary Methods and Results 1 for details) and thus do not explain the observed difference in sequestration. Another possibility is that the different GLS profiles of *A. thaliana* and *B. juncea* affected sequestration. *B. juncea* contains the same dominant GLS as horseradish, the natural host plant of *P. armoraciae* (Li and Kushad (2004), Table S1). Given the close relationship between *P. armoraciae* and their horseradish host plant, it is imaginable that *P. armoraciae* larvae selectively sequester allyl GLS, whereas the uptake of other GLS types may be less efficient. Since larvae were still able to sequester GLS from the myrosinase-deficient *tgg* mutant, the ingested GLS from *A. thaliana* wild type plants were likely hydrolysed by plant myrosinases. Although *P. armoraciae* adults were principally able to sequester other GLS types after they

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were shifted from *B. juncea* to *A. thaliana*, adults preferred to excrete the ingested GLS from *A. thaliana* and not the previously sequestered allyl GLS (Yang *et al.* 2020). Together, these results indicate that larvae and adults use different mechanisms to selectively sequester allyl GLS (Yang *et al.* 2020). This sequestration strategy might have played an important role in the evolution of the close association between *P. armoraciae* and horseradish.

Previous studies with GLS sequestering sawfly larvae of the genus *Athalia* demonstrated that in the absence of myrosinases, sequestered GLS have a low effect on predators (Müller, Boevé & Brakefield 2002; Müller & Brakefield 2003). We detected high myrosinase activity in *P. armoraciae* larvae, and found this enzyme to be co-localized with the sequestered GLS in the haemolymph. Thus, the fast deterrence of the predator after ingestion of the haemolymph indicates that GLS are rapidly converted to toxic isothiocyanates. This GLS hydrolysis appears to be primarily catalysed by *P. armoraciae* myrosinases because the detected levels of myrosinase activity in the predator gut were low. Previous studies with cyanogenic larvae of different burnet moth species revealed that the organization of the “cyanide bomb” differs within the genus *Zygaena* (Nahrstedt & Müller 1993; Pentzold *et al.* 2017). While cyanogenic glucosides and the cyanogenic \( \beta \)-glucosidase are compartmentalized in haemoplasma and haemocytes of *Zygaena filipendulae* (Pentzold *et al.* 2017), both components are co-localized in the haemoplasma of *Zygaena trifolii* larvae where enzyme activity is inhibited by Mg\(^{2+}\) and Ca\(^{2+}\) ions and pH conditions (Franzl, Ackermann & Nahrstedt 1989; Nahrstedt & Müller 1993). In cabbage aphids, sequestered GLS are localized in the haemolymph, whereas the aphid myrosinase is stored in crystalline microbodies in non-flight muscles in the head and thorax (Kazana *et al.* 2007). Thus, substantial injury is necessary to hydrolyse the sequestered GLS in aphids, which, in contrast to *P. armoraciae* larvae, usually do not survive the predator attack. We observed that cell damage induced by a freeze and thaw treatment of *P. armoraciae* larvae resulted in almost complete hydrolysis of stored GLS (refer to Supplementary Methods and Results 2 for details). Although this experiment provides initial evidence for a spatial separation of GLS and myrosinases in the *P. armoraciae* haemolymph, we still do not know how GLS get rapidly into contact with the myrosinase in the predator gut.

In summary, we show that the ability of *P. armoraciae* to benefit from sequestered plant metabolites strongly depends on the life stage, but how these ontogenetic differences in chemical defence influence the predation rates of different life stages in natural and agricultural
ecosystems remains to be determined. Our study emphasises that variation in insect chemical
defence should be considered in the context of relevant predator communities across insect
ontogeny.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1: Glucosinolate (GLS) concentration and myrosinase activity in different *P. armoraciae* life stages. (a) GLS were extracted from *P. armoraciae* and quantified by HPLC-UV (n = 7-8). (b) Myrosinase activity was determined in crude protein extracts using allyl GLS as substrate by quantifying released glucose (n = 5-9). Different letters indicate significant differences between life stages (generalized least squares method, *P* < 0.001). glc, glucose; FW, fresh weight; L3, third larval instar.

Figure 2: GLS concentrations in *P. armoraciae* larvae correlate with their survival rate in the presence of *H. axyridis* and affect the survival rate of the predator. (a) GLS concentrations in larvae fed with different food plants were analysed by HPLC-UV (generalized least squares method, *P* < 0.001, n = 12-20). (b) Survival rate of *P. armoraciae* larvae exposed to *H. axyridis* (log-rank test, *P* < 0.05, n = 60-61). Different letters indicate significant differences between groups. (c) Survival rate of *H. axyridis* larvae provided with wild type-fed and tgg-fed *P. armoraciae* larvae (log-rank test, ***P* < 0.001, n = 20). Each predator larva was supplied with one *P. armoraciae* larva per day. wild type, *A. thaliana* Col-0; mybcyp, *A. thaliana* myb28×myb29×cyp79B2×cyp79B3; tgg, *A. thaliana* tgg1×tgg2.

Figure 3: Predator-induced allyl isothiocyanate (AITC) formation in *P. armoraciae* larvae and pupae and distributions of sequestered GLS and myrosinase activity in different larval body parts. (a) Volatiles of *P. armoraciae* larvae and pupae that were exposed to *H. axyridis* larvae, were collected for four hours on Porapak-Q™ adsorbent, eluted with hexane, and emitted AITC was quantified by GC-MS (*m/z* 99). Volatile collections of flea beetle larvae and pupae served as controls. (b) *P. armoraciae* third instar larvae were dissected into gut, haemolymph and rest of body. Extracted GLS were analysed by HPLC-UV (n = 5) and myrosinase activity was determined by quantification of released glucose using allyl GLS as a substrate (n = 6). The levels of GLS and myrosinase activity detected in the different fractions are expressed relative to the total levels detected in all samples (set to 100%).

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Extracted ion chromatogram of m/z 99 (abundance x 1,000,000)

GLS and myrosinase activity distribution (% mean ± SD)

(a) Time (min)  
(b) Gut □ Rest of body □ Haemolymph □