The mechanism and the ecological function of plant glucoside sequestration in *Phyllotreta* flea beetles

Dissertation

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1. General Introduction

Phytophagous insects (herbivores) comprise approximately 25% of all species on this planet and have coexisted with their plant food sources for more than 400 million years (Scudder, 2009; Labandeira, 2013). Over time, plants and insect herbivores have coevolved dynamically in a so-called 'arms race' (Ehrlich and Raven, 1964) as plants developed mechanisms to prevent herbivory and insects adapted to those defenses with different counteradaptations in ongoing cycles. The process of coevolution is thought to be the major driver of the large species diversity and specializations we observe in insect herbivores today (Futuyma and Agrawal, 2009). Most insect herbivores are specialists (mono- or oligophages), accepting only closely related plant species of the same genus or family, whereas some are generalists (polyphages) that can use plants from different families as food sources (Ali and Agrawal, 2012). The large diversity of specialized metabolites produced by plants to fend off herbivores and pathogens plays an important role in the evolution of insect herbivores (Mithöfer and Boland, 2012). The distribution of these compounds can be widespread in the plant kingdom (e.g. terpenoids and flavonoids), or restricted to a plant order or several families (e.g. glucosinolates (GLSs) in Brassicales) (Fahey et al., 2001; Wink, 2008; Blažević et al., 2020). Defense metabolites target herbivores either directly by interference with basic processes (e.g. digestion, metabolism and signal transductions) or indirectly by attracting their natural enemies (Figure 1A and B) (Mithöfer and Boland, 2012; Fürstenberg-Hägg et al., 2013). Thus, plant specialized metabolites mediate interactions among multiple trophic levels, namely plants, herbivores, and their enemies.

The fitness and reproduction of insect herbivores depend on the quality of the host plant as food source, which contains beneficial carbon, nitrogen sources and trace elements, but also harmful defense metabolites (Awmack and Leather, 2002). In response to plant specialized metabolites, herbivores have developed behavioral and physiological counteradaptations to overcome the detrimental effects of this bottom-up selection pressure (Després et al., 2007; Heckel, 2014; War et al., 2018;War et al., 2019). These adaptations include the avoidance of highly protected plants or tissues, the excretion and thus elimination of toxic compounds, metabolic detoxification, and the tolerance of toxins by target-site insensitivity (Shroff et al., 2015; Jeschke et al., 2017; Karageorgi et al., 2019). Another adaptation is the sequestration of plant defense metabolites, *i.e.* selective accumulation in the insect body to fend off their own natural enemies (Figure 1C)

(Duffey, 1980; Nishida, 2002). This strategy enables insects to exploit the chemical defenses of their host plants (lower trophic level) to protect themselves against the top-down selection pressure by their natural enemies (higher trophic level) (Figure 1D). Since sequestration increases an herbivore's own defense and since sequestering herbivores are often specialist feeders, higher trophic levels are regarded as the driving force in selecting for such herbivore adaptations with their host plants (Dyer, 1995; Opitz and Müller, 2009; Petschenka and Agrawal, 2016). This thesis will focus on the sequestration of plant specialized metabolites by flea beetles and their redirection against flea beetle enemies.

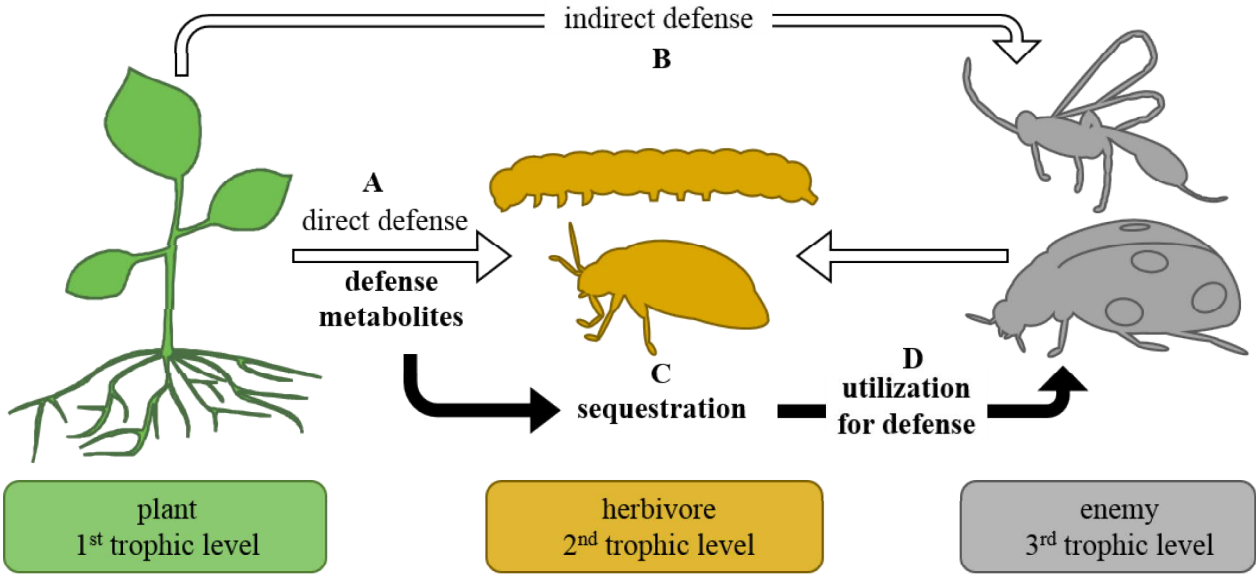

Figure 1: Scheme of plant-herbivore-enemy interactions mediated by plant defense metabolites. Herbivorous insects must simultaneously cope with their host plant defenses (lower trophic level) and their natural enemies (higher trophic level). The defense metabolites biosynthesized by plants against herbivory act either (A) directly on the attacking herbivore, or (B) indirectly by attracting its natural enemies, such as predators and parasitoids. Several specialist herbivores, however, evolved the ability to (C) sequester those plant defense metabolites to exploit them for their own protection by (D) redirecting them against their enemies.

1.1. Sequestration in insects

The sequestration of plant defense metabolites is widespread in insect herbivores as over 250 species, often specialist feeders in the orders Coleoptera, Lepidoptera, Hymenoptera, Heteroptera, Orthoptera and Sternorrhyncha, sequester defense metabolites from more than 40 plant families (Opitz and Müller, 2009). The main purpose of sequestration is defense against their own natural enemies, as it renders insect herbivores unpalatable or toxic for their antagonists (see Chapter 1.1.1)

(Opitz and Müller, 2009; Erb and Robert, 2016; Petschenka and Agrawal, 2016). In addition to their defensive value, the sequestered metabolites may also be involved in other processes like communication, reproduction, and sometimes nutrient recycling (Rowell-Rahier and Pasteels, 1986; Conner et al., 2000; Opitz and Müller, 2009). However, to store toxic metabolites in their bodies, insects require adaptive mechanisms to avoid autotoxicity (Erb and Robert, 2016; Petschenka and Agrawal, 2016). These adaptations include the reduction of internal exposure to sequestered toxins by their storage in specific structures or compartments (e.g. defense glands) or tolerance towards these metabolites by target-site insensitivity (Pasteels et al., 2003; Karageorgi et al., 2019).

1.1.1. Utilization of sequestered plant metabolites for defense

Insect herbivores are exposed to many kinds of natural enemies, comprising entomopathogens (e.g. bacteria and nematodes), parasitoids (e.g. ichneumon wasps and tachinid flies), and predators (arthropods, such as ladybirds and spiders, and vertebrates, such as birds). Sequestered plant metabolites were shown to have detrimental effects on non-adapted natural enemies ranging from deterrence to death (Aldrich et al., 1990; Fordyce, 2001; Boevé and Schaffner, 2003; Narberhaus et al., 2005; Kumar et al., 2014; Robert et al., 2017). However, the protective value of sequestration varies throughout the ontogeny of an insect. Sometimes, only the sequestering larval life stages are chemically protected because the plant metabolites are catabolized or eliminated during metamorphosis to the pupal and adult stage (Bowers, 1991; Bowers and Collinge, 1992; Bowers and Stamp, 1997; Bowers, 2003). In contrast, several insects transfer the sequestered metabolites throughout ontogeny and some even pass them on to the next generation by transmission into eggs (Bowers, 1991; Frick and Wink, 1995; Müller et al., 2001; Zagrobelny et al., 2007). Thereby, all life stages and even the offspring gain chemical protection. Nevertheless, sequestration does not provide a ubiquitous protection against all natural enemies, since adapted antagonists are able to overcome the chemical defenses and may even use these compounds as chemical cues to locate their prey (Boevé and Müller, 2005; Oberhauser et al., 2007; Baden et al., 2011; Zhang et al., 2019). In general, chemical defenses are most effective against generalist predators, but less effective against specialists, especially specialist parasitoids (Zvereva and Kozlov, 2016).

1.1.2. Processes involved in sequestration

The sequestration of plant specialized metabolites involves their uptake, transfer, and concentration in the hemolymph or specific tissues (e.g. defense glands) and may also include metabolic modification of those compounds (Duffey, 1980; Nishida, 2002; Opitz and Müller, 2009). In any case, sequestration requires transport processes. In the first step of sequestration, plant metabolites must be taken up from the gut lumen into the hemolymph. Non-polar compounds can be passively taken up by their diffusion across membranes (Lindigkeit et al., 1997), whereas polar compounds cannot and thus require a carrier-mediated uptake into the hemolymph. An active uptake was proposed in several systems, but no transporter has yet been identified in the gut of any sequestering insect (Narberhaus et al., 2004; Abdalsamee et al., 2014; Schmidt et al., 2019). If plant metabolites are not stored in the hemolymph, they are further transported to their storage locations, e.g. glands. The only identified transporters involved in sequestration are ATP binding cassette (ABC) transporters that mediate the transport of sequestered metabolites into specific body parts (Strauss et al., 2013; Kowalski et al., 2020). Although many insects sequester plant metabolites, the molecular mechanisms involved are still largely unknown (Opitz and Müller, 2009; Petschenka and Agrawal, 2016). However, regardless of the polarity of the metabolite, an active transport process must be involved in sequestration since these metabolites are accumulated against a concentration gradient to high concentrations.

1.1.3. Selective sequestration

Insects often accumulate specific plant metabolites out of a complex mixture of compounds ingested with their food plant, therefore the process of sequestration can be selective (Opitz and Müller, 2009). This selectivity can be achieved by behavioral, physical, and physiological mechanisms (Petschenka and Agrawal, 2016). Insects may choose or reject plants containing or lacking specific metabolites. The gut epithelium may prevent the entrance of polar compounds into the body cavity by passive diffusion and undesired compounds can be actively shuffled out of the gut cells back into the gut lumen by efflux transporters (Petschenka et al., 2013; Dobler et al., 2015). Furthermore, compounds can be metabolized in the gut before excretion or within the body cavity followed by the excretion via the Malpighian tubules, the main insect excretory organ (Heckel, 2014). The uptake of plant metabolites may be passive for non-polar molecules and active for polar ones, with transport processes being involved in the latter case showing specificity for

distinct metabolites or their stereochemistry (Wink et al., 1991; Lindigkeit et al., 1997; Kuhn et al., 2004; Zagrobelny et al., 2014). Altogether, these processes are involved in selective sequestration and the outcome of their interplay and specificity determines the type and the amount of metabolites will enter the insect body cavity and be retained therein.

1.2. Sequestration of plant glucosides

1.2.1. Glucosinolates and other glucosides in plant two-component chemical defense systems

Many plants conjugate specialized metabolites to a glucose molecule and store them as stable, non-toxic, and water-soluble protoxins separately from activating β-glucosidase enzymes (Figure 2). Examples for such glucosylated protoxin classes include two-component chemical defenses such as cyanogenic, benzoxazinoid, and iridoid glucosides, as well as salicinoids and GLSs (Figure 2) (Zagrobelny et al., 2004; Boeckler et al., 2011; Dobler et al., 2011; Wouters et al., 2016). In all two-component defenses, the glucosides and β-glucosidases are stored physically separated in intact plant tissues, either in different cells or in cellular compartments (Morant et al., 2008; Pentzold et al., 2014b). Upon tissue damage, caused for example by herbivory, both components come into contact, and the β-glucosidase cleaves the glucose moiety of the protoxin leading to the toxin formation (Figure 2). The reactive and toxic aglucones and their reaction products cause different effects in non-adapted herbivores ranging from deterrence to death (Pentzold et al., 2014b).

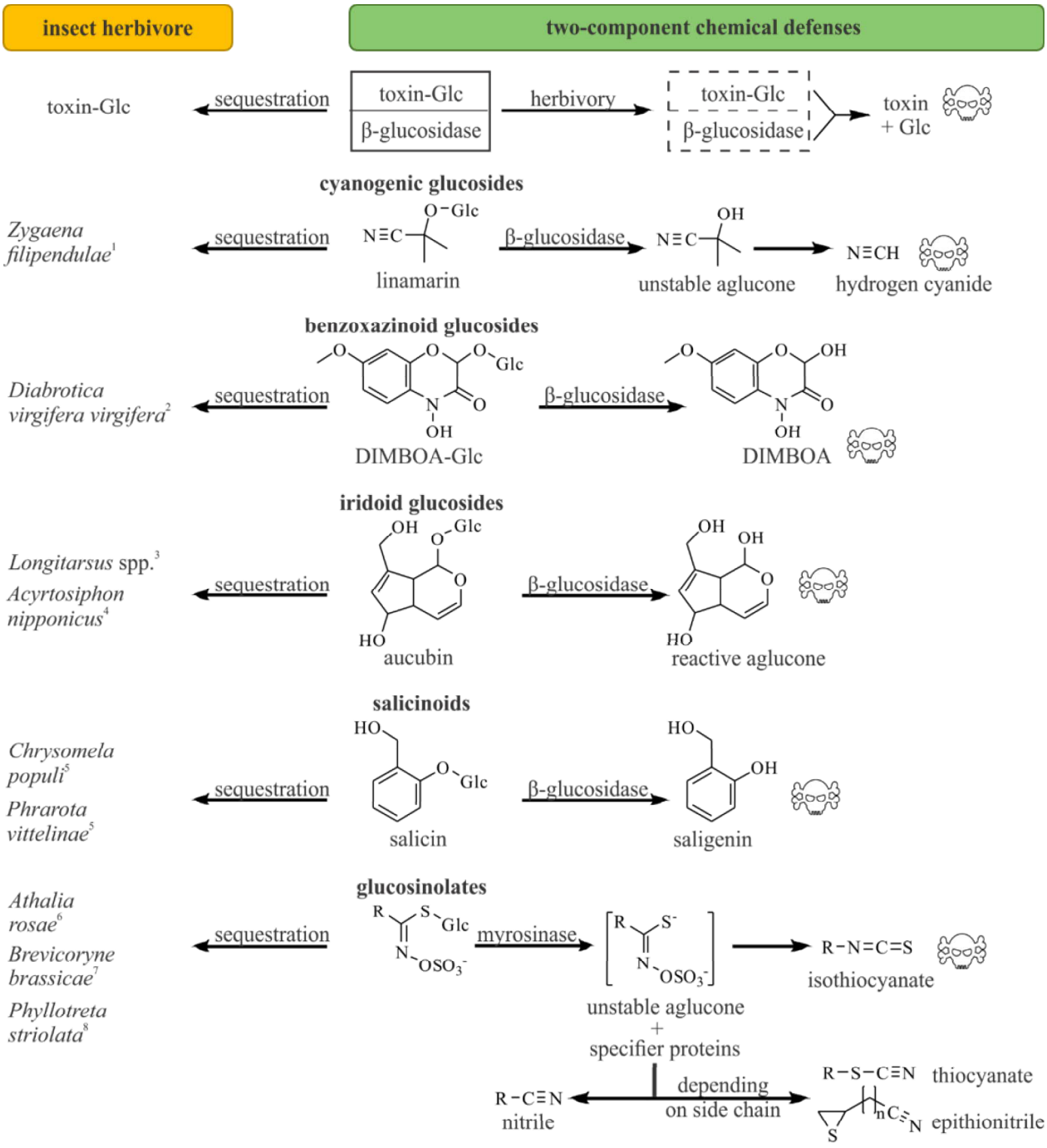

Figure 2: Scheme and examples of plant two-component chemical defenses and examples of insects sequestering plant glucosylated protoxins. Upon tissue damage, the non-toxic glucosylated protoxins (toxin-Glc) come into contact with corresponding β-glucosidases leading to the cleavage of the glucose (Glc) moiety and the formation of toxic aglucones (toxins). Examples for the breakdown of different glucosides are shown (adapted from (Pentzold et al., 2014b)). In the case of glucosinolate hydrolysis, alternative products are formed instead of isothiocyanates when specifier proteins are present (Wittstock et al., 2016). Examples for insect species that sequester glucosylated protoxins from their host plants. [1]Zagrobelny and Møller (2011), [2]Robert et al. (2017). [3]Willinger and Dobler (2001), [4]Nishida and Fukami (1989), [5]Pasteels et al. (1983), [6]Müller et al. (2001), [7]Bridges et al. (2002), [8]Beran et al. (2014).

The best studied two-component plant defense is the GLS-myrosinase system in Brassicales plants, also known as the 'mustard-oil bomb' (Matile, 1980; Halkier and Gershenzon, 2006). This system consists of the GLSs (thioglucosides) and β-thioglucosidase enzymes (myrosinases). Brassicales plants biosynthesize a large diversity of more than 130 different GLS structures with a common β-thioglucoside-*N*-hydroxysulfate backbone and an amino acid-derived side chain (Sønderby et al., 2010; Blažević et al., 2020). Depending on the amino acid precursor, they are broadly classified as aliphatic (derived from methionine, leucine, isoleucine, valine, and alanine), benzenic (derived from tyrosine and phenylalanine) and indolic (derived from tryptophan) GLSs. A variety of side-chain modifications, such as hydroxylation, esterification, and desaturation finally lead to the structure diversity of the known GLSs (Sønderby et al., 2010; Blažević et al., 2020). Many plants biosynthesize only a few GLSs, whereas 34 different GLS structures were found in leaves and seeds in a collection of *A. thaliana* ecotypes (Kliebenstein et al., 2001; Clarke, 2010). The GLS concentration and composition vary within a plant among organs and vary among plant species in both concentration and profile, affected by abiotic factors (e.g. drought and temperature) and biotic factors (e.g. by herbivores and pathogens) (Schweizer et al., 2013; Steindal et al., 2015; Witzel et al., 2015; Burow, 2016; Gill et al., 2016).

The myrosinases that degrade GLSs belong to glucoside hydrolase family 1 and are the only known *S*-glucoside hydrolases (Naumoff, 2011). Myrosinases use ascorbic acid as a cofactor, can form dimers by Zn^{2+} complexation and are highly stable enzymes due to their many salt bridges, disulfide bonds and strong glycosylation on their surfaces (Burmeister et al., 1997; Burmeister et al., 2000). Specific amino acid residues in the active center of myrosinases recognize and interact with the glucose and sulfur moiety, the GLS side chain, the cofactor, and the GLS aglucone formed (Burmeister et al., 1997; Burmeister et al., 2000; Rask et al., 2000). First, the glucose moiety is cleaved from the GLS and is covalently bound to the enzyme and subsequently, ascorbic acid promotes the hydrolysis of the glucose-enzyme bond by acting as a catalytic base. The released aglucone is unstable and its half-life ranges from a few seconds to a few minutes until further dissociation into toxic isothiocyanates (Mocniak et al., 2020). In the presence of specifier proteins and other factors, such as pH conditions and the presence of Fe^{2+}, breakdown products other than isothiocyanates are formed (Wittstock et al., 2016).

Isothiocyanates, the most reactive and toxic GLS breakdown products, protect plants from non-adapted herbivores and pathogens (Avato et al., 2013; Jeschke et al., 2016a; Pastorczyk and

Bednarek, 2016). Their toxicity is caused by their molecular character. The lipophilic property allows diffusion through membranes into cells and the electrophilic character causes biological damage due to their high reactivity towards thiols (-SH) and amine (-NH_2) groups in glutathione, peptides and proteins (Brown and Hampton, 2011). Thereby, isothiocyanates disrupt redox homeostasis, cause oxidative stress, and trigger cell death.

1.2.2. Mechanisms facilitating plant glucoside sequestration in insects

Despite the rapid hydrolysis of glucosinolates and other plant glucosides upon tissue disruption, many insects of different orders can sequester the glucosylated protoxins from two-component plant defenses (Opitz and Müller, 2009; Beran et al., 2014; Robert et al., 2017). Several examples are shown in Figure 2. The sequestration of a plant glucoside can be beneficial for the insect as it directly prevents toxic aglucone formation, by making non-toxic glucosides inaccessible to the activating enzymes remaining in the gut (Pentzold et al., 2014b). However, since plant β-glucosidases usually rapidly convert the glucosides into toxic aglucones upon tissue damage (Figure 2), sequestering insects require strategies to interrupt this reaction, thereby enabling the glucosides to be sequestered in their intact form. Several morphological, physiological, and metabolic strategies are known from glucoside-sequestering insects that prevent the hydrolysis of plant glucosylated protoxins by the corresponding β-glucosidases during feeding and digestion (Figure 3) (Abdalsamee et al., 2014; Pentzold et al., 2014b).

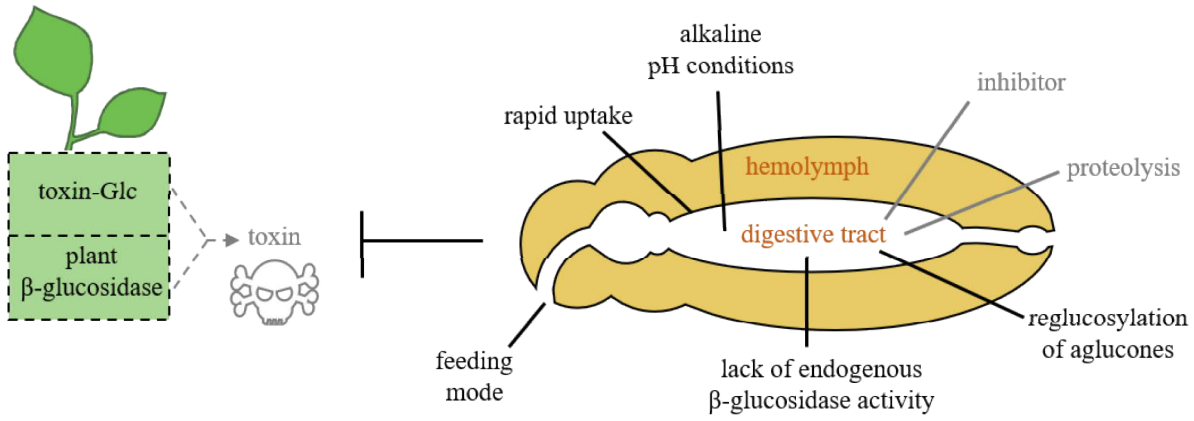

Figure 3: Adaptations of glucoside-sequestering insects to prevent glucoside hydrolysis during feeding and digestion. Known strategies from glucoside-sequestering insects are indicated in black and possible strategies in gray (adapted from (Pentzold et al., 2014b)).

The feeding mode of an insect determines the extent of plant damage and consequently the extent of glucoside hydrolysis and toxic aglucone formation during feeding (Figure 3). The level of damage increases from piercing-sucking to leaf-snipping, leaf mining and finally leaf chewing (Pentzold et al., 2014b). Piercing-sucking insects, such as aphids, pierce single cells with their stylet and thereby cause only minimal tissue damage, whereas chewing insects, such as beetles, grind plant tissues with their toothed mandibles and efficiently mix the contents of most plant cells.

Once the plant material is ingested, it comes into contact with the herbivore's digestive fluids. Since β-glucosidases are generally active in rather acidic pH conditions, highly alkaline conditions in the gut lumen can substantially reduce ingested plant β-glucosidase activity, for example by ionizing amino acid residues crucial for the enzymatic reaction (Figure 3) (Ketudat Cairns and Esen, 2010; Pentzold et al., 2014b). Such alkaline conditions are mostly found in lepidopteran larvae and were suggested to contribute to cyanogenic glucoside sequestration in the six-spot burnet moth *Zygaena filipendulae* (Terra and Ferreira, 2012; Pentzold et al., 2014a). Inhibition of β-glucosidases by a mechanism other than high pH may be another option, but to the best of my knowledge no insect-derived plant β-glucosidase inhibitor has been identified in any glucoside-sequestering insect so far (Figure 3). The only evidence for such a scenario was reported in the GLS-sequestering turnip sawfly larvae, where plant myrosinase activity was transiently reduced in the anterior gut (Abdalsamee et al., 2014).

Another possibility is a very fast glucoside absorption from the gut lumen facilitating its rapid uptake and separation from co-ingested β-glucosidases (Figure 3). This strategy was suggested for GLS sequestration in the turnip sawfly (Abdalsamee et al., 2014). Insects may also efficiently proteolyze plant defensive β-glucosidases in the digestive system, thereby preventing further glucoside hydrolysis (Figure 3). However, defensive plant β-glucosidases were shown to be highly resistant against digestion by a generalist lepidopteran larva (Vassão et al., 2018). Whether specialist glucoside-sequestering insects efficiently proteolyze plant β-glucosidases is unknown. If plant glucosides are hydrolyzed, insects may reglucosylate the aglucones formed (Figure 3) as was found for a benzoxazinoid aglucone in the gut of the western corn rootworm *Diabrotica virgifera virgifera* (Robert et al., 2017). For highly unstable aglucones that decompose to the final toxic products (e.g. cyanogenic glucosides and GLSs, Figure 2) this strategy may not be possible (Zagrobelny et al., 2008; Wittstock et al., 2016; Mocniak et al., 2020).

If not hydrolyzed by plant β-glucosidases, the ingested glucosides are exposed to endogenous insect β-glucosidases, which are involved in digestion and can thus contribute to aglucone formation in the gut (Pentzold et al., 2014b). However, a lack of substrate specificity for plant glucosides in the endogenous digestive β-glucosidases may prevent this additional breakdown (Figure 3)(Pentzold et al., 2014a).

In order to prevent the reaction of plant β-glucosidases with their corresponding glucosides, insect herbivores may not rely on one single strategy but combine different ones over the time course of feeding and digestion (Pentzold et al., 2014a; Pentzold et al., 2014b). However, for many insect herbivores, it is unclear how the sequestration of intact glucosides is facilitated.

1.2.3. Insect defense with sequestered plant glucosides

The glucosylated protoxins of two-component plant defenses are non-toxic and relatively stable. Some sequestering insects nevertheless benefit from the intact glucosides: for instance, the GLSs sequestered by turnip sawfly larvae that contribute to the hemolymph deterrence towards ants and predatory wasps (Müller et al., 2002; Müller and Brakefield, 2003). However, to reach their full bioactive potential, plant glucosides require conversion by β-glucosidases to the actual reactive products (Morant et al., 2008; Pentzold et al., 2014b). An endogenous β-glucosidase activity has been found in several sequestering herbivores that fulfills this purpose (Kazana et al., 2007; Opitz and Müller, 2009; Beran et al., 2014; Rahfeld et al., 2015; Robert et al., 2017; Zagrobelny et al., 2018). This allows those herbivores to form their own two-component defense systems and to hydrolyze the sequestered glucosides into the same detrimental defense metabolites as their host plants (Figure 4). A few insect β-glucosidases have been identified, characterized, and phylogenetically analyzed, showing that those enzymes evolved independently in insects (Jones et al., 2001; Jones et al., 2002; Husebye et al., 2005; Beran et al., 2014; Rahfeld et al., 2015; Pentzold et al., 2017).

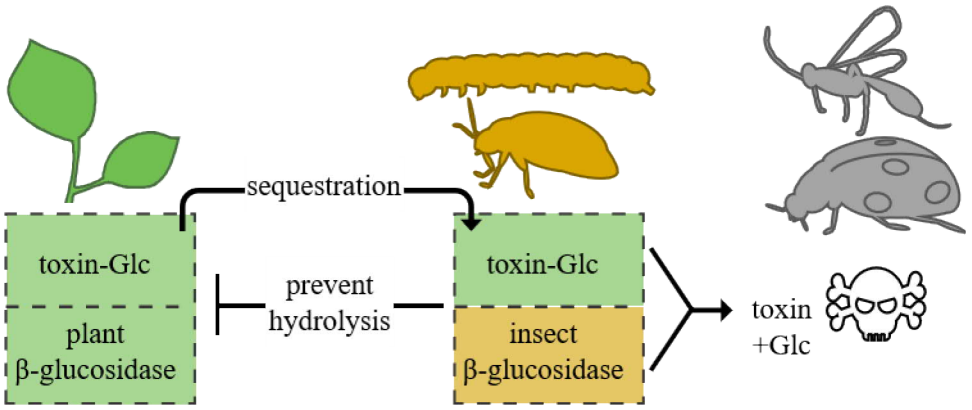

Figure 4: Scheme of herbivores forming their own two-component defense systems using sequestered protoxins and endogenous β-glucosidases. Herbivores prevent the hydrolysis of protoxins (toxin-Glc) during feeding and digestion to sequester them in their bodies. They phenocopy their host plants' defense systems by producing their own endogenous β-glucosidases to hydrolyze the plant-derived protoxins for defense against their own enemies.

In order to prevent an uncontrolled glucoside breakdown within their bodies, insects developed different mechanisms of safely storing both components in their bodies. This is achieved by storing both components outside of the body cavity in exocrine defense glands, compartmentalizing both components in different cells or tissues, or inhibiting the endogenous β-glucosidase when both components are stored together (Nahrstedt and Mueller, 1993; Kuhn et al., 2004; Kazana et al., 2007; Rahfeld et al., 2015).

For defense, the sequestered glucosides are hydrolyzed and toxic aglucones are released. Those aglucones were shown to successfully protect several insects against their enemies (Figure 4), for instance against bacteria, nematodes, ants or ladybirds (Rowell-Rahier and Pasteels, 1986; Kazana et al., 2007; Gross et al., 2008; Robert et al., 2017). Although it is assumed that the main role of insect two-component systems is defense, it is often unknown whether this is indeed the case. Moreover, it often remains unclear which predators are targeted and whether glucoside hydrolysis can protect all insect life stages.

1.3. *Phyllotreta* flea beetles and their interaction with their host plant

The striped flea beetle *Phyllotreta striolata* selectively sequesters intact GLSs and accumulates them to higher concentrations than present in their Brassicacae host plants (Beran et al., 2014). It also independently evolved an endogenous myrosinase that shows the highest activity towards the selectively sequestered GLSs and is consequently able to hydrolyze them to toxic isothiocyanates. Therefore, *P. striolata* is the first example of a beetle assembling its own GLS-myrosinase system.

However, questions remain on how intact GLSs are sequestered and what ecological functions they fulfill throughout their ontogeny. So far, studies have been performed with field-collected adults, which limits the experimental options and the accessibility of different life stages for studies. Unfortunately, several attempts to establish a laboratory *P. striolata* rearing have failed.

P. striolata is one out of approximately 240 species within the genus *Phyllotreta* (Coleoptera: Chrysomelidae: Alticini) with most species being specialized on plants in the Brassicales order (Gikonyo et al., 2019). The majority (62%) feed on the plant family Brassicacae, which include important crops as mustard, broccoli, and oilseed rape, as well as the model plant thale cress, *Arabidopsis thaliana*. Hence, these beetles are well-adapted to the GLS-myrosinase defense. Not only are they adapted to their host plants, but also react to and use the chemical cues from their host plant defense system. GLSs are feeding stimulants for adult *Phyllotreta* flea beetles and isothiocyanates are attractants presumably for host plant localization (Vincent and Stewart, 1984; Nielsen, 1988; Pivnick et al., 1992; Tóth et al., 2007). Moreover, feeding males emit an aggregation pheromone which contains the sesquiterpene (*6R,7S*)-himachala-9,11-diene as a major compound and attracts both sexes; the presence of isothiocyanates increases the attraction (Soroka et al., 2005; Tóth et al., 2012; Beran et al., 2016a).

The horseradish flea beetle *Phyllotreta armoraciae* is another member of the *Phyllotreta* genus that is monophagous on horseradish in nature but also accepts other Brassicacae food plants in the laboratory (Vig and Verdyck, 2001; Li and Kushad, 2004). Preliminary work revealed that, similar to *P. striolata*, *P. armoraciae* also sequesters GLSs and possesses an endogenous myrosinase activity (Körnig, 2015). In contrast to the oligophagous *P. striolata*, the establishment of a laboratory rearing was successful for this species. Throughout its life, *P. armoraciae* is closely associated with its host plant. Females oviposit their eggs in clutches, close to the plant in the soil or on the stem (Vig, 2004). Neonates move into the plant and feed internally in petioles and veins as leaf miners during their three larval instars. Mature larvae move into the soil and build an earthen pupation chamber. After eclosion, newly emerged adults move aboveground and feed externally on leaves. Based on their different habitats throughout the ontogeny, different life stages are exposed to different kinds of enemies. In this study, *P. armoraciae* was used as a model organism to investigate the sequestration and ecological function of GLSs in *Phyllotreta* flea beetles.

1.4. Aim of the study

The aim of this thesis was to investigate the mechanisms and processes involved in the sequestration of GLSs and their ecological role in *Phyllotreta* flea beetles. This was studied using the horseradish flea beetle *P. armoraciae*, which assembles its own GLS-myrosinase system by sequestering GLSs and producing an endogenous myrosinase (Körnig, 2015). Besides the main player *P. armoraciae*, the model plant *A. thaliana* was predominantly used as a food source and the Asian ladybird *Harmonia axyridis* as a generalist predator (Figure 5).

Phyllotreta flea beetles feed on many different plants in the family Brassicacae and thus encounter a variety of different structures, compositions, and concentrations of GLSs in the different host plants (Gikonyo et al., 2019). It is unknown how these factors influence sequestration. In **Manuscript I**, we analyzed the impact of variable plant GLS concentrations and compositions on the GLS sequestration in *P. armoraciae* adults by using the model plant *A. thaliana* that contains aliphatic and indolic GLSs and two mutant lines that lack either one of the GLS classes (Zhao et al., 2002; Sønderby et al., 2007). We examined the accumulation, metabolism, and excretion of individual GLSs and GLS classes to elucidate their role in selective GLS sequestration (Figure 5A).

Phyllotreta flea beetles ingest both GLSs and plant myrosinases when feeding on their host plants and sequester intact GLSs (Beran et al., 2014; Körnig, 2015). It is, however, unknown what impact plant myrosinases have on GLS sequestration and how the flea beetle copes with those plant enzymes to keep the GLSs intact for sequestration. In **Manuscript II**, I tested the influence of plant myrosinases on the metabolic fate of aliphatic GLSs in *P. armoraciae* and investigated the strategies that allow the sequestration of intact GLSs in this chewing insect (Figure 5A). Since aliphatic GLSs were partially hydrolyzed by plant myrosinases during beetle feeding, I additionally studied the impact of plant myrosinases, and thus GLS hydrolysis, on insect performance.

Phyllotreta flea beetles copy the two-component defense system of their host plants by sequestering GLSs and producing an endogenous myrosinase (Beran et al., 2014; Körnig, 2015). Analogously to the plant defense system, the conversion of sequestered GLSs to isothiocyanates would also be beneficial for *Phyllotreta* flea beetles to fend off their natural enemies. It is unclear, however, whether *Phyllotreta* beetles utilize this system to protect themselves from natural enemies by hydrolyzing the non-toxic GLSs to toxic isothiocyanates. Moreover, it is unknown whether all

life stages are protected by this chemical defense. In **Manuscript III,** I analyzed the GLS and endogenous myrosinase activity levels to determine whether *P. armoraciae* is able hydrolyze sequestered GLSs throughout its ontogeny (Figure 5B). I used *H. axyridis* as a generalist predator to test (1) whether the variable myrosinase activity in different life stages correlates with the isothiocyanate formation upon an attack and with the prey survival, and (2) whether sequestered GLSs indeed cause resistance to the generalist predator by assessing the effect of varying GLS levels on prey survival.

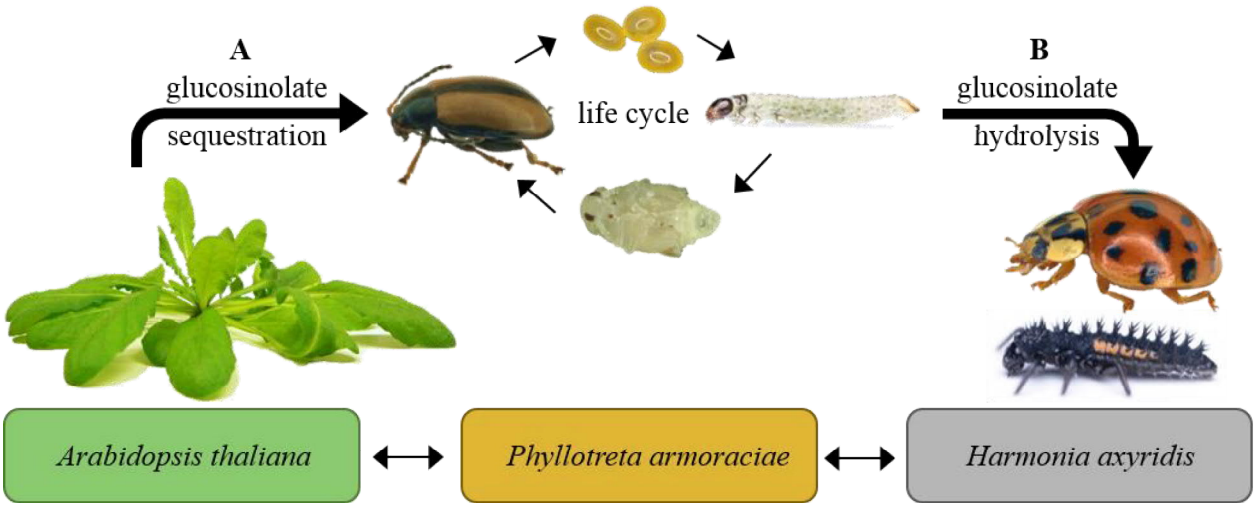

Figure 5: The three organisms in this thesis and overview of the addressed topics. (A) *Arabidopsis thaliana* was used to study the impact of GLS concentration and profile (**Manuscript I**), as well as of plant myrosinases (**Manuscript II**), on the sequestration of GLSs in *Phyllotreta armoraciae*. (B) The role of sequestered GLSs in defense was studied in different *P. armoraciae* life stages in predation assays using the Asian ladybird *Harmonia axyridis* as a generalist predator (**Manuscript III**).

2. Overview of Manuscripts

Manuscript I

Glucosinolate abundance and composition in Brassicaceae influence sequestration in a specialist flea beetle

Zhi-Ling Yang, Grit Kunert, Theresa Sporer, Johannes Körnig, Franziska Beran

Published in *Journal of Chemical Ecology* (2020), Volume 46: 186-197,
doi:10.1007/s10886-020-01144-y

Summary This study investigated how plant glucosinolates influence the glucosinolate sequestration in the horseradish flea beetle *Phyllotreta armoraciae*. We performed a feeding experiment with three *Arabidopsis thaliana* genotypes that differ in their glucosinolate levels and profiles and analyzed the metabolic fate of both ingested and previously stored glucosinolates in beetles. We found that beetles maintained a stable level of glucosinolates in their bodies by balancing the accumulation of new glucosinolates and the excretion of new and previously stored glucosinolates. Moreover, beetles accumulated new glucosinolates with a relatively broad substrate specificity and metabolized distinct glucosinolates. Approximately 40% of all ingested glucosinolates from wild type plants were recovered intact in beetles and feces suggesting that beetles partially overcome plant myrosinase activity. In summary, the variability in plant glucosinolates affects the glucosinolate profile in beetles but not their total levels and the results indicate that beetles maintain this glucosinolate homeostasis by uptake, metabolism and excretion.

Author contributions

Conceived project: TS (10%), ZLY, FB
Designed experiments: TS (10%), ZLY, FB
Performed experiments: TS (10%), ZLY, JK, FB
Chemical analyses: TS (10%), ZLY, JK
Data analyses: TS (10%), ZLY, GK, JK, FB
Manuscript writing: ZLY, FB

Manuscript II

How a glucosinolate-sequestering flea beetle copes with plant myrosinase

Theresa Sporer, Johannes Körnig, Natalie Wielsch, Steffi Gebauer-Jung, Michael Reichelt, Yvonne Hupfer, Franziska Beran

In preparation to be submitted to *Frontiers in Plant Science (Plant Pathogen Interactions)*

Summary This study assessed the impact of plant myrosinases on glucosinolate sequestration in *P. armoraciae* and the mechanisms in this insect to cope with plant myrosinases. Comparative feeding experiments with *Arabidopsis thaliana* genotypes with or without myrosinase activity showed that plant myrosinases partially hydrolyzed glucosinolates during beetle feeding and digestion and thus affected glucosinolate sequestration. Different mechanisms were investigated to elucidate how beetles prevent glucosinolate hydrolysis during feeding. A short-term feeding experiment revealed that beetles quickly separated glucosinolates from plant enzymes in the gut by rapid absorption across the gut epithelium. Furthermore, beetles excreted intact glucosinolates and only traces of myrosinase activity, although peptides corresponding to intact plant myrosinase were detected. Beetle gut extracts inhibited plant myrosinase activity in *in vitro* assays suggesting a permanent inhibition mechanisms during digestion. Taken together, *P. armoraciae* is able to largely prevent glucosinolate hydrolysis and is thus well adapted to the chemical defense of its brassicacaeous host plants.

Author contributions

Conceived project: TS (50%), FB

Designed experiments: TS (65%), FB, JK

Performed experiments: TS (50%), JK, YH, FB

Chemical analyses: TS (50%), JK, MR, NW

Data analyses: TS (65%), JK, NW

Bioinformatic analysis: SGJ

Manuscript writing: TS wrote the first draft of the manuscript, FB and JK contributed to the final draft

Manuscript III

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

Theresa Sporer, Johannes Körnig, Franziska Beran

Published in *Functional Ecology* (2020), Volume 34 (7): 1370-1379,
doi: 10.1111/1365-2435.13548

Summary In this study, we investigated the role of sequestered glucosinolates in the defense of the horseradish flea beetle *Phyllotreta armoraciae* against predation. Therefore, we compared the levels of glucosinolates and myrosinase activity in *P. armoraciae* across the whole life cycle and tested the effect of these factors on predation. All life stages contained glucosinolates in rather similar levels, whereas the levels of endogenous myrosinase activity strongly fluctuated throughout ontogeny. Larvae showed the highest activity, followed by adults, and eggs and pupae showed the lowest activity. The capacity to form toxic isothiocyanates for a successful defense thus strongly depended on the flea beetle's life stage. Predation experiments with a generalist arthropod predator, the Asian ladybird, confirmed that a low endogenous myrosinase activity caused a high mortality in pupae compared to larvae. Feeding larvae with different *Arabidopsis thaliana* genotypes with or without glucosinolates or plant myrosinase activity strongly affected the levels of sequestered glucosinolates in larvae. Subsequent predation assays showed that a lack of sequestered glucosinolates could not prevent predation and hence caused high mortality in the larvae. Taken together, the horseradish flea beetle depends on its food plant to obtain glucosinolates, which it successfully uses for its own chemical defense in a life stage-specific manner.

Author contributions

Conceived project: TS (50%), FB

Designed experiments: TS (60%), FB, JK

Performed experiments: TS (70%), JK

Chemical analyses: TS (90%), JK, FB

Data analyses: TS (60%), JK

Manuscript writing: TS (50%), JK, FB

3. Manuscripts

3.1. Manuscript I

Glucosinolate abundance and composition in Brassicaceae influence sequestration in a specialist flea beetle

Zhi-Ling Yang, Grit Kunert, Theresa Sporer, Johannes Körnig, Franziska Beran

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Glucosinolate Abundance and Composition in Brassicaceae Influence Sequestration in a Specialist Flea Beetle

Zhi-Ling Yang[1] · Grit Kunert[2] · Theresa Sporer[1] · Johannes Körnig[1] · Franziska Beran[1]

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Abstract
The horseradish flea beetle *Phyllotreta armoraciae* exclusively feeds on Brassicaceae, which contain glucosinolates as characteristic defense compounds. Although glucosinolates are usually degraded by plant enzymes (myrosinases) to toxic isothiocyanates after ingestion, *P. armoraciae* beetles sequester glucosinolates. Between and within brassicaceous plants, the glucosinolate content and composition can differ drastically. But how do these factors influence sequestration in *P. armoraciae*? To address this question, we performed a five-day feeding experiment with three *Arabidopsis thaliana* lines that differ four-fold in glucosinolate content and the composition of aliphatic and indolic glucosinolates. We quantified the amounts of ingested, sequestered, and excreted glucosinolates, and analyzed the changes in glucosinolate levels and composition in beetles before and after feeding on *Arabidopsis*. *P. armoraciae* accumulated almost all ingested glucosinolate types. However, some glucosinolates were accumulated more efficiently than others, and selected glucosinolates were modified by the beetles. The uptake of new glucosinolates correlated with a decrease in the level of stored glucosinolates so that the total glucosinolate content remained stable at around 35 nmol/mg beetle fresh weight. Beetles excreted previously stored as well as ingested glucosinolates from *Arabidopsis*, which suggests that *P. armoraciae* regulate their endogenous glucosinolate level by excretion. The metabolic fate of ingested glucosinolates, i.e. the proportions of sequestered and excreted glucosinolates, depended on glucosinolate type, content, and composition in the food plant. Overall, *P. armoraciae* sequestered and excreted up to 41% and 31% of the total ingested aliphatic and indolic glucosinolates from *Arabidopsis*, respectively. In summary, we show that glucosinolate variability in Brassicaceae influences the composition but not the level of sequestered glucosinolates in *P. armoraciae* beetles.

Keywords Plant-insect interaction · *Phyllotreta* · Sequestration · Glucosinolate · Excretion · Metabolism · Adaptation

Introduction

Many plants utilize two-component defenses that are activated upon tissue damage. These defense systems consist of glucosylated secondary metabolites, e.g. cyanogenic, iridoid, and benzoxazinoid glucosides that are separately stored from activating β-glucosidases in intact plant tissue (Pentzold et al. 2014b). A well-studied activated defense is the glucosinolate (GLS)-myrosinase system in plants of the order Brassicales, also known as the "mustard oil bomb" (Halkier and Gershenzon 2006; Lüthy and Matile 1984). When leaf damage disrupts the spatial separation between GLS and myrosinase, GLS are hydrolyzed to unstable aglucones that are further converted to breakdown products including highly reactive isothiocyanates (Wittstock et al. 2016). To date, more than 130 different GLS have been identified in plants, and have been broadly classified according to the structure of their amino acid-derived side chain as benzenic, indolic, or aliphatic GLS (Agerbirk and Olsen 2012).

Some herbivorous insects also possess activated defense systems that consist of glucosylated compounds, either sequestered from food plants or synthesized *de novo,* and insect-derived activating β-glucosidase enzymes (Beran et al. 2019). For example, larvae of the six spot burnet moth, *Zygaena filipendulae* (L.) (Zygaenidae), sequester and *de*

Zhi-Ling Yang and Grit Kunert contributed equally to this study.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10886-020-01144-y) contains supplementary material, which is available to authorized users.

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novo synthesize cyanogenic glucosides, and produce a cyanogenic β-glucosidase (Jensen et al. 2011; Zagrobelny et al. 2018). To sequester cyanogenic glucosides from their food plant, *Z. filipendulae* larvae prevent hydrolysis by plant cyanogenic *β*-glucosidases. For instance, reduced plant *β*-glucosidase activity under the alkaline gut pH conditions might facilitate the sequestration of ingested cyanogenic glucosides by *Z. filipendulae* larvae (Pentzold et al. 2014a). Another mechanism that allows insects to sequester glucosylated defense compounds is by absorbing them across the gut epithelium before they are activated in the gut lumen. This strategy was suggested to prevent the hydrolysis of ingested GLS in larvae of the turnip sawfly, *Athalia rosae* (L.) (Tenthrenidinae), although surprisingly, the sequestered GLS cannot be activated by the insect for its defense (Abdalsamee et al. 2014; Müller and Wittstock 2005). Thus, sequestration of glucosylated plant defense compounds might function as a detoxification strategy by preventing the formation of toxic breakdown products (Pentzold et al. 2014b) as an alternative to serving a defensive function.

We previously discovered that adults of the striped flea beetle, *Phyllotreta striolata* (Fabricius) (Chrysomelidae), have a high capacity to sequester certain aliphatic GLS from their brassicaceous food plants, and that the insects produce myrosinase that converts sequestered GLS to toxic isothiocyanates (Beran et al. 2014). *P. striolata* is an oligophagous species that feeds on many different cultivated and wild Brassicaceae plant species and thus encounters wide ranges of GLS concentrations and compositions in its food plants (Gikonyo et al. 2019). Analyses of the GLS sequestration patterns in beetles fed on different plant species revealed that the accumulation rate for a given GLS can depend on the food plant. These findings indicated that the plant's GLS composition can influence GLS sequestration in this species. However, whether rapid GLS sequestration can also prevent hydrolysis of ingested GLS in *P. striolata* is unknown. Several attempts to rear *P. striolata* in the laboratory for further studies failed, but we successfully established a laboratory colony of the horseradish flea beetle, *Phyllotreta armoraciae* (Koch) (Chrysomelidae), which we use as a model species to investigate the mechanism and function of GLS sequestration in the genus *Phyllotreta*.

Although *P. armoraciae* is monophagous on horseradish (*Armoracia rusticana*) in nature, the beetles accept several other Brassicaceae plant species as food in the laboratory (Nielsen 1978; Nielsen et al. 1979; Vig and Verdyck 2001). While beetles feed primarily on the leaf blade, larvae mine the petioles until they pupate in the soil (Vig 2004). In the laboratory, we rear *P. armoraciae* on a *Brassica juncea* cultivar that contains the same major GLS as horseradish, i.e. allyl GLS (Agneta et al. 2014; Beran et al. 2014). Preliminary studies revealed the presence of allyl GLS in all *P. armoraciae* life stages and showed that GLS were transferred from larvae through metamorphosis to the adult stage (Körnig 2015).

Here, we focused on GLS sequestration in adult *P. armoraciae* beetles. To elucidate where sequestered GLS are stored in *P. armoraciae*, we analyzed the distribution of sequestered GLS in the adult body. To understand how GLS levels and composition in food plants affect GLS sequestration, we performed a feeding experiment with newly emerged adults and three *Arabidopsis thaliana* lines. We used the *Arabidopsis* Col-0 wild type and two mutants in the Col-0 background. The plant lines differ about four-fold in total GLS levels and have different compositions of aliphatic and indolic GLS (Sønderby et al. 2007; Zhao et al. 2002). Specifically, we asked the following questions: (1) How are the GLS levels and composition in adult *P. armoraciae* beetles affected by ingested GLS? (2) Are ingested GLS selectively sequestered and metabolized? (3) Do *P. armoraciae* adults selectively excrete GLS? and (4) Does the metabolic fate of ingested GLS in *P. armoraciae* depend on GLS type, the total ingested GLS amount (influenced by the GLS level in the plant), and the GLS composition in the food plants?

Methods and Materials

***P. armoraciae* Rearing** The laboratory culture of *P. armoraciae* was established in 2012 using beetles collected from horseradish plants in Laasdorf, Thuringia, Germany. Adult *P. armoraciae* beetles were reared on three- to four-week old potted *Brassica juncea* cv. Bau Sin plants (Known-You Seed Co. Ltd., Kaohsiung, China) in a controlled environment chamber at 24 °C, 60% relative humidity, and a 14:10 h light:dark cycle. After one week, plants with eggs were transferred to a separate cage for larval development. Three weeks later, any remaining plant material was removed, and the soil containing pupae was kept in plastic containers (9 L volume, Lock&Lock, Seoul, South Korea) until adults emerged. Field-collected beetles were added to the colony every year to prevent an inbreeding depression. The experiments described here were carried out between 2014 and 2017.

Localization of Sequestered GLS in *P. armoraciae* Beetles To determine where sequestered GLS are stored in *P. armoraciae* beetles, we dissected adults collected from the laboratory colony. First, we collected hemolymph by inserting a thin glass capillary into the hemocoel between thorax and abdomen, and then separately collected head, legs, elytra, hindwings, thorax, integument, gut, fat body, and reproductive organs. Hemolymph and tissues of five males and five females were pooled in 500 μL of 80% (v/v) methanol on ice, and samples were stored at −20 °C until GLS analysis. Dissected tissues were homogenized using metal beads (2.4 mm diameter, Askubal, Korntal-Münchingen, Germany) using a TissueLyser II (QIAGEN, Hilden, Germany). After adding

50 nmol of 4-hydroxybenzyl GLS (sinalbin) as an internal standard to each sample, GLS were extracted, analyzed by HPLC-UV at 229 nm and quantified as previously described in Beran et al. (2014). The distribution of GLS in hemolymph and different beetle tissues was expressed relative to the total GLS amount detected in all samples, which was set to 100%.

Design of the GLS Sequestration Experiment with *P. armoraciae* and *A. thaliana* To determine how ingested GLS affect sequestration and excretion in *P. armoraciae*, we performed a feeding experiment with newly emerged beetles and three different *A. thaliana* genotypes that differ about four-fold in GLS contents and in their GLS composition. We used the Col-0 wild type of *Arabidopsis* that mainly produces 4-methylsulfinylbutyl (4MSOB) GLS and other methylsulfinylalkyl GLS as well as minor amounts of indolic GLS, and two double knock-out mutants, *myb28myb29* (*myb*) and *cyp79b2cyp79b3 (cyp)* in the Col-0 background, which are devoid of aliphatic and indolic GLS, respectively (Sønderby et al. 2007; Zhao et al. 2002). *Arabidopsis* plants were cultivated in a controlled environment chamber at 21 °C, 55% relative humidity and a 10:14 h light:dark cycle.

To compare GLS in *P. armoraciae* beetles before and after feeding on *Arabidopsis*, we randomly assigned newly emerged male and female beetles to the following four treatments, newly emerged (control before feeding), fed on wild type, fed on *myb*, and fed on *cyp*, each with ten replicates. Control beetles were immediately collected in groups of five beetles, weighed, frozen in liquid nitrogen, and stored at −20 °C for GLS analysis.

For feeding, we placed five beetles together with one detached leaf from a six- to seven-week old *Arabidopsis* plant into a Petri dish (60 mm diameter, Greiner Bio-One, Frickenhausen, Germany). The leaf petiole was inserted into a 0.2 mL reaction tube containing 0.1 mL ultrapure water to prevent wilting. Adults were provided with a new leaf taken from an undamaged *Arabidopsis* plant every day until day five, and were then starved for one additional day before sampling as described for the newly emerged adults.

To calculate how much plant tissue the beetles had ingested, we weighed each leaf before and after feeding. Because we noticed that the fresh weight of detached leaves increased by 23 ± 3% for *Arabidopsis* wild type, 22 ± 3% for *myb* mutant, and 15 ± 2% for *cyp* mutant, under our conditions (mean ± SD, $N = 8–9$), we corrected the initial leaf weight before calculating the amount of fed plant tissue. Fed leaves were frozen in liquid nitrogen and stored at −20 °C until they were freeze-dried for later GLS analysis. Feces were collected every day using 100 μL of ultrapure water per Petri dish. Each aqueous feces sample was mixed with 300 μL pure methanol (purity ≥99.9%, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and stored at −20 °C until GLS extraction.

P. armoraciae beetles were homogenized in 1 mL 80% (v/v) methanol containing 50 nmol sinalbin using a plastic pestle. Freeze-dried *Arabidopsis* leaves were homogenized using metal beads to a fine plant powder that was extracted with 1 mL 80% (v/v) methanol containing 50 nmol sinalbin. Feces samples were homogenized with metal beads and the volume was adjusted to 1 mL using 80% (v/v) methanol containing the internal standard sinalbin. The GLS extraction, analysis and quantification was done as described in Beran et al. (2014).

Question 1: How are the GLS levels and composition in adult P. armoraciae beetles affected by ingested GLS? The GLS compositions in the three *Arabidopsis* lines used in our sequestration experiment are shown in Table S1. We compared the individual and total GLS amounts (in nmol per beetle) and concentrations (in nmol per mg beetle) in newly emerged beetles with those in fed beetles by different statistical methods depending on the variance homogeneity and the normality of residuals. Comparisons by Student's t test, Mann-Whitney U test, and analysis of variance (ANOVA) were carried out in Sigma Plot 11.0 (Systat Software, Inc., Erkrath, Germany). Analyses using the method of generalized least squares were done in R 3.5.1 (nlme package, Pinheiro et al. 2019; R Core Team 2018). If necessary, data were transformed prior to analysis. For data analyzed with the generalized least squares method, the varIdent variance structure was applied, allowing each group to have a different variance. The P value was obtained by removing the explanatory variable and comparing both models using a likelihood ratio test (Zuur et al. 2009). Factor level reductions were used to reveal significant differences between groups (Crawley 2013). Details of statistical methods are provided in Supplementary Tables S2 and S3.

Since the amounts of previously sequestered allyl GLS were lower in fed beetles than in newly emerged beetles, we examined the influence of the total ingested GLS amount and the food plant on the allyl GLS levels in beetles and feces, respectively, by analysis of covariance (ANCOVA) in R (R Core Team 2018). The total ingested GLS amount was calculated based on the ingested amount of leaf tissue and the corresponding GLS concentration in each fed leaf. Although GLS are unevenly distributed in *Arabidopsis* rosette leaves (Shroff et al. 2008), adult feeding damage was randomly distributed across leaves in our experiment (Fig. S1). In both analyses, the ingested GLS amount per beetle was log-transformed. Allyl GLS amounts per beetle were log-transformed, and excreted allyl GLS amounts per beetle were square-root transformed in order to achieve homogeneity and normality of the residuals. Factor level reductions were used to reveal significant differences between groups (Crawley 2013). To elucidate which proportion of the lost allyl GLS was excreted, we expressed the allyl GLS amount detected in the feces relative to the lost allyl GLS amount in adults, which was set to 100%.

Question 2: Are ingested GLS from Arabidopsis wild type leaves selectively sequestered and metabolized in P. armoraciae adults? To determine whether *P. armoraciae* accumulated individual GLS from *Arabidopsis* wild type leaves selectively, we expressed the concentration of each GLS in adults relative to the average concentration in feeding-damaged leaves (set to 1). The relative (fold) accumulation of different GLS in *P. armoraciae* was compared using the generalized least squares method (nlme package, Pinheiro et al. 2019). Data were square-root-transformed prior to analysis.

The analysis of the relative accumulation of individual GLS from *Arabidopsis* wild type leaves in *P. armoraciae* revealed a disproportionately high accumulation of 4-methylthiobutyl (4MTB) GLS in beetles. In addition, these beetles contained significantly higher amounts of 3-butenyl (3But) GLS, although this GLS was not present in their food plant. To determine whether *P. armoraciae* converts 4MSOB GLS, the major aliphatic GLS in *Arabidopsis* wild type leaves, to 4MTB GLS and 3But GLS, we fed newly emerged adults with an aqueous solution containing 10 nmol 4MSOB GLS (purchased from Phytoplan, Heidelberg, Germany). We placed newly emerged beetles in a Petri dish with a 0.2 µl droplet containing the GLS, or pure water as a control, and observed each beetle until it had finished drinking the droplet under a microscope. To allow adults to metabolize the ingested 4MSOB GLS, they were fed for three days with detached *B. juncea* leaves, which do not contain 4MSOB GLS and 4MTB GLS (Beran et al. 2014). Afterwards, adults were frozen in liquid nitrogen and stored at −20 °C until GLS extraction. We analyzed four and six replicates for the control and 4MSOB GLS treatments, respectively, each consisting of five adults. Because the 4MSOB GLS solution fed to beetles contained a small amount of 4MTB GLS as contaminant, we compared the ingested 4MTB GLS amount (present in the fed 4MSOB

Table 1 Distribution of sequestered glucosinolates (GLS) in *P. armoraciae* adults

Body part/Tissue	Percentage of the total detected GLS
Hemolymph	29
Elytra	22
Legs	17
Head	10
Thorax	9
Hindwings	4
Gut	4
Integument	3
Reproductive organs	1
Fat body	1

Results are based on one sample derived from 5 females and 5 males

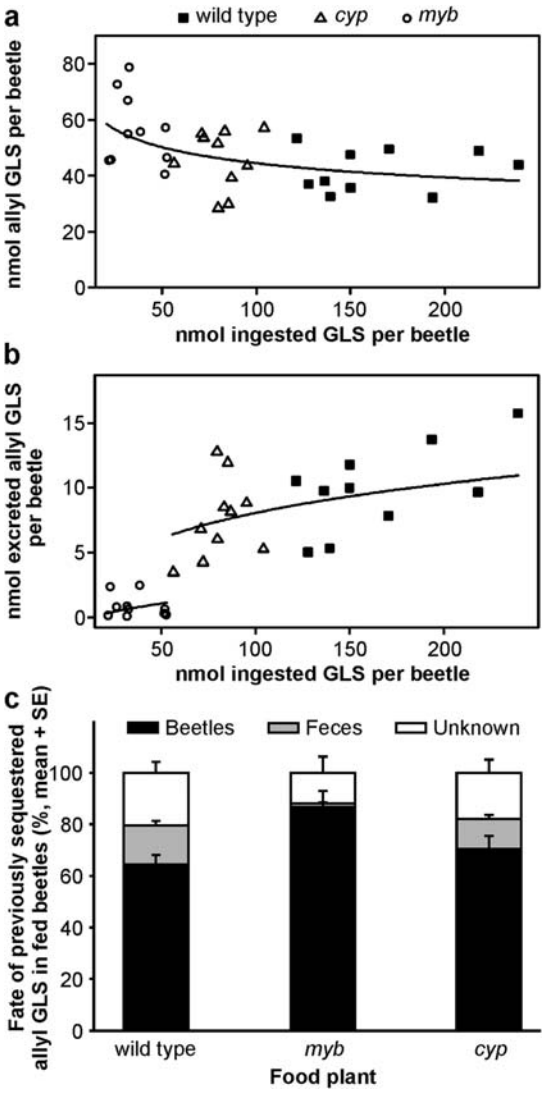
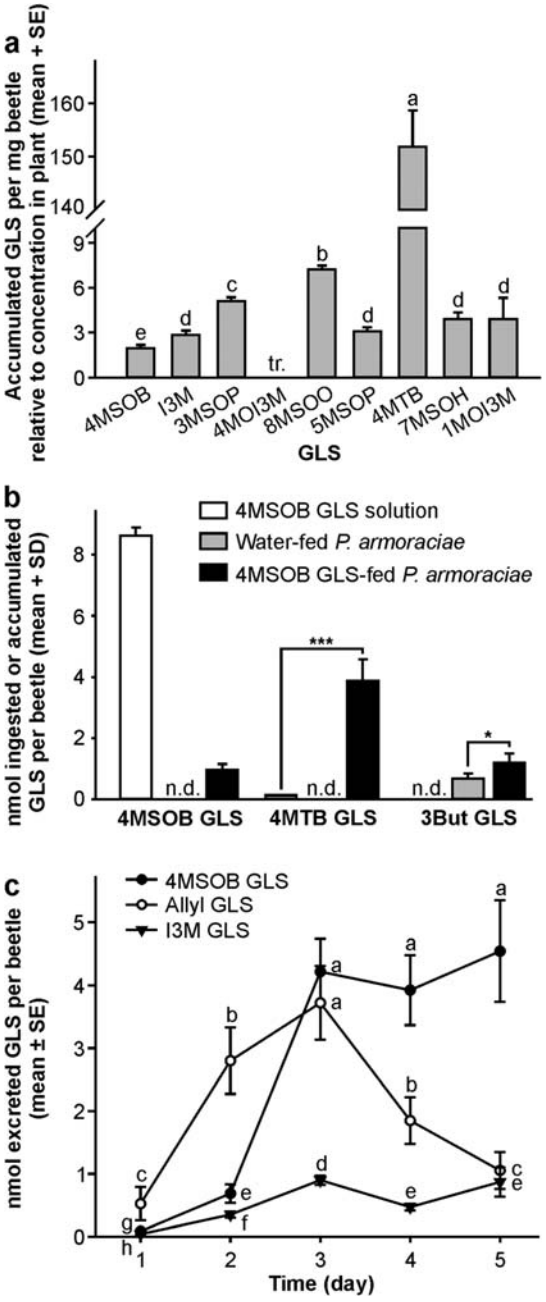


Fig. 1 Fate of previously sequestered allyl glucosinolate (GLS) in *P. armoraciae* beetles fed on different *Arabidopsis* lines for five days and starved for one day. **a** Relationship between the total ingested GLS amount and the amount of previously sequestered allyl GLS per beetle. **b** Relationship between the total ingested GLS amount and the amount of excreted allyl GLS per beetle. The statistical analyses were performed by ANCOVA using the total ingested GLS amount as a covariable. In the first analysis (**a**), both the total ingested GLS amount and allyl GLS amount were log transformed prior to analysis. In the second analysis (**b**), the total ingested GLS amount and the excreted allyl GLS amount was log transformed and square-root transformed, respectively, prior to analysis. The final estimates were back-transformed to their original scale, and plotted with regression lines. Data series that are not significantly different from each other were combined for regression line plotting. **c** Percentage of allyl GLS in fed beetles and feces relative to the allyl GLS amount in newly emerged beetles ($N = 10$). *myb*, *Arabidopsis myb28myb29* double knockout mutant; *cyp*, *Arabidopsis cyp79b2cyp79b3* double knockout mutant

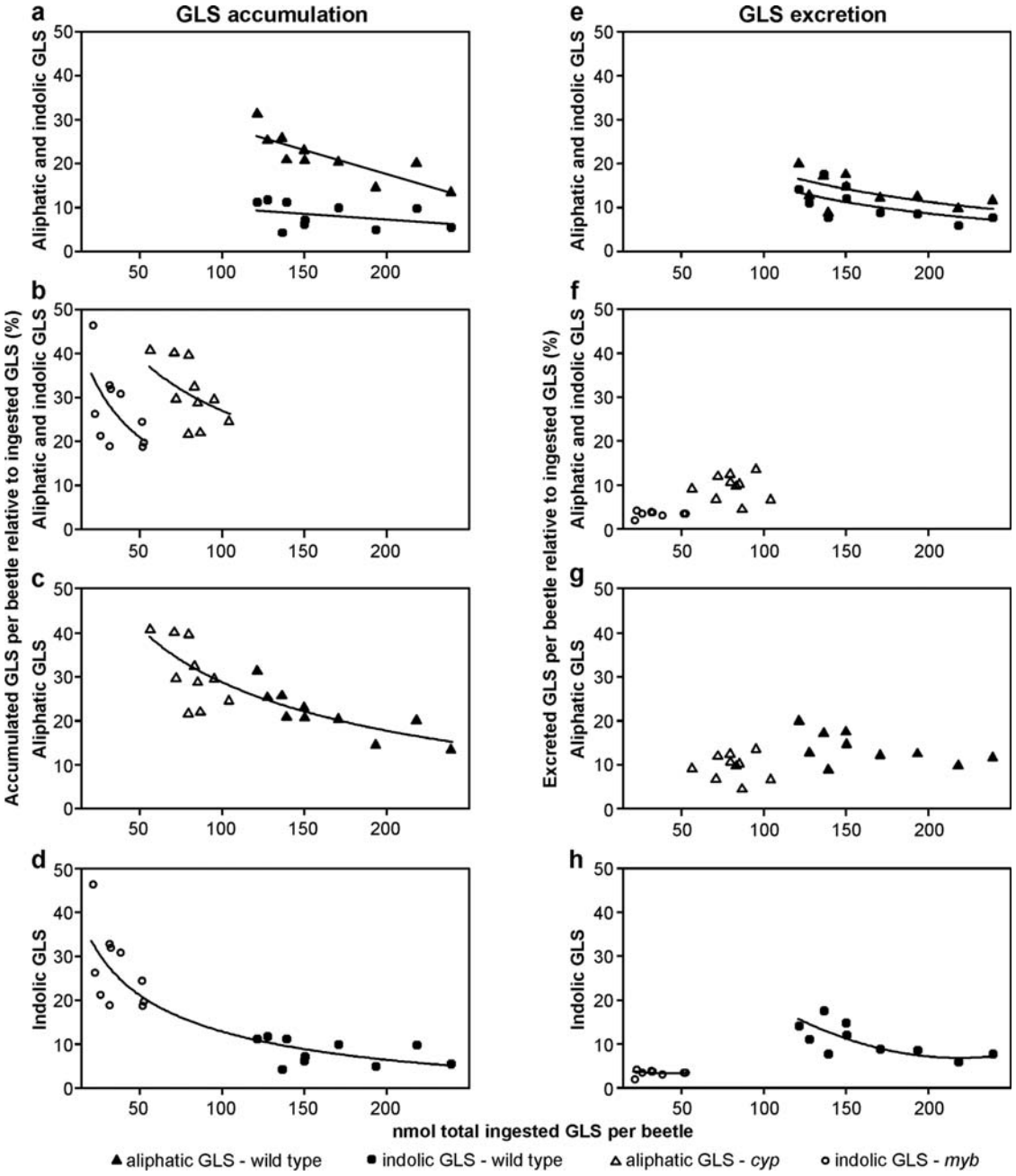


◀ **Fig. 2** Accumulation, metabolism and excretion of ingested GLS in *P. armoraciae* adults. **a** GLS accumulation pattern in *P. armoraciae* adults after five days feeding on *Arabidopsis* wild type leaves and one day starvation. To compare the accumulation of different GLS in *P. armoraciae*, the concentration of each GLS in adults is expressed relative to that in leaves (set to 1). GLS are sorted from the highest to the lowest concentration in *Arabidopsis*. Bars labeled with different letters are significantly different (generalized least squares method, $P < 0.05$, $N = 10$). **b** Metabolism of sequestered 4MSOB GLS in *P. armoraciae* adults. Adults were fed with an aqueous 4MSOB GLS solution or water as a control, and harvested for GLS extraction after feeding on *B. juncea* for three days ($N = 4$–6). The amounts of 4MTB GLS and 3But GLS between different groups were compared by Student's *t* test. A small amount of 4MTB GLS was detected as a contaminant in the 4MSOB GLS solution, but 4MTB GLS amounts in 4MSOB GLS-fed adults were significantly higher ($t = -8.241$, $P \leq 0.001$). **c** Time course of GLS excretion over five days feeding on *Arabidopsis* wild type leaves. The graph shows the excreted amounts of previously sequestered allyl GLS and newly ingested 4MSOB GLS and I3M GLS on each day. Different letters indicate significant differences between different days and GLS (linear mixed effects model, $P < 0.05$, $N = 10$). 3But, 3-butenyl; 4MSOB, 4-methylsulfinylbutyl; 4MTB, 4-methylthiobutyl; 8MSOO, 8-methylsulfinyloctyl; 3MSOP, 3-methylsulfinylpropyl; 5MSOP, 5-methylsulfinylpentyl; 7MSOH, 7-methylsulfinylheptyl; I3M, indol-3-ylmethyl; 4MOI3M, 4-methoxyindol-3-ylmethyl; 1MOI3M, 1-methoxyindol-3-ylmethyl; tr., traces; n.d., not detected; $^{*}P < 0.05$; $^{***}P < 0.001$

GLS solution) with the amount detected in fed beetles by Student's *t* test. Because 3But GLS was detected in control and fed beetles, we compared the levels in both groups by Student's *t* test. In addition to 4MSOB GLS, *Arabidopsis* contains also other methylsulfinylalkyl GLS, including 7-methylsulfinylheptyl GLS and 8-methylsulfinyloctyl GLS, which might be converted to the corresponding methylthioalkyl GLS. Since the chromatographic conditions used for the GLS analysis in beetles and feces did not allow the detection of 7-methylthioheptyl- and 8-methylthiooctyl GLS, we analyzed several samples by HPLC-UV at

Fig. 3 Metabolic fate of the ingested aliphatic and indolic GLS in ▶ *P. armoraciae* adults. The amount of aliphatic and/or indolic GLS ingested from *Arabidopsis* wild type, *myb*, and *cyp* rosette leaves, respectively, was set to 100%, and the corresponding percentages of aliphatic and indolic GLS detected in beetles and feces were calculated. The plots show the accumulated and excreted proportions of ingested aliphatic and/or indolic GLS by *P. armoraciae* adults relative to the total ingested GLS amount per beetle. **a** Accumulation of aliphatic and indolic GLS ingested from *Arabidopsis* wild type. **b** Accumulation of aliphatic and indolic GLS ingested from the *cyp* or *myb* mutant, respectively. **c** Accumulation of aliphatic GLS ingested from wild type plant and *cyp* mutant, respectively. **d** Accumulation of indolic GLS ingested from wild type plant and *myb* mutant, respectively. **e** Excretion of aliphatic and indolic GLS ingested from *Arabidopsis* wild type. **f** Excretion of aliphatic and indolic GLS ingested from *cyp* and *myb* mutant, respectively. **g** Excretion of aliphatic GLS ingested from wild type plant and *cyp* mutant, respectively. **h** Excretion of indolic GLS ingested from wild type plant and *myb* mutant, respectively. The statistical analyses were performed by ANCOVA, generalized least squares method, or linear mixed effects models using the total ingested GLS amount per beetle as covariable, with linear or quadratic regression. If necessary, data were transformed prior to analysis. The final estimates were back-transformed to their original scale, and plotted with regression lines. Data series that are not significantly different from each other were combined for regression line plotting. For methods and results of the statistical analyses, refer to Table 2. Aliphatic GLS: sum of 3MSOP GLS, 4MSOB GLS, 4MTB GLS, 5MSOP GLS, 7MSOH GLS, 8MSOO GLS; indolic GLS: sum of I3M GLS, 1MOI3M GLS, 4MOI3M GLS. For plant and GLS abbreviations, refer to legend of Figs. 1 and 2, respectively

229 nm using a modified solvent gradient of 0.2% formic acid (solvent A) and acetonitrile (solvent B): 1.5% (v/v) B (1 min), 1.5–5% (v/v) B (5 min), 5–7% (v/v) B (2 min), 7–21% (v/v) B (10 min), 21–29% (v/v) B (5 min), 29–43% (v/v) B (7 min), 43–100% (v/v) B (0.5 min), 100% (v/v) B (2.5 min), 100 to 1.5% (v/v) B (0.1 min), and 1.5% (v/v) B (4.9 min). In addition, we determined the presence of methylthiolalkyl GLS in beetle and feces samples by liquid chromatography coupled with mass spectrometry. Chromatographic analyses (as described above) were carried out on 1100 series equipment (Agilent Technologies, Waldbronn, Germany) coupled to an Esquire 6000 ESI-Ion



Trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in positive ionization mode in the range of *m/z* 60–1000, with a skimmer voltage of 52.8 V, capillary exit voltage of 117.3 V, capillary voltage of 3000 V, nebulizer pressure of 35 psi, drying gas of 11 L/min, and gas temperature of 330 °C. Elution was accomplished at a flow rate of 1 mL/min at 25 °C under chromatographic conditions as described above. Flow coming from the column was diverted at a ratio of 4:1 before reaching the electrospray ionization (ESI) unit. We detected 3-methylthiopropyl-, 7-methylthioheptyl- and 8-methylthiooctyl GLS as desulfo-GLS in samples by comparing the retention times, UV spectra, mass spectra and in-source fragmentation patterns to those of isolated standards (Brown et al. 2003). The presence of 5-methylthiopentyl GLS was analyzed according to its UV spectrum, mass spectrum, and in-source fragmentation pattern.

Question 3: Do P. armoraciae adults excrete GLS selectively? The amounts of individual and total GLS detected in feces were compared by different statistical methods as described above. Details of statistical analyses are provided in Table S4. To determine whether beetles excreted previously sequestered allyl GLS and ingested GLS selectively, we compared the amounts of allyl GLS, 4MSOB GLS, and indol-3-ylmethyl (I3M) GLS excreted by adults fed on *Arabidopsis* wild type leaves using a linear mixed effects model. The lme function (Pinheiro et al. 2019) was applied to account for the different beetle groups. GLS and day were treated as fixed effects, and beetle groups as random effect. The GLS amount was log-transformed prior to analysis. *P* values and significant differences between groups were obtained as described above.

Question 4: Does the metabolic fate of ingested GLS in P. armoraciae depend on GLS type, total ingested GLS amount, and the GLS composition in the food plant? To analyze the metabolic fate of ingested aliphatic and indolic GLS, we calculated the percentage of sequestered and excreted aliphatic and indolic GLS relative to the total amount of ingested aliphatic and indolic GLS, respectively (set to 100%). Because a low background of indolic GLS was present in newly emerged adults, we subtracted the average amount of each indolic GLS detected in newly emerged beetles from the corresponding GLS amounts detected after feeding on *Arabidopsis*. To analyze whether the total ingested GLS amount (covariable), the GLS type (aliphatic or indolic GLS as explanatory variable) or the GLS composition (*Arabidopsis* line as explanatory variable) affect the metabolic fate of ingested GLS, we performed ANCOVA or analyzed the data using the method of generalized least squares, or linear mixed effects models (nlme package, Pinheiro et al. 2019) with GLS ingestion and GLS type as fixed effects and beetle groups feeding on a certain plant as random intercept. ANCOVA analyses were conducted with type II variance partitioning of the car library (Fox and Weisberg 2011) to adjust each effect for other effects (Kabacoff 2011). If necessary, data were transformed prior to analysis. To determine the appropriate variance structure for the generalized least squares analyses, models fitted with different variance structures were compared based on the Akaike information criterion (AIC) (Zuur et al. 2009). *P* values were obtained as described above. We used the total ingested GLS amount instead of the ingested amounts of aliphatic and indolic GLS in our analyses, respectively, because a Spearman's rank correlation coefficient analysis showed a strong positive correlation between the total ingested GLS amount, the ingested aliphatic GLS amount and the ingested indolic GLS amount, respectively (rho $\geq$ 0.770, $P \leq 0.014$; Fig. S2). Details of statistical analyses are given in Table 2.

Results

Localization of sequestered GLS After hemolymph collection and dissection of *P. armoraciae* beetles, we found different quantities of GLS in all tissues. The highest proportion of GLS was detected in the hemolymph, which contained about one third of the total detected GLS. High proportions of GLS were also found in the elytra (22%), the legs (17%), and the head (10%), whereas only traces of GLS were found in reproductive organs and the fat body (Table 1).

Question 1: How are the GLS levels and composition in adult P. armoraciae beetles affected by ingested GLS? The concentrations and amounts of GLS detected in newly emerged and fed beetles are summarized in Tables S2 and S3, respectively. Newly emerged *P. armoraciae* adults contained mainly allyl GLS and minor amounts of 3But GLS and indolic GLS. This GLS composition largely corresponds to that in the rearing plant *B. juncea* (Beran et al. 2014). After feeding on different *Arabidopsis* lines, the total GLS concentrations and amounts in fed adults did not differ from those in newly emerged adults (GLS concentration: generalized least squares method, likelihood ratio = 6.309, $P = 0.098$; GLS amount: ANOVA, $F = 1.108$, $P = 0.359$). However, the GLS compositions in fed adults differed because of an accumulation of aliphatic and/or indolic GLS. This GLS uptake was balanced with a decrease of previously sequestered allyl GLS in beetles (Table S3). The allyl GLS levels in beetles were negatively correlated with the amount of ingested GLS, and did not depend on the food plant (Fig. 1a; ANCOVA, ingested GLS amount: $F = 8.391$, $P = 0.007$; plant: $F = 0.456$, $P = 0.639$; ingested GLS amount × plant: $F = 0.136$, $P = 0.873$). To determine whether beetles regulate their endogenous GLS levels by excreting GLS, we quantified the amounts of allyl GLS in feces (Table S4). Allyl GLS excretion was positively correlated with the ingested GLS amount and, in addition, depended on the food plant, because *myb*-fed adults excreted significantly less allyl GLS than wild

Table 2 Methods and results of statistical analyses of metabolic fate of ingested aliphatic and indolic GLS in *P. armoraciae* adults

Metabolic fate	Comparison	Statistical method	Equation form	Variance structure	Variable	Transformation	Statistics	*P* value
Accumulation	Aliphatic and indolic GLS from *Arabidopsis* wild type (Fig. 3a)	Linear mixed effects model	Linear	–	Accumulated percentage	–	–	–
					Ingested GLS amount	–	$LR = 8.790$	= 0.003
					GLS type	–	$LR = 27.778$	< 0.001
					Interaction	–	$LR = 6.860$	= 0.009
	Aliphatic GLS from *cyp* mutant, indolic GLS from *myb* mutant (Fig. 3b)	ANCOVA	Linear	–	Accumulated percentage	Arcsin-square-root	–	–
					Ingested GLS amount	Log	$F = 7.631$	= 0.013
					Plant	–	$F = 9.160$	= 0.008
					Interaction	–	$F = 0.687$	= 0.420
	Aliphatic GLS from *Arabidopsis* wild type and *cyp* mutant (Fig. 3c)	Generalized least squares	Linear	varComb (varIdent (form = ~ 1 | plant),varFixed(~ Ingested GLS amount))	Accumulated percentage	Arcsin-square-root	–	–
					Ingested GLS amount	Log	$LR = 24.058$	< 0.001
					Plant	–	$LR = 3.625$	= 0.057
					Interaction	–	$LR = 0.150$	= 0.698
	Indolic GLS from *Arabidopsis* wild type and *myb* mutant (Fig. 3d)	Generalized least squares	Linear	varPower (form = ~ log (Ingested GLS amount))	Accumulated percentage	Arcsin-square-root	–	–
					Ingested GLS amount	Log	$LR = 26.602$	< 0.001
					Plant	–	$LR = 1.095$	= 0.295
					Interaction	–	$LR = 0.560$	= 0.454
Excretion	Aliphatic and indolic GLS from *Arabidopsis* wild type (Fig. 3e)	Linear mixed effects model	Linear	–	Excreted percentage	Arcsin-square-root	–	–
					Ingested GLS amount	Log	$LR = 5.279$	= 0.022
					GLS type	–	$LR = 13.807$	< 0.001
					Interaction	–	$LR = 3.313$	= 0.069
	Aliphatic GLS from *cyp* mutant, indolic GLS from *myb* mutant (Fig. 3f)	Generalized least squares	Linear	varExp (form = ~ Ingested GLS amount | plant)	Excreted percentage	–	–	–
					Ingested GLS amount	–	$LR = 0.002$	= 0.964
					Plant	–	$LR = 15.311$	< 0.001
					Interaction	–	$LR = 0.321$	= 0.571
	Aliphatic GLS from *Arabidopsis* wild type and *cyp* mutant (Fig. 3g)	ANCOVA	Linear	–	Excreted percentage	–	–	–
					Ingested GLS amount	–	$F = 3.413$	= 0.082
					Plant	–	$F = 8.194$	= 0.010
					Interaction	–	$F = 0.142$	= 0.711
	Indolic GLS from *Arabidopsis* wild type and *myb* mutant (Fig. 3h)	Generalized least squares	Quadratic	varExp (form = ~ Ingested GLS amount | plant)	Excreted percentage	–	–	–
					Ingested GLS amount	–	$LR = 6.809$	0.009
					Plant	–	$LR = 8.359$	0.004
					Ingested GLS amount: Plant Interaction	–	$LR = 6.095$	0.014
					Ingested GLS amount^2	–	$LR = 5.659$	0.017
					Ingested GLS amount^2:Plant Interaction	–	$LR = 0.062$	0.803

LR, likelihood ratio

Table 3 Metabolic fate of the ingested aliphatic and indolic GLS in *P. armoraciae* adults that fed on leaves of different *Arabidopsis* lines for 5 days

Metabolic fate	Mean percentage[1] ± SD; $N = 10$			
	Aliphatic GLS wild type	Indolic GLS wild type	Aliphatic GLS *cyp*	Indolic GLS *myb*
Accumulation	21.5 ± 5.3	8.2 ± 2.9	30.9 ± 7.3	27.1 ± 8.7
Excretion	13.6 ± 3.6	10.8 ± 3.8	9.5 ± 2.9	3.4 ± 0.6
Total recovery[2]	35.1 ± 8.1	19.0 ± 4.0	40.3 ± 7.9	30.5 ± 8.3

[1] The amount of aliphatic and/or indolic GLS ingested from *Arabidopsis* wild type, *myb*, and *cyp* mutant, respectively, was set to 100%. The corresponding percentages of aliphatic and indolic GLS in adults (accumulation) and feces (excretion) were calculated. [2] The total recovery corresponds to the recovered proportion of ingested GLS, which was detected in adults (accumulation) and feces (excretion). Aliphatic GLS: sum of 3MSOP GLS, 4MSOB GLS, 4MTB GLS, 5MSOP GLS, 7MSOH GLS, 8MSOO GLS; indolic GLS: sum of I3M GLS, 1MOI3M GLS, 4MOI3M GLS. For plant and GLS abbreviations, refer to legend of Figs. 1 and 2, respectively

type- or *cyp*-fed adults (Fig. 1b; ANCOVA, ingested GLS amount: $F = 97.303$, $P < 0.001$, plant: $F = 7.852$, $P = 0.002$; ingested GLS amount × plant: $F = 2.527$, $P = 0.101$). In total, we recovered up to 40% of the lost allyl GLS (set to 100%) in feces (Fig. 1c).

Question 2: Are ingested GLS from Arabidopsis wild type leaves selectively sequestered and metabolized in P. armoraciae adults? P. armoraciae accumulated almost all GLS present in *Arabidopsis*, but at widely divergent efficiencies (Fig. 2a; generalized least squares method, likelihood ratio = 127.463, $P < 0.001$). While most GLS were concentrated between two and seven-fold in adults, the 4MTB GLS concentration was 152-fold higher in adults than in leaves. Since these 4MTB GLS amounts in beetles cannot be explained by direct accumulation from the food plant (ingestion of 321 mg wild type leaf per beetle would be necessary, but at most 70 mg of plant tissue was ingested per beetle), we hypothesized that *P. armoraciae* can convert sequestered 4MSOB GLS, the major aliphatic GLS in *Arabidopsis* wild type leaves, to 4MTB GLS. To test this hypothesis, we fed *P. armoraciae* with an aqueous 4MSOB GLS solution or water as a control. Three days later, we detected about four times more 4MTB GLS than 4MSOB GLS in these adults, whereas both GLS were below the detection limit in the control adults (Fig. 2b). Although a minor amount of 4MTB GLS was present in the 4MSOB GLS solution as a contaminant, significantly larger amounts of 4MTB GLS were detected in 4MSOB GLS-fed beetles (Student's *t* test, $t = -8.241$, $P \leq 0.001$). In addition, we found significantly more 3But GLS in 4MSOB GLS-fed adults than in the corresponding control adults, which indicates that *P. armoraciae* metabolize 4MSOB GLS to minor amounts of 3But GLS (Fig. 2b; Student's *t* test, $t = -3.044$, $P = 0.016$).

Since *Arabidopsis* contains other methylsulfinylalkyl GLS (Table S1), we searched for the corresponding methylthioalkyl GLS in beetles and feces, but detected only traces of 3-methylthiopropyl GLS in beetles.

Question 3: Do P. armoraciae adults excrete GLS selectively? A comparison of the GLS profiles in beetles and feces revealed that two GLS present in beetles, i.e. 3But- and 4MTB GLS, were not excreted (Tables S3, S4). We then analyzed the time-course of GLS excretion in wild type-fed adults by comparing the excreted amounts of allyl GLS with those of ingested 4MSOB GLS and I3M GLS on each day (Fig. 2c). We observed divergent excretion patterns for all three GLS, and found that the excreted amounts of each GLS depended on the day and the GLS type (linear mixed effects model, day: likelihood ratio = 97.331, $P < 0.001$; GLS: likelihood ratio = 80.494, $P < 0.001$; day × GLS: likelihood ratio = 44.642, $P < 0.001$). During the first two days of feeding, adults excreted primarily allyl GLS. The amounts of excreted 4MSOB GLS and I3M GLS increased over time and during the last two days, adults excreted significantly more 4MSOB GLS than allyl GLS.

Question 4: Does the metabolic fate of ingested GLS in P. armoraciae depend on GLS type, total ingested GLS amount, and GLS composition in the food plant? To analyze the metabolic fate of ingested GLS, we quantified how much of the total ingested aliphatic and indolic GLS were accumulated and excreted, respectively. In general, we found that adults accumulated and excreted significantly higher percentages of ingested aliphatic than indolic GLS (Fig. 3a, b, e, f, Table 2). However, *P. armoraciae* accumulated higher proportions of ingested GLS from mutants than from wild type plants (Fig. 3c, d, Table 2). This higher accumulation of ingested GLS from mutant leaves was due to the lower total ingested GLS amount, and not due to the different GLS compositions in mutant and wild type leaves (Fig. 3c, d, Table 2). In addition, the total ingested GLS amount negatively affected the accumulation rate of GLS in beetles (Fig. 3a–c, Table 2), as well as the excreted GLS proportion in wild type-fed adults (Fig. 3e–g, Table 2). However, the GLS composition, i.e. the presence of both GLS types, or only one GLS type in the food plant, affected the proportion of excreted GLS, but not the proportion of accumulated GLS (Fig. 3c, d, g, h; Table 2).

Specifically, adults excreted significantly higher proportions of ingested GLS from wild type than from mutant leaves (Fig. 3g, h).

In total, we recovered 35% of the ingested aliphatic GLS and 19% of the indolic GLS ingested from *Arabidopsis* wild type leaves, 40% of the aliphatic GLS ingested from *cyp* leaves, and 31% of the indolic GLS ingested from *myb* leaves (Table 3). The metabolic fate of the remaining ingested GLS is unknown.

Discussion

In this study, we analyzed the metabolic fate of both ingested and previously sequestered GLS in adult *P. armoraciae*. Our first major finding was that GLS sequestration in *P. armoraciae* saturates at levels of about 35 nmol GLS per mg beetle. To balance the accumulation of new aliphatic and indolic GLS, previously sequestered allyl GLS is lost (Table S3). This finding differs from previous results with *P. striolata* that showed that adults lost about 30% of their total sequestered GLS after feeding on *myb* plants for 18 days, whereas the total GLS levels increased three-fold when adults were shifted from *Arabidopsis myb* plants to *B. juncea* as food source (Beran et al. 2014). These findings suggest differences in the regulation of the total GLS levels in *P. armoraciae* and *P. striolata*, a topic that could be explored in comparative feeding studies with both *Phyllotreta* species.

One mechanism used by *P. armoraciae* to regulate its endogenous GLS levels is the excretion of sequestered GLS. A similar regulatory mechanism was observed in cabbage aphids, where decreasing GLS levels in juveniles developing into winged adult aphids were associated with the excretion of GLS (Kazana et al. 2007). The excretion of intact GLS implies that there is no plant myrosinase activity in the feces. This finding is remarkable given that ingested plant myrosinase enzyme was found to be highly resistant against digestive proteolysis in larvae of the generalist African cotton leafworm, *Spodoptera littoralis* (Boisduval) (Noctuidae) (Vassão et al. 2018). It will be interesting to elucidate whether plant defense proteins are digested more efficiently in the specialist *P. armoraciae* beetles than in the generalist *S. littoralis* larvae, or whether sequestering beetles inhibit plant myrosinase activity by other mechanisms.

P. armoraciae accumulated almost all types of GLS present in *Arabidopsis* wild type leaves, but some GLS were accumulated more than others. For example, *P. armoraciae* sequestered significantly more 8-methylsulfinyloctyl GLS than 7-methylsulfinylheptyl GLS (Fig. 2a). The different GLS accumulation efficiencies could be the result of selective GLS uptake from the gut and/or different rates of GLS metabolism and excretion. Similar to our previous study with *P. striolata*, we detected larger amounts of 4MTB GLS than could be accounted for by dietary intake in *P. armoraciae* (Beran et al. 2014). Here we show that adults metabolize 4MSOB GLS to 4MTB GLS and, in addition, to minor amounts of 3But GLS (Fig. 2b). In contrast, we found no evidence for a conversion of long-chain methylsulfinylalkyl GLS in *P. armoraciae*. This metabolism of 4MSOB GLS to 4MTB GLS and 3But GLS does not seem to be common for GLS-sequestering species since we did not observe the formation of 4MTB GLS and 3But GLS in the cabbage stem flea beetle *Psylliodes chrysocephala* (L.) (Chrysomelidae) (Fig. S3). A chemical reduction of methylsulfinylalkyl GLS, isothiocyanates, and nitriles to the corresponding methylthioalkyl metabolites was previously observed in several bacterial strains, but the functional significance of these metabolic conversions in bacteria and *P. armoraciae* remains unknown (Luang-In et al. 2014; Narbad and Rossiter 2018).

In addition to selective GLS accumulation and metabolism, we also observed selective excretion of GLS in *P. armoraciae*. Although 4MTB GLS is more abundant in adults than 4MSOB GLS, no 4MTB GLS was detected in feces, suggesting metabolic conversion of the ingested GLS. However, we cannot exclude that 4MTB GLS was further metabolized or decomposed after excretion and was therefore not detected. Furthermore, after feeding on wild type *Arabidopsis* for several days, adults selectively excreted the more recently ingested 4MSOB GLS rather than the previously stored allyl GLS (Fig. 2c). The excretion of previously sequestered GLS is likely mediated by the Malpighian tubules, the major organ responsible for the excretion of xenobiotics and plant toxins from the insect hemolymph (Dermauw and Van Leeuwen 2014; Maddrell and Gardiner 1976; Ruiz-Sanchez and O'Donnell 2015). GLS excretion may either be an active process as demonstrated for nicotine in the tobacco hornworm, *Manduca sexta* (L.) (Sphingidae), or may occur passively as observed for the cardiac glycoside ouabain in two polyphagous orthopterans (migratory locust *Locusta migratoria* (L.) (Acrididae) and variegated grasshopper *Zonocerus variegatus* (L.) (Pyrgomorphidae)) and the ouabain-sequestering milkweed bug, *Oncopeltus fasciatus* (Dallas) (Lygaeidae) (Gaertner et al. 1998; Meredith et al. 1984; Rafaeli-Bernstein and Mordue 1979; Rafaeli-Bernstein and Mordue 1978). Whether ingested GLS detected in the feces had previously been taken up into the body or had simply passed through the digestive system cannot be determined from the current study.

The metabolic fate of ingested GLS in *P. armoraciae* was influenced by several factors, i.e. GLS type, the total amount of ingested GLS (GLS level in the food plant), and GLS composition in the food plant (Fig. 3). In general, we recovered significantly less ingested indolic than aliphatic GLS, which indicates that indolic GLS were metabolized at a higher rate than aliphatic GLS in beetles or are sequestered in some unrecoverable form. GLS accumulation and excretion together accounted for the

metabolic fate of up to 41% and 31% of the total ingested aliphatic and indolic GLS from *Arabidopsis*, respectively. Independent of the GLS type, the total ingested GLS amount negatively influenced the GLS recovery. In other words, when *P. armoraciae* ingested higher levels of GLS, the proportion of accumulated and excreted GLS was lower (Fig. 3). One possible explanation for this result could be a limited capacity to stabilize ingested GLS at higher concentrations. The GLS composition in the food plant represents the third factor that influenced the metabolic fate of GLS in *P. armoraciae* because beetles excreted a higher proportion of ingested GLS from *Arabidopsis* wild type than from *myb* and *cyp* mutants, respectively. Our findings suggest that the concomitant ingestion of aliphatic and indolic GLS promotes the excretion of both GLS types by an unknown mechanism. However, the metabolic fate of more than 50% of the total ingested GLS remained unexplained in our study. To elucidate the reasons for this low recovery, in future work we will test the following hypotheses: i) the plant myrosinase hydrolyzes most ingested GLS during feeding and digestion, and ii) the beetle myrosinase hydrolyses sequestered GLS in *P. armoraciae*. Alternatively, GLS may also be metabolized by other pathways. There is still much to be learned about the fate of GLS in this sequestering insect.

In conclusion, we show that GLS variability in Brassicaceae influences the composition but not the level of sequestered GLS in *P. armoraciae* beetles. Our study revealed that *P. armoraciae* developed mechanisms to maintain stable GLS levels in their bodies by balancing uptake and excretion. The ecological consequences of different GLS accumulation patterns in beetles, in particular the effects on natural enemies or on intraspecific communication, remain to be determined in future studies.

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Authors' Contribution Z.-L.Y. and F.B. designed experiments, Z.-L.Y., T.S., and J.K. performed experiments, Z.-L.Y., G.K., T.S., J.K., and F.B. analyzed data, Z.-L.Y. and F.B. wrote the manuscript.

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3.1.Manuscript II

How a glucosinolate-sequestering flea beetle copes with plant myrosinase

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Abstract

Glucosinolates, the characteristic secondary metabolites of Brassicales, are hydrolyzed upon herbivory by myrosinase enzymes to form toxic and deterrent defense metabolites. The horseradish flea beetle, *Phyllotreta armoraciae*, is a specialist herbivore that sequesters glucosinolates despite myrosinase activity in its food plants, but how plant myrosinase activity influences sequestration and how beetles prevent glucosinolate hydrolysis during feeding and digestion is unknown. In feeding experiments performed with the myrosinase-deficient *Arabidopsis thaliana tgg1×tgg2* (*tgg*) mutant and the corresponding wild type, we observed a negative influence of plant myrosinase activity on glucosinolate sequestration. Consistently, we detected much higher levels of glucosinolate hydrolysis products in the body and feces of wild type-fed beetles compared to *tgg*-fed beetles. We assessed possible effects of exposure to glucosinolates hydrolysis products on beetles and found that wild type-fed beetles had a higher weight gain over a short period. However, there was no long-term effect on beetle performance. Since wild type-fed beetles consumed more plant material, these results suggest that beetles may compensate metabolic costs caused by glucosinolate hydrolysis with an increased food intake. Although *P. armoraciae* cannot fully prevent glucosinolate hydrolysis, beetles still sequester a major fraction of ingested glucosinolates. A short-term feeding experiment revealed that ingested glucosinolates are rapidly absorbed from the gut lumen – a strategy that might prevent glucosinolate hydrolysis. Moreover, gut content extracts of *P. armoraciae* suppressed myrosinase activity by up to 53% in *in vitro* assays, and, furthermore, almost no myrosinase activity was detected in the beetle feces. In summary, our results indicate that *P. armoraciae* is adapted to tolerate glucosinolate hydrolysis and uses several strategies to avoid myrosinase activity.

Key words

Plant-Insect Interaction, Plant Defense, Specialist Herbivore, Glucosinolate, Myrosinase, Adaptation, Sequestration, Excretion

Introduction

Many plants deter herbivores with a chemical defense that is activated upon tissue damage (Morant et al., 2008). The chemical defense compound is usually stored as an inactive glucose conjugate in the vacuole of plant cells. When plant tissue is damaged, the glucose moiety is hydrolyzed by a defensive β-glucosidase, originally localized separately from the glucose conjugate, which liberates toxic and deterrent compounds (Morant et al., 2008; Pentzold et al., 2014b). Herbivorous insects differ greatly in the degree of feeding damage they cause. Thus, activated defenses are usually more efficient in deterring chewing herbivores that cause extensive tissue damage than in deterring insects with less invasive feeding modes, such as sap suckers (Pentzold et al., 2014b).

Although activated chemical defenses protect plants from many herbivores, a number of insects evolved resistance against this plant defense strategy (Pentzold et al., 2014b). Some insects are even able to accumulate (sequester) plant glucosides in their body and deploy them for defense against predators (Kazana et al., 2007; Opitz and Müller, 2009; Beran et al., 2019). However, it is currently not well understood how in particular chewing insects sequester plant glucosides despite the presence of plant β-glucosidases. A study with turnip sawfly larvae, *Athalia rosae*, suggests that a rapid absorption of ingested glucosides (glucosinolates) across the gut epithelium prevents their hydrolysis in the gut; this is possibly facilitated by low plant β-glucosidase activity in the anterior gut (Abdalsamee et al., 2014). A rapid uptake mechanism was also proposed to enable western corn rootworm larvae, *Diabrotica virgifera virgifera* to sequester benzoxazinoid glucosides; however, there was no evidence for reduced β-glucosidase activity in the larval gut (Robert et al., 2017). The larvae of the cyanogenic glucoside-sequestering burnet moth, *Zygaena filipendulae*, avoid extensive plant β-glucosidase activity by a leaf-snipping feeding mode causing only minor tissue damage. Moreover, the common alkaline pH in the midgut lumen of lepidopteran larvae also inhibits plant β-glucosidase activity, whereas salivary extracts of burnet moth larvae did not prevent cyanogenic glucoside breakdown (Pentzold et al., 2014a).

Sequestering herbivores thus must avoid the hydrolysis of the ingested plant glucosides they wish to sequester, but the extent to which plant β-glucosidase activity influences glucoside sequestration has rarely been assessed. The brassicaceous model plant *Arabidopsis thaliana* offers an ideal system to address this question. The activated defense in *Arabidopsis* and other plants of the order Brassicales is the glucosinolate-myrosinase system (Halkier and Gershenzon, 2006; Blažević et

al., 2020). Glucosinolates are a structurally diverse group of amino acid-derived thioglucosides that are hydrolyzed by β-thioglucosidases called myrosinases. The resulting aglucone is unstable and rearranges spontaneously into a highly reactive isothiocyanate, which is toxic for small herbivores (Jeschke et al., 2016a). The *Arabidopsis tgg1×tgg2* double knock-out mutant mutant (*tgg*) is devoid of myrosinase activity in leaves (Barth and Jander, 2006). Therefore, the myrosinase-deficient *tgg* mutant and the corresponding *Arabidopsis* Col-0 wild type can be used for comparative studies in order to investigate the impact of plant myrosinase activity on glucosinolate sequestration. For example, in feeding experiments performed with the cabbage stem flea beetle, *Psylliodes chrysocephala*, adult beetles sequestered six times more glucosinolates from the myrosinase-deficient *Arabidopsis tgg* mutant than from the wild type (Beran et al., 2018). These results suggested that a major proportion of ingested glucosinolates was hydrolysed in *Arabidopsis* wild type-fed beetles. In a similar feeding experiment performed with larvae of the horseradish flea beetle, *Phyllotreta armoraciae*, only traces of sequestered glucosinolates were detected in wild type-fed larvae, whereas comparatively high glucosinlate levels were found in *tgg*-fed larvae (Sporer et al., 2020). In contrast to *P. armoraciae* larvae, adult beetles were able to sequester glucosinolates from *Arabidopsis* wild type leaves (Yang et al., 2020), suggesting that plant myrosinase activity has a stronger influence on glucosinolate sequestration in larvae compared to adults. Plant myrosinase activity influences not only sequestration, but also the feeding behavior of *Phyllotreta* flea beetles. The crucifer flea beetle, *Phyllotreta cruciferae*, caused less feeding damage on *Brassica rapa* plants selected for increased myrosinase activity than on plants selected for lower myrosinase activity under field conditions (Siemens and Mitchell-Olds, 1996).

Here, we investigated the influence of plant myrosinase activity on glucosinolate sequestration in the adult life stage of *P. armoraciae*. In a previous study, about 35% of the ingested glucosinolates from *Arabidopsis* wild type leaves were recovered intact from the body and feces of adults, whereas the metabolic fate of more than 60% of the total ingested glucosinolates remained unknown (Yang et al., 2020). One possible explanation is that the unrecovered glucosinolates were hydrolyzed by plant myrosinases during feeding and digestion. To investigate this possibility, we performed a series of comparative feeding experiments with myrosinase-deficient and wild type *Arabidopsis* plants. In nature, *P. armoraciae* is closely associated with horseradish, *Armoracia rusticana*, a plant species that is characterized by high levels of allyl glucosinolate (Li

and Kushad, 2004; Ciska et al., 2017). Therefore, we additionally investigated the influence of plant myrosinase activity on the sequestration of allyl glucosinolate by spiking the intact glucosinolate into *Arabidopsis* leaves. Our results revealed a negative influence of plant myrosinase activity on glucosinolate sequestration. Additionally, the detection of glucosinolate hydrolysis products in beetles and feces confirmed that a fraction of ingested glucosinolates is hydrolysed in *P. armoraciae*. We thus asked whether the exposure of *P. armoraciae* to toxic glucosinolate hydrolysis products is associated with metabolic costs. Further, we explored possible mechanisms that allow *P. armoraciae* to prevent glucosinolate hydrolysis during feeding and digestion, since a major proportion of ingested glucosinolates nevertheless remained intact.

Materials and Methods

Plants and insects

Food plants, *Brassica juncea* cv. "Bau Sin" and *Brassica rapa* cv. "Yu-Tsai-Sum" (Known-You Seed Co., Ltd., Taiwan) were cultivated in a controlled environment chamber (24°C, 55% relative humidity, 14-h light/10-h dark period). *Arabidopsis thaliana* plants were cultivated under short day conditions in a controlled environment chamber (21°C, 55% relative humidity, 10-h light/14-h dark period). The following genotypes were used: *A. thaliana* Col-0 (wild type), the myrosinase deficient *A. thaliana tgg1*×*tgg2* (*tgg*) double knockout mutant (Barth and Jander, 2006), and the *A. thaliana myb28*×*myb29* (*myb*) double knockout mutant which does not produce aliphatic glucosinolates (Sønderby et al., 2007).

Phyllotreta armoraciae was reared on potted three- to four-week old *B. juncea* or *B. rapa* plants in a controlled environment chamber (24°C, 60% relative humidity, 14-h light/10-h dark period). Adult beetles were provided with new plants every week and plants with eggs were kept separately for larval development. After three weeks, any remaining plant material was removed and the soil containing pupae was kept in plastic containers (9 L volume, Lock&Lock). Newly emerged adults were collected every two to three days. Unless stated otherwise, experiments were performed with newly emerged beetles that had been reared on *B. juncea* plants.

Sequestration experiments

To analyze whether plant myrosinase activity influences the sequestration of glucosinolates in *P. armoraciae* beetles, we performed sequestration experiments with the myrosinase-deficient *Arabidopsis tgg* mutant and the corresponding wild type Col-0.

In *Experiment 1*, we fed newly emerged beetles for one day with detached leaves of *Arabidopsis* wild type or *tgg* plants (n = 28 per genotype, two beetles per replicate). On the next day, the remaining leaves were weighed, frozen in liquid nitrogen, and stored at -20°C until they were freeze dried. To allow for metabolism of ingested aliphatic glucosinolates, we fed the beetles one additional day on *Arabidopsis myb* leaves before beetles were weighed, frozen in liquid nitrogen and stored at -20°C until extraction. Leaf and beetle samples were extracted, glucosinolates converted to desulfo-glucosinolates, and analyzed by high performance liquid chromatography coupled with diode array detection (HPLC-DAD) as described in Beran et al. (2014). Adult *P. armoraciae* beetles convert ingested 4-methylsulfinylbutyl (4MSOB) glucosinolate, the major aliphatic glucosinolate in *Arabidopsis* wild type and *tgg* plants, into 4-methylthiobutyl (4MTB) glucosinolate (Yang et al., 2020). Therefore, we summed up the concentrations of 4MSOB and 4MTB glucosinolate in each beetle sample and expressed this concentration relative to that of both glucosinolates in the corresponding leaf sample, which was set to 100%. To confirm that beetles feed equally on both *Arabidopsis* lines, we quantified the beetle feeding damage (2 beetles per leaf disc with 16 mm diameter) over one day using the software Fiji (Schindelin et al., 2012) (n = 8 per genotype).

In *Experiment 2*, we fed newly emerged beetles with *Arabidopsis* wild type, *tgg*, or *myb* leaves leaves for one day (n = 5, with 5 beetles per replicate). On the next day, feces were collected in 50 µL ultrapure water containing 0.1% (v/v) formic acid and mixed with 50 µL pure methanol and stored at -20 °C. Beetles were frozen in liquid nitrogen and stored at -20 °C until extraction. Remaining leaves were weighed, frozen in liquid nitrogen and freeze-dried. Feces samples were homogenized for 2 min at 25 Hz in a TissueLyzerII (Qiagen) using metal beads and beetles were homogenized in 500 µL 50% (v/v) methanol using plastic pestles. Freeze-dried leaves were homogenized to powder as described for feces samples and extracted with 800 µL 50% methanol. Samples were centrifuged at 4 C for 10 min at 16,000 × g and supernatants were stored at -20 °C until analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Identification and quantification of 4MSOB glucosinolate and 4MSOB glucosinolate-derived metabolites in samples was performed as described in Beran et al. (2018). After quantification, we subtracted the detected average amounts of each metabolite in *myb*-fed control beetles, which did not ingest 4MSOB glucosinolate, from those detected in wild type- or *tgg*-fed beetles.

In *Experiment 3*, we fed newly emerged beetles for one day with *Arabidopsis* wild type or *tgg* leaves that were each spiked with 300 nmol allyl glucosinolate (Carl Roth) as described in Schramm et al. (2012). This experiment was performed with adult beetles that had been reared on *B. rapa* plants, which do not produce allyl glucosinolate (Beran et al., 2018). The glucosinolate profile of unfed beetles reared on *B. rapa* was analyzed as described in Beran et al. (2014) (n = 20, with 5 beetles per replicate) to confirm that no allyl glucosinolate is present in beetles. To prevent wilting, we placed leaf petioles in reaction tubes filled with water. Newly emerged beetles were fed with spiked *Arabidopsis* leaves for one day (n = 10 per genotype, 5 beetles per replicate). Allyl glucosinolate-spiked leaves without beetles kept under the same conditions served as a recovery control (n = 8-10 per genotype). All leaf samples were frozen in liquid nitrogen, freeze-dried, and homogenized with metal beads (2.4 mm diameter, Askubal) for 2 min at 25 Hz in a TissueLyzerII (Qiagen). Beetles were weighed and frozen in liquid nitrogen. Feces were collected in 100 µL ultrapure water and mixed with 100 µL pure methanol. Leaf and beetle samples were homogenized in 1 mL and 800 µL 50% (v/v) methanol, respectively. After centrifugation for 10 min at 16,000 × g, supernatants were collected. Feces samples were homogenized as described for leaves, centrifuged at 4 C or 10 min at 16,000 × g, the supernatant collected, solvents evaporated using nitrogen and extracts were re-dissolved in 80 µL 50% methanol. Samples were stored at -20°C until LC-MS/MS as described in Malka et al. (2016) using a modified elution gradient. The gradient consisted of ultrapure water (solvent A) and acetonitrile (solvent B) and was carried out as follows: 1.5% (v/v) B (1 min), 1.5–5% (v/v) B (5 min), 5–7% (v/v) B (2 min), 7–12.6% (v/v) B (4 min), 12.6–100% (v/v) B (0.1 min), 100% (v/v) B (0.9 min), 100 to 1.5% (v/v) B (0.1 min), and 1.5% (v/v) B (3.85 min). Allyl glucosinolate was quantified using an external calibration curve. We recovered 97.8 ± 6.5% (mean ± SD) of the spiked glucosinolate from undamaged (control) wild type leaves and 109.5 ± 4.4% of the spiked allyl glucosinolate from undamaged (control) *tgg* leaves, showing that only small amounts of allyl glucosinolate were degraded by myrosinases in *Arabidopsis* leaves under our assay conditions. To determine how much allyl glucosinolate beetles had ingested, we subtracted the allyl glucosinolate amount detected in each fed leaf from the

average allyl glucosinolate amounts recovered from unfed control leaves of the corresponding genotype. The amounts of allyl glucosinolate that were recovered in beetles and feces were expressed relative to the total ingested amount, which was set to 100%.

In *Experiment 4*, we fed newly emerged beetles with allyl glucosinolate-spiked *Arabidopsis* wild type or *tgg* leaves (prepared as described in *Experiment 3*) for one day and simultaneously collected the headspace on Porapak-Q™ volatile collection traps (25 mg; ARS, Inc.) (n = 6–7 per genotype, 8 beetles per replicate). Leaves without beetles served as controls (n = 4 per genotype). The volatile collection and sample analysis by gas chromatography mass spectrometry (GC-MS) was performed as previously described in Sporer et al. (2020). Allyl isothiocyanate (AITC) was quantified in headspace samples using an external calibration curve prepared from an authentic standard (Sigma-Aldrich). The glucosinolate amount per fed beetle was determined as described in *Experiment 1*.

Performance experiment

Since our results suggested that a fraction of ingested glucosinolates is hydrolyzed to toxic isothiocyanates in *P. armoraciae*, we compared the weight gain, feeding amount, and the total levels of soluble protein, lipids, glycogen, and soluble carbohydrates in beetles that had fed for ten days on *Arabidopsis* wild type or *tgg* leaf discs. We separated newly emerged beetles into males and females and assigned them randomly to one of the two *Arabidopsis* genotypes (n = 10 females per genotype, n = 8-9 males per genotype). Each beetle was provided with a new leaf disc (16 mm diameter) excised from an undamaged *Arabidopsis* plant every day for 10 consecutive days. Fed leaf discs were photographed to determine the fed leaf area using the software Fiji. Each beetle was weighed before feeding and on day 4, 7 and 10. After ten days feeding, beetles were frozen in liquid nitrogen and stored at -20°C until analysis of energy reserves. The contents of soluble protein, total lipids, glycogen and soluble carbohydrates in individual beetles were determined as described in Foray et al. (2012) with minor modifications. Instead of a 96-well borosilicate microplate, we used a 96-well quartz glass microplate (Hellma Analytics) that was covered with MicroAmp clear adhesive film (Applied Biosystems). The plate was heated using a ThermoMixer (Eppendorf) and for measurements we used a Tecan Infinite 200 Reader (Tecan). As a control, we quantified the levels of soluble protein, amino acids, and sugars in rosette leaves of *Arabidopsis* wild type and *tgg* mutant plants (described in Supplementary Methods).

Short-term feeding experiment

In a short-term feeding experiment, we allowed newly emerged beetles to feed for 1 min on *Arabidopsis* leaves with (Col-0) and without (*tgg*) myrosinase activity. After 5 min, beetles were dissected into gut and remaining body (without head; n = 3, 3 beetles per replicate). Non-fed beetles were used as background control (n = 2-3, 3 beetles per replicate). Dissected guts were washed twice in phosphate-buffered saline (PBS) pH 7.4 (Bio-Rad) before sampling. Samples were homogenized in 500 µL 80% methanol containing 0.4 µM 4-hydroxybenzyl glucosinolate as internal standard using plastic pestles and stored at -20°C until extraction and analysis by LC-MS/MS as described above in *Experiment 2*. We quantified 4MSOB glucosinolate in each sample using an external standard curve and expressed the glucosinolate distribution in the gut and rest of the body relative to the total amount detected in both samples.

Myrosinase inhibition assays

To determine whether *P. armoraciae* can inhibit ingested myrosinase activity, we performed myrosinase activity assays with gut content extracts of *P. armoraciae*. The gut content of adults was collected as follows: dissected guts were washed in 20 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 5.2 containing protease inhibitors (cOmplete, EDTA-free), opened longitudinally using a microscissors, and the gut content was collected in 2.5 µL of the same buffer. For each sample, we pooled gut contents from 20 beetles, froze the samples in liquid nitrogen and stored them at -20°C until extraction (n = 4). Samples were homogenized with metal beads for 2 min at 25 Hz in a TissueLyzer II, centrifuged at 4°C for 10 min at 16,000 × g and the supernatant split into two subsamples of which one was boiled for 5 min at 99°C. Myrosinase from *S. alba* (Sigma-Aldrich) was partially purified before use as described in the Supplementary Methods.

Assays (50 µL total volume) consisted of 0.1 mM ascorbic acid (Fluka, Buchs, Switzerland), 0.2 mM 4MSOB glucosinolate substrate, 0.5 ng/µL myrosinase and a) gut content extract (corresponding to four beetles), b) boiled gut content extract, or c) pure 20 mM MES buffer, pH 5.2 as a control. Assays without myrosinase and without gut content extracts served as background controls. Assays were incubated for 15 min at 30°C, the reaction was stopped by 5 min boiling at 99 °C and extracted with 100 µL 80% methanol containing 4-hydroxybenzyl glucosinolate as an internal standard. Myrosinase activity was determined by quantifying the 4MSOB glucosinolate

235 substrate remaining in each assay after conversion to desulfo-glucosinolate and analysis by HPLC-
236 DAD as described in *Experiment 1*.

237 **Detection of myrosinase enzyme and activity in beetle feces**

238 To determine whether *P. armoraciae* can degrade ingested plant myrosinase enzymes, we
239 analyzed the fecal proteome. We collected feces of 90 adults that had fed on 24 *Arabidopsis* wild
240 type leaves for one day in a total volume of 1 mL 20 mM MES buffer pH 6.5 containing protease
241 inhibitors (cOmplete, EDTA-free, Roche). After homogenization with metal beads for 3 min at
242 25 Hz in a TissueLyzer II and centrifugation at 4°C for 10 min at 16,000 × g, we precipitated
243 soluble proteins in the supernatant with trichloroacetic acid and washed the pellet with acetone.
244 The protein pellet was dissolved in Laemmli buffer (BioRad), boiled for 15 min at 95°C, and
245 separated on a 12.5% Criterion Tris-HCl precast gel (Bio-Rad). Protein bands were stained with
246 colloidal Coomassie G250 (Carl Roth), excised from the gel, and digested with porcine trypsin
247 (Promega) as described in Shevchenko et al. (2006). Samples were re-dissolved in 30 µL 1% (v/v)
248 formic acid and 2 µL were analyzed by nano-UPLC-MS^E analysis as described in Vassão et al.
249 (2018). Data were acquired using data-independent acquisition (DIA), referred to as enhanced
250 MS^E. MS data were collected using MassLynx v4.1 software (Waters).

251 The processing of nano-UPLC-MS^E data and protein identification was performed as follows: the
252 acquired continuum of LC-MS^E data were processed using the ProteinLynx Global Server (PLGS)
253 version 2.5.2 (Waters) to generate product ion spectra for database searching according to the ion
254 accounting algorithm described in Li et al. (2009). Processed data were searched against a
255 reference sequence (Refseq) database containing *Arabidopsis thaliana* sequences (40785
256 sequences, downloaded from the Identical Protein Groups database at the National Center for
257 Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/refseq/ on February 28, 2020)
258 combined with a subdatabase containing common contaminants (O'Leary et al., 2016). Database
259 searching was performed at a false discovery rate (FDR) of 2% with the following parameters:
260 minimum numbers of fragments per peptide (3), peptides per protein (1), fragments per protein
261 (7), and maximum number of missed tryptic cleavage sites (1). Searches were restricted to tryptic
262 peptides with a fixed carbamidomethylation of cysteine residues along with variable oxidation of
263 methionine. Proteins were classified according to the algorithm described for PAnalyzer software

(Prieto et al., 2012) and divided into four groups: conclusive, indistinguishable, ambiguous, and non-conclusive. Conclusive and indistinguishable hits were considered as confident matches.

To determine whether *P. armoraciae* excretes active myrosinase enzyme, we analyzed myrosinase activity in feces homogenates and compared this activity with the corresponding ingested myrosinase activity in leaves. We used newly emerged *P. armoraciae* beetles that had been reared on *B. rapa*, and fed them for one day with *Arabidopsis myb* leaves containing myrosinase activity but no 4MSOB glucosinolate (n = 6, with 6 beetles per replicate). Leaves were weighed before and after feeding to determine the ingested plant fresh weight. Leaves were supplied with water during the experiment and the average proportional weight gain of intact leaves was used to correct the initial leaf weight (n = 16). Feces from each replicate were collected in 130 µl extraction buffer (20 mM MES buffer, pH 6.5, containing protease inhibitors (cOmplete, EDTA-free)). Feces and fed leaves were frozen in liquid nitrogen and stored at -80°C until extraction. Feces samples were homogenized with metal beads at 25 Hz for 2 min in a TissueLyzer II. The corresponding frozen leaf samples were homogenized with metal beads at 25 Hz for 2 min in a pre-cooled sample holder to prevent thawing. For each replicate, we calculated the ingested fresh weight and extracted the corresponding amount of homogenized plant tissue in the same buffer volume used for feces extracion (130 µL). After centrifugation at 4°C for 10 min at 16,000 × g, the supernatant was directly used for myrosinase activity assays. Assays (55 µL total volume) consisted of 25 µL extraction buffer, 5 µL of an aqueous 11 mM 4MSOB glucosinolate solution, and a) 25 µL feces homogenate or b) leaf extract. Assays containing 50 µL extraction buffer and substrate served as a control (n = 3). To test whether feces extracts have an inhibitory effect on plant myrosinase activity, we additionally performed assays in which we mixed 25 µL of feces homogenate with 25 µL of corresponding leaf extract and the glucosinolate substrate. Except for three assays with combined feces and leaf extracts, all assays were performed with two technical replicates. Assays were incubated for 30 min at 30°C, stopped by boiling for 5 min at 95°C, and 50 µL of 60% (v/v) methanol were added. After the activity assay, samples containing feces homogenates were centrifuged at 4°C for 10 min at 16,000 × g, the supernatant collected and final samples were stored at -20°C until LC-MS/MS. 4MSOB glucosinolate was quantified by LC-MS/MS as described in *Experiment 2* and myrosinase activity was expressed as nmol 4MSOB hydrolyzed per minute and mg (ingested) plant fresh weight.

294 **Statistical analyses**

295 Statistical analyses were performed in R3.3.1 (R Core Team, 2018) or SigmaPlot 11.0 (Systat
296 Software). Details of statistical analyses performed for each dataset are summarized in
297 Supplementary Table 1.

298 ## RESULTS

299 **Plant myrosinase activity influences glucosinolate sequestration**

300 *Experiment 1*: We examined the influence of plant myrosinase activity on glucosinolate
301 sequestration by feeding *P. armoraciae* adults with myrosinase-deficient (*tgg*) or corresponding
302 wild type *Arabidopsis* leaves, and quantified the levels of 4MSOB and 4MTB glucosinolate in
303 beetles relative to those in the food plant. Beetles that had fed on the myrosinase-deficient mutant
304 accumulated twofold higher levels of glucosinolates than wild type-fed beetles (Mann-Whitney
305 rank sum test, $U = 482.000$, $p < 0.001$; Figure 1A). However, beetle feeding rates and plant
306 glucosinolates levels did not differ between both genotypes (feeding rate: Student's *t*-test,
307 $t = 0.592$, $p = 0.564$; plant glucosinolates level: Mann-Whitney rank sum test, $U = 390.000$,
308 $p = 0.980$. Together, these results demonstrate a negative impact of plant myrosinase activity on
309 the sequestration of 4MSOB glucosinolate in *P. armoraciae*.

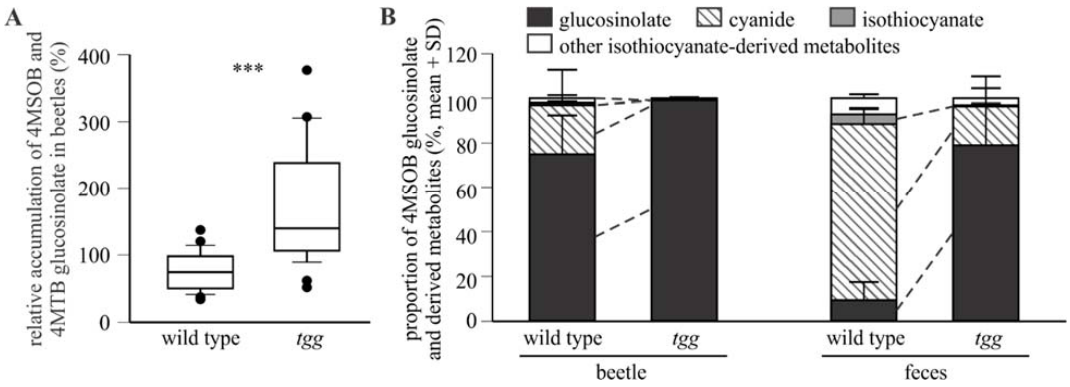

Figure 1: Plant myrosinase activity negatively influences glucosinolate sequestration in *P. armoraciae*. (A) Accumulation of 4-methylsulfinylbutyl (4MSOB) and 4-methylthiobutyl (4MTB) glucosinolate in *P. armoraciae* adults relative to the concentration in fed *Arabidopsis* wild type and myrosinase-deficient *tgg* leaves (n = 28). Glucosinolates were quantified after conversion to desfulo-glucosinolates by HPLC-DAD. The glucosinolate concentration in the plant was set to 100%. *** $p < 0.001$. (B) Proportions of 4MSOB glucosinolate and hydrolysis products detected in the body and feces of adult beetles fed with *Arabidopsis* wild type or myrosinase-deficient *tgg* leaves (n = 5). Glucosinolates and hydrolysis products were extracted with 50% methanol and analyzed by LC-MS/MS. Detected amounts of metabolites were expressed relative to the total amounts of all detected metabolites in beetles or feces (set to 100%). Dashed lines indicate significant differences between samples ($p < 0.05$). Statistical results are shown in Supplementary Table 1. 4MSOB cyanide corresponds to the nitrile hydrolysis product. Other isothiocyanate-derived metabolites comprise 4MSOB isothiocyanate-glutathione conjugate, 4MSOB isothiocyanate-cysteinylglycine conjugate, 4MSOB isothiocyanate-cysteine conjugate, 2-(4-(methylsulfinyl)butylamino)-4,5dihydrothiazole-carboxylic acid, 4MSOB amine, and 4MSOB acetamide.

Experiment 2: Since beetles sequestered less glucosinolates from wild type plants, we investigated whether ingested 4MSOB glucosinolate is hydrolyzed, by analyzing the bodies and feces of beetles that had fed on wild type or *tgg* leaves for the presence of hydrolysis products. Wild type-fed beetles contained significantly higher proportions of the nitrile hydrolysis product 4MSOB cyanide, 4MSOB isothiocyanate and 4MSOB isothiocyanate-derived metabolites, whereas the proportion of 4MSOB glucosinolate was lower compared to *tgg*-fed beetles (Figure 1B, Supplementary Table 1). Beetles that had fed on wild type plants also excreted significantly higher proportions of 4MSOB cyanide and 4MSOB isothiocyanate and correspondingly, a significantly lower proportion of 4MSOB glucosinolate than beetles from the *tgg* treatment (Figure 1B, Supplementary Table 1). Interestingly, in both beetles and feces from the wild type treatment, the detected proportions of 4MSOB cyanide were approximately seven-fold higher than that of 4MSOB isothiocyanate and other isothiocyanate-derived metabolites (Figure 1B). Moreover, despite the lack of ingested plant myrosinase activity in the *tgg*-treatment, beetles excreted

4MSOB cyanide and isothiocyanate (Figure 1B). We also analyzed the distinct metabolite amounts per beetle and found significantly higher amounts of 4MSOB cyanide and significantly lower amounts of other 4MSOB isothiocyanate-derived metabolites in wild type-fed beetles as in *tgg*-fed beetles (Supplementary Table 2). Further, wild type-fed beetles excreted significantly higher amounts of 4MSOB glucosinolate derived hydrolysis products compared to *tgg*-fed beetles (Supplementary Table 2). 4MSOB glucosinolate levels did not differ between wild type and *tgg* plants (Student's *t*-test, $t = 0.371$, $p = 0.720$). Together, these results show that plant myrosinases partially hydrolyzed 4MSOB glucosinolate during beetle feeding.

Experiment 3: We next investigated the impact of plant myrosinase activity on the sequestration of allyl glucosinolate, the major glucosinolate of the natural host plant of *P. armoraciae.*For this experiment, we used adult beetles reared on *B. rapa* were used because they do not contain allyl glucosinolate (Supplementary Table 3). Beetles were fed with wild type or myrosinase-deficient *Arabidopsis* leaves that had been spiked with allyl glucosinolate. *Tgg*-fed beetles again accumulated a significantly higher proportion of ingested allyl glucosinolate than wild type-fed beetles (Table 1). Both groups of beetles excreted less than 1% of the total ingested allyl glucosinolate in feces, and these levels did not differ between treatments (Table 1). Overall, plant myrosinase activity explained the fate of 9% of the ingested allyl glucosinolate, and the metabolic fate of 47% and 56% of the ingested allyl glucosinolate in *tgg*- and wild type-fed beetles remained unknown, respectively (Table 1).

Table 1: Proportions of ingested allyl glucosinolate detected in *P. armoraciae* beetles and feces. Adults were fed on *Arabidopsis* wild type and myrosinase-deficient *tgg* leaves, both spiked with ally glucosinolate. Shown is mean percentage ± SD (n = 10). Significant differences are marked in bold font.

	wild type-fed	*tgg*-fed	*t*	*p*
Beetle	43.8 ± 8.2	53.0 ± 4.8	-2.924	**0.009**
Feces	0.2 ± 0.3	0.3 ± 0.1	-0.655	0.521
Unknown	56.0 ± 8.4	46.7 ± 4.8	-2.883	**0.010**

Unknown, unrecovered proportion of ingested allyl glucosinolate

Experiment 4: To confirm that ingested allyl glucosinolate was hydrolyzed, we quantified the emission of the volatile glucosinolate hydrolysis product allyl isothiocyanate (AITC) during beetle feeding. We detected significantly higher amounts of AITC in the headspace samples of wild-type fed beetles as compared to *tgg*-fed beetles (wild type: 0.35 ± 0.06 nmol; *tgg*: 0.01 ± 0.01 nmol,

mean ± SD, Figure 2, Supplementary Table 1). The accumulated amounts of allyl glucosinolate per beetle did not differ between treatments (wild type: 4.0 ± 1.3 nmol; *tgg*: 5.3 ± 1.3 nmol, mean ± SD, Figure 2, Supplementary Table 1.

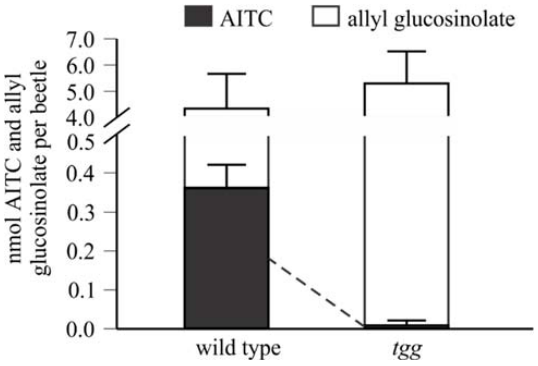

Figure 2: Detection of allyl glucosinolate and allyl isothiocyanate (AITC) in the bodies and headspace of *P. armoraciae* beetles fed with allyl glucosinolate-spiked *Arabidopsis* leaves with (wild type) and without (*tgg*) myrosinase activity. Headspace volatiles were collected for 24 h on Porapaq-Q^{TM} adsorbent, eluted with hexane, and AITC was quantified using gas chromatography-mass spectrometry (*m/z* 99). Detected amounts of AITC were corrected by subtracting the background emission detected in volatile collections performed without beetles, which served as controls. Allyl glucosinolate was quantified after conversion to desuflo-glucosinolates by HPLC-DAD. The dashed line indicates significant differences between samples ($p < 0.001$; n = 6-7). Statistical results are shown in Supplementary Table 1.

Plant myrosinase activity influences beetle weight gain and feeding activity

To determine whether exposure to glucosinolate hydrolysis products is associated with metabolic costs, we analyzed the fresh weight, weight gain and feeding rate of newly emerged beetles fed on *Arabidopsis* leaves with and without myrosinase activity over ten days. In both wild type and *tgg* treatments, beetle fresh weight differed between newly emerged (non-fed) beetles and beetles that had fed on *Arabidopsis* leaf discs for 4, 7 and 10 days (one-way repeated measurements ANOVA; wild type-fed: $F = 61.787$, $p < 0.001$; *tgg*-fed: $F = 42.213$, $p < 0.001$). The fresh weight of fed beetles was significantly higher than the starting weight, but the beetle weight did not differ significantly between day 4, 7 and 10 within the treatments. Relative to the starting weight, wild type-fed beetles gained significantly less weight until day 4 compared to *tgg*-fed beetles despite similar feeding amounts (Figure 3, Supplementary Table 1 and 4). However, the relative weight gain until day 10 did not differ between wild type- and *tgg*-fed beetles, but wild type-fed beetles consumed significantly more plant material than *tgg*-fed beetles (Figure 3B, Supplementary Table

390 4). To reveal metabolic costs of isothiocyanates detoxification in *P. armoraciae* , we compared
391 the levels total soluble protein, soluble carbohydrates, glycogen, and lipids in beetles after 10 days
392 feeding, but found no significant differences between *tgg*- and wild type-fed beetles
393 (Supplementary Table 5). As a control, we analyzed wild type and *tgg* plants for differences in
394 metabolites that could have influenced beetle feeding, performance and nutrition. We compared
395 the levels of soluble proteins, free amino acids, soluble sugars, and, additionally, glucosinolates in
396 rosette leaves in *tgg* and wild type plants, but found no differences in the total levels of these
397 metabolites and only minor differences for a few individual metabolites between both plant
398 genotypes (Supplementary Table 6). Together, these results suggest that plant myrosinase activity
399 negatively influences the efficiency of plant biomass conversion into body mass in *P. armoraciae*,
400 but adults beetles can compensate this effect by a higher feeding rate.

401

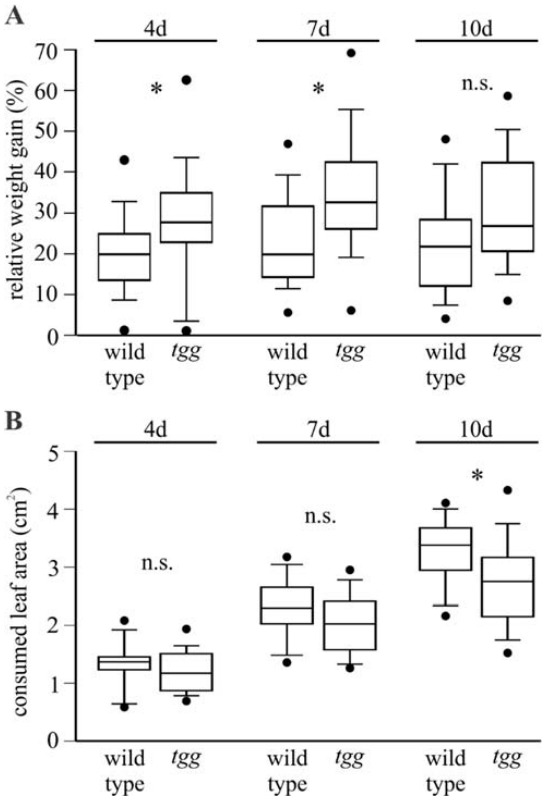

Figure 3: Plant myrosinase activity affects the relative weight gain and feeding rate of *P. armoraciae* beetles. Newly emerged beetles were provided with *Arabidopsis* wild type or myrosinase-deficient *tgg* leaf discs for 10 consecutive days (n = 18-19). (A) The weight gain relative to day 0 after 4, 7 and 10 days and (B) the consumed leaf material within 4, 7 and 10 days were compared between treatments, respectively. Statistical results are shown in Supplementary Table 4. *, $p < 0.05$; n.s., $p > 0.05$

P. armoraciae rapidly sequesters glucosinolates and suppresses plant myrosinase activity in the gut

Although our findings show that *P. armoraciae* cannot prevent glucosinolate hydrolysis completely, a major proportion of ingested glucosinolates remained intact and was sequestered (Figure 1 and 2, Table 1). One possible mechanism to prevent glucosinolate hydrolysis in the gut lumen is a rapid absorption of ingested glucosinolates across the gut epithelium. To investigate whether *P. armoraciae* rapidly absorbs ingested glucosinolates, we allowed beetles to feed for 1 min on *Arabidopsis* leaves with and without myrosinase activity and waited for 5 min to allow for glucosinolate sequestration and metabolism before dissecting beetles into gut and remaining body for chemical analysis. In both *tgg*- and wild type-fed beetles, we detected more than 80% of

the total detected 4MSOB glucosinolate in the remaining body, showing that *P. armoraciae* rapidly absorbs glucosinolates (Figure 4A, Supplementary Table 7). Accordingly, the proportions of glucosinolates recovered from gut samples did not differ between *tgg*- and wild type-fed beetles (Student's *t*-test, $t = 0.075$, $p = 0.944$, Figure 4A).

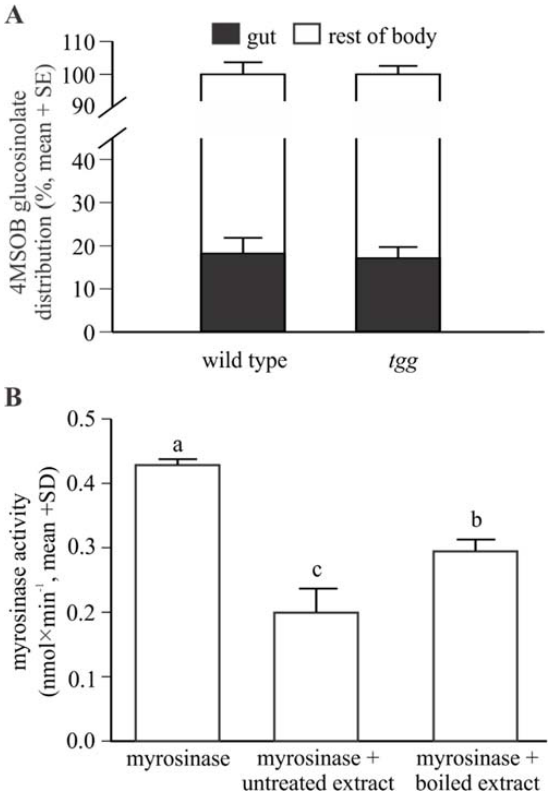

Figure 4: *P. armoraciae* rapidly absorb glucosinolates from the gut lumen into the body and gut content extracts inhibit plant myrosinase activity *in vitro*. (A) Beetles were allowed to feed for 1 min on *Arabidopsis* leaves with (wild type) or without (*tgg*) myrosinase activity and were dissected into gut and rest of body 5 min later (n = 3). Beetles were extracted with 80% methanol and 4MSOB glucosinolate was quantified by LC-MS/MS. The distribution of 4MSOB glucosinolate in gut and rest of body is expressed relative to the total amount detected in both samples (set to 100%). (B) Partially purified *Sinapis alba* myrosinase was affected by the supplementation of untreated and boiled gut content extracts in *in vitro* assays (n = 4). Myrosinase activity was determined by quantifying the 4MSOB glucosinolate substrate in each assay after conversion to desulfo-glucosinolate and analysis by HPLC-DAD. Assays without myrosinase served as background controls and activities were subtracted from the corresponding samples. Different letters indicate significant differences, $p < 0.001$.

To determine whether glucosinolate sequestration is facilitated by suppression of plant myrosinase activity in the gut lumen of *P. armoraciae*, we incubated beetle gut content extracts with partially

436 purified myrosinase enzyme from *S. alba* and 4MSOB glucosinolate. Compared to control assays,
437 gut content extracts significantly reduced myrosinase activity by up to 53% *in vitro* (ANOVA, F
438 = 85.639, $p < 0.001$; Figure 4B). Interestingly, boiled gut content extracts reduced myrosinases
439 activity significantly less than non-boiled gut content extracts.

440 ***P. armoraciae* excretes inactive myrosinase enzyme**

441 To investigate whether *P. armoraciae* is able to digest plant myrosinases, we analyzed the fecal
442 proteome by nano-UPLC-MS^E. We detected up to 14 peptides derived from the *Arabidopsis*
443 myrosinase TGG1 in two protein bands between approximately 55 and 70 kDa (Figure 5A,
444 Supplementary Table 8). The detected peptides covered up to 34% of the TGG1 amino acid
445 sequence (Figure 5B). For comparison, purified TGG1 from *Arabidopsis* leaves possesses an
446 apparent molecular weight of 75 kDa (Zhou et al., 2012), and the predicted unmodified molecular
447 mass of TGG1 is 61.1 kDa. The difference between predicted and apparent molecular weight is
448 due to glycosylation, as TGG1 possesses 9 glycosylation sites. Thus, the molecular weight range
449 in which we detected TGG1 peptides corresponds approximately to that of the intact glycosylated
450 and deglycosylated enzyme. No other members of glucoside hydrolase family 1 from *Arabidopsis*
451 were detected in beetle feces with our approach (Supplementary Table 9).

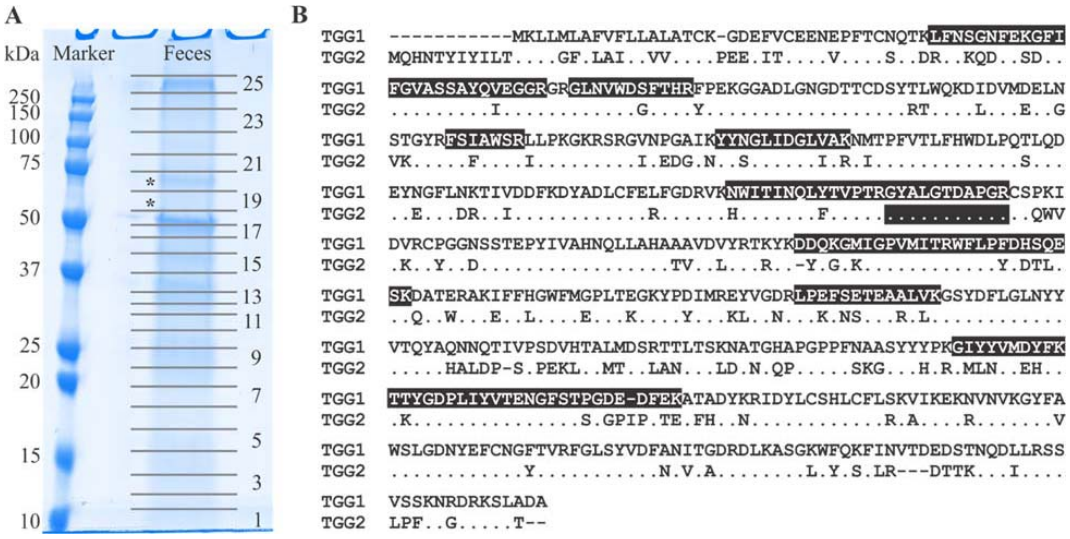

Figure 5: Detection of *Arabidopsis* myrosinase in the feces of *P. armoraciae.* After adult beetles had fed on *Arabidopsis* wild type leaves the feces proteome was analyzed. (A) Polyacrylamide gel electrophoresis (SDS-PAGE) gel of feces extract. Horizontal lines indicate cutting sites for protein bands which were numbered as shown on the right-hand side of the gel (only odd band numbers are shown). Proteins in the bands were digested with trypsin and peptides were analyzed by nano-UPLC-MS^E. In bands marked with an asterisk, TGG1-derived peptides were detected. (B) Amino acid sequence alignment of the *Arabidopsis* myrosinases TGG1 (AT5G26000.1) and TGG2 (AT5G25980.2). The peptides in the TGG1 sequence marked in bold font were detected in beetle feces by nano-UPLC-MS^E, and only one detected peptide matched the TGG2 sequence. Identical amino acids in the TGG2-sequence are represented by a dot.

Next, we compared the levels of myrosinase activity that beetles had ingested with those that were excreted. Myrosinase activity detected in feces corresponded to less than 4% of the ingested activity (Figure 6, $t = 10.449$, $p < 0.005$). We then tested whether beetle feces suppresses plant myrosinase activity by comparing the levels of myrosinase activity in leaf samples that were incubated with and without feces homogenates. However, leaf extracts that were incubated with feces homogenates did not show lower myrosinase activity than control leaf extracts (Figure 6, $t = 0.158$, $p = 1.000$).

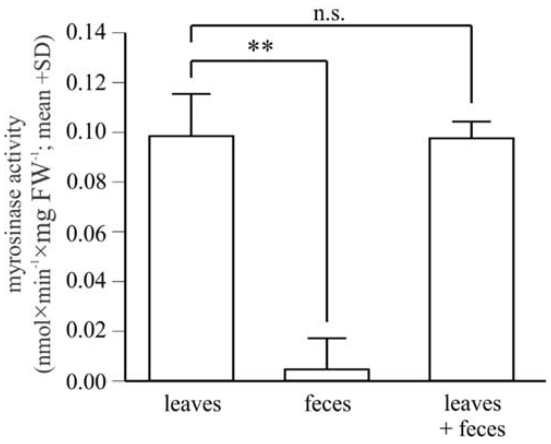

Figure 6: *Arabidopsis* myrosinase activity is strongly reduced after digestion. Enzymatic levels of myrosinase activity in *Arabidopsis myb* leaves were determined in non-ingested leaf material (leaves) and after beetle digestion (feces) of equivalent amounts. Samples containing leaf extracts, feces homogenates or both were spiked with 4MSOB glucosinolate and the reaction were stopped by heat inactivation after 30 min. Myrosinase activity was determined by quantifying the 4MSOB glucosinolate substrate remaining in the assays by LC-MS/MS. Co-incubation of non-ingested myrosinases with *P. armoraciae* feces homogenate did not affect myrosinase activity levels. **, $p < 0.005$; n.s., $p < 0.05$; n = 6. Statistical results are shown in Supplementary Table 1.

DISCUSSION

The enzymatic activity of defensive plant β-glucosidases is a major barrier to the sequestration of plant glucosides by herbivorous insects (Morant et al., 2008; Pentzold et al., 2014b). Here, we investigated the impact of plant myrosinase activity on the sequestration of glucosinolates in *P. armoraciae* and explored possible mechanisms that enable adult beetles to suppress glucosinolate hydrolysis during feeding and digestion. We demonstrate a negative influence of plant myrosinase activity on sequestration and confirmed that ingested glucosinolates are partially hydrolyzed to toxic isothiocyanates. Nevertheless, *P. armoraciae* prevented hydrolysis of a major proportion of ingested glucosinolates and sequestered them in the beetle body. We found two mechanisms that can reduce the glucosinolate hydrolysis rate in the gut: a rapid absorption of ingested glucosinolates across the gut epithelium and the suppression of plant myrosinase activity in the gut lumen.

Compared to previous studies with *P. chrysocephala* adults (Beran et al., 2018; Ahn et al., 2019), plant myrosinase activity had less impact on the sequestration and metabolism of ingested glucosinolates in *P. armoraciae* adults. In quantitative studies, approximately 75% of the total

494 ingested glucosinolates were hydrolyzed in *P. chrysocephala*, whereas only up 10% of ingested
495 allyl glucosinolate were hydrolyzed in *P. armoraciae*. These two specialist flea beetle species
496 belong to different genera that have likely adapted independently from each other to their
497 brassicaceous hosts (Gikonyo et al., 2019). However, *P. chrysocephala* and *P. armoraciae* adults
498 have similar body sizes and feeding modes and therefore cause comparable feeding damage. Our
499 results indicate that *P. armoraciae* adults are better adapted to overcome plant myrosinase activity
500 than *P. chrysocephala* adults.

501 Plant myrosinase activity negatively influenced the sequestration of 4MSOB glucosinolate and
502 allyl glucosinolate in *P. armoraciae* beetles. We detected significantly lower levels of sequestered
503 glucosinolates in wild type-fed compared to *tgg*-fed beetles. Consistently, we found significantly
504 higher levels of glucosinolate hydrolysis products when beetles fed on *Arabidopsis* wild type as in
505 *tgg* leaves, showing that plant myrosinases partially hydrolyze glucosinolates during beetle feeding
506 (Figure 1 and 2, Table 1). Although the sequestration rates of 4MSOB glucosinolate and allyl
507 glucosinolate cannot be compared directly because different methods were used for quantification
508 (Figure 1 and 2, Table 1), our results indicate a stronger influence of plant myrosinase activity on
509 4MSOB glucosinolate than on allyl glucosinolate. Biochemical studies with *Arabidopsis*
510 myrosinases TGG1 and TGG2 revealed similar activities of both enzymes towards these three
511 glucosinolates (Zhou et al., 2012), making it unlikely that the substrate preferences of *Arabidopsis*
512 myrosinases have affected glucosinolate sequestration in *P. armoraciae*. A similar glucosinolate-
513 dependent effect of plant myrosinase activity on sequestration was previously observed in
514 *P. armoraciae* larvae (Sporer et al., 2020). Larvae sequestered allyl glucosinolate from *B. juncea*
515 leaves but almost no glucosinolates from *Arabidopsis* wild type leaves, despite similar levels of
516 soluble myrosinase activity in both plant species. A feeding experiment with larvae and
517 *Arabidopsis* wild type and *tgg* plants, revealed that larvae only contained traces of 4MSOB
518 glucosinolate after feeding on wild type plants, whereas *tgg*-fed larvae accumulated significantly
519 higher 4MSOB glucosinolate levels (Supplementary Material, Supplementary Figure 1). In
520 accordance, higher levels of 4MSOB glucosinolate hydrolysis products were detected in wild-type
521 fed larvae, showing that the ability of *P. armoraciae* larvae and adults to prevent glucosinolate
522 hydrolysis differs. Together, the metabolic fate of ingested allyl glucosinolate in *P. armoraciae*
523 appears to be less affected by plant myrosinase activity than that of *Arabidopsis* glucosinolates.
524 Since allyl glucosinolate represents the dominant glucosinolate in horseradish, the natural host

plant of *P. armoraciae* (Li and Kushad, 2004), this specialist may have developed specific mechanisms to avoid the hydrolysis of allyl glucosinolate.

How chewing insects that cause extensive tissue damage prevent the hydrolysis of ingested plant glucosides is currently not well understood. One proposed mechanism is a rapid absorption of plant glucosides across the gut epithelium that separates substrate and enzyme. In other words, the process of plant glucoside sequestration, more specifically, the evolution of membrane transporters that mediate an efficient and fast glucoside uptake from the gut lumen into the gut epithelium followed by export into the hemolymph could represent an adaptation to overcome the activated host defense (Abdalsamee et al., 2014; Pentzold et al., 2014b; van Geem et al., 2014). In adult *P. armoraciae* beetles, we found 80% of the total detected glucosinolates five minutes after ingestion in the beetle body (without gut), but it is unclear whether this rapid glucosinolate absorption indeed prevents hydrolysis in the gut lumen. Moreover, we had expected to find less glucosinolates in the guts of wild type-fed beetles due to the presence of myrosinase activity, but we recovered similar proportions of glucosinolates in the guts of *tgg*- and wild type-fed beetles. However, we cannot rule out that glucosinolates detected in the gut were spatially separated from plant myrosinases, either in remaining intact plant tissue or in the gut epithelium. Up to now, a rapid absorption of ingested plant glucosides has only been demonstrated in the glucosinolate-sequestering turnip sawfly larvae. In addition, this mechanism has been proposed to enable western corn rootworm larvae to sequester benzoxazinoid glucosides from maize (Abdalsamee et al., 2014; Robert et al., 2017).

Another explanation for the detection of glucosinolates in the gut of *P. armoraciae* is that beetles are able to suppress the activity of ingested plant myrosinase enzymes. An important factor influencing plant enzyme activity is the gut pH, which varies considerably among insect species and additionally within species along the gut (Terra and Ferreira, 2012). For example, the highly alkaline pH of the midgut lumen of burnet moth larvae drastically reduced cyanogenic β-glucosidase activity in *Lotus corniculatus* leaf macerates (Pentzold et al., 2014a). In contrast, the neutral pH of gut homogenates of sequestering turnip sawfly larvae had only minor influence on ingested plant myrosinase activity (Abdalsamee et al., 2014). The pH of crude midgut homogenates of *P. armoraciae* beetles was 4.7 (details are described in the Supplementary Material); thus, the pH conditions in the beetle gut are unlikely to have a strong influence on plant

555 myrosinase activity. Several other factors are known to influence plant myrosinase activity
556 including ascorbic acid (cofactor of myrosinases), sulfate, sodium chloride and silver ions (Shikita
557 et al., 1999; Andersson et al., 2009; Bhat and Vyas, 2019; Marcinkowska and Jelen, 2020). In *in*
558 *vitro* assays, gut content extracts reduced plant myrosinase activity by 30 to 50%, with boiled
559 extracts inhibiting myrosinase activity significantly less than untreated extracts. These results
560 provide direct evidence for suppression of myrosinase activity in the gut of *P. armoraciae,* and,
561 furthermore, indicate that several factors contribute to myrosinase inhibition, of which some are
562 sensitive to heat.

563 Myrosinase from *S. alba* and other defensive β-glucosidases were largely resistant to digestion in
564 the larval gut of the generalist lepidopteran *Spodoptera littoralis* and thus retained most of their
565 activity after digestion (Vassão et al., 2018). Our proteomic analysis of beetle feces also indicates
566 that *Arabidopsis* myrosinase TGG1 resisted digestion in *P. armoraciae*, whereas TGG2 was not
567 detected in feces (Figure 5, Supplementary Table 8). Because *TGG2* expression is restricted to
568 phloem-associated cells (Barth and Jander, 2006), beetles likely ingested much less TGG2 than
569 TGG1 by avoiding the leaf midrib and veins (personal observation). Despite the detection of TGG1
570 enzyme, we found almost no myrosinase activity in feces of *P. armoraciae*. We tested for the
571 presence of myrosinase inhibitor(s) in feces homogenates, but observed no suppression of spiked
572 myrosinase activity under our assay conditions (Figure 6). Thus, we hypothesize that ingested
573 TGG1 has been inactivated during gut passage in *P. armoraciae*. Previous studies with the turnip
574 sawfly and the diamondback moth also indicated that plant myrosinases are not fully active in the
575 gut (Abdalsamee et al., 2014; Sun et al., 2019) sun). However, the underlying mechanism(s) of
576 myrosinase inhibition in the gut of specialist herbivores including *P. armoraciae* remain to be
577 determined.

578 The metabolic fate of about 50% of the total ingested allyl glucosinolate in our quantitative feeding
579 experiment remained unknown (Table 1). Moreover, adult beetles excreted 4MSOB glucosinolate-
580 derived hydrolysis products during feeding on the myrosinase-deficient *tgg* mutant (Figure 1B).
581 Since *P. armoraciae* larvae only sequestered glucosinolates from *Arabidopsis tgg* but not from
582 wild type plants (Sporer et al., 2020), we determined a similar feeding experiment for larvae (see
583 Supplementary Material). Also wild type-fed *P. armoraciae* larvae contained and excreted
584 4MSOB glucosinolate hydrolysis products (Supplementary Material, Supplementary Figure 1,

585 Supplementary Table 10). The levels of the hydrolysis products appear to be even higher in *tgg*-
586 fed larvae than in adults (Supplementary Figure 1, Figure 1B). These results suggest that ingested
587 glucosinolates are metabolized in *P. armoraciae* independent of plant myrosinases. *P. armoraciae*
588 possesses an endogenous beetle myrosinase that is likely responsible for the formation of AITC
589 detected in the headspace of *tgg*-fed beetles and the hydrolysis products of 4MSOB glucosinolate
590 detected in the feces of *tgg*-fed beetles and larvae (Figure 2, Supplementary Figure 1). Volatile
591 hydrolysis products derived from sequestered glucosinolates have also been detected in the
592 headspace of the striped flea beetle, *Phyllotreta striolata*, which led to the initial discovery of a
593 glucosinolate-myrosinase defense system in *Phyllotreta* flea beetles (Beran, 2011; Beran et al.,
594 2014). The AITC in the headspace of *P. armoraciae* adults would account only for a minor
595 proportion of unrecovered allyl glucosinolate in beetles. Considering the strong reactivity of free
596 isothiocyanates, they could have reacted with amino acid residues in peptides or proteins within
597 the body, thereby reducing the detectable levels of free isothiocyanates with our approach. The
598 role of the endogenous beetle myrosinase in the metabolism of sequestered glucosinolates remains
599 to be determined.

600 Isothiocyanates were shown to be the main hydrolysis product formed by *Arabidopsis* myrosinases
601 in damaged plant tissue (Schramm et al., 2012). Thus, the high proportion of 4MSOB cyanide
602 compared to 4MSOB isothiocyanate and derived metabolites detected in wild-type-fed beetles and
603 feces as well as in the feces of *tgg*-fed beetles was unexpected (Figure 1B). A similar observation
604 was also made in *P. armoraciae* larvae (Supplementary Material, Supplementary Figure 1), but
605 the cyanide proportion in wild type fed larvae were even higher than in beetles. The following
606 possibilities could explain the high levels of cyanide in *P. armoraciae*. Firstly, formed
607 isothiocyanates could have reacted with biological nucleophiles and were consequently not
608 recovered (Brown 2011). Secondly, isothiocyanates may have been metabolized by beetles or gut-
609 associated bacteria (Welte et al., 2016; van den Bosch et al., 2018; Friedrichs et al., 2020; Shukla
610 and Beran, 2020) and thirdly, *P. armoraciae* possesses a nitrile specifier protein that redirects the
611 glucosinolate hydrolysis towards less toxic cyanide (nitrile) (Wittstock et al., 2004). Further
612 studies are necessary to unravel the pathways in glucosinolate metabolism in *P. armoraciae*.

613 Our results showed that *P. armoraciae* adults cannot prevent glucosinolate hydrolysis completely
614 and are thus exposed to glucosinolate hydrolysis products that are known to negatively affect

615 growth and development of herbivorous insects (Agrawal and Kurashige, 2003; Jeschke et al.,
616 2016b; Jeschke et al., 2017; Sun et al., 2019). Despite similar feeding amounts, wild type-fed
617 beetles gained less weight that *tgg*-fed beetles until day 7. After 10 days feeding, weight gain of
618 wild type- and *tgg*-fed beetles did not differ anymore, but wild type-fed beetles had consumed
619 significantly more plant material than *tgg*-fed beetles over the entire feeding period. Together,
620 these results suggest that beetles compensate metabolic costs caused by glucosinolate hydrolysis
621 with an increased food intake. Compensatory feeding is a well-known reaction upon low food
622 quality (Timmins et al., 1988; Slansky Jr. and Wheeler, 1992; Lee et al., 2004).

623 Isothiocyanates are the most toxic and reactive glucosinolate hydrolysis products formed. A study
624 with *S. littoralis* larvae demonstrated that dietary isothiocyanates depleted cysteine and glutathione
625 levels and activated protein catabolism, resulting in lower levels of soluble proteins and increased
626 lipid content compared to control larvae that were not exposed to isothiocyanates (Jeschke et al.,
627 2016b). In contrast, we found no differences in the protein and lipid content of *P. armoraciae*
628 beetles that were fed with *Arabidopsis* leaves with and without myrosinase activity for ten days.
629 Although *P. armoraciae* larvae seem to be exposed to higher levels of glucosinolate breakdown
630 products than adults, they did not differ in developmental time, weight, and energy reserves when
631 they were reared on wild type or *tgg* leaves from the early second instar until the prepupal stage
632 (Supplementary Material, Supplementary Table 11). However, the major detected glucosinolate
633 hydrolysis product in both larvae and adults was the nitrile (4MSOB cyanide), and nitriles are
634 known to be less toxic than isothiocyanates (Wittstock et al., 2003). The redirection of
635 glucosinolate hydrolysis towards less toxic nitriles could explain why we did not observe a
636 negative long-term effect on *P. armoraciae*. Future studies are required to determine how
637 *P. armoraciae* copes with glucosinolate hydrolysis, specifically, by assessing the role of nitrile
638 formation and studying isothiocyanates metabolism. Regarding further long-term effects,
639 glucosinolate hydrolysis products could negatively affect other fitness parameters in
640 *P. armoraciae* that were not investigated in our study, such as female fecundity or egg hatching
641 rate (Sun et al., 2019). Overall, our results revealed no major impact of myrosinase activity on the
642 performance of *P. armoraciae* larvae and adults and therefore suggests that this specialist beetle
643 is well-adapted to cope with the chemical defense in its brassicaceous host plants.

CONCLUSION

An important strategy for insect herbivores to overcome two-component plant defenses is to prevent the glucoside hydrolysis by plant β-glucosidases during feeding. This avoids the formation of detrimental aglucones and allows insects to sequester intact and non-toxic glucosides from their host plants. Our study demonstrates that a specialist herbivore, *P. armoraciae*, prevents the hydrolysis of glucosinolates by plant myrosinases to a large extent. We suggest that two strategies, the rapid absorption of the glucosinolates from the gut and the inhibition of myrosinases in the gut, are the underlying mechanisms for this observation and thus facilitate the sequestration of intact glucosinolates. However, *P. armoraciae* cannot prevent glucosinolate hydrolysis completely and is exposed to glucosinolate hydrolysis products, which did not cause a negative long-term effect in this specialist beetle. Taken together, our results indicate that this specialist insect is well-adapted to the chemical defense in its brassicaceous host plants to both sequester glucosinolates and cope with detrimental metabolites.

AUTHOR CONTRIBUTIONS

TS, FB and JK designed experiments, TS, JK, YH and FB performed experiments, TS, JK, FB, MR, GK and NW analyzed data, SGJ performed the bioinformatic analysis, TS, FB and JK wrote the manuscript.

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832

3.1. Manuscript III

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

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RESEARCH ARTICLE

Functional Ecology

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

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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 the 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.

KEYWORDS

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 (Gross, 1993; Humphreys & Ruxton, 2019; Pasteels, Grégoire, & Rowell-Rahier, 1983; Rettenmeyer, 1970). 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 (Boege, Agrawal, & Thaler, 2019; Lindstedt, Murphy, & Mappes, 2019; Pasteels et al., 1983). Some insects that feed on chemically defended plants utilize plant defence compounds to deter predators by sequestering them in their bodies (Erb & Robert, 2016; Opitz & Müller, 2009; Petschenka & Agrawal, 2016). The fact that sequestering insects are frequently specialist feeders 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). Although sequestration of plant defensive chemicals appears to be widespread in herbivorous insects (Opitz & Müller, 2009), 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, Köllner, Gershenzon, & Tholl, 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, 1980, 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). *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).

Glucosinolates 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, Kurzbach, Herfurth, & Stauber, 2016). Isothiocyanates protect plants from non-adapted herbivores and pathogens due to their broad reactivity towards biological nucleophiles (Avato, D'Addabbo, Leonetti, & Argentieri, 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 et al., 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 (Beran et al., 2018; Müller & Wittstock, 2005; Opitz & Müller, 2009). 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, Giampà, Niehaus, & Müller, 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, Kunert, Sporer, Körnig, & Beran, 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 (Reddy et al., 2014; Xu, Clercq, Moens, Chen, & Han, 2010; Yan, Han, Moens, Chen, & Clercq, 2013; Yan, Lin, Huang, & Han, 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

Phyllotreta 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.), 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 3- to 4-week old *B. juncea* plants in a controlled-environment chamber at 24°C, 60% relative humidity and a 14/10-hr light/dark period. New plants were supplied every week and plants with eggs were kept separately for larval development. After 3 weeks, any remaining above-ground plant material was removed and the soil containing pupae was transferred to plastic containers (9 L volume; Lock & Lock). Emerging adults were collected every 2–3 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-hr 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-hr 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, Sønderby, Halkier, Jander, & Vos, 2009) and leaves of the *tgg1* × *tgg2* double knockout mutant (*tgg*) are lacking myrosinase activity (Barth & Jander, 2006).

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 1 day before sampling but larvae were not starved because they are more sensitive than adults. GLS were extracted, converted to desulfo-GLS and analysed using high performance liquid chromatography coupled to diode array detection (HPLC-DAD) 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) 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, Bates, DebRoy, & Sarkar, 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 11–13 days with rosette leaves of 6-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) camera mounted on a

Stemi 2000-C microscope (Zeiss). 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), and recorded their survival after 24 hr (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 those 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 in 30 min intervals over 6 hr exposure to third instar *H. axyridis* larvae. 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

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 using 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) for 4 hr (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.). Volatile traps were eluted twice with 100 µl of hexane (98% purity; Carl Roth) and samples were stored at −20°C until analysis using gas chromatography-mass spectrometry (GC-MS). Headspace samples were analysed using a 6890N gas chromatograph (Agilent Technologies) equipped with a Zebron ZB-5MSi capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) coupled to a 5,973 quadrupole mass spectrometer (Agilent Technologies). The carrier gas was helium at a constant flow rate of 1 ml/min. One microlitre per sample was injected in splitless mode. The front inlet temperature was set to 220°C. The oven program started at 40°C (held for 2 min), increased at 10°C/min to 100°C, and then with 50°C/min to 300°C (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).

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), 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. Samples for protein extraction were collected in PBS buffer containing proteinase inhibitors (cOmplete ethylenediaminetetraacetic acid [EDTA]-free; Roche). Protein extraction and myrosinase activity assays were performed immediately after dissection as described above. For each sample, tissues and haemolymph of 10 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 fresh weight and were thus generally higher than those detected in leaves of the rearing plant *B. juncea* (Figure 1A; Table S1, refer to Supporting Information 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, $LR = 57.077$, $p < 0.001$; Figure 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 (Figure 1B).

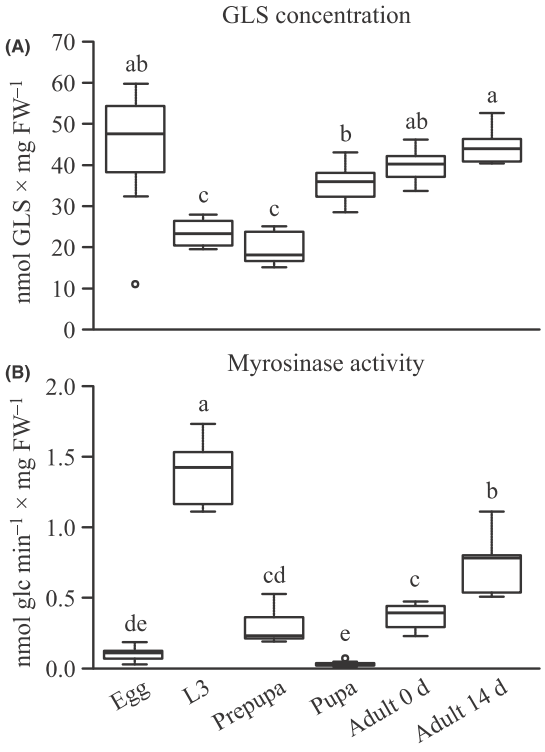

FIGURE 1 Glucosinolate (GLS) concentration and myrosinase activity in different *P. armoraciae* life stages. (A) GLS were extracted from *P. armoraciae* and quantified using high performance liquid chromatography coupled to diode array detection (HPLC-DAD) ($n = 7–8$). (B) Myrosinase activity was determined 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, $p < 0.001$). FW, fresh weight; glc, glucose; L3, third larval instar

3.2 | *Phyllotreta armoraciae* larvae deter the generalist predator *H. axyridis*

We analysed the interaction of different *P. armoraciae* life stages with a generalist predator by exposing larvae, pupae and adults 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 S1, S2 and S3 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 S1). 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, $LR = 74.500$, $p < 0.001$; Figure 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, $F = 0.383$, $p = 0.766$; Figure S1).

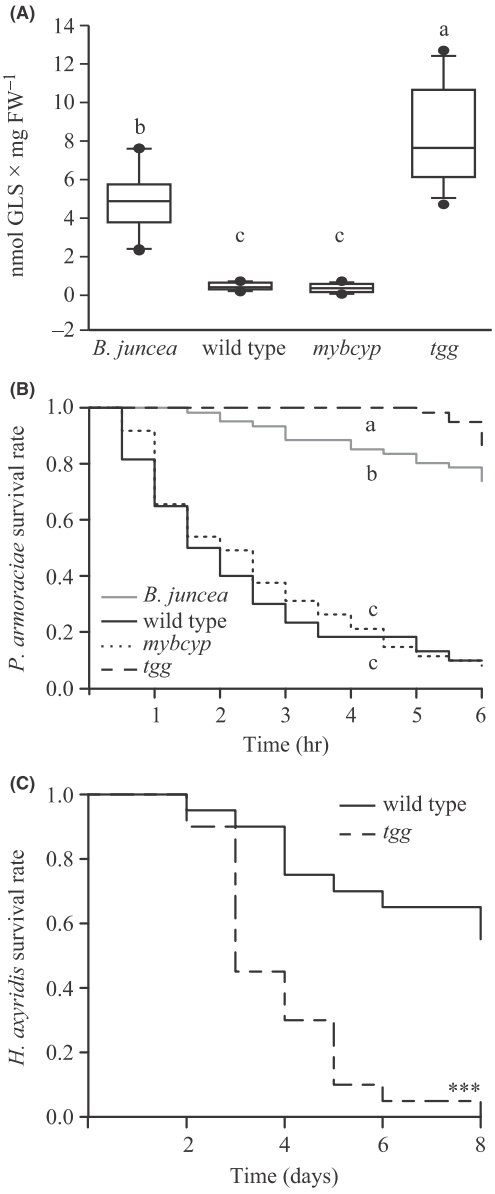

FIGURE 2 Glucosinolate (GLS) concentrations in *P. armoraciae* larvae correlate with their survival rate in the presence of *H. axyridis* and affect the survival rate of the predator. (A) GLS concentrations in larvae fed with different food plants were analysed using HPLC-DAD (generalized least squares 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*

We then exposed these larvae to *H. axyridis* and found that the survival rate strongly depended on the food plant (log-rank test, $p < 0.05$; Figure 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, $p < 0.001$; Figure 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, *M* ± *SD*). In the absence of *H. axyridis*, there was no detectable emission of AITC from larvae or pupae (Figure 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: $t = -15.242$, $p \leq 0.001$, myrosinase activity: $t = -9.442$, $p \leq 0.001$; Figure 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 $protein^{-1}$; *M* ± *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, Lognay, Wathelet, & Haubruge, 2002; Kazana et al., 2007). In *P. armoraciae*, distinct levels of myrosinase activity in larvae and pupae correlated with their predation risk

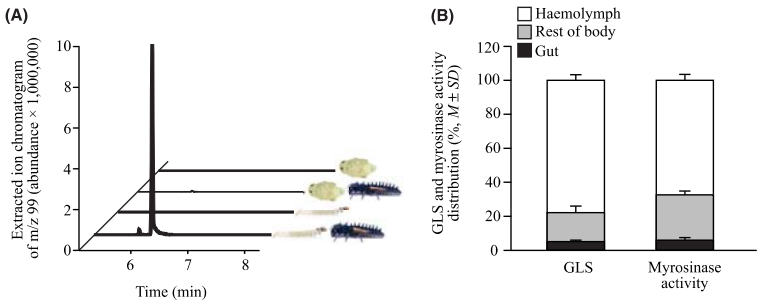

FIGURE 3 Predator-induced allyl isothiocyanate (AITC) formation in *P. armoraciae* larvae and pupae and distributions of sequestered GLS and myrosinase activity in different larval body parts. (A) Volatiles of *P. armoraciae* larvae and pupae that were exposed to *H. axyridis* larvae, were collected for 4 hr on Porapak-Q™ adsorbent, eluted with hexane and emitted AITC was quantified using gas chromatography-mass spectrometry (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 using HPLC-DAD ($n = 5$) 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%)

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 or trade-offs between different predator defence or avoidance mechanisms (Lindstedt et al., 2019; Pasteels et al., 1983). During development from egg to adult, *P. armoraciae* occupies different habitats and thus encounters different above- and below-ground 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 et al., 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 (Reddy et al., 2014; Xu et al., 2010; Yan et al., 2013, 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, but 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 but detected no difference between both plants under our assay conditions (refer to Supporting Information Methods and Results 1 for details). 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 & 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 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 β-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 Supporting Information 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 emphasizes that variation in insect chemical defence should be considered in the context of relevant predator communities across insect ontogeny.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

T.S. and F.B. designed the experiments; T.S., J.K. and F.B. performed experiments and analysed the data; T.S. and F.B. wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data are available from the Edmond Repository under https://doi.org/10.17617/3.1e (Sporer, Körnig, & Beran, 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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4. Unpublished Results

4.1. Identification and substrate specificity of the *Phyllotreta armoraciae* myrosinase

4.1.1. Material and methods

Cloning and heterologous expression of the P. armoraciae myrosinase (PaMYR): A candidate myrosinase gene from adult *P. armoraciae* beetles was identified in the in-house transcriptome based on sequence similarity to the previously identified myrosinase from *Phyllotreta. striolata* (Beran et al., 2014). The full-length open reading frame (ORF) of the candidate gene was amplified from *P. armoraciae* cDNA, cloned into the pCR4-TOPO TA vector (Thermo Fisher Scientific) and verified by Sanger sequencing. For protein expression, the open reading frame was cloned into the pIEx-4 expression vector (Novagen) without stop codon and sequenced. Primer sequences are listed in Table 1. One plasmid with the correct insert was used for transfection of High Five™ insect cells (Gibco) cultured in Express Five SFM medium (Gibco) supplemented with 20 mM glutamine (Gibco) and 50 µg/mL gentamicin (Gibco). Confluent insect cells were diluted 1:5, dispensed in 500 µL-aliquots into 24-well plates, and incubated at 27 °C. Cells were transfected on the next day using FuGENE HD Transfection Reagent (Promega) according to the manufacturer's protocol. Non-transfected cells were used as a negative control. Two days after transfection, the supernatants from two wells containing transfected or control cells were collected and combined, and 10 µL were sampled for Western blot analysis using an anit-6-His antibody (1:5,000, Invitrogen). Remaining samples were centrifuged at 4 °C for 10 min at 16,000 × g. The supernatants were concentrated to approximately 200 µL using Amicon Ultra-2 Centrifugal devices with a molecular weight cut-off of 10,000 kDa (Merck) according to the manufacturer's protocol. Subsequently, the concentrated supernatants were dialyzed in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 6.5 containing 150 mM NaCl in slide-A-Lyzer Dialysis cassette with a molecular weight cut-off of 10,000 kDa (Extra strength, Thermo Fisher Scientific). Protein concentration was determined using the Bradford protein assay (Bio-Rad).

Myrosinase activity assays: Myrosinase activity was determined using the using the Amplite Fluorimetric Glucose Quantitation Kit (AAT Bioquest) as described in Beran et al. (2018b) with 0.25 µg protein and a substrate concentration of 1.25 mM. The activity of the heterologously expressed enzyme was corrected by the background activities in protein extracts of non-transfected

cells. The following glucosinolates (GLSs) were used as substrates: allyl GLS, 3-butenyl (3But) GLS, 4-pentenyl (4Pent) GLS, 4-methylthiobutyl (4MTB) GLS, 4-methylsulfinylbutyl (4MSOB) GLS, benzyl GLS, 4-hydroxybenzyl (4OHBenzyl) and indol-3-ylmethyl (I3M) GLS (all obtained from Carl Roth or Phytoplan). Three technical replicates were performed for each substrate.

Function of the P. armoraciae myrosinase in vivo: RNA interference (RNAi) was used to verify the function of the identified myrosinase in *P. armoraciae*. Double stranded RNA (dsRNA) fragments of PaMYR (437-bp, dsMYR) and of a control, the inducible metalloproteinase inhibitor from the greater wax moth *Galleria mellonella* (AY330624.1) (223-bp, dsIMPI), were synthesized using the T7 RiboMAX™ Express RNAi System (Promega). No potentail off-target effects towards putative glycosidases were detected when all possible 21-mers of both RNA strands were searched against the local *P. armoraciae* transcriptome database allowing for two mismatches. One day after eclosion, *P. armoraciae* adults (reared on *Brassica rapa*) were injected with 100 nL containing 100 ng of dsPaMYR or 100 ng of dsIMPI, respectively, using a Nanoliter 2010 Injector (World Precision Instruments). Injected beetles were fed with leaves of three to four-week old *B. rapa* plants for five days. Beetles were starved for one day to allow digestion and excretion of plant material from the gut. Six days after dsRNA injection, beetles were collected for RNA extraction and qPCR as well as for protein extraction and myrosinase activity assays (n = 8, with 3 beetles per replicate). RNA extraction, purification, cDNA synthesis, and qPCR were performed as described in Beran et al. (2016b), and protein extraction and protein quantification were performed as described in Beran et al. (2018b) and myrosinase activity assays were performed as described above using 2 mM allyl GLS (Carl Roth) as substrate.

4.1.2. Results and discussion

The myrosinase candidate from *P. armoraciae* (PaMYR) was 86% identical in nucleotide sequence and 85% identical in amino acid sequence compared to the myrosinase from *P. striolata* (PsMYR) (Beran et al., 2014). The heterologously expressed PaMYR of adult *P. armoraciae* beetles showed large differences in substrate specificity regarding GLSs with different side chains (Figure 6) The highest activity was found for allyl GLS, which is the dominant GLS that is sequestered by *P. armoraciae* from the laboratory rearing plant *Brassica juncea* (Manuscript III). Allyl GLS is also the dominant GLS in the natural host plant horseradish (Li and Kushad, 2004). Overall, the recombinant PaMYR myrosinase shows a higher hydrolysis rate for several aliphatic GLSs than for the tested benzenic and indolic GLSs (Figure 6). In comparison, the substrate specificity of

PsMYR shows a similar pattern but larger differences in the hydrolysis rate between different GLS substrates (Beran et al., 2014). For example, the recombinant PsMYR hydrolyzes allyl GLS approximately at a 26-fold higher rate than the indolic I3M GLS, but the hydrolysis rate of PaMYR regarding allyl GLS was only 10-fold higher. These results suggest that the *P. armoraciae* myrosinase has a broader substrate specificity. In both *Phyllotreta* species, the hydrolysis rate of GLSs with different side chains matches the sequestration preference. While *P. armoraciae* has a relatively broad selectivity in the GLS sequestration pattern and a broader myrosinase substrate specificity (Manuscript I, Figure 2A), *P. striolata* shows a clear preference for the sequestration of aliphatic GLSs and a correspondingly narrower myrosinase substrate specificity (Beran et al., 2014). Sequestered GLSs (allyl GLS as well as GLSs with different structures) were shown to play a role in defense against predation in *P. armoraciae*. Both components (substrate and enzyme) are crucial to form isothiocyanates and successfully survive predation (Manuscript III, Figure 2A and B). A similar observation was also made in cabbage aphids, where the GLS sequestration pattern correlated with the endogenous myrosinase substrate specificity and the defense against natural enemies (Francis et al., 2001; 2002; Kazana et al., 2007; Kos et al., 2012; Goodey et al., 2015). Together, the congruence in the GLS sequestration and hydrolysis patterns in insect herbivores suggest adaptations in both the sequestration process and the activating enzymes that lead to a fine-tuned formation of insect two-component defense systems for increased fitness.

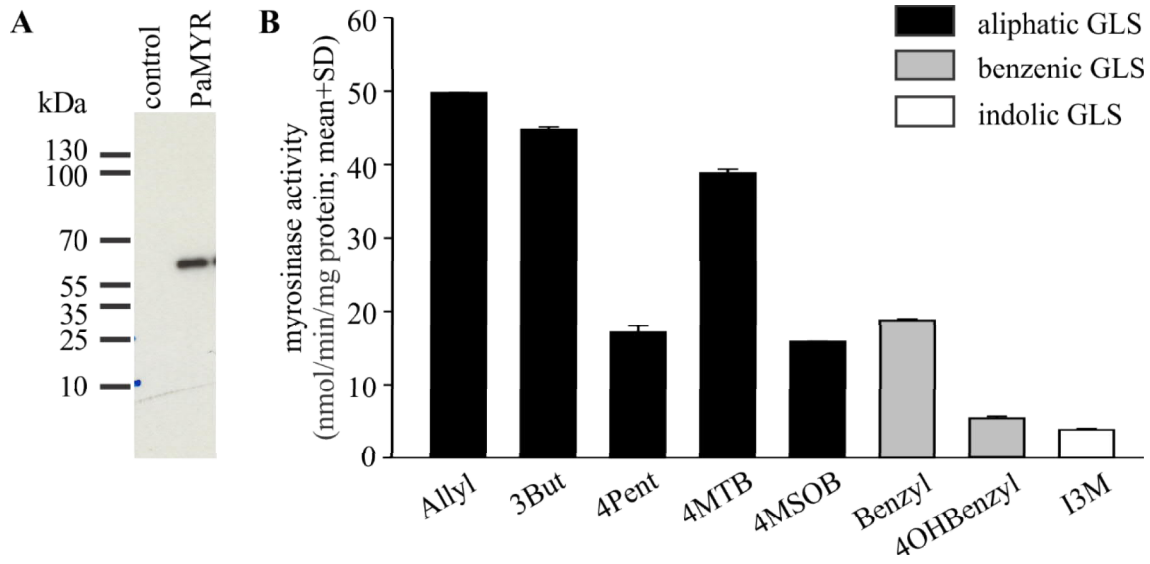

Figure 6: Detection and substrate specificity of the heterologously expressed *P. armoraciae* myrosinase. (A) Detection of the recombinant PaMYR in the supernatant of transfected High Five cells via Western blotting. The supernatant of non-transfected cells was used as control. (B) Activity of the heterologously expressed beetle myrosinase is shown for different glucosinolate (GLS) substrates (n = 3). GLS side chain abbreviations: 3But, 3-butenyl; 4Pent, 4-pentenyl; 4MTB, 4-methylthiobutyl; 4MSOB, 4-methylsulfinylbutyl; 4OHBenzyl, 4-hydroxybenzyl; I3M, indol-3-ylmethyl.

To determine whether the *P. armoraciae* myrosinase gene is responsible for the myrosinase activity in *P. armoraciae* adults, the gene expression was downregulated via RNAi. Downregulation of the *P. armoraciae* myrosinase resulted in 83% reduced expression level and in 76% reduced myrosinase activity as compared to control beetles (**Figure 7**). Thus, the expression level and enzyme activity are correlated and confirm that the identified myrosinase is indeed responsible for the myrosinase activity in *P. armoraciae* adults. Myrosinase-deficient *P. armoraciae* beetles can be further used to verify the protective function of isothiocyanates in defense or analyze the role of the endogenous myrosinase in the insect GLS metabolism or in the intraspecific communication with isothiocyanates.

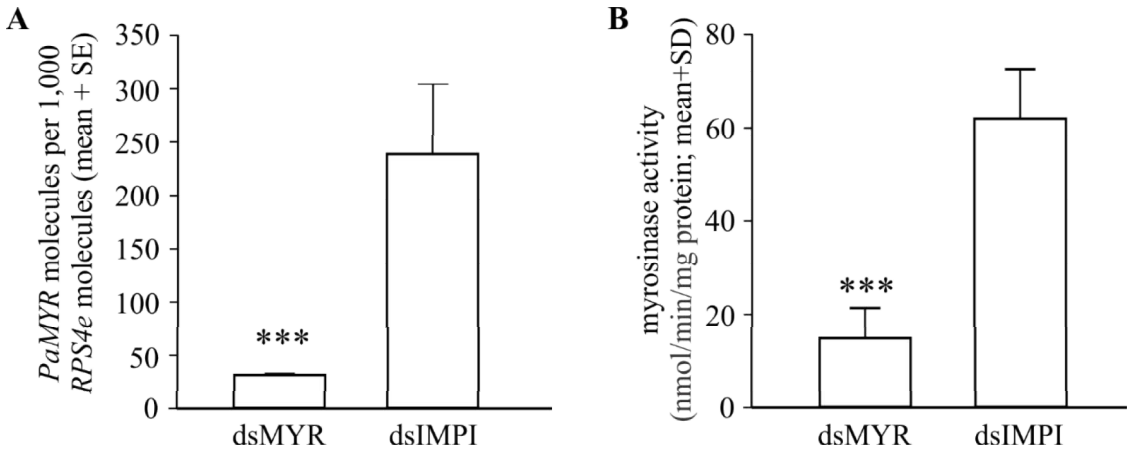

Figure 7: Knock-down of PaMYR gene expression in *P. armoraciae* using RNAi. Adult beetles were injected with double stranded RNAs targeting the P. armoraciae myrosinase (dsMYR) or a lepidopteran-specific inducible metalloproteinase inhibitor gene from the greater wax moth (dsIMPI) as a control. Six days after injection, (A) the gene expression of PaMYR relative to the expression of the reference gene RPS4e was significantly downregulated (Mann-Whitney rank sum test, $U = 3.000$) and (B) myrosinase activity was significantly reduced (Student's t-test, $t = -9.99$) in beetles. ***, $p \leq 0.001$.

Table 1: Primers used

Gene	Primer name	Primer sequence 5' - 3'	Purpose
PaMYR	UTR_Pa_C2654_F1	TCCGTTGAAATGAGCACCGA	full-length amplification from cDNA and cloning in pCR4 TOPO vector (forward)
	UTR_Pa_C2655_R1	CCTGCAATTTACTTAGCAAA CTGGA	full-length amplification from cDNA and cloning in in pCR4 TOPO vector (reverse)
	PaMYR-EX1	TGGATCCCATGCAGCAAAAA ATAGCATTCG	cloning in pIEx-4 vector for heterologous expression (forward)
	Pa-MYR-EX2	TGCGGCCGCCTTTACGTTAG CGCAATTTATACGTT	cloning in pIEx-4 vector for heterologous expression without stop codon (reverse)
	intPa_C2656_R2	GCCGGATGTGGTCAAGAAGA	internal sequencing (reverse)
	intPa_C2656_F2	TCGTACGGCGATGGAAAACA	internal sequencing (forward)
	intPa_C2656_F3	ACGGCGAAATCAGCTACAGA	internal sequencing(forward)
	qPaC2654_3F	AACGGTTACGCTGACACGAT	qPCR (forward)
	qPaC2654_3R	AAATACGGAATGGTGCCGGT	qPCR (reverse)
IMPI	T7-IMPI-F2	AATACGACTCACTATAGGGA GAG TAATGACAAGTGCTACTGTG AAGAT	amplification of DNA templates for dsRNA synthesis (forward)
	T7-IMPI-R2	TAATACGACTCACTATAGGG AGAG GGGAGTCAATGCAGGAAAA CT	amplification of DNA templates for dsRNA synthesis (reverse)
RPS4e	qPaRPS4e_F	CGTATTACTGCTGAAGAAGC	qPCR (forward)
	qPaRPS4e_R	ATCGTGGGTCACCAAGAACG	qPCR (reverse)

Author contributions

Conceived project: Theresa Sporer (70%), Franziska Beran

Designed experiments: Theresa Sporer (70%), Franziska Beran

Performed experiments: Theresa Sporer (50%), Johannes Körnig, Franziska Betzin, Franziska Beran

Data analyses: Theresa Sporer (50%), Johannes Körnig, Zhi-Ling Yang, Franziska Beran

Writing: Theresa Sporer

5. General Discussion

Insect herbivores are situated in the middle of trophic cascades and must therefore cope simultaneously with bottom-up selection pressure from the defenses of their host plants, and top-down selection pressure by their natural enemies. Specialist herbivores feeding on plants containing two-component chemical defenses, which consist of glucosylated protoxins and their corresponding β-glucosidases (Morant et al., 2008), have sometimes evolved the ability to cope with both selection pressures. They prevent the hydrolysis of the glucosylated protoxins during feeding, accumulate the glucosides in their bodies, and convert them to toxic aglucones for their self-defense using endogenous β-glucosidases (Pasteels et al., 1983; Kazana et al., 2007; Pentzold et al., 2017). From the perspective of multitrophic interactions it is important to understand the processes by which an organism acquires and uses a metabolite. However, for many insects it is unknown how they prevent glucoside degradation during feeding, and whether the hydrolysis of sequestered glucosides indeed plays a role in defense against their natural enemies. This thesis elucidates the mechanisms involved in glucosinolate (GLS) sequestration from brassicaceous plants in the studied organism, the horseradish flea beetle *Phyllotreta armoraciae*, and the role of sequestered GLSs in its defense against a generalist predator. Specifically, I focus on plant- and insect-derived factors that affect sequestration (Chapter 5.1), the mechanisms that facilitate the sequestration of intact plant glucosides in insects (Chapter 5.2), and the role of sequestered plant metabolites in defense and possibly other functions (Chapter 5.3 and 5.4).

5.1. Sequestration is a dynamic process involving multiple factors

Sequestration is a process involving the uptake, transfer, concentration, excretion, and metabolic modification of plant specialized metabolites in the insect body (Duffey, 1980; Nishida, 2002; Opitz and Müller, 2009). I investigated how the variability in different plant factors affects sequestration in *P. armoraciae.* The results showed that multiple host plant factors (GLS structure, profile and concentration (Manuscript I) and the GLS-activating enzyme (Manuscript II)) affect GLS sequestration by interacting with different sequestration processes (uptake, metabolism, and excretion) in the specialist insect (**Figure 8**).

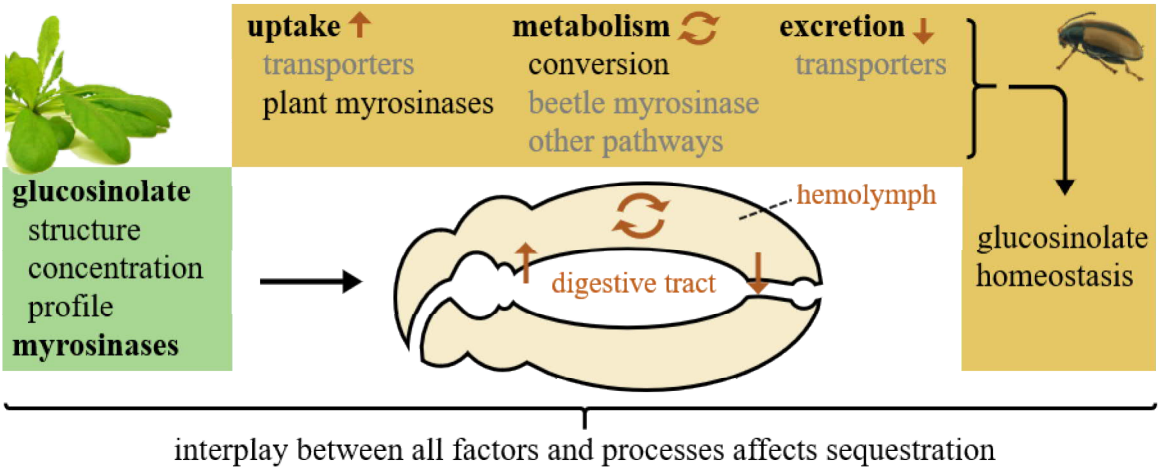

Figure 8: Plant factors affecting GLS sequestration by interacting with different sequestration processes in *P. armoraciae* adults. Plant factors (green box) comprise variability in GLSs and myrosinase activity, and beetle processes (orange box) the GLS uptake, enzymatic metabolism in beetles, and selective excretion. Beetles maintain a stable GLS level in their bodies by the interplay of uptake, metabolism, and excretion. How/if specific processes (indicated in gray) affect GLS sequestration and the metabolic fate of ingested GLSs in *P. armoraciae* remains to be determined.

By tracing the metabolic fate of ingested GLSs in *P. armoraciae*, several plant factors were found to affect the recovery of intact GLSs in beetles and feces, the GLS structure, profile, concentration (Manuscript I), as well as the myrosinase activity (Manuscript II). The impact of the GLS class and profile are discussed in Manuscript I. In this paragraph, I focus on the impact of plant myrosinases. Comparative feeding studies with *A. thaliana* wild type and myrosinase-deficient mutant plants revealed that plant myrosinases affect GLS sequestration in *P. armoraciae*, but adults largely prevent GLS hydrolysis during feeding and digestion (Manuscript II, Figure 1 and 2, Table 1). The negative impact depends on the GLS structure as the sequestration of allyl GLS seemed to be less affected than that of 4-methylsulfinylbutyl and 4-methylthiobutyl GLS. Moreover, the ability to prevent glucosinolate hydrolysis differed between *P. armoraciae* adults and larvae (Manuscripts I and II). In the presence of plant myrosinases, larvae only sequestered allyl GLS from *Brassica juncea* but no GLSs from *A. thaliana* (Manuscript III, Figure 2A). Since allyl GLS is the major GLS in the natural host plant horseradish, *P. armoraciae* may have developed specific mechanisms to stabilize this particular GLS (Li and Kushad, 2004). Moreover, the results suggest that the mechanisms or efficiencies to cope with plant myrosinases (see Chapter 5.2) differ between life stages. It is known that detrimental glucosinolate hydrolysis products negatively affect the growth and development of insect herbivores (Agrawal and Kurashige, 2003; Jeschke et al., 2016b; Jeschke et al., 2017; Sun et al., 2019). Although *P. armoraciae* is exposed to such metabolites, no

measurable negative long-term effects were found in both larvae and adults of this specialist beetle, suggesting that *P. armoraciae* is well adapted to the chemical defense of its brassicaceous host plants (Manuscript II). Plant β-glucosidases also affect the sequestration of plant glucosides in other chewing insects. The majority of the ingested 4-methylsulfinylbutyl GLS is degraded in the cabbage stem flea beetle (Beran et al., 2018b). Although no quantitative feeding study has been performed with larvae of the six-spot burnet moth, low cyanogenic glucoside amounts seem to be hydrolyzed during feeding by the corresponding *Lotus* β-glucosidases (Pentzold et al., 2014a). Consequently, the extent to which plant β-glucosidases affect glucoside sequestration varies between life stages and organisms, and depends on the substrate structures.

Irrespective of the GLS levels and profiles beetles ingested from the different food plants, *P. armoraciae* adults maintained a stable level of sequestered GLSs in their bodies over time (Manuscript I, Figure 1C). This GLS homeostasis involves both transport and metabolic processes. Beetles accumulated different GLSs with a relatively broad selectivity (Manuscript I, Figure 2A), suggesting that transporters mediating the uptake from the gut possess a broad substrate specificity. In contrast, the GLS excretion was selective and regulated as GLS excretion varied over time and some GLSs were not excreted (Manuscript I, Figure 2C). Therefore, the excretion process seems to play an important role for balancing GLS levels in *P. armoraciae* and may, additionally, regulate the selective accumulation of certain GLSs by eliminating non-desired ones from the body. In insects, this excretion is mediated *via* the Malpighian tubules. Based on the high GLS concentration in the hemolymph, the transport from the hemolymph into the Malpighian tubule lumen is presumably passive, as for cardiac glycosides in several insects (Rafaeli-Bernstein and Mordue, 1978; Rafaeli-Bernstein and Mordue, 1979; Meredith et al., 1984). A recent study identified a transporter in the Malpighian tubules of *P. armoraciae,* which mediates the absorption of GLSs that had been transported from the hemolymph into the Malpighian tubule lumen back into the hemolymph (Yang et al., under review). This process has been suggested to prevent GLS excretion and thereby promote retention in the body, a process which could be important in many sequestering insects. Moreover, selective transporters in the Malpighian tubules could contribute to the selective accumulation or retention of metabolites in the body. Unlike in *P. armoraciae*, the sequestered phenolic glucoside salicin is stored in the larval defense glands of the poplar leaf beetle, *Chrysomela populi*, thus an additional transport level is necessary from the hemolymph to the reservoir (Pasteels et al., 1983). In *C. populi*, both the uptake from the gut into the hemolymph and

the excretion from the hemolymph are unselective, whereas the import into the defense glands is salicin-specific (Kuhn et al., 2004; Kuhn et al., 2007; Discher et al., 2009; Strauss et al., 2013). Thus, transport processes differ depending on where the sequestered metabolites are stored (hemolymph or other structures). In any case, transport processes are crucial players for the accumulation of plant specialized metabolites in insect bodies.

Besides transport processes, metabolism also affects the level and profile of sequestered plant metabolites. Plant GLS concentration was negatively correlated with the recovery of intact GLSs in *P. armoraciae* beetles and feces (Manuscript II). Thus, beetles appear to have a limited capacity to stabilize GLSs. The efficacy of the mechanisms that prevent plant myrosinase-mediated GLS hydrolysis in the gut (Chapter 5.2) could be limited. Alternatively, this limitation could be due to a higher GLS turnover, as more GLSs are taken up in order to balance additional sequestration. In *P. armoraciae*, the uptake of new GLSs from *A. thaliana* correlated with a decrease in previously stored allyl GLS (Manuscript I). However, less than half of the allyl GLS decrease in the body was explained by excretion (Manuscript I). In addition, even in the absence of plant myrosinases, only half of the ingested allyl GLS was recovered in beetles and feces (Manuscript II, Table 1). Moreover *P. armoraciae* adults and larvae contained and excreted glucosinolate hydrolysis products during feeding on myrosinase-deficient *A. thaliana* plants (Manuscript II, Figure 1 and Supplementary Figure 1). Both observations indicate that *P. armoraciae* adults metabolize sequestered GLSs independent of plant enzymes, and thus metabolism likely contributes to the GLS homeostasis in beetles. Endogenous myrosinases may be involved in this turnover. In *P. armoraciae* adults, the myrosinase gene was identified by heterologous protein expression and its function was verified *in vivo* using RNAi (see unpublished data in Chapter 4). The substrate specificity of the recombinant myrosinase was relatively broad although several aliphatic GLSs were hydrolyzed at a higher rate than other GSLs (Chapter 4, Figure 1). These results suggest that insect myrosinase could contribute to the metabolism of the different GLSs that are sequestered by *P. armoraciae* adults, which could be assessed using myrosinase-deficient *P. armoraciae* beetles. Moreover, beetles were found to selectively convert methylsulfinylalkyl GLSs to methylthioalkyl GLSs, which is a reaction that was previously hypothesized for *P. striolata,* but the function in *Phyllotreta* remains elusive (Manuscript I, Figure 2B, Beran et al. (2014)). Besides this conversion and possibly the involvement of endogenous myrosinases, other pathways might contribute to GLS metabolism in *P. armoraciae* as in other insects. For example, turnip sawfly larvae metabolize sequestered GLSs to GLS-3-sulfates, a metabolic process most likely involving a GLS sulfatase

and a sulfotransferase enzyme (Müller and Wittstock, 2005; Opitz et al., 2011). Larvae of the mustard leaf beetle also metabolize benzenic GLSs independently of plant myrosinases into aromatic acids conjugated to aspartic acid (Friedrichs et al., 2020).

Together, these results show that the sequestration of specialized plant metabolites is a complex process involving the interaction of multiple plant- and insect-derived factors and includes transport and metabolic aspects (Figure 8). Transport processes, including uptake from the gut, transfer to the hemolymph, and excretion via Malpighian tubules, together with metabolic modification in insect bodies, regulate the levels and composition of sequestered plant defense compounds. The sequestration mechanism of each sequestering insect may be species-specific. The data presented in this work suggest that in *P. armoraciae*, specific levels of sequestered metabolites are maintained by regulatory fine-tuning, implying that a precise level may be of an ecological importance. On the one hand, low metabolite levels can be disadvantageous to the defense capacity against natural enemies (Chapter 5.3). On the other hand, the accumulation of excessive metabolite levels may be autotoxic or too costly to maintain. The results obtained lead to further questions: What are the mechanisms underlying the different sequestration abilities of different insect life stages? How do plant β-glucosidases affect the sequestration of different plant glucosides? Do transporters mediate selective sequestration? How and for which benefits are GLSs metabolized in *P. armoraciae*? What is the ecological relevance of different GLS accumulation patterns in beetles?

5.2. Uptake mechanisms of plant glucosides for sequestration in insects

In two-component plant defenses, tissue damage leads to the rapid hydrolysis of plant glucosides by β-glucosidases and thus to the formation of toxic aglucones (Morant et al., 2008). Therefore, chewing insects that sequester plant glucosides require mechanisms to prevent glucoside breakdown during feeding and digestion, such as a highly alkaline gut pH that inhibits plant β-glucosidases and the reglucosylation of the aglucones formed (Pentzold et al., 2014a; Pentzold et al., 2014b; Robert et al., 2017). Adult *P. armoraciae* beetles prevent GLS degradation during feeding and digestion to a large extent and sequester GLSs from Brassicaceae despite their chewing mouthparts, which led to the question of how this is accomplished in these beetles. Different strategies were analyzed in Manuscript II and the results strongly suggested that *P. armoraciae* combines two mechanisms to efficiently sequester intact GLSs, namely rapid GLS uptake from the gut and the inhibition of plant myrosinases. Beetles rapidly absorbed the majority of the ingested GLSs from the gut, thereby separating large proportions of the GLS substrate from the co-ingested

myrosinases and furthermore, gut extracts reduced plant myrosinase activity *in vitro* by half (Manuscript II, Figure 4).

The rapid sequestration of plant glucosides was first shown in larvae of the turnip sawfly *Athalia rosae* (Abdalsamee et al., 2014), a glucosinolate-sequestering insect, and this mechanism was also suggested for other sequestering herbivores with chewing mouthparts (Pankoke and Dobler, 2015; Robert et al., 2017). Due to their polarity, the uptake of plant glucosides across the gut epithelium must be mediated by membrane transporters that should be located in the anterior gut to ensure the quick separation of substrate and enzyme. Current studies have focused on the rapid uptake mechanisms and the elucidation of the uptake location in the gut (Yang et al., in preparation). So far, transporters facilitating glucoside uptake from the gut lumen into the body have not been identified in any sequestering insect. Transport across the gut epithelium involves two carrier-mediated steps: first, the import from the gut lumen into the gut epithelial cell, followed by the export from the cell into the hemolymph. In the well-studied salicin-sequestering poplar leaf beetles, a recent profiling of membrane proteins suggested that sugar porters, a family within the major facilitator superfamily (MFS), and ATP binding cassette (ABC) transporters are likely candidates for this uptake process from the gut (Schmidt et al., 2019). Both superfamilies contain importers as well as exporters and could therefore facilitate transport across the gut epithelium (Rees et al., 2009; Reddy et al., 2012). Members of these families may also be involved in GLS uptake in *Phyllotreta* adults and in plant glucoside uptake in other insects. In order to verify that a rapid uptake mechanism plays an essential role in the sequestration of intact GLSs, the responsible transporter(s) must be identified in the gut of *P. armoraciae* and knocked down, for example by RNA interference (RNAi). Once rapid uptake has been established for *P. armoraciae*, it would represents a good starting point to study sequestering systems in other insects, as rapid sequestration could be an important strategy for many glucoside sequestering organisms (Desroches et al., 1997; Kuhn et al., 2004; Pentzold et al., 2014a; Beran et al., 2018b). Glucoside sequestration has also been suggested as a detoxification strategy for two-component plant defenses as it prevents the formation of detrimental aglucones. Whether glucoside sequestration indeed represents a detoxification mechanism for those herbivores could likewise be tested with transporter-knockdown insects by analyzing insect growth and survival.

The inhibition of plant β-glucosidase activity by insects has never been demonstrated as clearly in any other insect as in the *in vitro* assays with the gut extracts of *P. armoraciae* adults (Manuscript

II, Figure 4B). The only previously elucidated mechanism for reducing plant β-glucosidase activity in insects has been highly alkaline pH conditions that lead to a lower degradation rate of co-ingested glucosides (Pentzold et al., 2014a). However, such alkaline gut conditions are typically found in lepidopteran larvae and not in coleopterans, which generally possess neutral to acidic gut lumina, as in *P. armoraciae* (Manuscript II) (Terra and Ferreira, 2012). A transient inhibition of plant myrosinase activity other than by pH conditions has so far been found in the anterior gut of the turnip sawfly *A. rosae* (Abdalsamee et al., 2014). However, no β-glucosidase inhibitor (or specific myrosinase inhibitor) was identified in any insect yet and also remains elusive for *P. armoraciae*. The inhibition could target the active center, mimic the transition state analog, or affect the enzyme activity by other chemical or biochemical mechanisms, for example by targeting the cofactor ascorbic acid. In any case, the inhibition mechanism should be specific for the respective plant β-glucosidase and must not affect the insect's own β-glycosidases to avoid the reduction of digestive efficiency (Terra and Ferreira, 2012). Future studies are necessary to identify the myrosinase inhibitor in *P. armoraciae*, which could be accomplished by bioassay-guided fractionation.

In *P. armoraciae* adults, other mechanisms to provide GLS uptake apart from the rapid glucoside absorption and the inhibition of plant β-glucosidases cannot be excluded. For example, a rapid reglucosylation of GLS aglucones (Robert et al., 2017; Mocniak et al., 2020) or a transient stabilization by chemical modifications could take place (Wang et al., 2012). However, I speculate that the two established strategies are the main ones to circumvent the activation of the plant two-component system and enable the uptake of intact plant glucosides. Since the ability to prevent glucosinolate hydrolysis differs between *P. armoraciae* adults and larvae (Manuscript II and III), this suggests differences in the mechanisms or efficiencies between life stages to cope with plant myrosinases. Future studies are necessary to unravel the underlying mechanisms for these differences.

5.3. Utilization of sequestered plant metabolites for defense

Insects accumulate toxic plant metabolites in their bodies mainly for defense against natural enemies. The underlying principle is based on the fact that sequestered plant metabolites or their metabolized products render the insect unpalatable or toxic to predators and parasitoids and unsuitable hosts for entomopathogens (Rowell-Rahier and Pasteels, 1986; Kazana et al., 2007; Gross et al., 2008; Robert et al., 2017).

Phyllotreta spp. sequester non-toxic plant glucosides (GLSs) and metabolize them into toxic breakdown products (isothiocyanates) using endogenous myrosinases (Manuscript III; Beran et al. (2014)). However, *P. armoraciae* cannot form isothiocyanates throughout its entire ontogeny. While all life stages contained the GLS substrate, endogenous myrosinase activity varied strongly, with larvae possessing the highest and pupae the lowest activity (Manuscript III, Figure 1). To assess the efficacy of the GLS-myrosinase system for defense in *P. armoraciae*, predation assays were performed using larvae of the Asian ladybird *Harmonia axyridis* as a generalist predator. The results showed that both GLSs and endogenous myrosinase activity must be present in the insect to form isothiocyanates, deter the predator, and allow *P. armoraciae* to survive predation (Manuscript III, Figure 2 and 3A, Video S1 and S2). Moreover, the GLS sequestration pattern and the substrate specificity pattern of the endogenous myrosinases in *Phyllotreta* flea beetles correlate suggesting a fine-tuned adaptation that increases insect fitness (see unpublished data in Chapter 4, (Beran et al., 2014)). Thus, GLS sequestration in *Phyllotreta* is an effective chemical defense against this arthropod predator, but the capability to fend off natural enemies using the GLS-myrosinase system is life stage-dependent. Regarding the ecological function of GLSs in defense, the predation experiments represent strong evidence that isothiocyanates cause the successful defense against predators. Predation assays with myrosinase-deficient *Phyllotreta* flea beetles, for example accomplished by RNAi, can verify this aspect in future research (see unpublished data in Chapter 4). Of course, *Phyllotreta* flea beetles encounter many other antagonists apart from predators, such as parasitoids, parasites, and pathogens. It is possible that the two-component system protects *Phyllotreta* from some of these other antagonists as well, but the assessment of the general effectiveness of GLSs in the chemical defense requires further studies.

During ontogeny, *Phyllotreta* beetles occupy different habitats and are thus exposed to different communities of natural enemies (Vig, 2004). Well-defended *P. armoraciae* larvae show a leaf-mining lifestyle and thus mostly avoid encounters with ladybirds and similar predators (Vig, 2004). Predators may encounter the last (third) larval instar, which leaves the plant, moves to the soil, and searches for a suitable place to dig into the ground for pupation. Under natural conditions, parasitoids are likely the most important threats to leaf-mining *Phyllotreta* larvae. In contrast to *P. armoraciae*, the larvae of most *Phyllotreta* spp. feed belowground on roots and consequently pathogens, parasites, and belowground predators (e.g. nematodes, viruses, bacteria, and fungi) may be especially relevant for these soil-borne life stages.

In the course of development, pupae cannot form isothiocyanates for protection (Manuscript III, Figure 1 and 3A, Video S2). It might be counter-intuitive that this non-mobile stage lacks chemical protection. However, during the pupal stage, metamorphosis takes place, a process during which organs and tissues are remodeled. Thus, pupae could be especially susceptible to autotoxicity by unintentionally hydrolyzed defense compounds. Another sequestered class of plant glucosides, iridoid glucosides, are catabolized or eliminated by insects during metamorphosis and are thus not transferred to the adult stage (Bowers, 1991; Bowers and Collinge, 1992; Bowers, 1993; Bowers, 2003). It is assumed that reorganization and metabolic activity make pupae sensitive to autotoxicity which can be prevented by the catabolism of sequestered plant compounds. In *P. armoraciae*, the non-toxic substrate is retained throughout metamorphosis, but the activating enzyme is downregulated (Manuscript III, Figure 1). A similar observation was made in a lepidopteran insect possessing a two-component system: *Z. filipendulae* larvae and adults emit high amounts of hydrogen cyanide, the toxic hydrolysis product from cyanogenic glucosides, whereas this emission is strongly reduced in pupae (Zagrobelny et al., 2015). I hypothesize that holometabolous insects with a two-component chemical defense must remove one component during metamorphosis as a mechanism to prevent autotoxicity. The lack of protection from a two-component system during metamorphosis can be compensated. Insect pupae may possess other defense metabolites, or use behavioral or physical defenses, like defensive movements, spines, cocoons, or in the case of *Phyllotreta,* building an earthen chamber as a mechanical barrier (Vig, 2004; Lindstedt et al., 2019).

After eclosion, adult *P. armoraciae* beetles are again equipped with a complete two-component system (Manuscript III, Figure 1). In addition to their chemical defense, adults have a higher mobility than all other life stages and can escape by jumping, dropping, flying, walking, and feigning death, and they possess a hard exoskeleton as mechanical protection from injuries. Like several other insects that transfer sequestered metabolites into the next generation (Pasteels et al., 1986; Dussourd et al., 1988; Tallamy et al., 2000), *P. armoraciae* adults also transfer GLSs into the eggs (Manuscript III, Figure 1). This benefits the offspring since the eggs are provided with the defense substrate right from the beginning and therefore only require the synthesis of the activating enzyme to be chemically protected. Until neonate hatching, eggs may be protected by their oviposition in clusters: if a predator consumes an egg from the cluster, intact GLSs may have a deterrent effect and additionally low amounts of toxic isothiocyanates may be produced, so that the

remaining eggs of the batch are avoided. This outcome was shown, for example, when green lacewing larvae probed and rejected egg clusters of *Utetheisa ornatrix* protected by pyrrolizidine alkaloids (Eisner et al., 2000).

Taken together, *P. armoraciae* can exploit sequestered plant glucosides for its own protection in a life-stage specific manner, but behavioral and morphological defenses may be important as well. For chemical defense, *Phyllotreta* flea beetles depend on their host plants to obtain the defensive substrates. The fact that the plant glucosides are transferred throughout ontogeny and into the next generation underlines the importance of these compounds for *Phyllotreta.* The role of sequestered plant glucosides in defense against higher trophic levels increases insect fitness and thus the selection pressure from natural enemies might explain the specialization of most *Phyllotreta* spp. on brassicaceous host plants (Gikonyo et al., 2019).

5.4. Other functions of sequestered plant metabolites in insects

Sequestered plant metabolites may not only be utilized by insects for defense but also represent resources that can be exploited for other purposes like communication or nutrition (Opitz and Müller, 2009). Sequestered compounds that are metabolized to volatile products, such as various plant glucoside-derived hydrolysis products (e.g. hydrogen cyanide, salicylaldehyde and several isothiocyanates), are well designed for an air-borne communication. In *Phyllotreta*, volatile isothiocyanatesact synergistically with the male aggregation pheromone blend, that contains the sesquiterpene (*6R,7S*)-himachala-9,11-diene as a dominant component, and increase the aggregation behavior of adult flea beetles (Soroka et al., 2005; Tóth et al., 2012; Beran et al., 2016a). However, since *Phyllotreta* adults only emit low amounts of GLS-derived isothiocyanates (Manuscript II, Beran et al. (2014)), they might be more relevant in a short-distance communication, for example for mating or mating choice. Similar examples have been shown or suggested in other insects (Conner et al., 1981; Iyengar et al., 2001; Hee and Tan, 2004; 2006). *U. ornatrix* males partially convert sequestered pyrrolizidine alkaloids into the courtship pheromone hydroxydanaidal, which is presented to females using abdominal brushes (Conner et al., 1981; Iyengar et al., 2001). Females appear to utilize pheromone quantity as a criterion to select their mating partner. *Bactrocera* fruit flies sequester the phenylpropanoid methyl eugenol and metabolize it into sex-pheromone components that are stored in the male rectal glands (Hee and Tan, 2004; 2006).

The fitness of insect herbivores strongly depends on the quality of their food plants with nutrients as a positive regulator on the one hand, and the presence of harmful defense compounds as a negative factor on the other hand (Awmack and Leather, 2002). By applying a strategy that allows sequestering insects to take up defense compounds without being harmed, they may be able to recycle valuable elements from those compounds. In *P. armoraciae,* the fate of the stored allyl GLS is only partially explained by excretion (Manuscript I) and beetles emitted only low amounts as isothiocyanate (Manuscript II, Figure 2). Hence, the recycling of nitrogen, sulfur or glucose from sequestered GLSs could be nutritionally relevant for other biological processes in *Phyllotreta* flea beetles. In brassy willow beetles, the glucose released from the hydrolysis of phenolic glucosides supplies up to 30% of the larval energy demand (Rowell-Rahier and Pasteels, 1986). Experimental removal of their defensive secretions leads to an increased glucose production due to substrate hydrolysis and to an increased growth. In *Z. filipendulae*, the turnover of cyanogenic glucosides during metamorphosis is expected to function in the reallocation of nitrogen to chitin synthesis (Zagrobelny and Møller, 2011; Zagrobelny et al., 2015).

Taken together, sequestration of plant metabolites can be a multi-layered strategy of herbivorous insects to cope with the defenses from their host plant and, at the same time, to exploit them for their own defense, nutrition, or communication. This thesis contributes knowledge on multiple factors and processes involved in the sequestration of plant specialized metabolites and their ecological role in *Phyllotreta*. Whether sequestration also fulfills other purposes in *Phyllotreta* besides a role in defense remains a topic for future studies.

6. Summary

The fitness and survival of insect herbivores depends on both the host plant quality, which encompasses nutrients, as well as toxins, and predation by natural enemies. Driven by these selection pressures, some insects have developed sophisticated strategies to cope with the challenges from both trophic levels, the plant chemical defense and predation. This thesis focuses on a beetle species, which feeds specifically on plants that are equipped with a two-component chemical defense. Such plant defense systems consist of two spatially separated components, non-toxic glucosylated metabolites (glucosides) and corresponding β-glucosidases. Upon tissue damage, the β-glucosidases hydrolyze the glucosides to toxic aglucones. However, some insect herbivores have developed mechanisms to prevent the glucoside breakdown, accumulate (sequester) the intact glucosides, and form an own two-component defense by producing endogenous β-glucosidases. This strategy interacts with both trophic levels and allows the insect to redirect plant defense metabolites against its own natural enemies. The so-called 'mustard-oil bomb' in plants of the order Brassicales is one of the best-studied two-component system. It is composed of glucosinolates (thioglucosides) and myrosinases (thioglucosidases). which hydrolyze the glucosinolates to toxic isothiocyanates. *Phyllotreta* flea beetles are specialized on plants in the family Brassicaceae, sequester intact glucosinolates and possess an endogenous myrosinase. However, the mechanisms and the ecological role of glucosinolate sequestrations are unknown. In my thesis, I therefore studied these aspects in the horseradish flea beetle *Phyllotreta armoraciae* using different *Arabidopsis thaliana* genotypes as food plants and the Asian ladybird *Harmonia axyridis* as a generalist predator (Figure 9).

Here, an analysis of the impact of varying host plant factors on glucosinolate sequestration in *P. armoraciae* revealed that glucosinolate structure, profile, and concentration, as well as plant myrosinase activity influence the sequestration. Moreover, adult beetles maintain a glucosinolate homeostasis in their bodies by the uptake, metabolism and excretion of glucosinolates. These results underline the complexity of sequestration in *P. armoraciae*, which results from an interplay between different host plant factors (substrate and enzyme) and insect processes (transport and metabolism).

Investigations on the extent to which glucosinolates are degraded by plant myrosinases during feeding and digestion revealed that *P. armoraciae* adults prevent glucosinolate hydrolysis by these

enzymes to a large extent. Based on investigations on the underlying mechanisms that prevent glucosinolate hydrolysis in *P. armoraciae* adults, I propose the contribution of two physiological processes. (1) Beetles rapidly absorb ingested glucosinolates from the gut, thereby separating the glucosinolate substrate from co-ingested plant myrosinases. (2) Beetles inhibit the activity of plant myrosinases in the gut. Both strategies may play a role in other glucoside-sequestering insects as well. Further, I showed that the sequestration ability differs strongly between larvae and adults of *P. armoraciae* suggesting different mechanisms or efficiencies to prevent glucosinolate degradation. Although *P. armoraciae* larvae and adults are exposed to detrimental glucosinolate hydrolysis products, there is no measurable negative long-term effect on the performance of this specialist beetle. Taken together, the obtained results demonstrate that this specialist insect is well-adapted to the chemical defense in its brassicaceous host plants.

For a successful chemical defense, the beetle relies on its host plant to obtain the defense substrates. I found differences in the two-component system between life stages of *P. armoraciae* and thus in the capability to utilize sequestered glucosinolates for defense. While glucosinolates are present throughout the entire ontogeny, myrosinase activity varies strongly between life stages, with larvae possessing the highest and pupae the lowest activity. Consequently, the capability to form toxic isothiocyanates for defense strongly fluctuates throughout the beetle's lifetime. Predation assays showed that both glucosinolates and endogenous myrosinase activity must be present in *P. armoraciae* for a successful defense and survival against the Asian ladybird, a generalist predator. Thus, sequestered plant metabolites selectively protect specific beetle life stages from predation.

In summary, this thesis provides insights into the tritrophic interactions of the horseradish flea beetle *P. armoraciae* with its brassicaceous host plants on one side, and a generalist predator on the other side. The sequestration of plant metabolites in beetles is a multi-layered process to overcome the defense from the host plant, and, at the same time exploit it for the beetle's own defense (Figure 9). This thesis contributes knowledge on multiple factors and processes involved in the sequestration of plant specialized metabolites and their ecological role in *Phyllotreta*, and represents a basis for further research in this area.

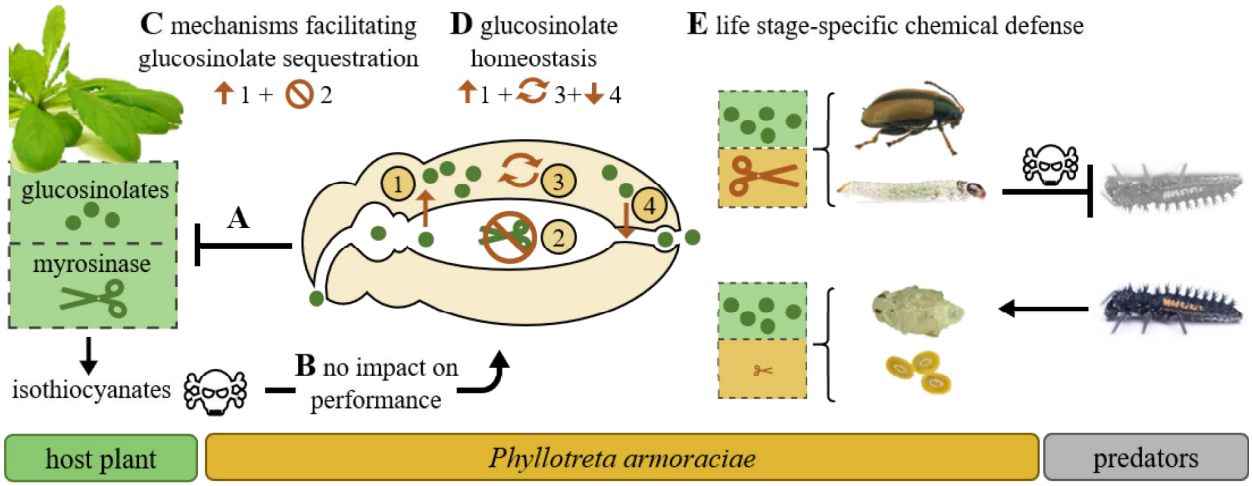

Figure 9: Graphical summary of the interactions of the horseradish flea beetle *Phyllotreta armoraciae* with brassicaceous host plants and the Asian ladybird *Harmonia axyridis*, a generalist predator. (A) Adult *P. armoraciae* beetles prevent glucosinolate hydrolysis by plant myrosinases during feeding and digestion to a large extent. (B) Although beetles are exposed to detrimental glucosinolate hydrolysis products, there is no long-term effect on insect performance. (C) The sequestration of intact glucosinolates is most likely facilitated by (1) the rapid glucosinolate absorption from the gut separating the substrate from plant myrosinase enzymes and (2) the inhibition of plant myrosinases. (D) Adult beetles balance the glucosinolate level in their bodies by glucosinolate (1) uptake, (3) metabolism, and (4) excretion. (E) Distinct life stages are equipped with a complete two-component chemical defense and hydrolyze sequestered glucosinolates with endogenous myrosinases (orange scissors) to toxic isothiocyanates. Both components must be present in *P. armoraciae* for a successful defense against the Asian ladybird. Thus, the successful chemical protection depends on the beetle life stage. In summary, *P. armoraciae* developed mechanisms to cope with the host plant defense, sequester intact plant glucosides, and utilize those compounds for defense in a life stage-specific manner.

7. Zusammenfassung

Die Fitness und das Überleben von pflanzenfressenden Insekten hängen einerseits von der Qualität der Wirtspflanze ab, die sowohl Nährstoffe als auch Toxine enthält, und andererseits von der Prädation durch natürliche Feinde. Aufgrund dieses zweiseitigen Selektionsdrucks entwickelten einige Insekten ausgeklügelte Strategien, um die Herausforderungen durch beide trophischen Ebenen, die chemische Abwehr von Pflanzen und Prädation, zu bewältigen. Diese Arbeit konzentrierte sich auf eine Käferart, die sich speziell von Pflanzen ernährt, welche mit einer chemischen Zweikomponentenabwehr ausgestattet sind. Solche pflanzlichen Abwehrsysteme bestehen aus zwei räumlich getrennten Komponenten, ungiftigen glucosylierten Metaboliten (Glucosiden) und dazugehörigen β-Glucosidasen, welche bei einer Verletzung des Gewebes mit den Glucosiden in Kontakt kommen und diese zu toxischen Aglucone hydrolysieren. Einige Insekten haben jedoch Mechanismen entwickelt, um den Glucosidabbau zu verhindern, die intakten Glucoside im Körper zu akkumulieren (sequestrieren) und ein eigenes Zweikomponentensystem zu bilden, indem sie endogene β-Glucosidasen produzieren. Diese Strategie interagiert mit beiden trophischen Ebenen und ermöglicht es dem Insekt, die pflanzlichen Abwehrmetabolite gegen seine eigenen natürlichen Feinde umzuleiten. Die sogenannte „Senfölbombe“ in Pflanzen aus der Ordnung Brassicales ist eines der am besten untersuchten Zweikomponentensysteme. Es besteht aus Glucosinolaten (Thioglucosiden) und Myrosinasen (Thioglucosidasen), welche die Glucosinolate in toxische Isothiocyanate umwandeln. Kohlerdflöhe der Gattung *Phyllotreta* sind auf Pflanzen der Familie der Brassicaceen spezialisiert. Diese Käfer sequestrieren intakte Glucosinolate und besitzen eine endogene Myrosinase. Die Mechanismen und die ökologische Funktion der Glucosinolatsequestrierung sind jedoch unbekannt. Daher habe ich in meiner Doktorarbeit diese Aspekte beim Meerrettich-Erdfloh *Phyllotreta armoraciae* mithilfe verschiedener *Arabidopsis thaliana* Genotypen als Futterpflanzen und des asiatischen Marienkäfers *Harmonia axyridis* als generalistischen Fraßfeind untersucht (Figure 9).

Die Analyse des Einflusses verschiedener Wirtspflanzenfaktoren auf die Glucosinolat-Sequestrierung in *P. armoraciae* zeigte, dass Glucosinolatstruktur, -profil und -konzentration sowie pflanzliche Myrosinaseaktivität die Sequestrierung beeinflussen. Darüber hinaus erhalten adulte Käfer durch Glucosinolataufnahme, -metabolismus und -exkretion eine Homöostase der sequestrierten Glucosinolate in ihrem Körper aufrecht. Diese Ergebnisse heben die Komplexität

der Sequestrierung in *P. armoraciae* hervor, welche auf das Zusammenspiel zwischen verschiedenen Faktoren der Wirtspflanze (Substrat und Enzym) und Prozesse innerhalb des Insekts (Transport und Metabolismus) zurückzuführen ist.

Untersuchungen darüber, wie viele Glucosinolate durch pflanzliche Myrosinasen während des Fressens und Verdauens abgebaut werden, ergaben, dass adulte *P. armoraciae* Käfer die Glucosinolathydrolyse durch Myrosinasen zum großen Teil verhindern können. Basierend auf Untersuchungen zu den zugrundeliegenden Mechanismen, welche die Glucosinolathydrolyse in *P. armoraciae* Käfern unterbinden, schlage ich die Beteiligung zweier physiologischer Prozesse vor: (1) Käfer absorbieren gefressene Glucosinolate schnell aus dem Darm, wodurch Glucosinolate von den zeitgleich aufgenommenen pflanzlichen Myrosinasen separiert werden. (2) Käfer inhibieren die Aktivität von pflanzliche Myrosiansen im Darm. Diese beiden Strategien könnten auch in anderen Glucosid-sequestrierenden Insekten eine Rolle spielen. Ich habe außerdem gezeigt, dass sich die Fähigkeit Glucosinolate zu sequestrieren stark zwischen Larven und Adulten von *P. armoraciae* unterscheidet. Diese Diskrepanz weist auf abweichende Mechanismen oder eine unterschiedliche Effizienz der beiden Lebensstadien in der Unterbindung des Glucosinolatabbaus hin. Obwohl *P. armoraciae* Larven und Adulte schädlichen Glucosinolathydrolyseprodukten ausgesetzt sind, gibt es keinen messbaren negativen Langzeiteffekt auf diesen spezialisierten Käfer. Zusammengefasst zeigen die erlangten Ergebnisse eine gute Anpassung dieses Spezialisten an die chemische Abwehr seiner Wirtspflanzen (Brassicaceen).

Für die erfolgreiche chemische Abwehr ist der Käfer auf seine Wirtspflanze angewiesen, um die Substrate zur Verteidigung zu erlangen. Ich fand allerdings Unterschiede zwischen Lebensstadien von *P. armoraciae* in der Fähigkeit sequestrierte Glucosinolate zur Abwehr zu nutzen. Während Glucosinolate in allen Lebenstadien vorhanden sind, variiert die endogene Myrosinaseaktivität stark zwischen den Lebensstadien. Larven besitzen die höchste Aktivität und Puppen die niedrigste. Folglich schwankt die Fähigkeit toxische Isothiocyanate zur Abwehr zu bilden innerhalb des Enzwicklungszyklus des Käfers sehr stark. Prädationsexperimente zeigten, dass sowohl Glucosinolate als auch endogene Myrosinaseaktivität in *P. armoraciae* vorhanden sein müssen, um eine erfolgreiche Abwehr und ein erfolgreiches Überleben gegen den asiatischen Marienkäfer, ein generalistischer Räuber, zu gewährleisten. Insgesamt zeigt dies, dass sequestrierte pflanzliche Metaboliten bestimmte Lebensstadien dieses Käfers selektiv vor Fraßfeinden schützt.

Diese Doktorarbeit trägt wesentliche Erkenntnisse über die tritrophischen Interaktionen des Meerrettich-Erdflohs *P. armoraciae* mit seinen Wirtspflanzen (Brassicaceen) auf der einen Seite und einem generalistischen Fraßfeind auf der anderen Seite bei. Die Sequestrierung von pflanzlichen Metaboliten in Käfern ist ein komplexer Prozess, um die Abwehr der Wirtspflanze zu überwinden und diese Abwehr gleichzeitig für die eigene Verteidigung zu nutzen (Figure 9). Diese Arbeit liefert Kenntnisse über verschiedene Faktoren und Prozesse, die an der Sequestrierung pflanzlicher Metaboliten beteiligt sind, sowie über deren ökologische Funktion in *Phyllotreta* und stellt eine Grundlage für weitere Forschung auf diesem Gebiert dar.

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10. Publications and Presentations

Publications

Kirsch, R., Vurmaz, E., Schaefer, C., Eberl, F., Sporer, T., Haeger, W., Pauchet, Y. (2020). Plants use identical inhibitors to protect their cell wall pectin against microbes and insects. Ecology and Evolution, 10(8), 3814-3824. doi:10.1002/ece3.6180.

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In preparation to be submitted to Frontiers in Plant Science (Plant Pathogen Interactions): Sporer, T, Körnig, J., Wielsch, N., Gebauer-Jung, S., Reichelt, M., Hupfer, Y., Beran F. How a glucosinolate-sequestering flea beetle copes with plant myrosinase.

Oral Presentations

Sporer T. (2019). Eat a bomb to be a bomb - how flea beetles defend themselves against predators. Talk presented at Institute Symposium, Max Planck Institute for Chemical Ecology, Jena, DE

Sporer T. (2017). How can a chewing insect sequester glucosinolates? Talk presented at 4th International Glucosinolate Conference 2017, Berlin, DE

Sporer T. (2017). How do flea beetles overcome the 'mustard-oil-bomb'? Talk presented at 16th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

Poster Presentations

Sporer T. (2019). Sequestered glucosinolates protect *Phyllotreta* flea beetles from a generalist predator. Poster presented at Gordon Research Conference - Plant-Herbivore Interaction: Advancing Plant-Herbivore Interactions Through Complementary Approaches and the Blurring of Disciplinary Boundaries, Gordon Research Conferences, Ventura, CA, US

Ren L.-L., Sporer T., Gikonyo M., Yang Z.-L., Beran F. (2018). How important is the detoxification of isothiocyanates by conjugation to glutathione in the cabbage stem flea beetle? Poster presented at Institute Symposium, MPI für Chemische Ökologie, Jena, DE

Sporer T., Yang Z.-L., Beran F. (2018). Assembling the beetle mustard-oil bomb: Glucosinolate sequestration and myrosinase activity in *Phyllotreta armoraciae*. Poster presented at 17th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

Sporer T., Yang Z.-L., Beran F. (2017). *Phyllotreta armoraciae* flea beetles combine two strategies to sequester intact glucosinolates from crucifer plants. Poster presented at 16th Symposium on Insect-Plant Interactions, Tours, FR

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Sporer T., Yang Z.-L., Gershenzon J., Heckel D.G., Beran F. (2016). How fast is the sequestration of dietary glucosinolates in *Phyllotreta armoraciae*? Poster presented at 15th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Sporer T., Gershenzon J., Heckel D.G., Beran F. (2015). Kidnapping (de)activated defense: how can *Phyllotreta* sequester intact glucosinolates? Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

11. Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die aktuell geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt ist. Entsprechend § 5 Abschnitt 4 der Promotionsordnung bestätige ich, dass ich diese Dissertation selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe. Alle verwendeten Hilfsmittel und Quellen sind in der Dissertation bzw. den entsprechenden Manuskripten eindeutig aufgeführt. Personen, welche bei der Erstellung und Auswertung der Versuche sowie der Manuskripte mitgewirkt haben, sind an den entsprechenden Stellen entweder als Autoren oder in der Danksagung aufgelistet. Des Weiteren versichere ich, dass keine kommerzielle Promotionsvermittlung in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir im Zusammenhang mit Inhalten der vorgelegten Dissertation erhalten haben. Die vorgelegte Dissertation wurde zu keinem früheren Zeitpunkt, weder in dieser noch in wesentlichen Teilen ähnliche staatliche oder wissenschaftliche Prüfungsarbeit, eingereicht.

Theresa Sporer Jena, den 14.12.2020

12. Supplementary Data

12.1. Manuscript I – Supplementary data

Supplementary Tables

Table S1 Glucosinolate (GLS) concentrations of 6-week-old feeding-damaged leaves of different *Arabidopsis* lines

GLS side chain	nmol GLS per mg plant fresh weight (mean ± SD; *N* = 50)		
	wild type	*myb*	*cyp*
3MSOP	0.222 ± 0.069	—	0.173 ± 0.067
4MSOB	1.829 ± 0.584	—	1.471 ± 0.598
4MTB	0.049 ± 0.034	—	0.038 ± 0.022
5MSOP	0.060 ± 0.017	—	0.057 ± 0.016
7MSOH	0.019 ± 0.007	—	0.022 ± 0.009
8MSOO	0.095 ± 0.049	—	0.093 ± 0.056
I3M	0.399 ± 0.112	0.520 ± 0.192	—
4MOI3M	0.108 ± 0.036	0.130 ± 0.040	—
1MOI3M	0.002 ± 0.001	0.004 ± 0.002	—
Total	2.781 ± 0.795	0.653 ± 0.215	1.855 ± 0.725

—, not detected. wild type, *Arabidopsis* Col-0; *myb*, *myb28myb29* mutant; *cyp*, *cyp79b2cyp79b3* mutant; GLS side chain abbreviations: 3MSOP, 3-methylsulfinylpropyl; 4MSOB, 4-methylsulfinylbutyl; 4MTB, 4-methylthiobutyl; 5MSOP, 5-methylsulfinylpentyl; 7MSOH, 7-methylsulfinylheptyl; 8MSOO, 8-methylsulfinyloctyl; I3M, indol-3-ylmethyl; 4MOI3M, 4-methoxyindol-3-ylmethyl; 1MOI3M, 1-methoxyindol-3-ylmethyl.

Table S2 GLS concentrations in newly emerged *P. armoraciae* adults and after five days of feeding on three different *Arabidopsis* lines

GLS side chain	nmol sequestered GLS per mg beetle (mean ± SD (median), *N* = 10)				Statistical method	Statistics	*P* value
	Newly emerged beetles	Beetles fed on wild type	Beetles fed on *myb*	Beetles fed on *cyp*			
Allyl	34.107 ± 6.430 (34.339) a	19.810 ± 1.735 (19.299) c	26.330 ± 5.096 (25.985) b	21.792 ± 5.003 (23.407) c	Generalized least squares	*LR* = 29.190	< 0.001
3But	0.496 ± 0.285 (0.439) a	0.760 ± 0.286 (0.780) a	0.097 ± 0.090 (0.059) b	0.627 ± 0.229 (0.535) a	ANOVA[1]	*F* = 25.489	< 0.001
3MSOP	—	1.133 ± 0.172 (1.206) a	—	0.859 ± 0.146 (0.851) b	Student's *t*-test	*t* = 3.847	= 0.001
4MSOB	—	3.579 ± 1.209 (3.412)	—	3.051 ± 0.983 (3.293)	Student's *t*-test	*t* = 1.071	= 0.298
4MTB	—	7.436 ± 1.067 (7.668)	—	6.557 ± 1.108 (6.330)	Student's *t*-test	*t* = 1.805	= 0.088
5MSOP	—	0.184 ± 0.053 (0.176)	—	0.197 ± 0.043 (0.213)	Student's *t*-test	*t* = -0.633	= 0.535
7MSOH	—	0.073 ± 0.027 (0.064) b	—	0.145 ± 0.064 (0.122) a	Mann-Whitney *U* test	*U* = 12.000	= 0.005
8MSOO	—	0.684 ± 0.079 (0.658)	—	0.865 ± 0.314 (0.814)	Mann-Whitney *U* test	*U* = 32.000	= 0.186
I3M	0.041 ± 0.034 (0.032) c	1.177 ± 0.350 (1.194) b	4.076 ± 1.060 (3.979) a	0.030 ± 0.023 (0.024) c	Generalized least squares	*LR* = 54.850	< 0.001
4MOI3M	0.025 ± 0.017 (0.022) c	0.046 ± 0.033 (0.037) b	0.296 ± 0.129 (0.282) a	0.008 ± 0.013 (0.000) d	Generalized least squares[2]	*LR* = 49.157	< 0.001
1MOI3M	—	0.008 ± 0.009 (0.005) b	0.049 ± 0.017 (0.050) a	—	Student's *t*-test	*t* = -6.677	< 0.001
Total	34.668 ± 6.611 (34.915)	34.889 ± 1.994 (34.637)	30.847 ± 4.315 (30.718)	34.131 ± 5.136 (33.858)	Generalized least squares	*LR* = 6.309	= 0.098

[1]Data were square-root transformed prior to analysis. [2]Data were log transformed prior to analysis. GLS concentrations labeled with different letters are significantly different ($P < 0.05$). LR, likelihood ratio; —, not detected; 3But, 3-butenyl; for other abbreviations refer to the legend of Table S1

Table S3 GLS amounts of newly emerged *P. armoraciae* adults and after five days of feeding on three different *Arabidospis* lines

GLS side chain	nmol sequestered GLS per adult (mean ± SD (median); $N = 10$)				Statistical method	Statistics	*P* value
	Newly emerged beetles	Beetles fed on wild type	Beetles fed on *myb*	Beetles fed on *cyp*			
Allyl	65.060 ± 10.849 (66.440) a	41.818 ± 7.758 (40.890) c	56.437 ± 12.800 (55.330) ab	45.775 ± 10.630 (47.865) bc	ANOVA	$F = 9.746$	< 0.001
3But	0.947 ± 0.515 (0.884) b	1.597 ± 0.635 (1.575) a	0.193 ± 0.162 (0.120) c	1.332 ± 0.515 (1.155) ab	ANOVA[1]	$F = 25.891$	< 0.001
3MSOP	—	2.371 ± 0.417 (2.404) a	—	1.819 ± 0.400 (1.762) b	Student's *t*-test	$t = 3.021$	= 0.007
4MSOB	—	7.366 ± 1.925 (6.943)	—	6.299 ± 1.627 (6.729)	Student's *t*-test	$t = 1.340$	= 0.197
4MTB	—	15.738 ± 3.466 (16.099)	—	13.973 ± 3.293 (13.954)	Student's *t*-test	$t = 1.167$	= 0.258
5MSOP	—	0.381 ± 0.108 (0.367)	—	0.415 ± 0.095 (0.426)	Student's *t*-test	$t = -0.738$	= 0.470
7MSOH	—	0.150 ± 0.048 (0.146) b	—	0.301 ± 0.120 (0.283) a	Mann-Whitney *U* test	$U = 6.000$	= 0.001
8MSOO	—	1.438 ± 0.248 (1.397)	—	1.798 ± 0.578 (1.744)	Mann-Whitney *U* test	$U = 30.000$	= 0.140
I3M	0.084 ± 0.080 (0.060) c	2.476 ± 0.822 (2.574) b	8.754 ± 2.423 (9.534) a	0.061 ± 0.047 (0.053) c	Generalized least squares	$LR = 51.434$	< 0.001
4MOI3M	0.048 ± 0.033 (0.044) b	0.093 ± 0.056 (0.078) b	0.626 ± 0.263 (0.584) a	0.016 ± 0.027 (0.000) c	ANOVA[2]	$F = 54.521$	< 0.001
1MOI3M	—	0.017 ± 0.021 (0.009) b	0.106 ± 0.040 (0.108) a	—	Mann-Whitney *U* test	$U = 1.500$	< 0.001
Total	66.139 ± 11.220 (67.185)	73.446 ± 11.438 (71.724)	66.116 ± 12.010 (66.353)	71.788 ± 11.063 (71.739)	ANOVA	$F = 1.108$	= 0.359

[1]Data were square-root transformed prior to analysis. [2]Data were log transformed prior to analysis. GLS amounts labeled with different letters are significantly different ($P < 0.05$). LR, likelihood ratio; —, not detected. For GLS and host plant abbreviations, refer to legend of Table S1 and Table S2.

Table S4 GLS amounts detected in *P. armoraciae* feces after feeding on different *Arabidopsis* lines for five days

GLS side chain	nmol excreted GLS per beetle (mean ± SD (median); *N* = 10)			Statistical method	Statistics	*P* value
	wild type	*myb*	*cyp*			
Allyl	9.946 ± 3.364 (9.896) a	0.879 ± 0.873 (0.648) b	7.601 ± 3.069 (7.475) a	ANOVA[2]	$F = 54.564$	< 0.001
3MSOP	1.737 ± 0.406 (1.833) a	—	0.748 ± 0.330 (0.809) b	Student's *t*-test	$t = 5.983$	< 0.001
4MSOB	13.456 ± 2.961 (13.967) a	—	5.341 ± 1.986 (5.768) b	Student's *t*-test	$t = 7.197$	< 0.001
5MSOP	1.172 ± 0.264 (1.083) a	—	0.823 ± 0.324 (0.797) b	Student's *t*-test	$t = 2.638$	= 0.017
7MSOH	0.409 ± 0.085 (0.382) a	—	0.242 ± 0.088 (0.245) b	Student's *t*-test	$t = 4.325$	< 0.001
8MSOO	0.912 ± 0.234 (0.958) a	—	0.539 ± 0.232 (0.537) b	Student's *t*-test	$t = 3.582$	= 0.002
I3M	2.632 ± 0.709 (2.545) a	0.741 ± 0.335 (0.700) b	*0.000 ± 0.000* (*0.000*)[1]	Student's *t*-test	$t = 7.629$	< 0.001
4MOI3M	0.525 ± 0.101 (0.527)	0.498 ± 0.147 (0.480)	*0.001 ± 0.004* (0.000)[1]	Student's *t*-test	$t = 0.477$	= 0.639
Total	30.789 ± 7.344 (33.095) a	2.118 ± 0.902 (2.068) c	15.295 ± 4.851 (13.631) b	ANOVA[3]	$F = 136.860$	< 0.001

—, not detected. [1]If a GLS was only detected in one sample, the mean is written in italics and data was not included in the statistical analysis. [2]Data were square-root transformed prior to analysis. [3]Data were log transformed prior to analysis. GLS amounts labeled with different letters are significantly different ($P < 0.05$). For GLS and food plant abbreviations, refer to legend of Table S1 and S2.

Supplementary Figures

Fig. S1 Representative feeding patterns of five *P. armoraciae* adults on *Arabidopsis* wild type, *myb28myb29* (*myb*), and *cyp79b2cyp79b3* (*cyp*), respectively, for one day.

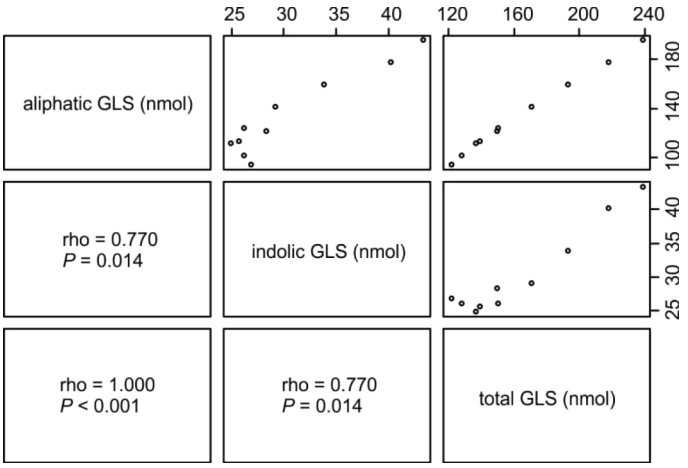


Fig. S2 Spearman's rank correlation matrix between the ingested aliphatic GLS, indolic GLS and total GLS amounts by *P. armoraciae* adults from *Arabidopsis* wild type in five days. The correlation coefficients and significance, and scatterplot matrices are show on the left and right side of the diagonal, respectively. Aliphatic GLS: sum of 3MSOP GLS, 4MSOB GLS, 4MTB GLS, 5MSOP GLS, 7MSOH GLS, 8MSOO GLS; indolic GLS: sum of I3M GLS, 1MOI3M GLS, 4MOI3M GLS. For GLS abbreviations, refer to legend of Table S1.

Supplementary Information and Figure on 4-methylsulfinylbutyl GLS metabolism in the cabbage stem flea beetle, *Psylliodes chrysoecphala*

Metabolism of 4-methylsulfinylbutyl GLS in the cabbage stem flea beetle Psylliodes chrysocephala

To determine whether the cabbage stem flea beetle, *Psylliodes chrysocephala* (L.) (Chrysomelidae), metabolizes sequestered 4-methylsulfinylbutyl (4MSOB) GLS to 4-methylthiobutyl- (4MTB) and 3-butenyl (3But) GLS, we performed a feeding experiment with newly emerged beetles. *P. chrysocephala* adults were reared on three to four-week-old potted *Brassica rapa* cv. Yu Tsai Sum plants (Known-You Seed Co. Ltd., Kaohsiung, China) as described in Beran et al. (2018a). Newly emerged adults were placed dorsally onto sticky tape and forced to drink 0.2 µL of an aqueous solution containing 10 nmol of 4MSOB GLS (purchased from Phytoplan, Heidelberg, Germany) using a pipette. Beetles fed with pure water served as a control. To allow the metabolism of ingested and sequestered 4MSOB GLS, fed beetles were kept in Petri dishes with detached leaves of *B. rapa* for three days. Afterwards, adults were frozen in liquid nitrogen and stored at -20°C until GLS analysis. There were four and six biological replicates for the control and 4MSOB GLS treatments, respectively, each consisting of five adults.

We detected a minor amount of 4MTB GLS as a contaminant in the 4MSOB GLS solution that was fed to *P. chrysocephala*. However, the 4MTB GLS amount detected in *P. chrysocephala* beetles was significantly lower than that detected in the fed 4MSOB GLS solution (Fig. S3; Student's *t*-test, 4MTB GLS: $t = 37.269$, $P < 0.001$), which indicates that *P. chrysocephala* does not metabolize significant quantities of sequestered 4MSOB GLS to 4MTB GLS. 3But GLS is known to be present in *P. chrysocephala* beetles reared on *B. rapa* (Beran et al., 2018). In our experiment, we found no difference between the 3But GLS levels in control beetles and 4MSOB GLS-fed beetles (Fig. S3; Student's *t*-test, 3But GLS: $t = 0.919$, $P = 0.385$), making it unlikely that *P. chrysocephala* adults convert 4MSOB GLS to 3But GLS.

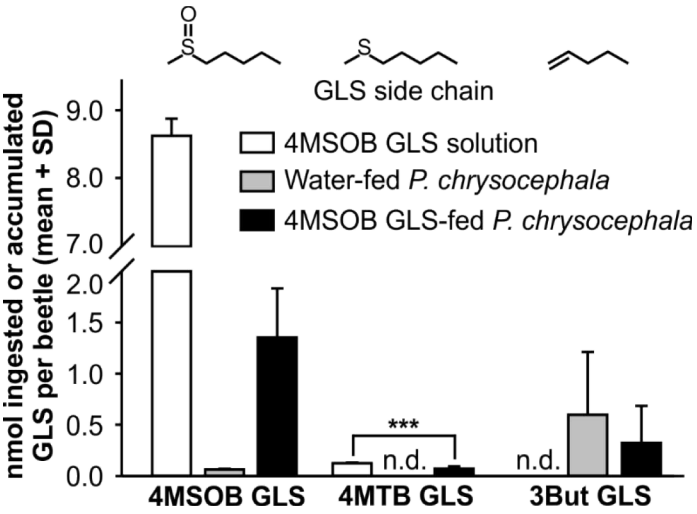


Fig. S3 *Psylliodes chrysocephala* adults do not metabolize sequestered 4-methylsulfinylbutyl (4MSOB) GLS. Adults were fed with an aqueous 4MSOB GLS solution or water as a control, and harvested for GLS extraction after three days feeding on *Brassica rapa* leaves ($N = 4 – 6$). The 4MSOB GLS solution fed to adults contained a small amount of 4-methylthiobutyl (4MTB) GLS as a contaminant. The 4MTB GLS amount in the fed 4MSOB GLS solution was compared with the 4MTB GLS amount detected in *P. chrysocephala* by Student's *t*-test. 3-Butenyl (3But) GLS was not detected in the 4MSOB GLS solution, but was detected in control beetles and fed beetles. 3But GLS amounts in adults were log-transformed and compared by Student's *t*-test. n.d., not detected, ***$P < 0.001$.

Reference

Beran, F., Sporer, T., Paetz, C., Ahn, S.-J., Betzin, F., Kunert, G., Shekhov, A., Vassão, D.G., Bartram, S., Lorenz, S. & Reichelt, M. (2018) One pathway is not enough: The cabbage stem flea beetle *Psylliodes chrysocephala* uses multiple strategies to overcome the glucosinolate-myrosinase defense in its host plants. *Frontiers in Plant Science,* **9,** 1754.

12.2. Manuscript II – Supplementary data

SUPPLEMENTARY METHODS AND RESULTS

Chemical analyses of *A. thaliana* wild type and *tgg* plants

For chemical analyses of *A. thaliana* wild type and *tgg* plants, we harvested whole rosettes of six-week old plants (n = 10). Rosettes were frozen in liquid nitrogen, freeze-dried and homogenized using metal beads (2.4 mm diameter, Askubal) for 2 min at 25 Hz in a TissueLyser II (Qiagen). Glucosinolates were extracted from 20 mg plant powder, converted to desulfo-glucosinolates and analyzed by HPLC-DAD as described in Beran et al. (2014). To determine the soluble protein content, 10 mg plant powder were extracted with 900 µL of 20 mM MES buffer pH 6.5 by shaking with metal beads for 2 min at 20 Hz in a TissueLyser II, followed by centrifugation at 4°C for 10 min at 16,000 × g. Protein levels in supernatants were determined using the Bradford protein assay (Bio-Rad). Amino acids and sugars were analyzed by LC-MS/MS. Therefore, 15 mg plant powder were extracted with 1 mL of 80% MeOH by shaking and centrifugation as described above. The supernatant was diluted 1:10 with water containing a mix of $^{15}N/^{13}C$ labeled algal amino acids in a concentration of 10 µg × mL^{-1} (Isotec). Amino acids were analyzed by LC-MS/MS on a Zorbax Eclipse C18-column (XDB-C18, 50 x 4.6 mm x 1.8 µm; Agilent, Santa Clara, CA, USA) (for details, refer to Crocoll et al. (2016)). Each amino acid was quantified relative to the peak area of its corresponding labeled amino acid, except for tryptophan (using phenylalanine and applying a response factor of 0.42) and asparagine (using aspartate and a response factor of 1.0). Soluble sugars were analyzed from the 1:10-diluted extract by LC-MS/MS on a hydrophilic interaction liquid chromatography (HILIC)-column (apHera-NH_2 Polymer; 15 × 4.6 mm, 5 µm; Supelco) as described in Madsen et al. (2015). Sugars were quantified using an external standard curve prepared from authentic standards of glucose, fructose, sucrose (all obtained from Sigma-Aldrich) and raffinose (Fluka).

Partial purification of myrosinase from *S. alba*

We purified commercially available myrosinase enzyme (100 units (U) per g solid, isolated from *Sinapis alba* seeds; Sigma Aldrich) by fast protein liquid chromatography. The crude enzyme extract (ca. 25 U) was dissolved in 1 mL buffer (20 mM Tris·HCl, 0.15 M NaCl, pH 8, containing protease inhibitors (cOmplete, EDTA-free)) and subjected to size exclusion chromatography using

a Superdex 200 10/300 GL column (GE Healthcare) as described in Beran et al. (2014). Fractions were tested for myrosinase activity as described below. Active fractions were pooled and desalted using Zeba Spin Desalting Columns (7KDa MWCO, 5mL; Thermo Fisher Scientific) equilibrated with 20 mM Tris HCl pH 8 before anion exchange chromatography using a 1-mL ResourceQ column (GE Healthcare) as described in Beran et al. (2014). Fractions containing myrosinase activity were pooled and dialyzed overnight against 20 mM MES buffer pH 6.5 containing 20% (v/v) glycerol at 4°C using a Slide-A-Lyzer Dialysis Cassette (10K MWCO, 3 mL; Thermo Fisher Scientific). After dialysis, protease inhibitors (cOmplete, EDTA-free, Roche) were added, the protein concentration determined using the Bradford protein assay (Bio-Rad) and the extract was stored at 4°C.

Protein fractions were screened for myrosinase activity using a protocol modified from Travers-Martin et al. (2008). Assays consisted of 5 µL sample, 45 µL 20 mM MES buffer (pH 6.5) containing 2.78 mM allyl glucosinolate (Carl Roth) as substrate, and 50 µL assay reagent (20 mM MES buffer (pH 6.5) containing 57 U/ml glucose-oxidase (E.C.1.1.3.4, from *Aspergillus niger*, Serva), 5.6 U/ml peroxidase (E.C.1.11.1.7, from horseradish, Serva), 30.7 mM phenol (Sigma Aldrich) and 2.8 mM 4-aminoantipyrine (Sigma Aldrich)). Assays were incubated at room temperature for 30 min in transparent polystyrene 96-well microplates (Nunc, Thermo Fisher Scientific). Myrosinase activity was visually detected (pink assay color).

pH measurements of *P. armoraciae* gut homogenates

We determined the midgut pH of adults collected from *B. juncea* rearing cages (n = 6). Dissected midguts containing plant material were homogenized in 40 µL deionized water and the pH was measured using an InLab Micro electrode (Mettler-Toledo, Schwerzenbach, Switzerland). The pH of midgut homogenates was 4.7 ± 0.2 (mean ± SD).

Sequestration experiment with *P. armoraciae* larvae

Previous research showed that *P. armoraciae* larvae sequestered glucosinolates from myrosinase-deficient *tgg* plants, but only traces of glucosinolates from *Arabidopsis* wild type plants (Sporer et al., 2020). To determine whether ingested 4MSOB glucosinolate is hydrolyzed during feeding and digestion in *P. armoraciae* larvae, we performed a feeding experiment with *Arabidopsis* wild type, *tgg* and *myb* control leaves. The feeding experiment was performed as described for *Experiment 2* (see main manuscript) except that the midrib of *Arabidopsis* leaves was carefully removed using a

scalpel to prevent larvae from mining. Larvae, feces, and remaining leaves were sampled, extracted and analyzed by LC-MS/MS (for 4MSOB glucosinolate and derived metabolites) as described for adult beetles (n = 5, with 5 larvae per replicate).

In contrast to adult beetles (Figure 1), larvae fed on wild type plants contained only traces of 4MSOB glucosinolate and the corresponding proportion was significantly higher in *tgg*-fed than in wild type-fed larvae (Supplementary Figure 1, Supplementary Table 1 and 10). 4MSOB cyanide was the major proportion of all detected metabolites in wild type-fed larvae, being significantly higher in this treatment as compared to the proportion in *tgg*-fed larvae (Supplementary Figure 1, Supplementary Table 1). Moreover, the proportions of 4MSOB isothiocyanate and 4MSOB isothiocyanate-derived metabolites were significantly higher in *tgg*-fed than in wild type-fed larvae. The distinct proportions of all metabolites excreted by larvae, 4MSOB glucosinolate and derived metabolites, did not differ between treatments, but the major proportion excreted in both treatments was 4MSOB cyanide (Supplementary Figure 1, Supplementary Table 1). Regarding the total amounts of the distinct metabolites detected in both larvae and feces, only 4MSOB isothiocyanate and derived metabolites were significantly higher in *tgg*-fed compared to wild type fed larvae, whereas the remaining metabolites in larvae and feces did not differ between treatments (Supplementary Table 10). Plant 4MSOB glucosinolate levels did not differ between both genotypes (Student's *t*-test, $t = -0.132$, $p = 0.898$). Taken together, these results showed that plant myrosinases hydrolyzed the majority of 4MSOB glucosinolate during larval feeding and digestion, and independent of plant myrosinases, the major proportions detected in both larvae and feces were 4MSOB glucosinolate-derived hydrolysis products.

Performance experiment with *P. armoraciae* larvae

Since glucosinolates are hydrolyzed during feeding of larvae on *Arabidopsis* wild type plants, we conducted a similar long-term performance experiment with *Arabidopsis* wild type and *tgg* plants as for adults (see main manuscript) with some modifications: early second instar larvae were randomly assigned to one of the two *Arabidopsis* genotypes and provided with a steady supply of new leaves cut from undamaged plants until development to mature larvae (prepupae). The time until the development to the prepupal stage was recorded and each prepupa (n = 68-74) was weighed, frozen in liquid nitrogen and stored at -20°C until the analysis of energy reserves (i.e. total levels of soluble protein, lipids, glycogen, and soluble carbohydrates, see performance experiment in the main manuscript). Prepupal energy reserves were analyzed in 22 randomly

selected prepupae from each treatment. Two individuals were pooled and analyzed (n = 11 per *Arabidopsis* genotype).

Larvae did not differ in developmental time from early second instar larvae to prepupae between both wild type and *tgg* treatments (wild type-fed: 33 ± 2 days, *tgg*-fed: 34 ± 3 days, mean ± SD; Mann-Whitney rank sum test, U = 2,319.500, $p = 0.420$, n = 68-74). Prepupae did not differ in the fresh weight and the analyzed energy reserves between treatments (Supplementary Table 11).

SUPPLEMENTARY TABLES

Supplementary Table 1: Methods and results of statistical analyses.

Experiment	Comparison	Statistical method	Variable	Statistics	*p* value
Experiment 1	Relative 4MSOB and 4MTB glucosinolate accumulation in beetles (wild type vs. *tgg*)	Mann-Whitney rank sum test	Percentage[A]	$U = 482.000$	< 0.001
	Beetle feeding rate (cm^2) (wild type vs. *tgg*)	Student's *t*-test	Leaf area	$t = 0.592$	= 0.564
	Plant 4MSOB and 4MTB glucosinolate levels (wild type vs. *tgg*)	Mann-Whitney rank sum test	Concentration	$U = 390.000$	= 0.980
Experiment 2	4MSOB glucosinolate in beetles (wild type vs. *tgg*)	Student's *t*-test[B]	Percentage[A]	$t = 3.931$	= 0.016
	4MSOB-cyanide in beetles (wild type vs. *tgg*)			$t = 3.963$	= 0.016
	4MSOB isothiocyanate in beetles (wild type vs. *tgg*)	Mann-Whitney rank sum test[B]		$U = 0.000$	= 0.032
	Other 4MSOB isothiocyanate derived metabolites in beetles (wild type vs. *tgg*)			$U = 0.000$	= 0.032
	4MSOB glucosinolate in feces (wild type vs. *tgg*)	Student's *t*-test[B]		$t = 6.389$	= 0.004
	4MSOB-cyanide in feces (wild type vs. *tgg*)			$t = 6.686$	= 0.004
	4MSOB isothiocyanate in feces (wild type vs. *tgg*)			$t = 3.913$	= 0.016
	Other 4MSOB isothiocyanate derived metabolites in (wild type vs. *tgg*)	Mann-Whitney rank sum test[B]		$U = 5.000$	= 0.604
	4MSOB glucosinolate and derived metabolites in beetles (wild type vs. *tgg*)	Student's *t*-test, Mann-Whitney rank sum test	Amount	see Supplementary Table 2	
	4MSOB glucosinolate and derived metabolites in beetle feces (wild type vs. *tgg*)				
	Plant 4MSOB glucosinolate levels (wild type vs. *tgg*)	Student's *t*-test	Concentration	$t = 0.371$	= 0.720
Experiment 3	Recovery of ingested allyl glucosinolate in beetles and feces (wild type vs. *tgg*)	Student's *t*-test	Percentage[A]	see Table 1	
Experiment 4	Emitted AITC per beetle (allyl glucosinolate-spiked wild type vs. *tgg*)	Mann-Whitney rank sum test	Amount	$U = 0.000$	= 0.001
	Sequestered allyl glucosinolate per beetle (allyl glucosinolate-spiked wild type vs. *tgg*)	Student's *t*-test	Amount	$t = 1.689$	= 0.119
Long term feeding experiment	Beetle fresh weight before and after 4, 7 and 10 days of feeding (wild type)	one-way repeated measurements ANOVA	Fresh weight	$F = 61.787$	< 0.001
	Beetle fresh weight before and after 4, and 10 days of feeding (*tgg*)			$F = 42.213$	< 0.001

	Relative weight gain of beetles (wild type vs. *tgg*)	Mann-Whitney rank sum test	Percentage[A] 4d	$U = 92.000$	$= 0.017$
		Student's *t*-test	7d	$t = -2.699$	$= 0.011$
			10d	$t = 1.867$	$= 0.070$
	Beetle feeding rate (wild type vs. *tgg*)	Student's *t*-test	Leaf area 4d	$t = 1.451$	$= 0.156$
			7d	$t = 1.960$	$= 0.058$
			10d	$t = 2.631$	$= 0.013$
	Soluble protein and other metabolites (wild type vs. *tgg*)	Student's *t*-test, Mann-Whitney rank sum test	Concentration	see Supplementary Table 6	
Short-term feeding experiment and inhibition assays	4MSOB glucosinolate level in beetle gut (wild type vs. *tgg*)	Student's *t*-test	Percentage[A]	$t = 0.161$	$= 0.880$
	Plant myrosinase inhibition by gut extracts	one-way ANOVA	Activity	$F = 85.639$	< 0.01
Myrosinase activity in feces	Ingested and excreted myrosinase activity	Paired *t*-test[B]	Activity	$t = 10.449$	< 0.005
	Co-incubation of plant myrosinases with feces homogenates	Paired *t*-test		$t = 0.158$	$= 1.000$
Supplementary feeding experiment with larvae	4MSOB glucosinolate in larvae (wild type vs. *tgg*)	Mann-Whitney rank sum test[B]	Percentage[A]	$U = 0.000$	$= 0.032$
	4MSOB-cyanide in larvae (wild type vs. *tgg*)	Student's *t*-test[B]		$t = 17.788$	$= 0.004$
	4MSOB isothiocyanate in larvae (wild type vs. *tgg*)			$t = 15.341$	$= 0.004$
	Other 4MSOB isothiocyanate derived metabolites in larvae (wild type vs. *tgg*)			$t = 6.300$	$= 0.004$
	4MSOB glucosinolate in feces (wild type vs. *tgg*)			$t = 2.897$	$= 0.080$
	4MSOB-cyanide in larva feces (wild type vs. *tgg*)			$t = 2.359$	$= 0.184$
	4MSOB isothiocyanate in larva feces (wild type vs. *tgg*)			$t = 1.859$	$= 0.400$
	Other 4MSOB isothiocyanate derived metabolites in larva feces (wild type vs. *tgg*)			$t = 1.156$	$= 1.000$
	4MSOB glucosinolate and derived metabolites in larvae (wild type vs. *tgg*)	Student's *t*-test, Mann-Whitney rank sum test	Amount	see Supplementary Table 10	
	4MSOB glucosinolate and derived metabolites in larval feces (wild type vs. *tgg*)			see Supplementary Table 10	
	Plant 4MSOB glucosinolate levels (wild type vs. *tgg*)	Student's *t*-test	Concentration	$t = 0.132$	$= 0.898$
Supplementary long term feeding experiment with larvae	Larval development time (wild type vs. *tgg*)	Student's *t*-test	Days	$U = 2{,}319.500$	$= 0.420$
	Prepupal weight and energy reserves (wild type vs. *tgg*)	Student's *t*-test, Mann-Whitney rank sum test	Fresh weight or concentration	see Supplementary Table 11	

Wild type, *Arabidopsis* wild type; *tgg*, *Arabidopsis tgg;* A, Arcsin-square-root transformed; B, *p*-values adjusted for false discovery rate in multiple hypothesis testing with Benjamini–Hochberg method

Supplementary Table 2: Detected amounts of 4MSOB glucosinolate and derived metabolites in adult *P. armoraciae* beetles and feces after feeding on *Arabidopsis* wild type and *tgg* leaves for one day. Shown is the amount (nmol) per individual as mean ± SD (n = 5). Significant differences are marked in bold font.

	Beetle				Feces			
Metabolite	wild type-fed	*tgg*-fed	Statistics	*p*	wild type-fed	*tgg*-fed	Statistics	*p*
4MSOB glucosinolate	3.734 ± 2.862	6.229 ± 2.953	$t = -1.213$	0.260	0.129 ± 0.166	0.867 ± 0.917	$U = 5.000$	0.151
4MSOB cyanide[1]	0.483 ± 0.208	0.050 ± 0.048	$U = 1.000$	**0.016**	0.809 ± 0.460	0.078 ± 0.054	$U = 1.000$	**0.016**
4MSOB isothiocyanate	0.025 ± 0.020	0.006 ± 0.002	$U = 4.000$	0.095	0.040 ± 0.028	0.002 ± 0.001	$U = 0.000$	**0.008**
Other 4MSOB isothiocyanate-derived metabolites[2]	0.048 ± 0.025	0.008 ±0.004	$U = 2.000$	**0.032**	0.073 ± 0.038	0.013 ±0.017	$t = 2.882$	**0.020**

[1]corresponds to 4MSOB nitrile; [2]comprise 4MSOB isothiocyanate-glutathione conjugate, 4MSOB isothiocyanate-cysteinylglycine conjugate, 4MSOB isothiocyanate-cysteine conjugate. 2-(4-(methylsulfinyl)butylamino)-4,5dihydrothiazole-carboxylic acid, 4MSOB mine, 4MSOB acetamide

Supplementary Data

Supplementary Table 3: Glucosinolate concentration and profile in newly emerged *P. armoraciae* adults reared on *B. rapa.* Shown is mean ± SD (n = 20).

Glucosinolate sidechain	nmol glucosinolate × mg^{-1} fresh weight
3-butenyl	0.48 ± 0.53
4-pentenyl	0.90 ± 0.68
2-hydroxy-3-butenyl	3.66 ± 1.65
2-hydroxy-4-pentenyl	0.82 ± 0.54
5-methythiopentyl	1.53 ± 0.54
benzyl	0.19 ± 0.14
2-phenylethyl	0.35 ± 0.30
Indol-3-ylmethyl	0.23 ± 0.11
4methoxyindol-3-ylmethyl	0.06 ± 0.03
1-methoxyindol-3-ylmethyl	0.22 ± 0.08
Total	8.42 ± 3.26

Supplementary Table 4: Relative weight gain and feeding rate of newly emerged *P. armoraciae* adults after 4, 7 and 10 days feeding on *A. thaliana* wild type and *tgg* leaf discs (n = 18-19, mean ± SD). Significant differences are marked in bold font.

Parameter	4d			7d			10d		
	wild type-fed	*tgg*-fed	Statistics	wild type-fed	*tgg*-fed	Statistics	wild type-fed	*tgg*-fed	Statistics
Relative weight gain (%)	21.2 ± 8.4	28.3 ± 12.4	$U = 92.000$ **$p = 0.017$**	23.1 ± 10.7	34.5 ± 13.7	$t = -2.699$ **$p = 0.011$**	23.0 ± 11.7	30.6 ± 12.9	$t = -1.867$ $p = 0.070$
Consumed leaf area (cm^2)	1.4 ± 0.4	1.2 ± 0.3	$t = 1.451$ $p = 0.156$	2.3 ± 0.5	2.0 ± 0.5	$t = 1.960$ $p = 0.058$	3.3 ± 0.5	2.7 ± 0.7	$t = 2.631$ **$p = 0.013$**

Supplementary Data

Supplementary Table 5: Energy reserves of newly emerged *P. armoraciae* adults after feeding on six-week old *A. thaliana* wild type and *tgg* leaf discs for 10 days (mean ± SD).

Parameter	Sex	n	wild type-fed	*tgg*-fed	Statistics	*p*
µg soluble protein × mg FW^{-1}	both	18-19	61.35 ± 14.67	65.69 ± 15.51	$U = 145.000$	0.438
	male	8-9	52.20 ± 6.65	51.36 ± 3.87	$U = 34.000$	0.885
	female	10	69.59 ± 15.03	77.15 ± 11.19	$t = -1.210$	0.242
µg total lipids × mg FW^{-1}	both	18-19	3.06 ± 2.70	3.86 ± 3.69	$U = 151.000$	0.553
	male	8-9	1.34 ± 1.55	1.86 ± 3.16	$U = 34.000$	0.885
	female	10	4.61 ± 2.57	5.45 ± 3.29	$t = -0.604$	0.554
µg glycogen × mg FW^{-1}	both	18-19	19.52 ± 5.99	21.15 ± 8.82	$U = 354.000$	0.727
	male	8-9	22.07 ± 5.59	26.72 ± 10.22	$U = 27.000$	0.413
	female	10	17.22 ± 5.38	16.70 ± 3.46	$t = 1.099$	0.286
µg soluble carbohydrates × mg FW^{-1}	both	18-19	3.77 ± 2.13	2.93 ± 1.83	$U = 132.000$	0.242
	male	8-9	3.79 ± 2.87	2.77 ± 2.12	$U = 25.000$	0.312
	female	10	3.75 ± 1.08	3.06 ± 1.53	$U = 47.000$	0.850

Supplementary Table 6: Protein and metabolite concentrations in leaf material of six-week old *A. thaliana* wild type and *tgg* plants (n = 10; mean ± SD). Significant differences are marked in bold font.

		Arabidopsis wild type	*Arabidopsis tgg*	Statistics	*p*
Soluble protein (mg × g dry weight^{-1})		37.010 ±6.028	36.805 ± 7.269	t = 0.069	0.946
Amino acids (µmol × g dry weight^{-1})	Alanine	8.040 ± 1.418	8.016 ± 1.365	t = 0.038	0.970
	Arginine*	1.498 ± 0.509	1.207 ± 0.187	U = 30.000	0.140
	Asparagine	9.540 ± 2.831	8.687 ± 1.458	U = 44.000	0.678
	Aspartic acid	6.433 ± 0.764	6.862 ± 0.627	t = -1.302	0.209
	Glutamic acid	49.035 ± 5.004	50.804 ± 4.426	t = -0.794	0.437
	Glutamine	100.721 ± 17.043	109.767 ± 13.346	t = -1.254	0.226
	Histidine*	2.741 ± 0.341	2.909 ± 0.392	t = -0.968	0.346
	Isoleucine*	1.100 ± 0.188	1.041 ± 0.093	t = 0.847	0.408
	Leucine*	0.959 ± 0.128	0.898 ± 0.134	t = 0.991	0.335
	Lysine*	0.781 ± 0.088	0.747 ± 0.082	t = 0.836	0.414
	Methionine*	0.253 ± 0.022	0.237 ± 0.035	t = 1.185	0.252
	Phenylalanine*	0.800 ± 0.226	0.727 ± 0.092	U = 43.000	0.623
	Proline	3.984 ± 1.007	4.906 ± 1.017	U = 18.000	**0.017**
	Serine	16.839 ± 2.716	16.025 ± 0.996	U = 45.000	0.734
	Threonine*	11.012 ± 1.184	11.675 ± 0.850	t = -1.365	0.189
	Tryptophane*	0.139 ± 0.039	0.130 ± 0.016	U = 46.000	0.791
	Tyrosine	0.457 ± 0.087	0.392 ± 0.042	t = 2.013	0.059
	Valine*	1.820 ± 0.265	1.764 ± 0.128	t = 0.562	0.581
	Total essential amino acids*	21.103 ± 2.171	21.336 ± 1.176	t = -0.283	0.781
	Total non-essential amino acids	185.508 ± 23.637	196.770 ± 15.005	t = -1.207	0.243
	Total amino acids	206.611 ± 24.816	218.107 ± 15.327	t = -1.182	0.252
Soluble sugars (mg × g dry weight^{-1})	Glucose	0.154 ± 0.045	0.152 ± 0.044	t = 0.090	0.929
	Fructose	0.069 ± 0.019	0.049 ± 0.011	t = -2.796	**0.012**
	Sucrose	0.125 ±0.027	0.145 ± 0.016	t = 2.186	**0.042**
	Raffinose	0.080 ± 0.021	0.087 ± 0.009	U = 45.000	0.307
	Total sugars	0.428 ± 0.079	0.433 ± 0.060	t = 0.538	0.597
Glucosinolates (µmol × g dry weight^{-1})	3MSOP glucosinolate	1.924 ± 0.251	1.834 ± 0.130	U = 38.000	0.385
	4MSOB glucosinolate	15.071 ± 1.537	14.811 ± 1.253	t = 0.393	0.699
	5MSOP glucosinolate	0.510 ± 0.047	0.513 ± 0.042	t = -0.146	0.885
	7MSOH glucosinolate	0.249 ± 0.032	0.231 ± 0.016	t = 1.543	0.140
	8MSOO glucosinolate	1.209 ± 0.094	1.263 ± 0.082	t = -1.298	0.211
	4MTB glucosinolate	1.200 ± 0.132	1.183 ± 0.126	t = 0.285	0.779
	4OHI3M glucosinolate	0.022 ± 0.011	0.016 ± 0.010	t = 1.216	0.240
	I3M glucosinolate	1.756 ± 0.218	1.532 ± 0.138	t = 2.598	**0.018**
	4MOI3M glucosinolate	0.394 ± 0.035	0.368 ± 0.039	U = 28.000	0.104
	1MOI3M glucosinolate	0.088 ± 0.035	0.127 ± 0.105	U = 37.000	0.345
	Total glucosinolates	22.422 ± 1.902	21.877 ± 1.414	t = 0.689	0.500

Supplementary Table 6 continued: *, essential amino acids; 3MSOP, 3-methylsulfinylpropyl; 4MSOB, 4-methylsulfinylbutyl; 5MSOP, 5-methylsulfinylpentyl; 7MSOH, 7-methylsulfinylheptyl; 8MSOO, 8-

methylsulfinyloctyl; 4MTB, 4-methylthiobutyl; 4OHI3M, 4-hydroxyindol-3-ylmethyl; I3M, indol-3-ylmethyl; 4MOI3M, 4-methoxyindol-3-ylmethyl; 1MOI3M, 1-methoxyindol-3-ylmethyl;

Supplementary Table 7: Distribution of dietary glucosinolates in the gut and remaining body of *P. armoraciae* adults after 1 min feeding and 5 min digestion. Data were arcsine square root transformed before statistical analysis by Student's *t*-test (n = 3). Significant differences are marked in bold font

Treatment	4MSOB glucosinolate proportion (mean % ± SE)			
	Gut	Rest of body	*t*	*p*
wild type-fed	16.5 ± 2.6	83.5 ± 2.6	-8.529	**0.013**
tgg-fed	16.1 ± 2.2	83.9 ± 2.2	-10.103	**0.010**

Supplementary Table 8: *A. thaliana* TGG1-derived peptides detected by nano-UPLC-MS^E in feces extracts of *P. armoraciae* adults that had fed on *A. thaliana* wild type leaves. The numbers of the corresponding bands of the SDS-PAGE gel are listed

Amino acid sequence	Detected in protein band number	Sequence identity
LFNSGNFEK	20	TGG1
GFIFGVASSAYQVEGGR	20	TGG1
GLNVWDSFTHR	20	TGG1
GGADLGNGDTTCDSYTLWQK	19	TGG1
FSIAWSR	19, 20	TGG1 and TGG6
YYNGLIDGLVAK	19, 20	TGG1
NWITINQLYTVPTR	19, 20	TGG1
GYALGTDAPGR	19, 20	TGG1 and TGG2
DDQKGMIGPVMITR*	19	TGG1
GMIGPVMITR	19, 20	TGG1
WFLPFDHSQESK	19, 20	TGG1
LPEFSETEAALVK	19, 20	TGG1
GIYYVMDYFK	19, 20	TGG1
TTYGDPLIYVTENGFSTPGDEDFEK	20	TGG1

Supplementary Table 9: Proteins detected in the fecal proteome of *P. armoraciae* adults that had fed on *Arabidopsis* wild type leaves (Please, refer to the excel sheet "Supplementary Table 9" in the folder "Manuscript II – Supplements" on the attached CD).

Supplementary Table 10: Detected amounts of 4MSOB glucosinolate and derived metabolites in *P. armoraciae* larvae and feces after feeding on *Arabidopsis* wild type and *tgg* leaves after one day feeding. Shown is the amount (nmol) per individual as mean + SD (n = 5). Significant differences are marked in bold font.

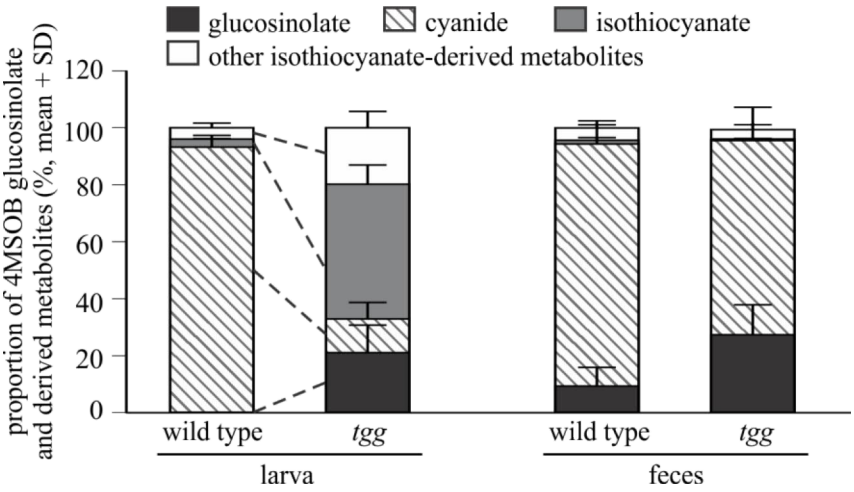
	Larva				Feces			
Metabolite	wild type-fed	*tgg*-fed	Statistics	*p*	wild type-fed	*tgg*-fed	Statistics	*p*
4MSOB glucosinolate	0.001 ± 0.001	0.372 ± 0.290	t = -2.558	0.034	0.215 ± 0.165	0.100 ± 0.078	t = -1.262	0.242
4MSOB cyanide[1]	0.684 ± 0.415	0.207 ± 0.130	U = 5.000	0.151	0.537 ± 0.342	1.158 ± 0.628	t = 1.737	0.121
4MSOB isothiocyanate	0.016 ± 0.008	0.728 ± 0.286	U = 0.000	**0.008**	0.004 ± 0.003	0.013 ± 0.007	t = 2.231	0.056
Other 4MSOB isothiocyanate-derived metabolites[2]	0.023 ± 0.011	0.334 ± 0.162	U = 0.000	**0.008**	0.027 ±0.030	0.051 ±0.027	t = 1.193	0.267

[1]corresponds to 4MSOB nitrile; [2]comprise 4MSOB isothiocyanate-glutathione conjugate, 4MSOB isothiocyanate-cysteinylglycine conjugate, 4MSOB isothiocyanate-cysteine conjugate. 2-(4-(methylsulfinyl)butylamino)-4,5dihydrothiazole-carboxylic acid, 4MSOB mine, 4MSOB acetamide

Supplementary Table 11: Fresh weight and energy reserves of *P. armoraciae* prepupae (mature larvae) that developed on six-week old *A. thaliana* wild type and *tgg* leaves from the early second instar. Mean ± SD are listed.

Parameter	n	wild type-fed	*tgg*-fed	Statistics	*p*
mg FW × prepupa^{-1}	68-74	1.34 ± 0.23	1.32 ± 0.22	t = 0.331	0.741
µg soluble protein × mg FW^{-1}	11	72.78 ± 6.91	77.47 ± 13.06	U = 49.000	0.470
µg total lipids × mg FW^{-1}	11	51.87 ± 3.42	41.77 ± 3.46	t = -1.586	0.129
µg glycogen × mg FW^{-1}	11	157.58 ± 34.98	128.68 ± 17.56	U = 52.000	0.599
µg soluble carbohydrates × mg FW^{-1}	11	55.52 ± 2.88	51.24 ± 4.96	t = 0.526	0.605

SUPPLEMENTARY FIGURES



Supplementary Figure 1: Proportions of 4-methylsulfinylbuty (4MSOB) glucosinolate and derived hydrolysis products detected in the body and feces of *P. armoraciae* larvae fed with *Arabidopsis* wild type or myrosinase-deficient *tgg* leaves (n = 5). Glucosinolates and hydrolysis products were extracted with 50% methanol and analyzed by LC-MS/MS. Detected amounts of metabolites were expressed relative to the total amounts of all detected metabolites in larvae or feces (set to 100%). Dashed lines indicate significant differences between samples ($p < 0.05$). Statistical results are shown in Supplementary Table 1. 4MSOB cyanide corresponds to the 4MSOB nitrile. Other isothiocyanate-derived metabolites comprise 4MSOB isothiocyanate-glutathione conjugate, 4MSOB isothiocyanate-cysteinylglycine conjugate, 4MSOB isothiocyanate-cysteine conjugate, 2-(4-(methylsulfinyl)butylamino)-4,5dihydrothiazole-carboxylic acid, 4MSOB amine, and 4MSOB acetamide.

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12.3. Manuscript III – Supplementary data

SUPPLEMENTARY METHODS AND RESULTS

1. Comparison of GLS levels and myrosinase activity in leaves of *B. juncea* and *A. thaliana*

GLS levels were determined in four-week old *B. juncea* plants that had been fed upon by *P. armoraciae* adults for one week (about 10 beetles per plant; $n = 23$). From each plant, leaf discs (one leaf disc per true leaf, 20 mm diameter) were pooled, weighed, frozen in liquid nitrogen, and stored at -20°C. Samples were lyophilized and GLS were extracted and analysed by HPLC-DAD as described in Beran *et al.* (2014). The GLS concentration in feeding-damaged *B. juncea* leaves was lower than in all *P. armoraciae* life stages (Table S1).

To confirm the GLS levels of plants used for feeding of *P. armoraciae* larvae (described in section 2.2.1), we harvested one rosette leaf per *A. thaliana* plant ($n = 26$) and two leaf discs (20 mm diameter) per *B. juncea* plant ($n = 25$). Plant samples were processed as described above. The GLS concentrations in leaves of *B. juncea*, *A. thaliana* wild type, *tgg* and *mybcyp* mutant plants differed significantly (Table S5).

To compare the levels of myrosinase activity in *A. thaliana* wild type and *B. juncea* leaves, we harvested three rosette leaves per *A. thaliana* plant ($n = 7$) and six leaf discs (20 mm diameter, two leaf discs per third, fourth and fifth true leaf) per *B. juncea* plant ($n = 6$). The collected leaf material was weighed, frozen in liquid nitrogen and stored at -80°C until protein extraction. To determine soluble myrosinase activity, frozen plant material (ca. 350 mg fresh weight) was homogenised to a fine powder using metal beads (2.4 mm diameter, Askubal, Korntal-Münchingen, Germany) at 25 Hz in a precooled TissueLyzer II (Qiagen, Hilden, Germany) and extracted three times with 900 µL of 20 mM MES buffer pH 6.5 containing protease inhibitors (cOmplete, EDTA-free, Roche, Mannheim, Germany). One millilitre of the resulting crude extract was used for subsequent purification steps as described in Beran *et al.* (2018). Myrosinase activity assays were performed with 0.25 µg total protein and allyl GLS as a substrate. Although allyl GLS is not present in *A. thaliana* wild type leaves, purified myrosinase enzymes (TGG1 and TGG2) from *A. thaliana* were similarly active towards 4-methylsulfinylbutyl GLS and allyl GLS (Zhou *et al.* 2012). Under our assay conditions, plant myrosinase activity did not differ between *A. thaliana* wild type and

B. juncea leaves (0.07 ± 0.03 and 0.08 ± 0.02 nmol × min^{-1} × mg plant fresh $weight^{-1}$, respectively, mean ± SD; Student's *t*-test, $t = -1.108$, $p = 0.291$)

2. Compartmentalisation of GLS and myrosinase in the haemolymph of *P. armoraciae* larvae

Sequestered GLS and myrosinase activity were found to be co-localized in the haemolymph of *P. armoraciae* larvae, which raises questions about how *P. armoraciae* control GLS hydrolysis. In cyanogenic larvae of the six-spot burnet moth *Zygaena filipendulae*, cyanogenic glucosides are stored in the haemoplasma, whereas the corresponding cyanogenic β-glucosidase is localized in the haemocytes (Pentzold *et al.* 2017). To obtain evidence for a spatial separation of GLS and myrosinase in *P. armoraciae* larvae, we tested whether cell disruption by freezing and thawing induces GLS hydrolysis. Frozen third instar larvae were transferred into a volatile collection setup at room temperature for 6 hrs to quantify the amounts of emitted allyl isothiocyanate (AITC) as described in section 2.4. Afterwards, larvae were removed from the setup, frozen in liquid nitrogen, and stored at -20°C until GLS extraction (described in section 2.2). Larvae that were kept frozen served as a control. Each sample consisted of three larvae ($n = 12$). Thawed larvae emitted high amounts of AITC, which accounted for 73 ± 9% (mean ± SE) of the previously sequestered allyl GLS. The amounts of allyl GLS detected in thawed larvae corresponded to less than 1% of those detected in control larvae.

SUPPLEMENTARY TABLES

Table S1: Glucosinolate (GLS) profiles of feeding-damaged *B. juncea* leaves and different life stages of *P. armoraciae*.

GLS sidechain	Mean GLS concentration [nmol GLS × mg^{-1} fresh weight ± SD]						
	B. juncea	**Egg**	**L3**	**Prepupa**	**Pupa**	**Adult 0 d**	**Adult 14 d**
Allyl	7.52 ± 1.83	41.40 ± 15.32	23.15 ± 3.24	19.62 ± 3.86	34.97 ± 4.56	39.03 ± 4.05	42.91 ± 3.65
3But	0.50 ± 0.28	0.51 ± 0.26	0.15 ± 0.10	0.14 ± 0.07	0.36 ± 0.24	0.56 ± 0.31	0.59 ± 0.34
4OHI3M	0.01 ± 0.01	0.30 ± 0.13	0.03 ± 0.01	0.02 ± 0.02	0.01 ± 0.02	0.01 ± 0.02	n.d.
I3M	0.06 ± 0.02	tr.	0.10 ± 0.00	0.06 ± 0.03	0.04 ± 0.01	0.13 ± 0.05	0.61 ± 0.13
4MOI3M	tr.	tr.	0.08 ± 0.04	0.07 ± 0.03	0.08 ± 0.05	0.07 ± 0.03	0.07 ± 0.03
Benzyl	0.33 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	0.24 ± 0.03
1MOI3M	tr.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	8.13 ± 2.05	43.38 ± 15.58	23.50 ± 3.28	19.91 ± 3.86	35.47 ± 4.70	39.80 ± 3.96	44.43 ± 3.89

L3, third instar larva; n.d., not detected; tr., traces; GLS sidechains: 3But, 3-butenyl; 4OHI3M, 4-hydroxyindol-3-ylmethyl; I3M, indol-3-ylmethyl; 4MOI3M, 4-methoxyindol-3-ylmethyl; 1MOI3M, 1-methoxyindol-3-ylmethyl.

Table S2: Myrosinase activity in different life stages of *P. armoraciae*.

Life stage	**Mean myrosinase activity [nmol glucose × min^{-1} × mg^{-1} protein ± SD]**
Egg	9.69 ± 6.84
Third instar larva	69.72 ± 12.90
Prepupa	20.84 ± 4.97
Pupa	3.16 ± 1.17
Adult 0 d	48.95 ± 9.65
Adult 14 d	33.07 ± 4.25

Table S3: Interaction between *H. axyridis* and *P. armoraciae*: number of predator attacks and feeding time per attack within 10 min observation time.

Observation	**Number of predator attacks (feeding time per attack in seconds)**		
	***P. armoraciae* larva**	***P. armoraciae* pupa**	***P. armoraciae* adult**
1	5 (191, 14, 11, 201, 86)	1 (542)	0 (-)
2	2 (1, 5)	2 (2, 412)	0 (-)
3	2 (15, 4)	1 (497)	1* (-)
4	1 (1)	1 (34)	0 (-)
5	1 (10)	0 (-)	0 (-)
6	2 (5, 1)	1 (297)	0 (-)
7	4 (2, 1, 1, 3)	1 (535)	0 (-)
8	0 (-)	1 (275)	0 (-)
9	1 (1)	0 (-)	0 (-)
10	0 (-)	1 (1)	0 (-)
11	1 (1)	0 (-)	0 (-)
12	1 (12)	0 (-)	0 (-)
13	2 (54, 3)	1 (490)	0 (-)
14	3 (1, 1, 1)	1 (588)	0 (-)
15	1 (52)	0 (-)	0 (-)
16	1 (1)	1 (227)	0 (-)

**P. armoraciae* adult was attacked but not injured by *H. axyridis*.

Table S4: Survivorship of *P. armoraciae* pupae and third instar larvae in predator choice assays.

Survivorship observations (n)		**larva**	
		alive	killed
pupa	alive	8	2
	killed	30	15

Table S5: Total GLS concentrations in *B. juncea* and different *A. thaliana* lines and corresponding GLS profiles of fed *P. armoraciae* larvae.

	GLS sidechain	Mean GLS concentration [nmol × g^{-1} fresh weight ± SD]				Statistical method	Statistics	*p*-value
		B. juncea	***A. thaliana* wild type**	***A. thaliana mybcyp***	***A. thaliana tgg***			
Plants	Total	1.29 ± 0.96 b	1.41 ± 0.73 b	n.d.	2.53 ± 1.02 a	ANOVA	$F = 14.009$	≤ 0.001
P. armoraciae larvae fed on different plants	Allyl	4.82 ± 1.60 a	0.03 ± 0.07 c	0.10 ± 0.18 c	0.91 ± 0.59 b	Generalized least squares method	$LR = 61.955$	< 0.001
	4MTB	n.d.	0.03 ± 0.03 b	0.02 ± 0.03 b	1.60 ± 0.95 a	Kruskal Wallis ANOVA	$H = 27.477$	≤ 0.001
	3MSOP	n.d.	n.d.	n.d.	1.24 ± 0.28	-	-	-
	4MSOB	n.d.	0.26 ± 0.10 b	0.21 ± 0.21 b	4.01 ± 0.85 a	Kruskal Wallis ANOVA	$H = 26.032$	≤ 0.001
	5MSOP	n.d.	n.d.	n.d.	0.15 ± 0.04	-	-	-
	8MSOO	n.d.	0.02 ± 0.02 b	n.d.	0.17 ± 0.12 a	Mann-Whitney rank sum test	$U = 0.000$	≤ 0.001
	I3M	0.02 ± 0.02 b	0.03 ± 0.01 b	0.03 ± 0.02 b	0.16 ± 0.05 a	Kruskal Wallis ANOVA	$H = 29.256$	≤ 0.001
	4MOI3M	n.d.	0.01 ± 0.01	n.d.	0.02 ± 0.02	Mann-Whitney rank sum test	$U = 64.500$	= 0.411
	1MOI3M	n.d.	0.09 ± 0.02	n.d.	0.10 ± 0.02	Student's *t*-test	$t = -0.599$	= 0.555
	Total	4.84 ± 1.60 b	0.46 ± 0.18 c	0.36 ± 0.21 c	8.33 ± 2.42 a	Generalized least squares method	$LR = 74.500$,	< 0.001

GLS concentrations labeled with different letters are significantly different between treatments; *A. thaliana* wild type, *A. thaliana* Col-0; *A. thaliana mybcyp*, *A. thaliana myb28×myb29×cyp79B2×cyp79B3* quadruple knock-out mutant (no GLS, myrosinase); *A. thaliana tgg*, *A. thaliana tgg1×tgg2* double knock-out mutant (GLS, no myrosinase); n.d., not detected; GLS sidechains: 4MTB, 4-methylthiobutyl; 3MSOP, 3-methylsulfinylpropyl; 4MSOB, 4-methylsulfinylbutyl; 5MSOP, 5-methylsulfinylpentyl; 8MSOO, 8-methylsulfinyloctyl; I3M, indol-3-ylmethyl; 4MOI3M, 4-methoxyindol-3-ylmethyl; 1MOI3M, 1-methoxyindol-3-ylmethyl.

Table S6: Fresh weight of *H. axyridis* third instar larvae fed with *P. armoraciae* larvae containing low or high GLS levels for eight consecutive days.

Day	**Weight of *H. axyridis* larvae relative to the initial weight [mg, mean ± SE]**	
	Fed with low-GLS *P. armoraciae* larvae	**Fed with high-GLS *P. armoraciae* larvae**
2	+0.15 ± 0.2 (*n* = 19)	-0.50 ± 0.11 (*n* = 18)
3	+0.52 ± 0.14 (*n* = 18)	-0.58 ± 0.18 (*n* = 9)
4	+0.50 ± 0.29 (*n* = 15)	-0.77 ± 0.14 (*n* = 6)
5	+1.0 ± 0.23 (*n* = 14)	-0.67 ± 0.37 (*n* = 2)
6	+1.03 ± 0.27 (*n* = 13)	-0.58 (*n* = 1)
7	+1.02 ± 0.28 (*n* = 13)	-1.21 (*n* = 1)
8	+0.99 ± 0.31 (*n* = 11)	-

Table S7: GLS amounts and myrosinase activity in different body parts of *P. armoraciae* third instar larvae.

Body part	**Mean GLS amount [nmol GLS × individual^{-1}± SD]**	**Mean myrosinase activity [nmol glucose × min^{-1} × mg^{-1} protein ± SD]**
Gut	5.1 ± 0.8	8.1 ± 1.9
Haemolymph	77.9 ± 3.3	57.3 ± 6.3
Rest of body	17.0 ± 3.9	9.3 ± 2.1

SUPPLEMENTARY FIGURES

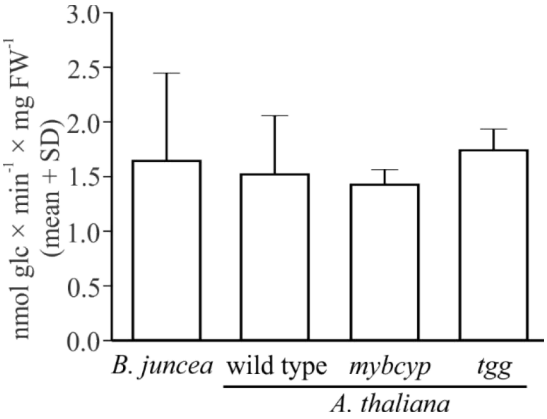

Figure S1: Myrosinase activity in *P. armoraciae* larvae fed on different plants. Soluble myrosinase activity was determined in crude protein extracts of *P. armoraciae* larvae that fed up to 13 days on *B. juncea* or on one of the three different *A. thaliana* lines. Myrosinase activity was determined as nmol released glucose (glc) per min and mg fresh weight using allyl GLS as substrate. Activities did not differ between treatments (ANOVA, $F = 0.383$, $p = 0.766$, $n = 6$). FW, fresh weight; *A. thaliana* wild type, *A. thaliana* Col-0; *A. thaliana mybcyp*, *A. thaliana myb28×myb29×cyp79B2×cyp79B3* quadruple knock-out mutant (no GLS, myrosinase); *A. thaliana tgg*, *A. thaliana tgg1×tgg2* double knock-out mutant (contains GLS but no myrosinase).

SUPPLEMENTARY VIDEO FILES

Video S1: Interaction of *H. axyridis* larvae and *P. armoraciae* larvae. (Please, refer to the video file “VideoS1.mp4” in the folder “Manuscript III - Supplements” on the attached CD)

Video S2: Interaction of *H. axyridis* larvae and *P. armoraciae* pupae. (Please, refer to the video file “VideoS2.mp4” in the folder “Manuscript III - Supplements” on the attached CD)

Video S3: Interaction of *H. axyridis* larvae and *P. armoraciae* adults. (Please, refer to the video file “VideoS3.mp4” in the folder “Manuscript III - Supplements” on the attached CD)

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