Novel glucosinolate metabolism in larvae of the leaf beetle
*Phaedon cochleariae*

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**ARTICLE INFO**

**Keywords:**
- *Phaedon cochleariae*
- Glucosinolate
- Detoxification
- Metabolism
- Leaf beetle

**ABSTRACT**

Plants of the Brassicaceae are defended by a binary system, in which glucosinolates are degraded by myrosinases, forming toxic breakdown products such as isothiocyanates and nitriles. Various detoxification pathways and avoidance strategies have been found that allow different herbivorous insect taxa to deal with the glucosinolate-myrosinase system of their host plants. Here, we investigated how larvae of the leaf beetle species *Phaedon cochleariae* (Coleoptera: Chrysomelidae), a feeding specialist on Brassicaceae, cope with this binary defence. We performed feeding experiments using leaves of watercress (*Nasturtium officinale*, containing 2-phenylethyl glucosinolate as major glucosinolate and myrosinases) and pea (*Pisum sativum*, lacking glucosinolates and myrosinases), to which benzenic glucosinolates (benzyl- or 4-hydroxybenzyl glucosinolate) were applied. Performing comparative metabolomics using UHPLC-QTOF-MS/MS, \(\text{N}-(\text{phenylacetyl})\) aspartic acid, \(\text{N}-(\text{benzoyl})\) aspartic acid and \(\text{N}-(\text{4-hydroxybenzoyl})\) aspartic acid were identified as major metabolites of 2-phenylethyl-, benzyl- and 4-hydroxybenzyl glucosinolate, respectively, in larvae and faeces. This suggests that larvae of *P. cochleariae* metabolise isothiocyanates or nitriles to aspartic acid conjugates of aromatic acids derived from the ingested benzenic glucosinolates. Myrosinase measurements revealed activity only in second-instar larvae that were fed with watercress, but not in freshly moulted and starved second-instar larvae fed with pea leaves. Our results indicate that the predicted pathway can occur independently of the presence of plant myrosinases, because the same major glucosinolate-breakdown metabolites were found in the larvae feeding on treated watercress and pea leaves. A conjugation of glucosinolate-derived compounds with aspartic acid is a novel metabolic pathway that has not been described for other herbivores.

1. **Introduction**

Plants have evolved a broad spectrum of defence mechanisms that restrict damage by insect antagonists. In addition to physical defences, plants produce various classes of specialised (or secondary) metabolites (Mitrofer and Boland, 2012) that act as repellents, deterrents or toxins to herbivores (Agnihotri et al., 2018; Bruce, 2014; Kim and Jander, 2007). Several of these compounds are stored in the plants as glucosides, such as benzoxazinoid, cyanogenic and iridoid glucosides as well as glucosinolates (Dobler et al., 2011; Morant et al., 2008; Winde and Wittstock, 2011). Upon plant tissue disruption due to herbivore attack, these metabolites come into contact with plant \(\beta\)-glucosidases, which are usually stored separately. These \(\beta\)-glucosidases cleave the glucose and the resulting aglucons are further metabolised into often toxic compounds (Kissen et al., 2009; Morant et al., 2008; Thangstad et al., 2004). Nevertheless, many insects can feed on plants expressing these binary defence mechanisms, because they possess various morphological, behavioural or physiological adaptations (Pentzold et al., 2014b). Intriguingly, particularly with regard to physiological means of detoxification, multiple mechanisms have evolved to circumvent the same class of plant defences (Heckel, 2014; Jeschke et al., 2016; Pentzold et al., 2014a, 2014b), but not all of these mechanisms are yet fully explored.

The Brassicaceae are an agriculturally important plant family, which is well known for the glucosinolate-myrosinase system, also frequently referred to as the ‘mustard oil bomb’ (Matile, 1980). Glucosinolates are...
anions composed of thiocyanoxyanates with an S-linked β-glucopyr-
anosyl residue and an O-linked sulfate residue in addition to a variable 
side chain, which is derived from different amino acids (Halkier, 2016; 
Blazević et al., 2020). Myrosinases are thioglucoside glucosidases 
generally stored separately from glucosinolates. When coming into 
contact, the S-glucosidic bond of the glucosinolate is hydrolysed and an 
unstable aglucon formed, which is further re-arranged. Depending on 
the pH, the structural type of the glucosinolate side chain and the 
presence of specifier proteins, different biologically active metabolites 
arise, including isothiocyanates, thiocyanates, nitriles or other classes of 
hydrolysis products (Lambrix et al., 2001; Wittstock et al., 2016) 
(Fig. 1A). However, there are also instances of endogenous catabolism of 
glucosinolates without prior tissue disruption (Bednarek et al., 2009; 
Petersen et al., 2002). Moreover, microbiota localised in the guts of 
various organisms can metabolise plant glucosinolates (Hammer and 
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Numerous herbivorous insect species have evolved physiological 
counter-adaptations to the glucosinolate-myrosinase system of the 
plants (Abdalsamee et al., 2014; Jeschke et al., 2016; Wünde and Witt-
stock, 2011). To circumvent the ‘mustard oil bomb’ some species 
sequester glucosinolates into body tissues, thereby separating them from 
plant myrosinases. This mechanism has been found in insects with 
piercing-sucking feeding style such as Athalia rosae (Hymenoptera, 
Aphididae) (Francis et al., 2001), Murgantia histrionica (Hemiptera, 
Pentatomidae) (Aliabadi et al., 2002) as well as in species with 
chewing-biting mouthparts such as Athalia rosae (Hymenoptera, Ten-
thredinidae) (Müller et al., 2001), Phyllostreta striolata and Psylliodes 
chrysocephala (Coleoptera, Chrysomelidae) (Beran et al., 2014, 2018). 
Aphids of B. brassicae and beetles of P. striolata express their own myr-
osinases, forming a binary defence together with the sequestered glu-
cosinolates (Beran et al., 2014; Kazana et al., 2007). In larvae of Pieris 
rapae (Lepidoptera, Pieridae), a nitrile-specifier protein (NSP) redirects 
the hydrolysis of glucosinolates to less toxic nitriles (Wittstock et al., 2004). The feeding generalist Schistocerca gregaria (Orthoptera, Acridi-
dae) and the specialist Plutella xylostella (Lepidoptera, Plutellidae) pro-
cede a sulfatase that hydrolyses the glucosinolates to 
desulfoglucosinolates (Falk and Gershenzon, 2007; Ratżka et al., 2002), 
which cannot be cleaved by myrosinases. Likewise, in other insect taxa 
myrosinase sulfatase activity has been found (Beran et al., 2018; 
Malka et al., 2016; Opitz et al., 2011). In contrast, some Lepidopteran 
feeding generalists as well as the chrysomelid P. chrysocephala conjugate 
isothiocyanates with the pseudo-tripeptide glutathione via a glutathione 
S-transferase and excrete the resulting non-toxic conjugates (Beran 
et al., 2018; Schramm et al., 2012; Wadleigh and Yu, 1988). These 
examples highlight the huge diversity and species-specificity of glucosi-
nolate metabolism by insect herbivores.

In the present study, we investigated the glucosinolate metabolism in 
larvae of the mustard leaf beetle Phaedon cochleariae (Coleoptera: 
Chrysomelidae), which is a feeding specialist on plants of the Brassica-
ceae. The beetles rely on glucosinolates for host plant recognition 
(Nielsen, 1978; Reifenrath and Müller, 2008), but it was unclear how 
this species deals with the glucosinolate-myrosinase system upon 
feeding. To study which metabolites result from glucosinolate meta-
bolism in P. cochleariae, larvae were offered leaves of a Brassicaceae 
host, watercress (Nasturtium officinale), or a non-host, pea (Pisum sat-
vium, Fabaceae). Pea lacks glucosinolates and myrosinases but is 
accepted by P. cochleariae when glucosinolates are applied on the leaves 
(Reifenrath et al., 2005). The benzenic glucosinolates benzyl glucosi-
nolate (glucotropaeolin) and 4-hydroxybenzyl glucosinolate (gluco-
nalbin), which do not naturally occur in watercress (Reifenrath et al., 
2005), were administered on leaves of both plant species and larvae and 
their faeces analysed for potential glucosinolate breakdown metabolites. 
Due to the highly species-specific detoxification pathways for the 
glucosinolate-myrosinase system known from different insects, we 
anticipated that P. cochleariae evolved yet another way to cope with this 
defence. Furthermore, if the metabolism of glucosinolates in 
P. cochleariae depends on plant myrosinases, we predicted that gluco-
sinolates are metabolised in larvae feeding on treated watercress leaves 
but remain intact in larvae fed with treated pea leaves.

2. Materials and methods

2.1. Plant and insect rearing

A breeding stock of P. cochleariae was kept in the laboratory under 
constant climate conditions (20 °C, 65% r.h., 16:8 h light:dark). The 
stock consisted of 500–600 individuals and every year about 100 wild 
individuals were collected from watercress plants at the river Ems in 
Germany (51°51’21” N, 8°41’37” E) and crossed into the stock. Beetles 
were reared in plastic boxes (20 × 20 × 6.5 cm; 50–100 individuals per
box) and were provided *ad libitum* with 7–8 weeks old non-flowering watercress plants (seeds from Volmary GmbH, Münster, Germany). Under these rearing conditions, the larvae take about 15–17 d for their entire larval development with three larval instars and the pupal duration lasts for 6 d (Kühne and Müller, 2011). The glucosinolate profile of watercress leaves is dominated by 2-phenylethyl glucosinolate (gluco- nasturtin) (>85% of the total glucosinolate concentration) as well as two indole glucosinolates, 4-methoxyindol-3-ylmethyl glucosinolate and indol-3-ylmethyl glucosinolate, while a few other glucosinolates occur only in minor quantities (Reifenrath et al., 2005). For feeding experiments (see below), seven-to-eight-week-old non-flowering watercress and six-week-old flowering pea plants (seeds from Kiepenkerl, Bruno Nebelung GmbH, Konken, Germany) were used. All plants were grown in pots (12 cm diameter) on composted soil in a greenhouse (60% r.h., 16:8 h light:dark).

2.2. Feeding experiments

To investigate the metabolism of benzenic glucosinolates in *P. cochleariae*, larvae of the second instar (about 6 mm long; Fig. 1B) were offered leaflets of either watercress or pea treated with 40 mM solutions of benzyl glucosinolate (glucotropaeolin, 1, Fig. 2) or 4-hydroxybenzyl glucosinolate (glucosinalbin, 3, both glucosinolates >99%, PHYtoplan GmbH, Heidelberg, Germany) in a mixture of methanol, millipore water and dichloromethane (64:30:6 v:v:v; organic solvents from Fisher Scientific, Loughborough, UK). Single leaflets of watercress or pea with a diameter of around 2.2 cm were treated with 50 μL of either benzyl glucosinolate or 4-hydroxybenzyl glucosinolate solution (resulting in 2 μmol per leaflet) or the respective solvent mixture only and offered in a Petri dish (55 mm diameter) lined with moistened filter paper. The solutions or solvent were pipetted on the leaf surface and the solvent allowed to evaporate for 40 min. The applied glucosinolate amount was chosen, because it equals about ten times the mean concentration of benzyl glucosinolate in young leaves of white mustard (*Sinapis alba*) (roughly 2 μmol/g dry weight) (Martin and Müller, 2007; Neubauer et al., 2014), a potential host of *P. cochleariae* (Reifenrath and Müller, 2008). We aimed to provoke sufficient amounts of glucosinolate breakdown metabolites for identification but to stay in a range that is not too far from concentrations larvae experience in nature.

2.3. Recovery of glucosinolates from treated leaves

To quantify the glucosinolate amounts that could be recovered from treated leaflets after an experimental period of 23 h without any impact by larvae, leaflets of watercress and pea were treated with glucosinolate solutions as described above (2.2. Feeding experiments). After evaporation of the solvents, the leaflets were kept in Petri dishes lined with wet filter paper for 23 h without larvae and then frozen in liquid nitrogen (six replicates per plant species and per glucosinolate solution). As controls, 50 μL of the 40 mM solutions of benzyl glucosinolate as well as 4-hydroxybenzyl glucosinolate (one replicate per glucosinolate) were put in Eppendorf tubes and directly frozen in liquid nitrogen. All samples were stored at −80 °C and freeze-dried.

Glucosinolates were extracted from this material by a threefold extraction in 80% methanol (v:v) and trapped on ion exchange columns (diethylaminoethyl (DEAE) Sephadex A-25 (Sigma Aldrich, St. Louis, MO, USA) swelled in acetic acid buffer, for details see Abdalsamee and Müller, 2012). Allyl glucosinolate (sinigrin, PHYtoplan GmbH) was added as an internal standard at the first extraction. After incubation with a sulfatase overnight, desulfoglucosinolates were eluted with water and analysed using a high performance liquid chromatograph coupled to a diode array detector (1200 Series, Agilent Technologies, Inc., Santa Clara, CA, USA) and equipped with a Supelcosil LC-18 column (3 μm, 150 × 3 mm, Supelco, Bellefonte, PA, USA) at a gradient from 5% to 95% methanol in water (as in Abdalsamee and Müller, 2012). Peaks of desulfoglucosinolates were quantified at 229 nm and response factors taken into account (0.95 for benzyl glucosinolate, 0.5 for 4-hydroxybenzyl glucosinolate and 1 for allyl glucosinolate). Recovery was calculated as percentage of exogenous glucosinolate extracted from treated plant material relative to the amount detected in control samples (set to 100%). Recovery from watercress versus pea leaves was compared for each glucosinolate with Mann-Whitney U-tests (with continuity correction due to ties in the data) in R Studio v. February 1, 5033 with required R v. 3.6.3. (R Developmental Core Team, 2020).

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**Fig. 2.** Glucosinolates applied in feeding assays (benzyl glucosinolate (1) and 4-hydroxybenzyl glucosinolate (3)) and the main glucosinolate of watercress [2-phenylethyl glucosinolate (7)] as well as their major metabolites in larvae of *Pheidon cochleariae*. Molecular formulas, molecular weights (MW) and main ions found using UHPLC-ESI-QTOF-MS/MS (MS mode) are shown.
2.4. Analysis of metabolites in larvae and faeces

To identify potential metabolites of ingested glucosinolates in *P. cochlearia*, larvae were taken directly after moult to the second instar (11–12 days old), starved for 8 h and then offered leaflets of watercress or pea treated with glucosinolate solution or solvent control in groups of three larvae in Petri dishes (as described in 2.2. Feeding experiments; *n* = 6 replicate Petri dishes per plant species and glucosinolate or solvent control). After a feeding period of 23 h, three groups of three larvae per treatment combination were dissected on ice into guts and remaining body tissues and the guts were shortly rinsed in millipore water to clean them from haemolymph. Body parts were then put in 2 ml Eppendorf tubes and frozen in liquid nitrogen. The remaining three groups of three larvae were transferred into 2 ml Eppendorf tubes and left for 3 h without food to collect their faeces. Afterwards these larvae with emptied guts were transferred to new tubes and frozen (“whole larvae”). In addition, watercress and pea leaflets treated with benzyl glucosinolate or 4-hydroxybenzyl glucosinolate solution or the solvent control (*n* = 3 per treatment combination) were taken at the beginning of the experiment. All samples were frozen in liquid nitrogen, stored at −80 °C and lyophilised.

For further processing, samples were extracted in 300 μl 90% (v:v) ice-cold methanol containing hydrocortisone (≥98%, Sigma-Aldrich Chemie GmbH, Steinheim Germany) as internal standard and centrifuged for 5 min at 4 °C. The supernatants were collected, filtered with 0.2 μm polytetrafluoroethylene membrane syringe filters (Phenomenex, Torrance, CA, USA) and stored at −80 °C. Metabolome analyses were carried out using a ultra-high performance liquid chromatography coupled to a quadrupole time of flight mass spectrometer (UHPLC-QTOF-MS/MS; UHPLC: Dionex UltiMate 3000, Thermo Fisher Scientific, San José, CA, USA; Q-TOF: compact, Bruker Daltonics, Bremen, Germany) equipped with a Kinetex XB-C18 column (1.7 μm, 150 × 2.1 mm, with guard column, Phenomenex, Torrance, CA, USA). Chromatographic separation was done following Schrieber et al. (2019) with slight modifications, using a gradient of water with 0.1% (v:v) formic acid (FA; eluent additive for LC-MS, ~98%, Sigma-Aldrich) (eluent A) to acetonitrile (LC-MS grade, Th. Geyer, Renningen, Germany) with 0.1% (v:v) FA (eluent B) with a flow rate of 0.5 ml min⁻¹, with 2% B to 30% B within 20 min, then to 75% B within 9 min, followed by column cleaning and equilibration. Line spectra (50–1300 m/z) were acquired in MS and MS/MS mode using negative electrospray ionisation (ESI) at 8 Hz with end plate offset: 500 V, capillary voltage: 3000 V, nebuliser (N₂) pressure: 3 bar, dry gas (N₂) flow and temperature: 12.1 min⁻¹ at 275 °C, quadrupole ion energy: 4 eV, low mass: 90 m/z, collision energy in MS mode: 7 eV, transfer time: 75 μs, pre-pulse storage: 6 μs. Fragmentation of the most intense ions was performed using N₂ as collision gas in Auto-MS/MS mode, applying a ramping of collision energies and isolation widths along with increasing m/z. To enable mass axis recalibration, a sodium formate calibration solution was introduced to the ESI sprayer preceding each sample. Ten blanks were measured using the same method.

Mass axis recalibration and picking of molecular features (each described by a retention time and m/z) were done in Compas Data-Analysis 4.4 (Bruker Daltonics) using the Find Molecular Features algorithm including spectral background subtraction. The settings used were signal-to-noise threshold: 3, correlation coefficient threshold: 0.75, minimum compounds length: 25 spectra, smoothing width: 10. For further generation (i.e., sorting of features belonging to the same metabolite together) the ion types allowed were: [M-H], [M+H-O-H]⁻, [M+Cl]⁻, [M+HCOOH-H]⁻, [M+CH₂COOH-H]⁻, [2M+H]⁺, [2M+HCOOH-H]⁻, [2M+CH₂COOH-H]⁻, [3M-H]⁻. Compass ProfileAnalysis 2.3 (Bruker Daltonics) was used for alignment of buckets across samples, allowing deviations of 0.1 min (retention time) and 5 mDa (m/z), respectively. The intensities (peak heights) of the most intense metabolic features within each bucket were related to the intensities of the [M+HCOOH-H]⁻ ion produced by hydrocortisone. Fold changes of features (i.e., their mean intensities in samples of larvae fed with glucosinolate-treated leaflets divided by their mean intensities in samples of larvae fed with control leaflets) were computed. To screen for potential degradation products of glucosinolates, intensities of features in the different treatment groups (plant species × treatment of the leaflet × type of larval sample) were inspected and only features that fulfilled all the following criteria were left in the dataset: 1) their mean intensity in at least one treatment group was at least 50 x higher than their mean intensity in the blanks, 2) they occurred in at least two of the three replicate samples of at least one treatment group, 3) their fold change was >2 or they only occurred in samples of larvae fed with glucosinolate-treated leaflets (but not in the corresponding control samples), 4) their mean intensity in at least one group of samples (of glucosinolate-fed larvae) was >1000 counts.

For the identification of relevant metabolites that differed between samples taken from larvae fed with glucosinolate- versus solvent-treated leaves (metabolites 1–8, Tables 1 and S1), samples in which these metabolites were abundant were again analysed by UHPLC-QTOF-MS/MS. To further trace the ions formed in MS mode and their fragments in MS/MS mode, lower spectra rates (2 Hz) were used and multiple reaction monitoring (with isolation widths and collision energies adjusted for each parent ion m/z) was applied both in negative (parameter settings as described above) and positive (capillary voltage: 4500 V) ESI mode. Molecular formulas of parent and daughter ions were created with Smart Formula (3D) in DataAnalysis and ranked according to their m/z deviation and isotopic pattern fit. Moreover, MetFrag (Rutkies et al., 2016) was used for in-silico fragmentation and matching of spectra against the MassBank of North America (https://mona.fiehnlab.ucdavis.edu/) to obtain suggestions for structural formulas. Retention times, UV/VIS spectra as well as MS and MS/MS spectra were compared to those of reference standards included in an in-house database that were measured under the same conditions. To specifically search for isothiocyanates and nitriles that could have been formed from phenylethyl glucosinolate (in watercress) and the glucosinolates applied on the leaflets, several standards (2-phenylethylisothiocyanate, 3-phenylpropanenitrile, phenylacetonitrile, 4-hydroxyphenylacetonitrile) were measured under the same conditions and larval samples screened for these metabolites.

2.5. Purification and structure elucidation of major metabolite (2) of benzyl glucosinolate

To purify the main metabolite (2) of benzyl glucosinolate (1), additional feeding experiments were carried out. Groups of three third instar larvae (16 days old, to gain more material) were fed with watercress leaves treated with benzyl glucosinolate solution for 23 h in Petri dishes. After the feeding period, larvae were transferred into 2 ml Eppendorf tubes and allowed to defaecate for 4 h. The faeces left in the corresponding Petri dishes were also added to the tubes. The extraction of these pooled larvae and their faeces was performed from lyophilised samples as described in 2.4. The metabolite (2) was purified by semi-preparative HPLC-DAD (1200 Series, Agilent Technologies) equipped with a Kinetex XB-C18 column (5 μm, 250 × 4.6 mm, with guard column, Phenomenex). Per run 25 μl of a sample were separated using a gradient of eluent A (water with 0.1% v:v FA) and eluent B (acetonitrile with 0.1% v:v FA) at a flow rate of 1 ml min⁻¹ with 2% B to 20% B within 20 min, then 20% B to 100% B within 5 min, followed by column cleaning and equilibration. When metabolite (2) was visible at 229 nm (at approximately 16 min), it was collected manually for 1 s in a 5 ml glass flask. In total, collections of 56 runs were pooled and freeze-dried in between collection bouts.

For analysis by nuclear magnetic resonance (NMR), metabolite (2) was dissolved in 0.5 ml of D₂O (Deutero GmbH, Kastellaun, Germany). NMR spectra were recorded on a Bruker Avance FT NMR spectrometer operating at a proton resonance frequency of 600.13 MHz. The instrument was equipped with a 5 mm BBO SmartProbe. Proton NMR data
were collected at room temperature using a pulse sequence including presaturation of the water resonance. Chemical shifts are given in parts per million (ppm).

2.6. Measurements of myrosinase activities

To test for active myrosinases in samples, larvae were starved for different durations and subsequently fed with leaflets of watercress or pea as described in 2.2. (Feeding experiments). For the first batch, mid-second-instar larvae were starved for 3.5 h and then either further processed or fed untreated watercress or pea leaves for 23 h. In the second batch, freshly moulted second-instar larvae were starved for 8.5 h and then either further processed or fed untreated watercress or pea leaves for 24 h. Material from groups of three larvae was pooled directly after the starvation phase (n = 1) or after the corresponding feeding time on watercress or pea leaves (n = 3 replicates per treatment combination). The larvae were dissected and the guts rinsed in millipore water to clean them from haemolymph. Guts as well as the remaining bodies were collected in separate 2 ml Eppendorf tubes. All dissection steps were performed on ice. In addition, leaves of watercress and pea (n = 3 per species) as well as pupae developed from larvae kept on watercress (n = 1, pooled from 3 individuals) were taken. All samples were stored at –20 °C until activity measurements of myrosinases.

To test for activities of soluble myrosinases, samples were macerated in extraction buffer (200 mM Tris, 10 mM ethylenediaminetetraacetic acid, pH 5.5) on ice and centrifuged. Glucosinolates present in the supernatants were removed using DEAE Sephadex A-25 columns, as described above. The purified extracts were then measured in 96-well microplates on a microplate photometer (Multiskan EX, Thermo Electron, China) at 492 nm for 45 min at room temperature after addition of 1 mM benzyl glucosinolate dissolved in phosphate buffer (pH 6.5) as substrate or phosphate buffer as controls. The kinetics of glucose release due to myrosinase activity were measured by adding a mixture of glucose oxidase, peroxidase, 4-aminophenylpyrin and phenol as colour reagent, following the protocol described in Travers-Martin et al. (2008). A calibration curve of glucose was measured on each plate and used for quantification. Soluble protein concentrations of the purified extracts were determined according to Bradford (1976) using bovine serum albumin as standard and myrosinase activities related to those.

3. Results

3.1. Main metabolites identified in samples of larvae and faeces

Across all samples, eight compounds (1–8, Table 1) were identified via UHPLC-QTOF-MS/MS that differed between samples of the different treatments. The identities of the applied benzyl glucosinolate (1; eluting at 5.35 min and producing a major ion with 408.436 m/z in ESI− MS mode) and 4-hydroxybenzyl glucosinolate (3; at 2.30 min, 424.0386 m/z) as well as of the major glucosinolate of watercress, 2-phenylethyl glucosinolate (7; at 7.90 min, 422.0583 m/z) were confirmed by comparison to reference standards. Besides the dominant deprotonated molecular ions in MS mode (see m/z values above), the glucosinolates produced characteristic fragments derived from the sulfate residues, i.e., [SO3]−, [SO4]2− and [HSO3]− ions (Table S1). The glucosinolates were not visible in ESI+ MS mode.

One main metabolite with 236.0556 m/z in ESI− mode (at 5.75 min, 2; Table 1) was found exclusively in larvae fed with added benzyl glucosinolate (1). In larvae fed with leaves treated with 4-hydroxybenzyl glucosinolate (3), three abundant metabolites with 252.0512 m/z (at 3.20 min, 4), 137.0242 m/z (at 4.13 min, 5) and 121.0294 m/z (at 5.45 min, 6) were detected. Exclusively in samples of larvae that had fed on watercress with 2-phenylethyl glucosinolate (7) as dominant glucosinolate, a metabolite with 250.0730 m/z (at 6.78 min, 8) was detected. For all these metabolites, the m/z difference of ca. 2 Da between the dominant ions in the ESI− and the ESI+ mode indicated that the m/z values given above represent deprotonated ions (Table 1).

The m/z difference between the glucosinolates (1, 3 and 7) and the main metabolites found in the respective larval samples (2, 4 and 8) was consistently about −172 m/z (Table 1). The molecular formulas assigned to these main metabolites revealed that they contained, in contrast to the glucosinolates, no sulfur (Table 1). Analyses of the fragmentation patterns revealed that these three metabolites contained aspartic acid residues, as indicated by the common fragments with an m/z of 115 m/z ([C6H12O5]SO3− (2, 4 and 8) and 132 m/z ([C6H12O5]− (for 4 and 8) (Table S1). The compounds were putatively identified as N-(benzoyl) aspartic acid (2) derived from benzyl glucosinolate (1), N-(4-hydroxybenzoyl) aspartic acid (4) derived from 4-hydroxybenzyl glucosinolate (3) and N-(phenylacetyl) aspartic acid (8) derived from 2-phenylethyl glucosinolate (7) (Table 1, Fig. 2).

No reference standards were available for these three metabolites. However, the identity of N-(benzoyl) aspartic acid (2) was further confirmed by NMR spectroscopy after purification of the metabolite. The 1H NMR spectrum showed corresponding signals for the two subunits of the molecule (Fig. S1). The resonances at δH 7.72, δH 7.75, δH 7.53, δH 7.45 (pseudo-t, δH 7.53, δH 7.45, 7H, 2H) could be assigned to five protons bound to a phenyl ring. The signals of the protons at ortho and meta positions were shifted downfield, indicating the presence of an electron-withdrawing group. For the aspartic acid moiety, three resonances were obtained in the spectrum exhibiting relative intensities of 1:1:1, as expected. The resonance at δH 4.53 [dd, δH 3.7 and 9.6 Hz, 1H] could be attributed to H-(C(α)). For the diastereotopic protons at C(β) two separate resonances were obtained at δH 2.72 [dd, δH 15.6 Hz, 3J(H,H) = 3.7 Hz, 1H] and δH 2.57 [dd, δH 15.6 Hz, 3J(H,H) = 9.6 Hz, 1H]. Besides the signals of metabolite (2), further resonances were obtained which have to be attributed to impurities.

Benzyl glucosinolate (1) and 4-hydroxybenzyl glucosinolate (3), which had been applied to the leaves, were found in several samples of the different respective insect parts (Table 2). The leaf-endogenous 2-phenylethyl glucosinolate (7) was found in four out of six faecal samples and in one out of six bodies as well as one whole larva that had been

Table 1
Glucosinolates (benzyl glucosinolate, 4-hydroxybenzyl glucosinolate, 2-phenylethyl glucosinate) and their breakdown metabolites found in samples of larvae of Phaedon cochlarearum, with molecular formulas, retention times (RT), ions (with types, m/z and formulas indicated) in negative (ESI−) and positive (ESI+) electrospray ionisation mode detected by UHPLC-QTOF-MS/MS. For further details on metabolites see Table S1.

<table>
<thead>
<tr>
<th>ID</th>
<th>Metabolite</th>
<th>Molecular formula</th>
<th>RT [min] average</th>
<th>ESI−</th>
<th>Ion type</th>
<th>Ion formula</th>
<th>ESI+</th>
<th>Ion type</th>
<th>Ion formula</th>
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<tr>
<td>1</td>
<td>Benzyl glucosinolate</td>
<td>C13H14O5N2S2</td>
<td>5.35</td>
<td>[M-H]</td>
<td>408.436</td>
<td>[C13H14O5N2S2]−</td>
<td>[M-H]+</td>
<td>238.0717</td>
<td>[C13H12N2O5]1+</td>
</tr>
<tr>
<td>3</td>
<td>4-Hydroxybenzyl glucosinolate</td>
<td>C14H15O5N2S2</td>
<td>2.30</td>
<td>[M-H]</td>
<td>424.0386</td>
<td>[C14H15O5N2S2]−</td>
<td>[M-H]+</td>
<td>238.0717</td>
<td>[C13H12N2O5]1+</td>
</tr>
<tr>
<td>5</td>
<td>4-Hydroxybenzolic acid</td>
<td>C8H9O2</td>
<td>4.13</td>
<td>[M-H]</td>
<td>121.0294</td>
<td>[C8H9O2]−</td>
<td>[M-H]+</td>
<td>123.0441</td>
<td>[C13H10O3]2+</td>
</tr>
<tr>
<td>6</td>
<td>4-Hydroxybenzaldehyde</td>
<td>C8H8O2</td>
<td>5.45</td>
<td>[M-H]</td>
<td>422.0583</td>
<td>[C8H8O2]−</td>
<td>[M-H]+</td>
<td>252.0855</td>
<td>[C13H10O3]2+</td>
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<tr>
<td>7</td>
<td>2-Phenylethyl glucosinolate</td>
<td>C13H14O5N2S2</td>
<td>7.90</td>
<td>[M-H]</td>
<td>250.0730</td>
<td>[C13H14O5N2S2]−</td>
<td>[M-H]+</td>
<td>252.0855</td>
<td>[C13H10O3]2+</td>
</tr>
</tbody>
</table>
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Table 2

Number of samples of (parts of) larvae of Phaedon cochleariae (total 3 replicates) or their faeces, in which metabolites were detected. Benzy1 glucosinolate (1), (Benzoyl) aspartic acid (2), 4-Hydroxybenzyl glucosinolate (3), (4-Hydroxybenzoyl) aspartic acid (4), 4-Hydroxybenzoic acid (5), 4-Hydroxybenzaldehyde (6), (8-Phenylacetyl) aspartic acid (7), N-[β-(4-Hydroxybenzoyl)aspartic acid (8), N-[β-(Phenylacetyl)aspartic acid (9), N-[β-(Methoxybenzyl)aspartic acid (10), N-[β-(Methoxybenzydimethyl) aspartic acid (11), and N-[β-(Methoxybenzylidene)aspartic acid (12), were applied on leaves of watercress (containing 2-phenylethyl glucosinolate, 7) or pea and offered to freshly moulted second-instar larvae for 23 h.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Watercress</th>
<th>Pea</th>
<th>Watercress</th>
<th>Pea</th>
<th>Watercress</th>
<th>Pea</th>
<th>Watercress</th>
<th>Pea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>benzyl glucosinolate</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>(1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(Benzoyl) aspartic acid</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>4-Hydroxybenzyl glucosinolate</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4-(4-Hydroxybenzoyl) aspartic acid</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4-Hydroxybenzoic acid</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4-Hydroxybenzaldehyde</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(8-Phenylacetyl) aspartic acid</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>N-[β-(4-Hydroxybenzoyl)aspartic acid</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>N-[β-(Phenylacetyl)aspartic acid</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>N-[β-(Methoxybenzyl)aspartic acid</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>N-[β-(Methoxybenzydimethyl)aspartic acid</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>N-[β-(Methoxybenzylidene)aspartic acid</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* If compound was not detected, its concentration may have been too low or it was not present.

*b* Most likely contaminations.

Fed with differently treated watercress leaves (Table 2). The main metabolites 2, 4 and 8 were found in almost all samples of the corresponding bodies and faeces of P. cochleariae larvae which had ingested glucosinolates 1, 3, or 7, respectively (Table 2), but were not found in any of the plant samples (data not shown).

In addition to (4), metabolites with 137.0246 m/z (at 4.13 min, 5) and 121.0294 m/z (at 5.45 min, 6) were detected in samples of larvae fed with leaves treated with 4-hydroxybenzyl glucosinolate (3). These compounds showed a common characteristic fragment in ESI+ mode [93.0346 m/z ([C$_6$H$_7$O$_2$]) (5, 6)]. Moreover, fragmentation of the major ion in ESI+ mode (i.e., 139.0389 m/z) of metabolite (5) revealed a dominant fragment [121.0284 m/z ([C$_7$H$_8$O$_2$])]. By comparison to reference standards, these metabolites were identified as 4-hydroxybenzoic acid (5) and 4-hydroxybenzaldehyde (6) (Tables 1 and S1). The compounds were detected in whole bodies and compound (5) also in the gut, but they were not found in faeces (Table 2). Moreover, (5) was also detected in leaf samples of watercress and pea to which 4-hydroxybenzyl glucosinolate had been applied and traces of (6) were detected in watercress leaves with applied glucosinolates.

3.2. Stability of applied glucosinolates on leaves

Glucosinolates applied to leaves were still intact after 23 h under the conditions of the feeding tests, but without larvae. Recovery of the two glucosinolates was high on both plant species, but somewhat lower for benzyl glucosinolate (1) on leaves of watercress [89.7% (83.9–92.4], median (IQR), n = 61] compared to pea [100% (100–100); p = 0.003, Mann-Whitney U-test]. 4-Hydroxybenzyl glucosinolate (3) was recovered at >90% from leaves of both plant species [watercress: 100% (100–100); pea: 100% (90.02–100), p = 0.176].

3.3. Myrosinase activities in leaves and larval parts

As expected, leaves of watercress had a high myrosinase activity, whereas leaves of pea had no myrosinase activity (Table 3). In guts of mid second-instar larvae starved and fed subsequently with watercress, likewise a high myrosinase activity was detectable. In starved mid second-instar larvae and larvae fed with pea leaves instead of watercress after starvation, a rather low myrosinase activity was detected (Table 3). In contrast, no myrosinase activity was detectable in guts of larvae that had starved directly after molting to the second instar or were treated with differently treated watercress leaves.

Table 3

Myrosinase activity in leaves of watercress and pea as well as in guts and remaining bodies of the second-instar (L2) larvae or in pupae of Phaedon cochleariae (three individuals pooled per sample). All larvae were fed with watercress until molting to the L2 stage. Freshly moulted L2 larvae were offered watercress or pea leaves after 8.5 h of starvation. Mid L2 larvae fed on watercress were starved for 3.5 h and then fed with either watercress or pea leaves. The plant species in the columns refer to the final food provided for 24 h after the starvation period.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Myrosinase activity, mean ± SD (n = 3) [nmol glucose µg protein$^{-1} \times$ min$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>0.083 ± 0.034 n.d.$^b$</td>
</tr>
</tbody>
</table>
| Mid L2 larvae (starved for 3.5 h) | Guts 0.014$^a$ 0.038 ± 0.032 0.012 ± 0.009  
| Bodies (without gut) | n.d. n.d. |
| Freshly-moulted L2 larvae (starved for 8.5 h) | Guts n.d.$^b$ 0.052 ± 0.032  
| Bodies (without gut) | n.d. n.d. |
| Pupae  | n.d.$^b$                                                        |

*a* n.d. – no activity detectable.

$b$ Only one replicate measured.
the same but allowed to feed on pea leaves after the starvation period. Likewise, in remaining larval bodies without guts as well as in pupae no myrosinase activity could be detected (Table 3).

4. Discussion

Feeding experiments with *P. cochleariae* larvae and subsequent chemical analysis support our hypothesis that *P. cochleariae* evolved a way that is different from the known glucosinolate metabolism pathways in other insect species (Abdalsamee et al., 2014; Jeschke et al., 2016; Winde and Wittstock, 2011) to deal with the 'mustard oil bomb'.

To investigate the metabolism of glucosinolates in *P. cochleariae* larvae, we offered larvae leaves to which glucosinolates had been added. After almost one day, most of the glucosinolates that had been applied to the leaves were still intact in a control experiment without larvae. Thus, the glucosinolate solutions were quite stable and metabolites of glucosinolates found in larval and faecal samples can predominantly be assigned to processes that occurred in the larvae. Slightly lower recovery from leaves of watercress than from pea may be related to the presence of myrosinases only in the former. Due to different feeding amounts of leaves by the individual larvae, we had to pool three larvae per sample. Therefore, we focus on qualitative rather than quantitative analyses of the glucosinolate metabolism in *P. cochleariae*.

Comparative metabolomics analysis of insect samples from our feeding experiments revealed metabolites whose presence depended on the kind of glucosinolate ingested by the larvae. For each of the tested glucosinolates (1, 3, 7), one specific major metabolite (2, 4, 8) was detected in almost all samples derived from the corresponding larvae and faeces, but none of those were found in any of the plant samples. Hence, these metabolites are likely formed by larval metabolism of glucosinolates or of plant-derived glucosinolate breakdown products. The common difference of about ~172 m/z between the ions of the glucosinolates and their main metabolites suggests that these three benzenic glucosinolates undergo the same metabolic pathway in larvae of *P. cochleariae* (Fig. 2). The main metabolites were identified as N-(benzoyl) aspartic acid (2) derived from benzyl glucosinolate (1), N-4-(hydroxybenzoyl) aspartic acid (4) derived from 4-hydroxybenzyl glucosinolate (3) and N-(phenylethyl) aspartic acid (8) derived from 2-phenylethyl glucosinolate (7). The structure of N-(benzoyl) aspartic acid (2) was confirmed by NMR spectroscopy (Fig. S1). Thus, larvae of *P. cochleariae* obviously conjugate the amino acid aspartic acid to aromatic acids derived from ingested benzenic glucosinolates. A similar route of benzenic glucosinolate metabolism was identified in caterpillars of *P. rapae*, where glycine serves as conjugation partner (Vergara et al., 2006, 2007). A conjugation of glucosinolate-derived metabolites with aspartic acid, as found in the current study in larvae of *P. cochleariae*, is, to our knowledge, not known from any other species feeding on Brassicaceae.

While in feeding experiments with benzyl- and 2-phenylethyl glucosinolate (1, 7) only one major metabolite for each glucosinolate was found, two further metabolites were detected in bodies, but not faeces, of *P. cochleariae*, when larvae had ingested 4-hydroxybenzyl glucosinolate (3) (Table 2). These metabolites were identified as 4-hydroxybenzoic acid (5) and 4-hydroxybenzaldehyde (6) and might represent pathway intermediates (Tables 1 and S1; Fig. 3). Resulting from hydrolysis of 4-hydroxybenzyl glucosinolate, 4-hydroxybenzyl isothiocyanate may be hydrolysed in *P. cochleariae* into 4-hydroxybenzyl alcohol and subsequently converted into 4-hydroxybenzaldehyde (6) and then into 4-hydroxybenzoic acid (5). Further steps of amino acid conjugation may then take place. Alternatively, 4-hydroxybenzyl glucosinolate may react to 4-hydroxyphenylacetocyanitrile, 4-hydroxymandelonitrile and finally to 4-hydroxybenzaldehyde by splitting off a cyanide, followed by the above-described pathway (Fig. 3). Thus, the metabolites detected in larval samples of *P. cochleariae* after ingestion of benzene-based glucosinolates on watercress or pea leaves could arise from both isothiocyanates or nitriles as initial glucosinolate breakdown metabolites.

Strikingly, neither isothiocyanates nor nitriles were detected in any of the samples, indicating rapid conversion to other products.

In caterpillars of *P. rapae*, ingestion of benzyl glucosinolate-containing plant material leads from phenylacetocyanitile, the product of joint action of plant myrosinases and larval NSP, via α-hydroxylation and cyanide release to benzaldehyde. After oxidation to benzoic acid, a conjugation with glycine takes place (Staubert et al., 2012; Wittstock et al., 2004). Formation of nitriles upon myrosinase-catalysed glucosinolate hydrolysis has been observed at low pH (<5) or high iron concentrations (>0.01 mM) as well as upon catalysis by plant or insect NSPs.
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is needed to test whether benzenic isothiocyanates may be decomposed 4-hydroxybenzyl glucosinolate (Agerbirk et al., 2008). Future research corresponding isothiocyanate under release of a thiocyanate ion (SCN−) (Agerbirk et al., 2008). However, at least in plant homogenates, SCN− formation has not been observed with e.g. benzyl glucosinolate, as this and similar glucosinolates give rise to more stable isothiocyanates than 4-hydroxybenzyl glucosinolate (Agerbirk et al., 2008). Future research is needed to test whether benzenic isothiocyanates may be decomposed accompanied by SCN− release upon ingestion of the corresponding glucosinolates by P. coelestis.

The pathways discussed above imply that the ingested glucosinolates are hydrolysed by myrosinases from either plant or insect. Unexpectedly, the major metabolites 2, 4 and 8 were also found in freshly-moulded larvae of P. coelestis fed with glucosinolate-treated leaves of pea (Table 2) that lack myrosinases. A low myrosinase activity was found in guts of second-instar larvae that were fed with watercress, starved and then fed with pea. In contrast, no myrosinase activity was found in guts of second-instar larvae that had starved directly after moult and then fed with pea. Insects appear to empty their guts entirely before each moult (Beament, 1968). This may explain the lack of detectable myrosinase activity in freshly moulded larvae feeding on pea and indicate that the myrosinase activity found in mid second-instar larvae that were switched from watercress to pea may be due to some remaining myrosinase activity from watercress tissue.

Based on these data, plant myrosinases likely contributed to glucosinolate hydrolysis when P. coelestis larvae fed on watercress leaves. In addition, the larvae may induce an own myrosinase once they feed on glucosinolate-containing plants, as known for the induction of a glucosinolate sulfatase upon feeding on Brassicaceae in S. gregaria (Falk and Gershenzon, 2007). Alternatively, larvae of P. coelestis may carry microorganisms in their digestive system that contribute to glucosinolate hydrolysis and may be responsible for the formation of iso-thiocyanates and/or nitriles. In fact, bacteria that convert glucosinolates to isothiocyanates or nitriles have been found in the guts of humans and rats (Liu et al., 2020; Liu et al., 2017; Mullaney et al., 2013). Microbial symbionts offer several benefits for insects, because they improve insect nutrition by producing essential amino acids, vitamins and digestive enzymes and are involved in the detoxification or modification of plant allelochemicals (Beran et al., 2014, 2018). In addition, larvae of P. coelestis obtained from matrix-assisted laser desorption ionisation mass spectrometry imaging revealed indeed increased abundances of N-(benzoyl) aspartic acid (4) and N-(phenylacetyl) aspartic acid (8) in the Malpighian tubules (data not shown).

The applied glucosinolates (1, 3) and the major glucosinolate of watercress, 2-phenethyl glucosinolate (7), were also found unmodified in different insect parts and faeces, but there was no consistent accumulation of the respective glucosinolates in whole larvae and body samples without gut (Table 2). In the present experiments, large amounts of the glucosinolates were applied on the leaves, which may not have been fully converted by P. coelestis. Thus, there is no clear evidence that larvae of P. coelestis sequester these glucosinolates. In contrast, two other chrysomelid beetle species that are specialists on Brassicaceae, P. striolata and P. chrysolina, selectively sequester various glucosinolates from their host plants (Beran et al., 2014, 2018). This illustrates nicely the diversity of insect mechanisms directed at coping with the plant glucosinolate-myrosinase system, even within one beetle family.

In summary, P. coelestis has evolved another way of dealing with the ‘mustard oil bomb’. Larvae metabolise glucosinolate-derived compounds, likely isothiocyanates or nitriles, to aromatic acids, which they then conjugate with aspartic acid. The glucosinolate metabolism seems thereby not necessarily to be dependent on plant myrosinases. Future studies are needed to test whether the proposed metabolic pathway also applies to other glucosinolates and to further elucidate the postulated pathway.

Declaration of competing interest

None.

Acknowledgements

We thank the gardeners of Bielefeld University for help in plant rearing, Laura Lachenicht and Martin Zumbrunnen for their help in insect rearing, Leon Brüggemann and Sarah Rhodes for their help in conducting some of the experiments. This work was funded by the grant MU 1829/20–1 to CM of the Deutsche Forschungsgemeinschaft.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2020.103431.

References