# Friedrich Schiller University Jena

Faculty of Biological Sciences

Prepared at the Max Planck Institute for Chemical Ecology

# "A comparative physiological and morphological study of plant glucoside uptake across three leaf beetle species"

**Master's Thesis**

to gain the academic grade as a

*Master of Science* in the Study Program *Evolution, Ecology and Systematics* (EES)

(M. Sc.)

Submitted by

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# Abbreviations

M = molar = mol per litre
mM = milimolar

pmol = picomole
nmol = nanomole

nL = nanolitre
µL = microlitre
mL = millilitre

s = seconds
min = minutes
h = hours
d = days

m/z = mass-to-charge ratio

°C = degrees Celsius

mg = milligram

# Chemicals used

### Glucosinolates

Sinigrin = allyl glucosinolate

4MSOB-gls=
4MSOB-glucosinolate =
4-Methylsulfinylbutyl-glucosinolate =
Glucoraphanin

Sinalbin = pOHB-glucosinolate=
p-Hydroxybenzyl-glucosinolate =
Glucoraphasatin

4MTB-glucosinolate =
4-methylthio-3-butenyl glucosinolate

### Other glucosides

Linamarin

Salicin

Catalpol

### Other chemicals

DEAE= Diethylaminoethyl

Amaranthe dye; sodium;3-hydroxy-4-[(4-sulfonaphthalen-1-yl)diazenyl]naphthalene-2,7-disulfonic acid

### Fixatives

Dubosq Brasil = 150 mL 80% ethanol, 1 g picric acid, 60 mL 38% formaldehyde, 15 mL acetic acid, provided by Dr. H. Pohl, Universität Jena (Tröger et al., 2019)

Karnovskys Fixative: 4% Formaldehyde, 2.5% Glutaraldehyde in 0.1M Sodium-Cacodylate Buffer; pH= 7.4
Recipe by EMZ Jena

### Stain

Richardson blue = 1:1 mixture of 1% Azur II in deionized water and 1% Methylene Blue in 1% Borax solution (Richardson et al., 1960)

### Buffer

PBS-Buffer: Phosphate buffered saline

MES-buffer: 2-(N-morpholino) ethanesulfonic acid - buffer

# Organisms used in this study

**Plants**

*Brassica juncea* (L.) CZERN. cv. Bau Sin – Chinese mustard

*Brassica rapa* L. cv. Yu-Tsai-Sum – Edible rape

*Brassica rapa* subsp. *pekinensis* (LOUR.) HANELT – Napa cabbage

*Arabidopsis thaliana* (L.) HEYNH. – thale cress, mouse-ear cress

**Beetles**

*Phaedon cochleariae* (FABRICIUS, 1792) – Mustard leaf beetle

*Psylliodes chrysocephala* (LINNAEUS, 1758) – Cabbage-stem flea beetle

*Phyllotreta armoraciae* (KOCH, 1803) – Horseradish flea beetle

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# Summary

Many insects are able to take up and accumulate plant defence glucosides to use them for their own defence, a process known as sequestration. The physiology of the uptake of plant glucosides from the gut lumen into the haemocoel is largely unknown. To investigate, whether this uptake is selective, I conducted a comparative experiment, in which I fed three leaf beetle species with different plant defence glucosides. These species were *Phyllotreta armoraciae* and *Psylliodes chrysocephala*, which both sequester glucosinolates, the characteristic defence compounds of the plant order Brassicales, and *Phaedon cochleariae*, which does not sequester glucosinolates. Uptake in *P. armoraciae* was highly selective to glucosinolates with only small amounts of other plant glucosides taken up. In *P. chrysocephala* the uptake was also selective towards glucosinolates, but other plant glucosides were also sequestered by this species. A small proportion of glucosinolates was also taken up by *P. cochleariae* and overall glucosinolate recovery was low in this species. The uptake of glucosinolates in *P. armoraciae* has to happen against a concentration gradient of glucosinolates, which are stored in high concentrations in the haemolymph ($c = 61$ mM) of this species. This requires the presence of an active uptake mechanism. The expression of glucosinolate-specific transporter in the foregut of *P. armoraciae* indicated, that glucosinolate uptake might happen in this gut part. To test this hypothesis, I conducted a short term feeding experiment with *P. armoraciae*, during which plant material was only present in the foregut. After 15 s of feeding, over 50% of total detected glucosinolates were found in the haemocoel. I conducted the same experiment with a plant, devoid of the glucosinolate-activating enzyme and found no significant difference between the two treatments, which shows that this rapid uptake is independent of this enzyme. The insect foregut is usually not involved in uptake processes of hydrophilic compounds, due to a continuous cuticle known as intima. I investigated the morphology of the crop, a foregut part used for storage of ingested material, in a comparative approach. To confirm the presence of chitin in the crop, I excited the autofluorescence of chitin with confocal laser scanning microscopy. This confirmed that all three species possessed a chitinous cuticle in their crops. The fluorescence response of the crop differed between species in intensity and structure. To visualize this structure in more detail, I analysed the crop ultrastructure with transmission electron microscopy. The intima was continuous in all three species, but differed in thickness between species. The procuticle of *P. armoraciae* and *P. chrysocephala* was much thinner and had less laminae than that of *P. cochleariae*. This thinner intima might be an adaptation to facilitate glucosinolate uptake.

# Zusammenfassung

Zahlreiche Insekten können pflanzliche Abwehrstoffe aus ihrer Nahrung aufnehmen, sie in ihren Körpern anreichern und zu ihrer eigenen Verteidigung nutzen. Dieser Prozess wird Sequestrierung genannt. Die Physiologie der Aufnahme von Pflanzenglucosiden aus dem Darmlumen in das Hämocoel ist wenig erforscht. Um die Selektivität dieser Aufnahme zu untersuchen, habe ich ein vergleichendes Experiment mit drei Blattkäferarten durchgeführt, die ich mit verschiedenen pflanzlichen Glucosiden fütterte. Die untersuchten Arten waren *Phyllotreta armoraciae* und *Psylliodes chrysocephala*, die beide Glucosinolate, die charakteristischen Abwehrstoffe der Pflanzenfamilie Brassicales, sequestrieren und *Phaedon cochleariae*, welcher keine Glucosinolate sequestriert. Die Glucosidaufnahme von *P. armoraciae* war stark selektiv für Glucosinolate und nur geringe Anteile von anderen Glucosiden wurden aufgenommen. Auch die Aufnahme durch *P. chrysocephala* war selektiv für Glucosinolate, doch sequestrierte diese Art auch andere Glucoside. Ein kleiner Anteil an Glucosinolaten wurde auch durch *P. cochleariae* aufgenommen, doch war die Wiederfindungsrate von Glucosinolaten niedrig. Die Aufnahme von Glucosinolaten in *P. armoraciae* muss gegen einen Konzentrationsgradienten erfolgen, da in dieser Art hohe Konzentrationen von Glucosinolaten in der Hämolymphe gespeichert werden (c = 61 mM). Dies erfordert einen aktiven Aufnahmemechanismus. Glucosinolat-spezifische Transporterproteine sind im Vorderdarm von *P. armoraciae* exprimiert, was darauf hinweist, dass Glucosinolataufnahme in diesem Teil des Darmes stattfindet. Ich verifizierte diese Hypothese durch ein Fütterungsexperiment, während welchem sich Pflanzenmaterial ausschließlich im Vorderdarm von *P. armoraciae* befand. Nach 15 s Fressen, waren über 50% der gesamt detektierten Glucosinolate bereits im Haemocoel. Dasselbe Experiment wurde mit einer Pflanze durchgeführt, der das Glucosinolat-aktivierende Enzym fehlt. Zwischen Käfern, die auf den beiden Pflanzen fraßen, wurde kein signifikanter Unterschied gefunden, was darauf hindeutet, dass die Aufnahme unabhängig von der Anwesenheit dieses Enzymes ist. Der Vorderdarm von Insekten ist für gewöhnlich nicht an Aufnahmeprozessen hydrophiler Stoffe beteiligt, da er eine chitinöse Cuticula besitzt. Ich untersuchte die Morphologie des Kropfes, eines Vorderdarmteiles mit Speicherfunktion, in einem vergleichenden Ansatz. Die Anwesenheit von Chitin verifizierte ich durch Anregung seiner Autofluoreszenz mit einem konfokalen Laser-Scanning Mikroskop. Die Fluoreszensantwort unterschied sich in Intensität und Struktur zwischen den Arten. Um die Kropf Strukturen detaillierter zu untersuchen, nutzte ich Transmissionselektronenmikroskopie. Große Unterschiede existierten in der Dicke der Cuticula zwischen den Arten. Die Procuticula von *P. armoracie* und *P. chrysocephala* war sehr viel dünner und hatte weniger Lamellen als die von *P. cochleariae*. Diese dünnere Intima könnte eine Anpassung sein, die die Aufnahme von Glucosinolaten aus der Nahrung erleichtert.

# 1. Introduction

## 1.1. Plant-Insect interaction

Plants and insects have been coexisting and affecting the evolution of each other for 400 million years (Labandeira, 2013, Fürstenberg-Hägg et al., 2013, Evans and Kitson, 2020). Plants benefit from insects as pollinators (Woodcock et al., 2019), but are also under threat from herbivory by phytophagous insects (Davies, 1988, Moreira et al., 2019). Selective pressure through herbivory lead to plant defence adaptations which in turn acted as selective pressures on the herbivores. This reciprocal influencing is known as coevolution (Ehrlich and Raven, 1964). Coevolution led to a plethora of mechanical adaptations such as increased toughness and hardness (Lucas et al., 2000), the evolution of pubescence, waxes (Wilson Fernandes, 1994), spinescence or sclerophylly (Hanley et al., 2007). A greater influence on insect biodiversity had chemical plant defence (Ehrlich and Raven, 1964). Chemical plant defence is a significant obstacle to herbivore feeding (Mithöfer and Boland, 2012). Defence compounds are produced as response to an herbivore attack (Harborne, 1999, Paxton, 1981), or are stored in plant tissue in anticipation of an herbivore attack (Mithöfer and Boland, 2012, Pentzold et al., 2014, VanEtten et al., 1994). A widespread form of stored plant defence are activated binary defence systems (Pentzold et al., 2014). They consist of a protoxin, in form of a glucoside, and a separately stored, activating $\beta$-glucosidase enzyme (Morant et al., 2008, Pentzold et al., 2014). Upon tissue damage, e.g. through herbivore feeding, the two components get in contact and the enzyme hydrolyses the glucoside, resulting in the release of a glucose moiety and a reactive aglucone (Morant et al., 2008, Pentzold et al., 2014, Halkier, 2016, Halkier and Gershenzon, 2006). Many insects evolved adaptations and strategies to cope with chemical plant defence, like specialized feeding behaviours, enzymatic detoxification, target-site mutations or effector-mediated suppression of plant signalling (Després et al., 2007, War et al., 2012, Erb and Reymond, 2019, Duffey, 1980).

## 1.2. The glucosinolate myrosinase system

One well studied activated binary defence system is the glucosinolate-myrosinase system, the characteristic defence system of the plant order brassicales. It is also known as the "mustard oil bomb" (Halkier and Gershenzon, 2006, Bhat and Vyas, 2019, Blažević et al., 2020, Frohne and Jensen, 1998, Rodman et al., 1998, Singh, 2004). Glucosinolates are amino acid derivatives and are classified based on their side chains structure as aliphatic, benzenic or indolic glucosinolates (Blažević et al., 2020). More than 130 structurally different glucosinolates have been reported as of 2018 (Blažević et al., 2020). Glucosinolates and the

activating $\beta$-glucosidase enzyme myrosinase are spatially separated in plants. (Luthy and Matile, 1984, Kelly et al., 1998, Koroleva et al., 2000, Koroleva et al., 2010, Shirakawa et al., 2014, Shirakawa and Hara-Nishimura, 2018, Nintemann et al., 2018). If herbivore feeding destroys this separation, the myrosinase hydrolyses the glucosinolate at the thioglucoside linkage, resulting in the release of a glucose moiety and an unstable aglucone (Halkier and Gershenzon, 2006)(Fig. 1). The aglucone subsequently undergoes a Lossen rearrangement (Ettlinger and Lundeen, 1957, Bones and Rossiter, 1996) which usually results in the formation of toxic isothiocyanates (Halkier, 2016). Isothiocyanates can cross cellular membranes (Jeschke et al., 2015) and inhibit and crosslink free amino acids and proteins, by reacting with their thiol, sulphide and amino groups (Kawakishi and Kaneko, 1985, Kawakishi and Kaneko, 1987, Brown and Hampton, 2011). Isothiocyanates are toxic for insects (Lichtenstein et al., 1964, Seo and Tang, 1982, Erickson and Feeny, 1974, Blau et al., 1978, Wadleigh and Yu, 1988, Winde and Wittstock, 2011, Jeschke et al., 2017, de Souza et al., 2018), nematicidal (Tsao et al., 2000), fungicidal (Walker et al., 1937, Vig et al., 2009) and bactericidal (Fan et al., 2011). Many different insect strategies to cope with the glucosinolate myrosinase system exist (Fig. 1; see also supplement 7.1).

## 1.3. Sequestration

Some herbivorous insects can exploit chemical plant defence compounds and accumulate them in their bodies to defend themselves against their own enemies. This widespread phenomenon is termed sequestration (Heckel, 2014, Duffey, 1980, Petschenka and Agrawal, 2016, Erb and Robert, 2016, Opitz and Müller, 2009). As of 2009, sequestration has been described from over 250 species from different insect orders and from at least 40 plant families (Opitz and Müller, 2009). It is prominently known from coleopterans (beetles) and lepidopterans (moths and butterflies) (Opitz and Müller, 2009, Nishida, 2002) but also occurs in other insect orders (Table 1). One of the most popular examples of a sequestering insect is the monarch butterfly *Danaus plexippus* (LINNAEUS, 1758 ), which sequesters cardenolides from plants of the milkweed family, Asclepiadaceae, (Reichstein et al., 1968) and thus becomes unpalatable for avian predators (Brower et al., 1968).

Sequestration is a physiologically complex process and requires a multitude of adaptations. The insect requires resistance against the sequestered toxin and an appropriate mechanism to take up the compound from the gut into the body (Duffey, 1980, Erb and Robert, 2016, Petschenka and Agrawal, 2015). The uptake by a carrier, specific to a defence compound can have detoxifying function, since it can physically remove a particular chemical from a solution e.g. an intact glucoside from the gut lumen before it can be activated by a plant $\beta$-

glucosidase enzyme (Duffey, 1980, Erb and Robert, 2016, Abdalsamee et al., 2014). A taken up compound only accumulates in the body if more of it is taken up, then is metabolised or excreted (Duffey, 1980).

Unselective uptake mechanisms might also play a role in sequestration. Larvae of some beetles from subfamily Chrysomelinae *de novo* produce a defence secrete (Søe et al., 2004) but are also able to sequester precursor compounds, if these are ingested (Feld et al., 2001). In this group, a general, unselective uptake of plant glucosides from the midgut into the haemolymph has been found (Discher et al., 2009). From the haemolymph, compounds are either imported into defence secretion with high selectivity (Strauss et al., 2013) or are excreted by the Malpighian tubules (Discher et al., 2009,(Boland, 2015).

### 1.3.1. Sequestration of glucosinolates

Larvae of the sawfly *Athalia rosae* (Linnaeus, 1758 ) (Müller et al., 2001) and other *Athalia* species (Opitz et al., 2012) sequester plant glucosinolates into their haemolymph. The haemolymph is emitted (reflex bleeding) to deter predators (Müller et al., 2002, Müller and Brakefield, 2003, Opitz et al., 2010). Glucosinolates are also sequestered by the two Brassicales specialist aphids, the cabbage aphid *Brevicoryne brassicae* (Linnaeus, 1758) and the turnip aphid *Lipaphis erysimi* (Kaltenbach, 1843). Both aphids sequester intact glucosinolates in their haemolymph (Bridges et al., 2002, Kazana et al., 2007) and possess an aphid myrosinase enzyme (Macgibbon and Beuzenberg, 1978, Jones et al., 2001, Pontoppidan et al., 2001, Francis et al., 2002), to constitute their own 'mustard oil bomb' (Bridges et al., 2002) as defence against predators (Pratt et al., 2008). Two glucosinolate sequestering flea beetles, the striped flea beetle *Phyllotreta striolata* (Fabricius, 1801) and the horseradish flea beetle *Phyllotreta armoraciae* (Koch, 1803), also possess a myrosinase enzyme (Beran et al., 2014, Sporer et al., 2020)

### 1.3.2. Glucosinolate sequestration in *Phyllotreta armoraciae*

*P. armoraciae* is monophagous on *Armoracia rusticana* (Horseradish) in the wild, but readily accepts other glucosinolate containing plants under laboratory conditions (Nielsen et al., 1979). *P. armoraciae* selectively sequester glucosinolates from their host plant into their haemolymph and retain them there (Yang et al., 2020). The total glucosinolate concentration in adults can reach up to 44 nmol/mg fresh weight (Sporer et al., 2020). Total glucosinolate levels remain constant in feeding adults, while the composition is influenced by the uptake of new glucosinolates (Yang et al., 2020). This equilibrium is maintained, at least partially, by selective excretion of intact glucosinolates (Yang et al., 2020). Recently, glucosinolate

specific transporters have been described from *P. armoraciae* (Yang et al., *under review*). They belong to the sugar porter family and evidence suggests, that they play a key role in glucosinolate homeostasis, by reabsorbing glucosinolates from the Malpighian tubule lumen back into the haemolymph (Yang et al., *under review*). Interestingly, the two transporters with the broadest spectrum of glucosinolates transported are expressed in the beetle foregut, but not the midgut (Yang et al., *under review*). *P. armoraciae* possesses a beetle myrosinase enzyme (Sporer et al., 2020). In larval *P. armoraciae*, the beetle myrosinase is localised in the haemolymph and creates a mustard oil bomb, which deters the Asian ladybeetle *Harmonia axyridis* (PALLAS, 1773), a generalist predator (Sporer et al., 2020).

### 1.3.3. Glucosinolate sequestration in *Psylliodes chrysocephala*

The cabbage stem flea beetle *P. chrysocephala* (LINNAEUS, 1758) is a pest species on brassicaceous crops, especially on oilseed rape, *Brassica napus* (Bonnemaison, 1965, Williams, 2010). It selectively sequesters glucosinolates into its haemolymph and stores them throughout their life cycle (Beran et al., 2018). Total glucosinolate concentration in adult *P. chrysocephala* is around 4 nmol/mg fresh weight (Beran et al., 2018). No beetle myrosinase activity has been found in this species (Beran et al., 2018). Isothiocyanates formed during feeding are detoxified either through conjugation with glutathione (Beran et al., 2018) or through gut symbionts (Shukla and Beran, 2020). *P. chrysocephala* possesses a gut membrane associated sulfatase, which desulfates benzenic and indolic glucosinolates to desulfo-glucosinolates (Ahn et al., 2019), which cannot be activated by plant myrosinase anymore (Fig.1) (Ettlinger et al., 1961).
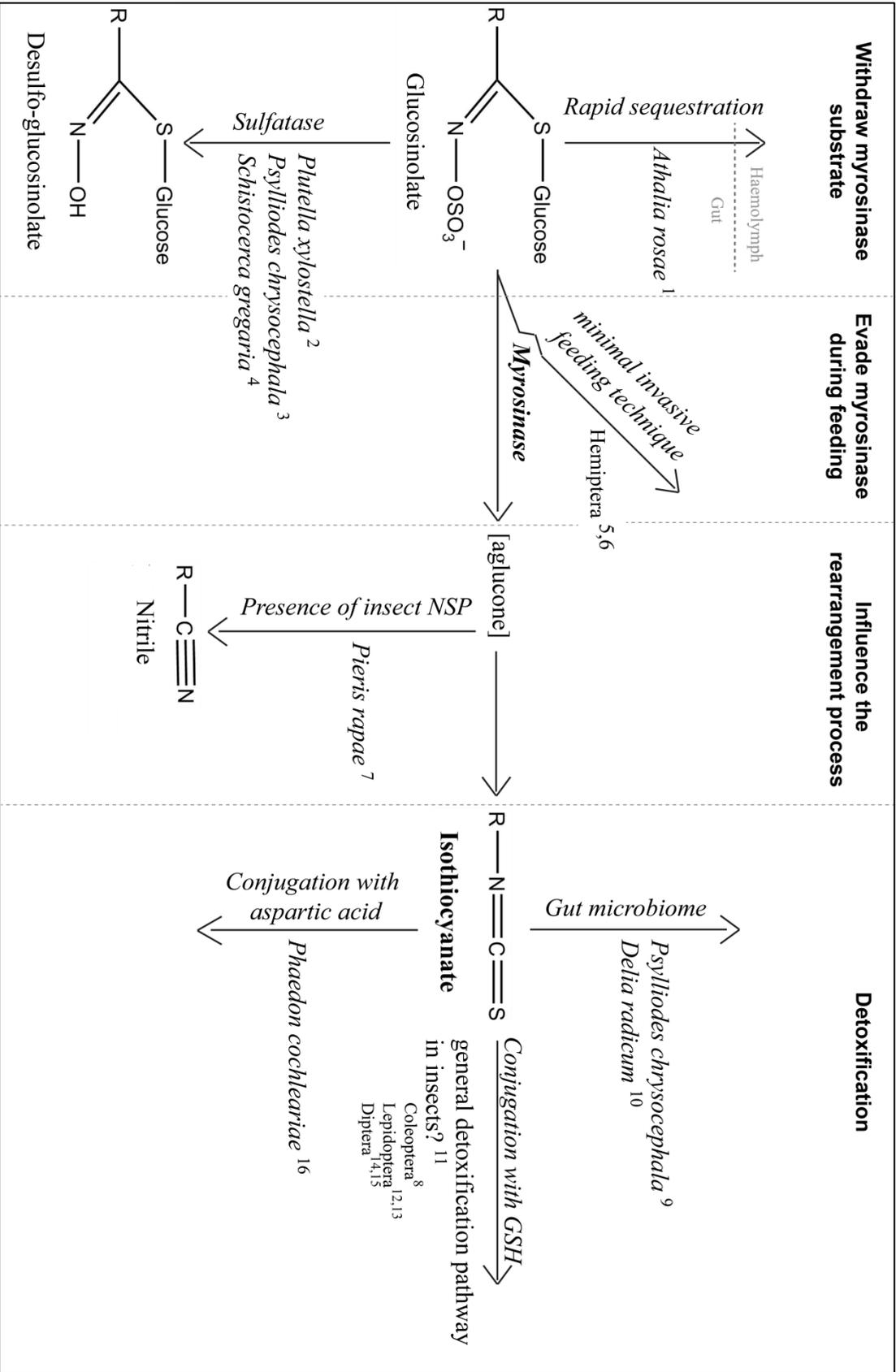
**Figure 1: The glucosinolate myrosinase system and different insect adaptations/strategies to prevent glucosinolate hydrolysis or detoxify isothiocyanates.** 1(Abdalsamee et al., 2014); 2(Ratzka et al., 2002); 3(Ahn et al., 2019); 4 (Falk and Gershenzon, 2007); 5(Bridges et al., 2002); 6(Aliabadi et al., 2002); 7(Wittstock et al., 2004); 8(Beran et al., 2018); 9(Shukla and Beran, 2020); 10(Welte et al., 2016a); 11(Kawakishi and Kaneko, 1985); 12(Wadleigh and Yu, 1988); 13(Schramm et al., 2012); 14(Vanhaelen et al., 2001); 15(Gloss et al., 2014); 16(Friedrichs et al., 2020). NSP= nitrile specifier protein; GSH = Glutathione. See also Supplement II.

Withdraw myrosinase substrate

Evade myrosinase during feeding

Influence the rearrangement process

Detoxification

Haemolymph

Gut

Glucosinolate

*Rapid sequestration*

*Athalia rosae* [1]

Sulfatase

Desulfo-glucosinolate

*Plutella xylostella* [2]
*Psylliodes chrysocephala* [3]
*Schistocerca gregaria* [4]

**Myrosinase**

minimal invasive feeding technique

Hemiptera [5,6]

[aglucone]

*Presence of insect NSP*

*Pieris rapae* [7]

Nitrile

**Isothiocyanate**

*Gut microbiome*

*Psylliodes chrysocephala* [9]
*Delia radicum* [10]

*Conjugation with GSH*

general detoxification pathway in insects? [11]
Coleoptera [8]
Lepidoptera [12,13]
Diptera [14,15]

*Conjugation with aspartic acid*

*Phaedon cochleariae* [16]

17

## 1.4. The insect gut

The expression of glucosinolate specific transporters in the foregut but not the midgut of *P. armoraciae* (Yang et al., *under review*) is surprising. Uptake of hydrophilic compounds present in ingested material happens usually in the insect midgut (Nation, 2016, Turunen, 1985). The insect fore- and hindgut are of ectodermal origin and therefore possess a chitinous cuticle known as intima (Chapman et al., 2013, Beutel et al., 2014). The foregut intima has an overall very low permeability for water and organic compounds (Fig. 2) and is thus considered a barrier for uptake processes (Treherne, 1967, Maddrell and Gardiner, 1980). Some instances of uptake of lipophilic compounds in the foregut are known (Hoffman and Downer, 1976, Joshi and Agarwal, 1977). But Glucosinolates are polar compounds (Nguyen et al., 2020), so if uptake is happening in the crop, it needs to be active and supposedly against a concentration gradient, since *P. armoraciae* stores glucosinolates in its haemolymph (Sporer et al., 2020)(Yang et al., 2020). The impermeability of the insect cuticle to hydrophilic compounds is attributed to the epicuticular wax layer (Wigglesworth, 1985, Locke, 1965, Treherne, 1957), which consists of waxes and sclerotin/cuticulin (Wigglesworth, 1985, Moussian, 2013, Wigglesworth, 1990).
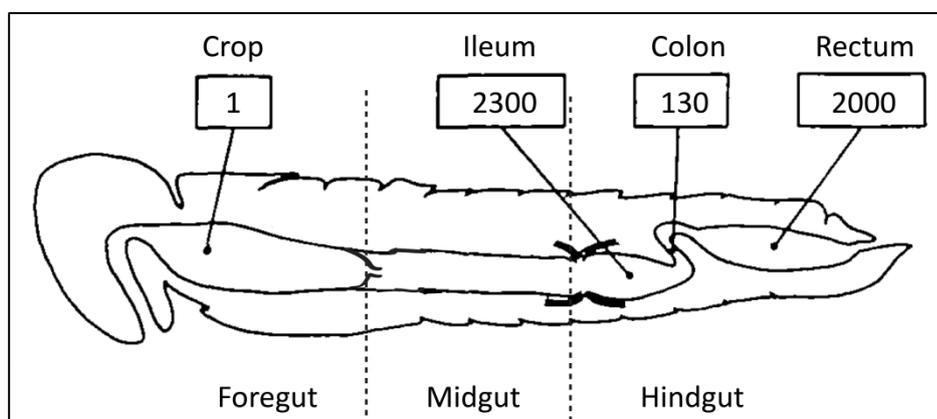


**Figure 2: Schematic insect gut structure with relative permeability of the cuticular linings of ectodermal gut parts of the desert locust *Schistocerca gregaria* (STÅL, 1873) for organic compounds**. Modified after (Maddrell and Gardiner, 1980)

Besides the foregut, the insect gut consists of two other distinct parts, the midgut and the hindgut (Fig.2). The midgut cells (Fig. S10) are involved in production and secretion of digestive enzymes, and take up water and nutrients from the ingested material into the haemolymph (Turunen and Crailsheim, 1996, Terra et al., 2019, Chapman et al., 2013). Compounds are taken up from the midgut into the haemolymph lumen either passively through trans- or paracellular diffusion, or actively through transporter mediated transport or

endocytosis (Denecke et al., 2018, Turunen, 1985). The hindgut consists of different parts; pylorus, ileum, colon and rectum (Fig.2). Ileum and rectum are the major sides of water and ion reabsorption (Turunen, 1985, Phillips et al., 1987a, Phillips, 1981, Maddrell, 1981). They are of ectodermal origin and possess an intima, which is more permeable for water, small ions and molecules than that of the foregut (Treherne, 1967, Maddrell and Gardiner, 1980, Turunen, 1985).

## 1.5. Aim of the study

### 1.5.1. Haemolymph analysis

To determine the actual glucosinolate levels in the haemolymph of the focal species *P. armoraciae* against which an uptake has to take place, I extracted haemolymph and analysed it with high-performance liquid chromatography coupled mass spectroscopy (HPLC/MS) for the presence of glucosinolates. Additionally, the concentration of amino acids and sugars in the samples were determined. Sugars are an important part of the insect haemolymph and function as energy source and in osmoregulation (Wyatt, 1961, Nation, 2016). The disaccharide trehalose is the major sugar in the haemolymph of higher insects (Nation, 2016, Gillott, 2005, Bedford, 1977, Kanost, 2009, Wyatt, 1961). Amino acids also play a key role in osmoregulation. Insects are known to have large amounts of free amino acids in their haemolymph (Nation, 2016, Woodring and Blakeney, 1980, Kanost, 2009).. Proline is usually one of the highest concentrated amino acids in the haemolymph (Wyatt, 1961, Kanost, 2009) and can be an energy source for flight muscles (Gäde and Auerswald, 2002, Kanost, 2009)

### 1.5.2. Are glucosinolates selectively taken up by different brassicales-specialist leaf beetle species?

Glucosinolate accumulation in the flea beetle species *P. armoraciae* and *P. chrysocephala* is well studied. Both species selectively accumulate different glucosinolate types in their haemolymph (Beran et al., 2018, Yang et al., 2020). The glucosinolate concentration in *P. armoraciae* adults is around ten times higher than in *P. chrysocephala* (Beran et al., 2018, Yang et al., 2020, Sporer et al., 2020). The physiological background of the glucosinolate uptake from the gut lumen into the haemolymph is still unknown. To test, if the selectivity with which plant glucosides are taken up differs between species, I performed a comparative feeding experiment. I used *P. armoraciae* and *P. chrysocephala*, which both sequester glucosinolates and the mustard leaf beetle *Phaedon cochleariae* (FABRICIUS, 1792) from the subfamily Chrysomelinae. *P. cochleariae* is a Brassicales specialist leaf beetle (Bogdanov-

Katjkov, 1923) and not known to sequester glucosinolates (Friedrichs et al., 2020). I compared the uptake of three different glucosinolates and three glucosides from other binary defence systems (Table 1; Fig. 4).

Because the recovery of intact glucosides was lowest from *P. cochleariae*, an additional experiment was performed with this species, to determine in which body part intact glucosides are degraded.
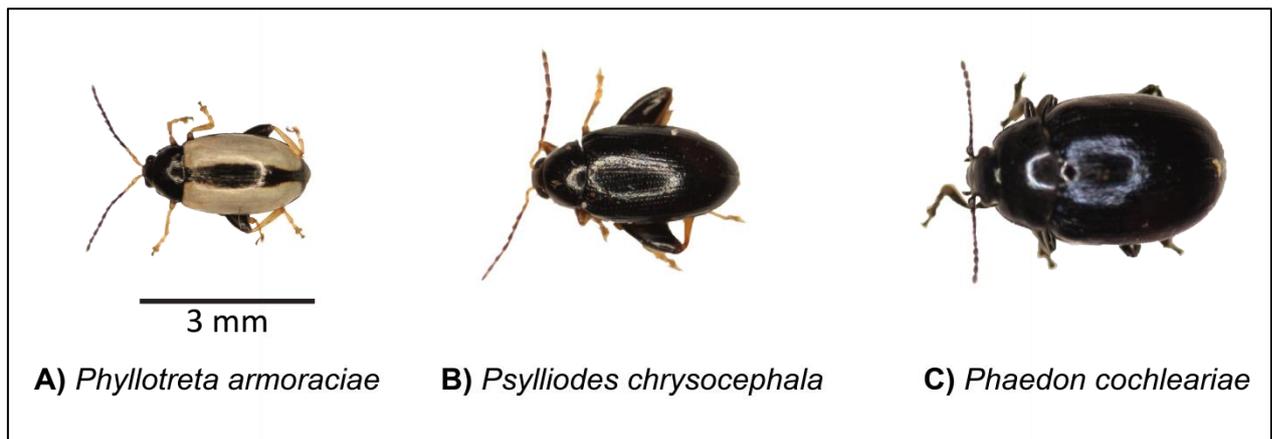


**Figure 3: The three leaf beetle species used in experiments:** A) *Phyllotreta armoraciae*, B) *Psylliodes chrysocephala* and C) *Phaedon cochleariae*

### 1.5.3. Are glucosinolates taken up in the foregut of *Phyllotreta armoraciae*?

The expression of glucosinolate specific transporters in the foregut of *P. armoraciae* suggests that this gut part plays a role in glucosinolate uptake (Yang et al., *under review*). To test this, I performed a short term feeding experiment, during which ingested plant material was only present in the foregut.

### 1.5.4. Does the crop structure of the three leaf beetles differ and does it show morphological adaptation for glucosinolate uptake?

Since the short-term feeding experiment showed, that a large proportion of ingested glucosinolates were taken up in the foregut of *P. armoraciae*, I investigated the morphology of the crop of this species in comparison with those of *P. cochleariae* and *P. chrysocephala*. The 3D structure of the alimentary system was reconstructed from micro-computed tomography (µCT) images. The chitinous crop structure was visualized by exciting the chitin autofluorescence using confocal laser scanning microscopy (CLSM) and the crop ultrastructure was investigated with transmission electron microscopy (TEM).

**Table 1: Overview over four binary defence systems, their occurrence in nature, a chemical example structure, their toxicity and reported cases of sequestration.** The glucosides presented as example structures were used in the "Selectivity of uptake" experiment.

| Binary defence system | Occurrence | Structures known/ Biosynthesis | Example structure | Toxin & toxicity | Sequestration | Further information on example structure |
|---|---|---|---|---|---|---|
| Cyanogenic glucosides | ferns, gymnosperms and angiosperms[1] | Around 50 known structures; biosynthesized from Amino acids[1,2,3] |  Linamarin | Cyanide[4], binding to cytochrome oxidase, cyanide is inhibiting cellular respiration[5] | **Lepidoptera:** Zygaena filipendulae[6,7] | Linamarin is e.g. found in Linum usitatissimum (Linaceae)[8,9] or Lotus corniculatus (Fabaceae)[7] |
| Salicinoids | Salicaceaes[10] | Around 20 known structures[10], biosynthesized from L-phenylalanine[11] |  Salicin | Formation of protein-binding quinones and tissue-damaging reactive oxygen species[11,12] | **Lepidoptera:** Limenitis archippus[13] **Coleoptera:** Chrysomela sp. & Phratora sp. [14,15,16] | Salicin and the derived salicylic acid are used as analgetic and anti-inflammatory agent in human pharmacology[10,17] |
| Iridoid glucosides | Asterids[18] | Several hundred structures are known, all sharing a similar skeleton derived from cyclopentanoid monoterpenes[19,20,21,22] |  Catalpol | The open-chain aglucones forms irreversible bonds with the nucleophilic sites of proteins, crosslinking them and inhibiting enzymes[22,23] | **Lepidoptera:** Euphydras phaeton[24] Ceratomia catalpa[25,26] **Hymenoptera:** Athalia circularis & A. cordata[27] **Coleoptera:** Longitarsus spp. [28] | Catalpol is one of the most abundant naturally occurring iridoids[29] e.g. it co-occurs with aucubin in Plantago species[30] |
| Glucosinolates | Brassicales; [and genus Drypetes (Euphorbiacae)][31] | >130[32], Biosynthesis from amino acids[33] |  Sinigrin | Isothiocyanate, (nitriles, epithionitriles) Isothiocyanates have strong affinity towards thiol, sulphide and amino groups of free proteins and amino acids[34,35,36] | **Hymenoptera:** Athalia rosae[37] A. liberta & A. lugens[38] **Hemiptera:** Brevicoryne brassicae & Lipaphis erysimi[39,40] Murgantia histrionica[41] **Coleoptera:** Psylliodes chrysocephala[42] Phyllotreta striolata[43] & P. armoraciae[44,45] | Sinigrin was the first described glucosinolate structure[47]. It is the major glucosinolate in Armoraciae rusticana[48] and Brassica juncea cv. Bau sin[43] |
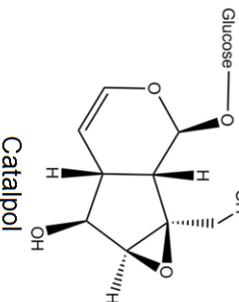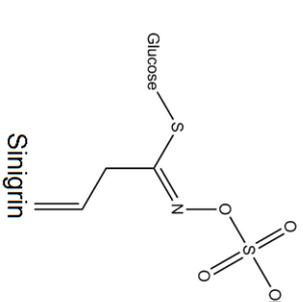
# 2. Material & Methods

## 2.1. Plant rearing

*Arabidopsis thaliana* Col – 0 wildtype plants and plants of the double knockout mutant *tgg1* x *tgg2* in the Col-0 background were cultivated under short-day conditions in a controlled environment chamber (55% humidity, 21°C, light/dark: 10 h/14 h). The *tgg1 x tgg2* mutant is virtually devoid of myrosinase activity in above ground plant tissues (Barth and Jander, 2006).

*Brassica rapa* cv. Yu-Tsai-Sum (purchased from "Known-You Seed", Kaohsiung, Taiwan), Pak choi, were cultivated in a controlled environment chamber (55% humidity, 21°C, light/dark: 14 h/10 h).

*Brassica juncea* cv. Bau Sin (purchased from "Known-You Seeds", Kaohsiung, Taiwan), Chinese mustard were cultivated in a controlled environment (60% humidity, 24°C, light/dark: 14 h/10 h).

## 2.2. Beetle rearing

*Phaedon cochleariae*

The lab population of *P. cochleariae* was established on Chinese cabbage, *Brassica rapa* ssp. *pekinensis* (commercial product from the local supermarket) and was reared for over 20 generations under laboratory conditions (60% humidity, 15°C, light/dark 16 h/8 h) (see (Kirsch et al., 2020)). All life stages were reared together. Eggs were collected regularly to establish new generations.

*Psylliodes chrysocephala*

The lab population was established in 2012 from beetles collected in Laasdorf, Thuringia, Germany. Adults were reared on three to four week old *B. rapa* in a controlled environment chamber (75% humidity, 24°C, light/dark: 16 h/8 h). After one week, the plants with the deposited eggs were transferred to a new cage for larval development. After three weeks, the soil containing the pupae was transferred to plastic containers (9 l volume, Lock&Lock) with a lid modified to enable air circulation. Plants were watered daily and newly emerged adults were collected thrice per week.

*Phyllotreta armoraciae*

The laboratory population of *P. armoraciae* was established in 2012 with beetles collected from *A. rusticana* plants in Laasdorf, Thuringia, Germany. Beetles were reared on *B. juncea* cv. "Bau Sin", which has sinigrin as the major glucosinolate (Beran et al., 2014) like the natural host plant of this species (Vig and Verdyck, 2001, Li and Kushad, 2004). In 2016, a second population feeding on *B. rapa* cv. "Yu-Tsai-Sum", was established with offspring of the *B. juncea* lab population. *B. rapa* has a different glucosinolate profile than *B. juncea* (Beran et al., 2014). Adults were reared on three to four week old plants in a controlled environment chamber (60% humidity, 24°C, light/dark: 14 h/10 h). After one week, the plants with the deposited eggs were transferred to a different cage for larval development. After three weeks, the soil, containing the pupae was transferred to plastic containers (9 l volume, Lock&Lock) with a lid modified to enable air circulation. Plants were watered daily and newly emerged beetle adults were collected thrice per week.

## 2.3.  Software usage

All data analyses were performed with "Microsoft Excel 2010".

Statistical tests were performed with SigmaPlot 11.0 (Systat Software Inc.).

HPLC/MS data sets were analysed with "SCIEX Analyst 1.6.3" and "Microsoft Excel 2010".

Figures were created using SigmaPlot 11.0 (Systat Software Inc.), CorelDRAW X6 and Adobe Illustrator CS5.Chemical structures were created using ChemDraw Professional 17.1 (PerkinElmer).

Image processing was conducted with ZEN software (ZEN 2011, Zeiss), Adobe Photoshop CS5 and Fiji (Schindelin et al., 2012)/ ImageJ (1.53p) with Bio-formats plugin (Linkert et al., 2010).

Segmentation for 3D reconstruction was done with Amira 6.0.1 (Thermo Fisher Scientific).

## 2.4.  Chemical analyses with HPLC/MS

High pressure liquid chromatography coupled mass spectrometry (HPLC/MS) analyses were either performed on an "Agilent 1260 Series" (Agilent Technologies, Böblingen, Germany) coupled to an "AB SCIEX API 5000 tandem mass spectrometer" (Applied Biosystems Darmstadt, Germany) or on an "Agilent 1200 Series" (Agilent Technologies, Böblingen, Germany) system connected to an "AB SCIEX API 3200 tandem mass spectrometer" (Applied Biosystems Darmstadt, Germany).

### 2.4.1. Glucosinolates

Glucosinolates in *P. armoraciae* haemolymph were analysed on an "Agilent 1200 HPLC" connected to an "API 3200". "Selectivity of glucoside uptake", "Glucoside degradation in *P.cochleariae* body parts" and "Short term feeding experiment" glucosinolate analyses were performed on "Agilent 1260 Series" coupled to an "API 5000".

For the separation of glucosinolates a "Nucleodur Sphinx RP" (length: 250 x 4.6 mm, particle size: 5 µm, Machrey-Nagel, Düren, Germany) column was used. The mobile phase consisted of 0.2% formic acid in ultrapure water as solvent A and acetonitrile as solvent B at a flow rate of 1 ml/min. Elution gradient was: 0-1 min, 1.5% B; 1-6 min, 1.5-5% B; 6-8 min, 5-7%; 8-18 min, 7-21% B; 18-23 min, 21-29%B; 23-23.1 min, 29-100% B; 23.1-24 min, 100% B; 24-24.1 min, 100-1.5%B; 24.1-28 min, 1.5% B. The ionization source was set to negative mode; ion spray voltage was maintained at -4,500 eV. Gas temperature was set to 700 °C, nebulizing gas to 70 psi, drying gas to 60 psi, curtain gas to 20 psi and collision gas to 10 psi. The method used in the mass spectrometer was MRM (multiple reaction monitoring). See Table 2 for compound identification parameters.

**Table 2: HPLC/MS parameter for the detection of intact glucosinolates**. Retention time in method file and measured retention time of the glucosinolates in standard are shown. RT= Retention time; DP = declustering potential; CE = collision Energy; gls = glucosinolate; std = standard

| Name | Q1 [m/z] | Q3 [m/z] | RT (in min) method | RT (in min) of std | DP (in V) | CE (in V) |
|------|----------|----------|--------------------|--------------------|-----------|-----------|
| Sinigrin | 358 | 95.9 | 10 | 9.1 | -65 | -60 |
| 4MSOB-gls | 435 | 95.8 | 8.6 | 9.15 | -65 | -60 |
| I3M-gls | 447 | 95.8 | 21.9 | 21.9 | -65 | -50 |
| Sinalbin | 424 | 95.9 | 13.3 | 13.4 | -65 | -60 |
| 3-Butenyl-gls | 372 | 95.9 | 14 | 13.4 | -65 | -60 |
| Benzyl-gls | 408 | 95.9 | 20.5 | 19.5 | -65 | -60 |
| 2-PE gls | 421 | 95.9 | 24.5 | 23.5 | -65 | -50 |
| 4OHI3M gls | 463 | 95.8 | 17 | 15.6 | -65 | -50 |

### 2.4.2. Non-glucosinolate plant glucosides.

Analyses of the non-host glucosides linamarin, salicin and catalpol were performed on an "Agilent Technologies 1260 Series" coupled to an "AB SCIEX API 5000".

Glucosides were separated on an "Agilent XDB-C18 column" (5 cm × 4.6 mm, 1.8 μm particle size) using a binary solvent system consisting of 0.05% (v/v) formic acid in water as solvent A and acetonitrile as solvent B with a flow rate of 1.1 mL/min at 25 °C. Elution gradient was: 0-0.5 min, 5% B; 0.5-2.5 min, 5-31% B; 2.5-2.52 min, 31-100% B; 2.25-3.5 min, 100% B; 3.5-3.51 min, 100-5% B, 3.51-6 min, 5% B. The ionspray voltage was maintained at -4200 eV. The turbo gas temperature was set at 630 °C. Nebulizing gas was set at 60 psi, curtain gas at 30 psi, and collision gas at 5 psi. The method used by the mass spectrometer was multiple reactions monitoring (MRM). Glucosides were quantified by external standard curves. See Table 3 for compound identification parameters.

**Table 3: HPLC/MS parameter for the detection of intact non-glucosinolate glucosides**. Retention time in method file and measured retention time of the glucosinolates in standard are shown. RT= Retention time; DP = declustering potential; CE = collision Energy

| Name | Q1 [m/z] | Q3 [m/z] | RT (in min) | DP (in V) | CE (in V) |
|---|---|---|---|---|---|
| Salicin | 284.874 | 122.898 | 2.6 | -50 | -18 |
| Linamarin form 161 | 292 | 161 | 1.2 | -50 | -20 |
| Linamarin form 45 | 292 | 45 | 1.2 | -50 | -26 |
| Catalpol | 361 | 199 | 0.8 | -50 | -10 |
| Catalpol - formiate | 407 | 199 | 0.8 | -50 | -10 |

### 2.4.3. Sugars

An "Agilent 1200 HPLC" system connected to an "API 3200" was used for sugar analysis. Separation happened on an "apHera NH2 Polymer" (length: 15 x 4.6 mm, particle size: 5 μm, Supelco) column, using a mobile phase consisting of ultrapure water as solvent A and Acetonitrile acetonitrile as solvent B at flow rate of 1.0 mL/min. Elution gradient was: 0-0.5 min, 80%B; 0.5-13 min, 80-55% B; 13-14 min, 55-80%; 14-18 min, 80%B. The ionspray voltage was maintained at -4500 eV. The turbo gas temperature was set at 600 °C. Nebulizing gas was set at 50 psi, curtain gas at 20 psi, heating gas at 60 psi and collision gas at 5 psi. The

method used by the mass spectrometer was multiple reactions monitoring (MRM). Sugars were quantified by external standard curves. See table 4 for compound identification parameters.

**Table 4: HPLC/MS parameters for the detection of sugars.** RT= Retention time; DP = declustering potential; CE = collision Energy

| Name | Q1 [m/z] | Q3 [m/z] | RT (in min) method | DP (in V) | CE (in V) |
|---|---|---|---|---|---|
| Glucose | 178 | 89 | 7.4 | -25 | -12 |
| Sucrose | 340 | 59 | 8.9 | -55 | -45 |
| Trehalose | 340 | 59 | 9.75 | -55 | -45 |
| Sorbitol | 180 | 89 | 6.8 | -35 | -22 |
| Unknown sugar-alcohol | 180 | 89 | 6.05 | -35 | -22 |

## 2.5. Haemolymph Analysis

Haemolymph was collected from one week old *P. armoraciae* adults from the *B. juncea* population and analysed for glucosinolates, sugars and amino acids. Beetles fed on their host plants until haemolymph extraction. The beetles were put in a petri dish with their elytra on sticky tape (Tesa® Powerstrips® Large). One or two hind legs were removed at the coxa, using forceps and the emerging haemolymph droplet was collected in a 0.5 µL glass capillary (Hirschmann® minicaps® End-to-end). The capillaries were marked in 1 mm sections (corresponding to 15.6 nL) to determine the collected haemolymph volume. The collected haemolymph was pipetted into 500 µL methanol (90%) to stop enzymatic activity. A total of six replicates were collected, each containing haemolymph of 50 beetles.

Sample processing started with homogenization at 30 Hz for 1 min (Qiagen TissueLyser II). Next, the samples were boiled at 95 °C for 5 min ("Eppendorf Thermomixer comfort") to denature all proteins. After cooling, the samples were centrifuged twice for 10 min at 18,312 $\times$ *g*. The pellet was discarded and the supernatant was vacuum dried ("Eppendorf Concentrator 5301"). The dried sample was redissolved in 50 µL methanol (50%), vortexed for 30 s, left for 5 min, vortexed for 30 s and centrifuged for 1 min at 18,312 $\times$ *g*. The concentrations of glucosinolates and sugars were analysed separately in different dilutions using HPLC/MS (See 2.4.)

## 2.6. Amino acid detection after derivatisation with FMOC

Samples for amino acid analysis were derivatised with fluorenylmethyloxycarbonyl (FMOC, Fluka, Germany) to make them less polar and enhance separation (see (Pande et al., 2014) for method). 2 µL of haemolymph sample was spiked with 98 µL standard solution containing 16 isotopically labelled amino acids (algal amino acids 13C, 15N, Isotec, Miamisburg, US; c = 10 µg/mL in ultrapure water). 100 µL Borate Buffer (0.8 M, pH = 10) and 200 µL FMOC-Acetonitrile (30 mM) were added to the spiked samples and left to react for 5 min. 800 µL of hexane were added and after 20 min, 200 µL of the lower, aqueous phase was taken and analysed by HPLC/MS. The samples were quantified by internal standards, which did not contain methionine, histidine, lysine, cysteine, tyrosine and tryptophan, therefore no data for these amino acids was available. Furthermore isoleucine and leucine form a combined peak.

For the analysis an "Agilent Technologies 1290 Infinity" (Agilent Technologies, Böblingen, Germany) HPLC system connected to an "API 5000" (AB Sciex, Darmstadt, Germany) were used (See table 5 for detailed parameters of the HPLC/MS analysis of amino acids). The column used for separation was a "Zorbax Eclipse XDB-C18 column" (length: 50x4.6 mm, particle size: 1.8 µm, Agilent Technologies, Germany). Formic acid (0.05%) in water was solvent A and acetonitrile solvent B of the mobile phase which had a flow rate of 1.1 ml/min. The elution profile was: 0-0.5 min, 10% B; 0.5-4.5 min, 10-90%; 4.5-6 min, 90-100% B; 6-6.5 min; 100% B; 6.5-6.51 min, 100-10%; 6.51-9 min, 10%B. The turbo spray ion source operated in negative ionization mode; ionspray voltage was -4,500 eV; turbo gas temperature was 700 °C. Nebulising gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. Multiple reactions monitoring (MRM) was used.

**Table 5: HPLC/MS parameters for the detection of FMOC-derivatised amino acids and the isotopically labelled internal standard in the haemolymph of *P. armoraciae*.** RT= Retention time; DP = declustering potential; CE = collision Energy; std = standard

| Name | Q1 [m/z] | Q3 [m/z] | RT (min) method | RT (min) of std | DP | CE | Internal std | Q1 [m/z] | Q3 [m/z] |
|---|---|---|---|---|---|---|---|---|---|
| Ala-FMOC | 310 | 88 | 4.5 | 4.39 | -55 | -10 | labAla-FMOC | 314 | 92 |
| Ser-FMOC | 326 | 130 | 4 | 3.95 | -55 | -14 | labSer-FMOC | 330 | 134 |
| Val-FMOC | 338 | 116 | 4.8 | 4.75 | -60 | -10 | labVal-FMOC | 344 | 122 |
| Thr-FMOC | 340 | 144 | 4.2 | 4.1 | -65 | -14 | labThr-FMOC | 345 | 149 |
| Leu-FMOC | 352 | 130 | 5 | 4.96 | -60 | -10 | labLeu-FMOC | 359 | 137 |
| Asn-FMOC | 353 | 157 | 5 | 4.96 | -65 | -12 | labAsn-FMOC | 359 | 163 |
| Asp-FMOC | 354 | 157,8 | 4.1 | 3.99 | -70 | -16 | labAsp-FMOC | 359 | 162,8 |
| Gln-FMOC | 367 | 145,001 | 4.1 | 4.22 | -65 | -12 | labGln-FMOC | 374 | 152 |
| Glu-FMOC | 368 | 172 | 4.1 | 4.01 | -70 | -14 | labGlu-FMOC | 374 | 178 |
| Phe-FMOC | 386 | 164 | 5 | 4.93 | -60 | -10 | labPhe-FMOC | 396 | 174 |
| Pro-FMOC | 336 | 114 | 4.6 | 4.53 | -60 | -10 | labPro-FMOC | 342 | 120 |
| Arg-FMOC | 395 | 173 | 3.5 | | -75 | -18 | labArg-FMOC | 405 | 183 |
| Trp-FMOC | 425 | 203 | 4.7 | | -55 | -12 | | | |

## 2.7. Selectivity of glucoside uptake in three leaf beetle species

To determine if the uptake of plant glucosides is selective in leaf beetles, a comparative feeding experiment was performed with *P. armoraciae* (reared on *B. rapa*), *P. chrysocephala* and *P. cochleariae*. All beetles were collected as newly emerged adults and kept on a moist tissue with leaf material of *B. rapa* for 2 d. 24 h before the experiment, beetles were kept on dry tissue to ensure an empty gut and stimulate drinking behaviour.

An equimolar 1.5 mM glucoside mixture containing the three glucosinolates sinigrin (Fig. 4 A I), 4MSOB-glucosinolate (Fig. 4 A II) and sinalbin (Fig. 4 A III) and the three non-host glucosides linamarin (Fig. 4 B), catalpol (Fig. 4 C) and salicin (Fig. 4 D) was prepared (see also Table 1). Amaranthe dye in a concentration of 0.2% (w/v) was also included in the glucoside mixture as an indicator for the uptake of the droplet into the gut and for tissue intactness during dissection.
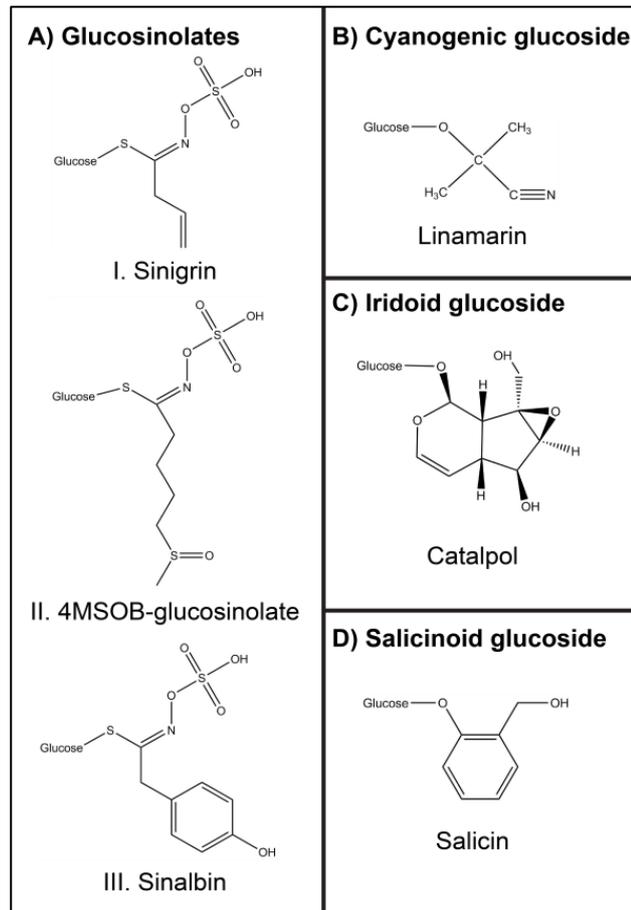
**Figure 4: The glucosides in the equimolar glucoside mixture.** A) Glucosinolates, B) Cyanogenic glucoside, C) Iridoid glucoside, D) Salicinoid

*P. chrysocephala* refused to drink the mixture. Through presentation of the individual compounds in the mix, catalpol was identified as the deterrent and excluded from experiments with this species.

The beetles were put in a small petri dish with their elytra onto sticky tape (Tesa® Powerstrips® Large). They were allowed to drink two 50.6 nL droplets of the equimolar 1.5 mM glucoside mixture (Fig. 4), that were provided using a microinjector ("Nanoliter 2010", World Precision Instruments). If a droplet was not taken up properly by the beetle, the beetle was discarded. After the uptake of the second droplet, the beetles remained on sticky tape for 4 min and were subsequently dissected into head, gut and 'Rest body'. The gut was washed twice in PBS-buffer (pH = 6.8). If fore – and midgut were not intact until the hindgut, the sample was discarded. A total of 10 replicates per species were collected, each contained body parts of three beetles. 210 µL (head, gut) and 280 µL ('Rest body') 80% methanol were added to the replicates. They were homogenized using plastic pestles.

For each replicate, a control replicate was taken with beetles from the same batch. Controls were not fed but dissected like the replicates, to determine the background of glucosinolate in the beetles. Each control replicate comprised three beetles. Additionally, to determine if loss by dissection occurred, from each beetle batch a 'recovery control' was taken. Theses beetles were fed as described above but homogenized without being dissected. The waiting period between ingestion and homogenisation was extended to 5 min to account for the time required for dissection. To determine the real ingested glucoside amount ingested by the beetles, samples of the glucoside mix were taken by injecting six droplets (50.6 nL) into a 5 µL drop of ultrapure water. After a waiting period of 5 min it was collected in 280 µL 80% methanol.

For the extraction, samples were vortexed for at least 30 s and centrifuged for 10 min at $16,100 \times g$. The pellet was discarded and the supernatant was dried in a vacuum centrifuge ("Eppendorf Concentrator 5301") at 30 °C. The dried sample was dissolved in 60 µL ultrapure water, vortexed for 30 s and centrifuged for 30 s with $6,700 \times g$. After waiting 5 min the samples were vortexed for at least 1 min, briefly centrifuged and transferred to plastic vials for HPLC-MS/MS analysis. (See 2.4.)

## 2.8. Glucoside degradation by body part homogenates of *Phaedon cochleariae.*

Recovery of intact glucosides was low from *P. cochleariae*. To determine where glucosides are degraded in *P. cochleariae*, incubation experiments with homogenates of the body parts head, gut and 'Rest body' were performed.

*P. cochleariae* were collected as pupae. After moulting into adult stage, they were kept on *B. rapa* plant material for 2 d. Beetles were starved for 1 d prior to the experiment. Beetles were dissected in air into head, gut and 'Rest body'. Heads and guts were washed in PBS buffer (pH = 6.8) twice after dissection. Each replicate contained the respective body part of two beetles. They were homogenized in ultrapure water (head & gut: 20 µL, 'Rest body': 50 µL) using pestles. Subsequently four 50.6 nL droplets of the equimolar 1.5 mM glucoside mixture (see Fig. 4) were placed on the side of each Eppendorf tube. The tubes were centrifuged for 1 s to mix the droplets with the homogenate and briefly centrifuged. After 5 min incubation, the replicates were frozen in liquid nitrogen and subsequently stored in -20 °C. A total of 6 replicates for each body part were taken. As a control, four droplets of the equimolar glucoside mix were injected into 5 µL ultrapure water by the above mentioned method.

Extractions were performed by adding pure methanol to the frozen samples to stop enzymatic activity whilst thawing. The methanol volume was calculated, so that the final concentration (v/v) of methanol was 75% (volume of methanol head & gut: 60 µL, 'Rest body': 150 µL; controls: 15 µL). Replicates were vortexed for 30 s and centrifuged for 10 min at $16,100 \times g$. The supernatants were transferred to new tubes. The pellets were extracted with 75% (v/v) methanol twice more (volume added to head & gut: 60 µL, 'Rest body': 100 µL; controls: 10 µL) and the extracts were pooled. The pellets were discarded and the extracts were dried by vacuum centrifugation at 30 °C ("Eppendorf Concentrator 5301"). The replicates were each redissolved in 60 µL ultrapure water and vortexed for 30 s, centrifuged for 30 s at $6,700 \times g$ left for 5 min and then again vortexed for > 1min. Replicates were transferred to plastic vials and analysed by HPLC/MS (see 2.4.).

### 2.9. Location of glucosinolate uptake in *P. armoraciae*

To determine if glucosinolates are taken up in the foregut of *P. armoraciae*, I conducted a short-term feeding experiment, during which plant material was only present in the foregut. *P. armoraciae* beetles (*B. juncea* population) were collected as newly emerged adults and kept with leaf material of *B. juncea* for 40 h. Beetles were starved 24 h before the experiment to ensure an empty gut and stimulate feeding.

Beetles were allowed to feed on a leaf of *A. thaliana* Col-0 wildtype (fresh weight determined prior to experiment) for 15 s. If a beetle fed for 15 s, it was immediately dissected. If not, it was discarded. Dissection was performed in air on a cold metal dissection dish. Head, fore- and midgut were pulled from the 'Rest body' and washed twice in PBS buffer (pH = 6.8). The 'Rest body' was put in liquid nitrogen. 70 µL 80% methanol was added to head, fore- and midgut on the dissection dish. The head was removed and put in liquid nitrogen. The gut in methanol was taken up with a pipette and immediately homogenized using a plastic pestle. A total of 10 replicates were taken, each containing 3 beetles which fed on the same leaf. After adding the third 'Rest body' to the replicate, 300 µL 80% methanol were added and the replicate was homogenized using a plastic pestle. 200 µL 80% methanol was added to head replicates and it was homogenized with a plastic pestle. The leaves used for the experiment were frozen and analysed for their glucosinolate content by HPLC/UV. Detection and quantification focused on 4MSOB-glucosinolate, the dominant aliphatic glucosinolate of the *A. thaliana* Col-0 line (Kliebenstein et al., 2001). 10 control replicates were taken of beetles not fed on *A. thaliana* to determine the glucosinolate background in the beetles. Replicates were analysed by HPLC/MS (see 2.4.).

### 2.9.1. Influence of Myrosinase

To determine, if plant myrosinase has an influence on sequestration, the same experiment was also conducted with the *A. thaliana* Col-0 *tgg1/tgg2* double knock-out mutant which does not have myrosinase activity (Barth and Jander, 2006).

### 2.9.2. Extraction of plant glucosinolates as desulfo-glucosinolates and subsequent HPLC-UV analysis

The leaves used for the experiments were tested for their glucosinolate content and –profile. Glucosinolates were extracted as desulfo-glucosinolates and analysed by HPLC-UV.

The frozen leaf samples were freeze dried (Alpha 2-4 LD, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode im Harz) for 3 d. The dried leaf material was homogenized for 3 min at 25 Hz (Qiagen Tissue Lyser). 1 mL 80% methanol containing sinigrin as an internal standard (c = 30 µM) was added. Afterwards the samples were heated to 95 °C for 5 min to denature proteins. Samples were centrifuged for 10 min at 16,100 x *g*, the supernatant transferred to a new tube and the pellet discarded.

A 96 column plate was prepared with DEAE sephadex A-25 (Sigma Aldrich), which was conditioned with 1 mL ultrapure water and two times 500 µL 80% methanol per column. After conditioning, the sephadex columns were loaded with the extract. The sephadex bound the glucosinolates, so as 500 µL 80% methanol and two times 1 mL ultrapure water were subsequently added to the column; all compounds soluble in these liquids were washed out. Afterwards 500 µL MES buffer were added. Erroneously 0.5 M MES buffer was used instead of 0.02 M. To account for this, two additional 500 µL loads of 0.02 M MES buffer (pH = 5.2) were added to the columns. The pH of the MES buffer caused an acidic pH environment in the sephadex column. 30 µL sulfatase -solution from *Helix pomatia* (LINNAEUS, 1758) (prepared after the method of Graser et al., 2001 was added to each column. This sulfatase works best in acidic pH. The columns were closed with parafilm and incubated overnight. On the next day, the desulfo-glucosinolates were eluted into a 96 well plate, using 500 µL ultrapure water. The glucosinolates in the plant samples were detected by HPLC-UV.

HPLC/UV analysis was performed as described in Beran et al. 2014. Samples were analysed by HPLC ("Agilent Technologies HP1100 Series") coupled to a photodiode array detector and a reversed phase column ("NUCLEODUR Sphinx RP", $250 \times 4.6$ mm, 5 µm particle size; Macherey–Nagel) using an ultrapure water (solvent A) and acetonitrile (solvent B) gradient with a flow rate of 1 mL/min. The injection volume was 50 µL. The elution gradient was: 0-1 min, 1.5% B; 1-6 min, 1.5-5% B; 6-8 min, 5-7% B; 8-18 min, 7-21% B;18-23 min, 21-29% B; 23-23.5 min, 29-100% B; 23.5-26 min, 100% B; 26-26.01 min: 100- 1.5% B; 26.01-31 min: 1.5% B

Identification of glucosinolates was done by Dr. M. Reichelt. The concentration of 4MSOB-glucosinolate per fresh weight was determined by quantifying the peaks relative to the internal standard taking response factors (Clarke, 2010) into account.

## 2.10. Gut structure morphology

### 2.10.1. Light microscopy

Newly emerged adults of *P. armoraciae*, *P. chrysocephala* and *P. cochleariae* were collected and kept on *B. rapa* plants for 2 d. 24 h before dissection, the beetles were starved to ensure an empty gut. The beetles were dissected in air. Guts were placed on a slide with well in a PBS triton-X-100 mix. Triton was added to decrease surface tension and prevent the gut from floating. Darkfield pictures were taken with an "Axiocam 506 color" (Zeiss, Oberkochen, Germany) on an "Axio Zoom.V16", (Zeiss, Oberkochen, Germany) 506 with a "PlanNeoFluar Z 1.0x" (Zeiss, Oberkochen, Germany) Objective.

### 2.10.2. Micro computed tomatography/ 3D reconstruction

*P. armoraciae* and *P. chrysocephala* were collected as newly emerged adults. *P. cochleariae* were collected as adults and starved for 2 d prior to fixation. Beetles were fixed in "Dubosq Brasil" (Provided by Dr. H. Pohl, FSU Jena) for 24 h. To ensure thorough penetration of the inner tissues, the legs were removed at the coxae. After fixation, the samples were washed with 70% ethanol and dehydrated by an ethanol-series with ascending ethanol concentrations (70%, 80%, 90%, 96%, 100%). The samples remained in each concentration for 20 min. The 100% concentration step was repeated thrice. The samples were stained with iodine ($I_2$ 1% (w/v) in 100% ethanol) for 4 d. After staining, the samples were critical point dried (EmiTech K850 Critical Point Dryer, Quorum Technologies Ltd., Ashford, England). Micro CT pictures were taken on a Bruker Skyscan 2211 μCT scanner (Bruker, Belgium) at the Max-Planck-Institut für Menschheitsgeschichte, Jena,Germany, equipped with a high-resolution (4,000×2,600 pixel) X-ray sensitive CCD camera. For beam strength, exposure time, image pixel size and rotation steps in 360° scan see table 6. Segmentation was done in Amira 6.0.1.

**Table 6: µCT parameters**

| Species | Source voltage (kV) | Source current (µA) | Exposure time | Image pixel size (µm) | Rotation step (degrees) |
|---|---|---|---|---|---|
| Phaedon cochleariae | 40 | 300 | 1800 | 1.8 | 0.15 |
| Psylliodes chrysocephala | 40 | 300 | 1800 | 1.4 | 0.15 |
| Phyllotreta armoraciae | 30 | 170 | 1600 | 2.1 | 0.1 |

### 2.10.3. Confocal laser scanning microscopy

To investigate the chitinous structure of their crops, confocal laser scanning microscopy (CLSM) was performed with *P. armoraciae* (from *B. juncea* population), *P. chrysocephala* and *P. cochleariae*. The autofluorescent properties of chitin were used (see Pentzold et al., 2019). Beetles were collected as adults and fed for at least 24 h on their respective host plant to ensure a fully developed gut. Beetles were starved for 24 h before dissection. Dissection was conducted in air. The dissected guts were placed in pure glycerine or 1:1 glycerine - PBS buffer on a slide. The confocal laser scanning microscope used was a "LSM 880" (Zeiss, Oberkochen, Germany), with different objectives and magnifications (see table S8). Chitin autofluorescence was excited by a 405 nm laser diode (Diode 405-30) with a light source power of 30% and fitting main beam splitter. Emission was detected between 410 nm and 695 nm (see Table S8 for detailed parameters).

### 2.10.4. Transmission electron microscopy (and semi thin sections)

The ultrastructure of the crop of *P. amoraciae*, *P. chrysocephala* and *P. cochleariae* was investigated using Transmission Electron Microscopy (TEM). Primary fixations and dissections were conducted at the Max-Planck-Institute for chemical Ecology and secondary fixation, staining, embedding, trimming and microscopy was done at the Elektronenmikroskopischen Zentrum Jena (EMZ). *P. armoraciae* and *P. chrysocephala* used were newly emerged adults; *P. cochleariae* was starved for 3 d prior to fixation to ensure the absence of plant material in the crop. One *P. cochleariae,* one *P. chrysocephala* and two *P. armoraciae* specimens were investigated.

Primary fixation was done in Karnovsky's fixative (4% Formaldehyde, 2.5% Glutaraldehyde in 0.1 M Sodium-Cacodylate (Na-Cacodylate) Buffer; pH = 7.4; recipe by Dr. S. Nietzsche, EMZ Jena). Femurs of the beetles were broken in the middle to enable penetration of fixative into the body cavity. Each individual remained in 1 mL of fixative for 24 h at room temperature. Each sample was washed thrice with 0.1 M Na-Cacodylate buffer in 20 min intervals and left in the buffer overnight at room temperature. The first specimen of *P. armoraciae* and the *P. cochleariae* were erroneously washed in 0.25 M Na-Cacodylate buffer. The samples were washed a fourth time with Na-Cacodylate buffer and dissected in the same buffer. Secondary fixation, contrasting and embedding were conducted by C. Kämnitz or Dr. S. Nietzsche at the EMZ Jena. Secondary fixation and first contrasting was performed with osmium tetroxide (1% OsO4 in 0.1 M Na-Cacodylate buffer) for 2 h. Afterwards three 10 min washing steps in 0.1 M Na-Cacodylate followed. Subsequently the samples were dehydrated with an ethanol series (10 min in 30% ethanol, 5 min in 50% ethanol). Dehydration was intermitted by the second contrasting step, for which the samples were placed in 2% uranyl acetate in 50% ethanol for 1 h in the dark. The dehydration was continued (10 min in 50% ethanol; 10 min in 70% ethanol). Overnight, the samples remained in 70% ethanol. On the next day the dehydration was completed (3x 10 min in 96% ethanol; 3x 10 min in 100% ethanol). As a final step of dehydration, the samples were transferred to propylene oxide for 2 min. Samples were transferred to a series of Araldite and propylene oxide mixtures of different ratios to accomplish a gradual infiltration of the samples with Araldite, remaining in each step for at least 1 h (1:2 Araldit:-propylene oxide mix; 1:1; 2:1). Lastly, the samples were placed in pure Araldite for 0.5 h and then into an Araldite filled silicone mould and left to harden for 72 h in a drying cabinet at 60 °C. The embedding medium Araldite (Plano GmbH Wetzlar, Germany. Art. Nr. R1030) was prepared by mixing epoxy resin (Araldite Cy212, 22 g), DDSA (Dodecenylsuccinic anhydride, 22 g) and Dibuthylphthalate (1 g). This mixture was stored in the fridge. Shortly before the embedding, the hardener, 2.4% BDMA (Benzyldimethylamin), was added to the mix of the other three ingredients.

Trimming, sectioning, and electron image recording were conducted by Dr. S. Nietzsche of the EMZ Jena. Semi thin sections were performed for overview and orientation purposes and were stained with Richardson Blue (1:1 mixture of 1% Azur II in deionized water and 1% Methylene Blue in 1% Borax solution after Richardson et al., 1960). The electron microscope used was a Zeiss-EM 900 digital (Zeiss, Oberkochen, Germany) with a resolution of 0.6 nm, operating with 80 kV. Photodocumentation was done with a 2k slow scan CCD Camera (TRS, Moorenweis, Germany).

# 3. Results

## 3.1. Analysis of the haemolymph composition of *Phyllotreta armoraciae*

The haemolymph of one week old *P. armoraciae* adults (reared on *B. juncea*) was collected and analysed for its composition of glucosinolates, amino acids and sugars. Haemolymph volume of replicates was between 2.5 µL and 3.2 µL extracted from 50 beetles.

### 3.1.1. Glucosinolates

Total concentration of glucosinolates in the haemolymph was $60.9 \pm 17.2$ mM. Sinigrin was the major glucosinolate with a concentration of $56 \pm 15.4$ mM, and accounted for around 92% of the total glucosinolate concentration (Fig. 5A). High concentrations of 3-butenyl glucosinolate ($2.4 \pm 1.6$ mM) and I3M glucosinolate ($1.7 \pm 0.9$ mM) were also detected in the haemolymph, while only traces of benzyl-glucosinolate and 2-phenylethyl glucosinolate were found.

### 3.1.2. Amino acids

Histidine, lysine, methionine, cysteine, tyrosine and tryptophan were not included in the internal standard and could not be detected. Furthermore isoleucine and leucin formed a combined peak and could not be distinguished from each other. The concentration of detected amino acids in the haemolymph was $66.1 \pm 12.2$ mM. The most abundant amino acid was proline (Fig. 5B) with a concentration of $25.2 \pm 4.9$ mM, which accounted for 38% of total detected amino acids. Together with the second highest concentrated amino acid glutamine ($10.6 \pm 2.9$ mM) they accounted for 54% of total detected amino acids. Proline and glutamine concentrations also had the highest variability of all detected amino acids.

### 3.1.3. Sugars

The total sugar concentration in the haemolymph was $10.6 \pm 4.9$ mM. The most prevalent sugar found was glucose (Fig. 5C) with a concentration of $9.1 \pm 4$ mM, which accounted for 86% of total detected sugar concentration. Sucrose ($0.8 \pm 1.4$ mM) was more abundant than the trehalose, which was only detected in trace amounts ($0.02 \pm 0.01$ mM). An unknown sugar alcohol with a retention time around 6.03 min was also found and quantified using the sorbitol standard curve. It had a peak distinctly different from both, manitol and myo-inisitol (*data not shown*). Furthermore, fructose was also detected in traces in preliminary experiments (*data not shown*).

**A)** <u>Glucosinolates</u>



**B)** <u>Amino acids</u>



**C)** <u>Sugars</u>



mM in *Phyllotreta armoraciae* haemolymph
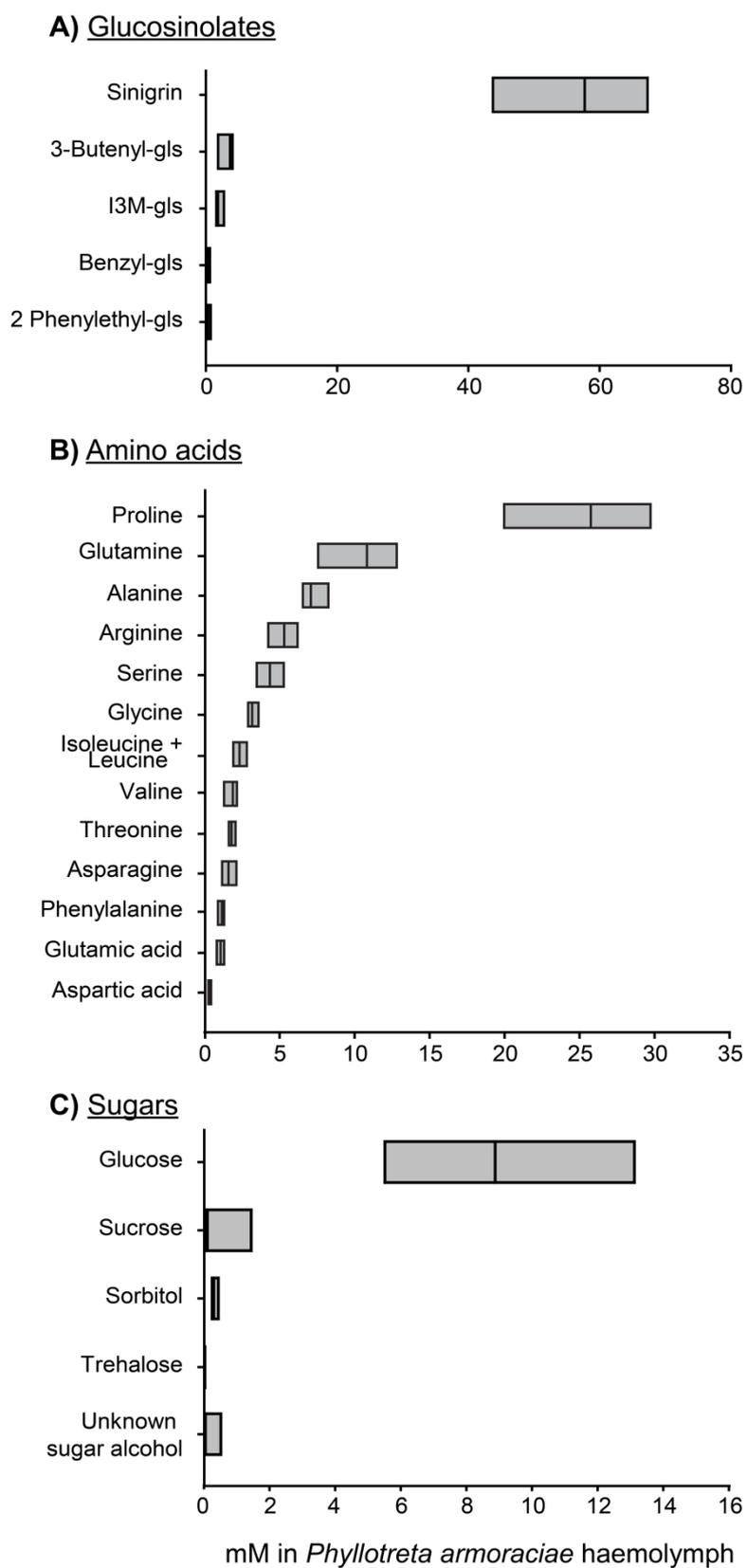
**Figure 5: Concentrations of A) Glucosinolates, B) Amino acids and C) Sugars in the haemolymph of *P. armoraciae* in mM**. N=6. gls = glucosinolate

### 3.2. Selectivity of plant glucoside uptake in three leaf beetle species

To analyse whether glucosides are selectively taken up by chrysomelid beetles, I fed a mixture of different plant glucosides to three leaf beetles species: The two glucosinolate sequestering species *P. armoraciae* and *P. chrysocephala*, and the non-glucosinolate sequestering *P. cochleariae*. The glucoside mixture contained three glucosinolates (sinigrin, 4MSOB-glucosinolate and sinalbin), and three non-host glucosides (linamarin, salicin and catalpol) (Fig.4). *P. chrysocephala* refused to drink the glucoside mixture when catalpol was present. Thus catalpol was excluded from the experiments with *P. chrysocephala*.

All six glucosides were found in the "Rest body" of all three species but in different proportions (Fig. 6A, B). If an intact glucoside was recovered from the 'Rest body', it was regarded as having been sequestered. Sequestration differed between the three species. A significantly lower sequestration of all three glucosinolates was found in *P. cochleariae* compared with the other two species (Fig.6A), Between *P. armoraciae* and *P. chrysocephala*, sinalbin was the only glucosinolate of which the sequestration differed significantly (Fig. 6A). It was found to be higher in *P. chrysocephala*. In all three species, glucosinolates were more sequestered than non-host glucosides (Fig. S2, Fig. 6A). *P. chrysocephala* sequestered significantly more of the two non-host glucosides linamarin and salicin than the other two species (Fig. 6B). Salicin was sequestered by *P. chrysocephala* with the same efficiency as the glucosinolates (Fig. S3). *P. armoraciae* sequestered significantly more salicin and catalpol than *P. cochleariae* (Fig. 6B).

The recoveries of ingested intact glucosides differed significantly between species and glucosides (Fig.1C, D, Fig. S3). The recovery relative to ingested amount of all three glucosinolates was lowest from *P. cochleariae* (Fig. 6C). Some glucosinolates loss in *P. cochleariae* occurred by dissection (Fig. S2). Between the other two species, significant differences were also found: Of sinigrin, significantly more was recovered from *P. chrysocephala* than from *P. armoraciae* and of sinalbin significantly more was recovered from *P. armoraciae* than from *P. chrysocephala* (Fig. 6C). No significant difference was found in the recovery of linamarin between all three species (Fig. 6D). Salicin recovery was significantly lowest from *P. cochleariae* and did not differ significantly between the other two species (Fig. 6D). Catalpol recovery was low in *P. cochleariae* and *P. armoraciae* and did not differ significantly between these two species.
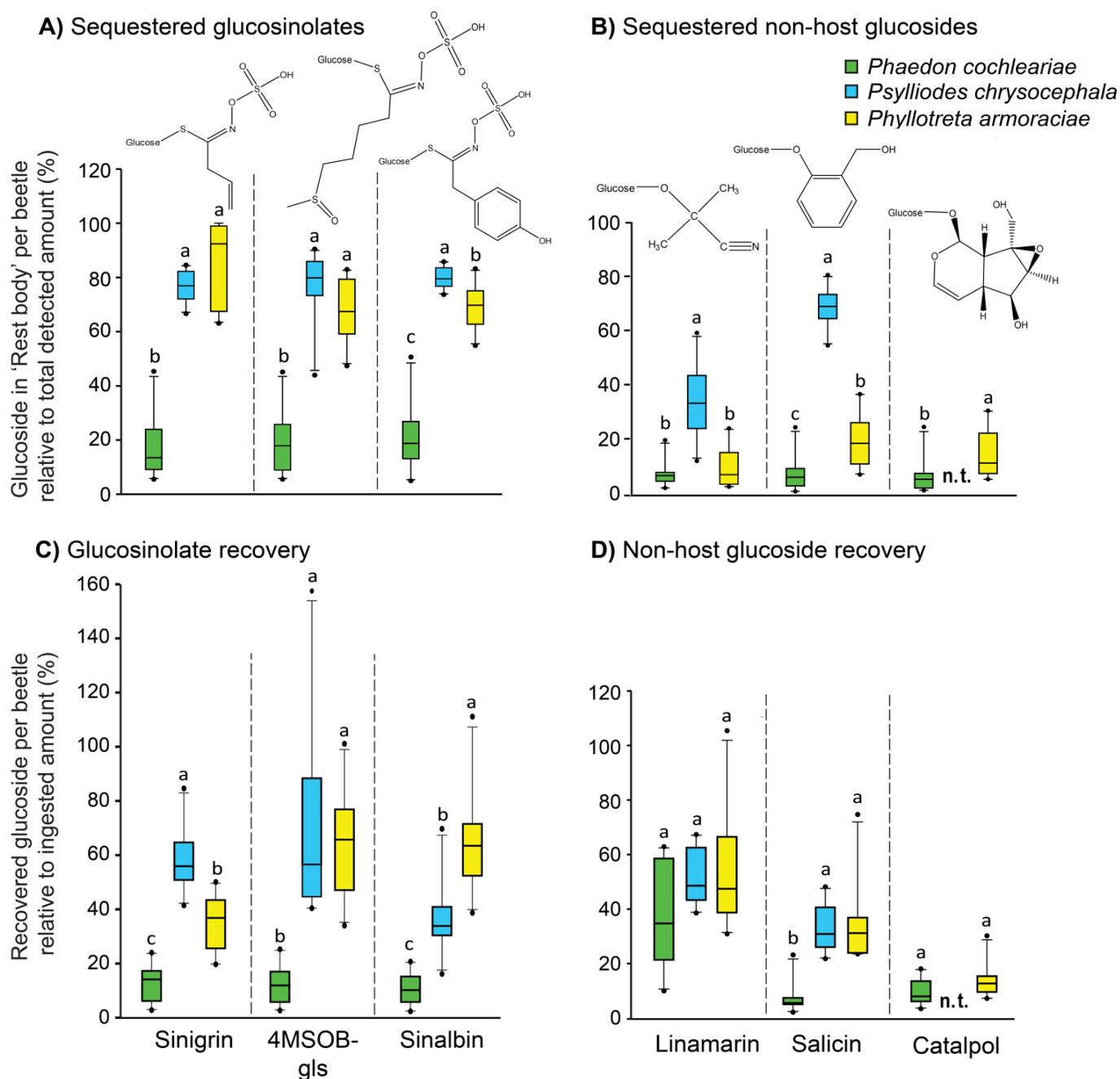
**Figure 6: Relative sequestered and recovered glucosides from the three leaf beetle species.** A) Sequestered glucosinolates and B) sequestered non-host glucosides relative to total detected amount (set to 100%). C) Recovered glucosinolates and D) recovered non-host glucosides relative to ingested amount (set to 100%). Chemical glucoside structures are shown above A) and B). Lower boxplot border shows 25th percentile of data, line in boxplot shows median and upper quartile border shows 75th percentile. Whiskers show 10th respectively 90th percentile and filled black dots show outliers. Differences between species were investigated using One-Way ANOVAs, *t*-tests (Catalpol in B and D) and a Kruskal Wallis One-way ANOVA on Ranks (Salicin in D). Different letters indicate significant differences between species. N = 10/species. n. t. = not tested; gls = glucosinolate. See Table S3 for details on statistics and data transformation.

### 3.3. Degradation of glucosides in *P. cochleariae* body part homogenates

Because the recovery of the three glucosinolates and salicin was lowest from *P. cochleariae*, an incubation experiment with body part homogenates was performed to determine in which body part the glucosides were degraded.

Recovery of all three glucosinolates was low ($< 40\%$) from all three body part incubates. It was lowest from head incubates, though not significantly (Fig. 7 A-C). Linamarin and salicin recovery were similar to each other, with a high recovery from head and gut incubates, but significantly lower recovery from 'Rest body' incubates (Fig. 7 D. E). Catalpol recovery was lower than that of the other two glucosides and also significantly lowest from 'Rest body' (Fig. 7 F).
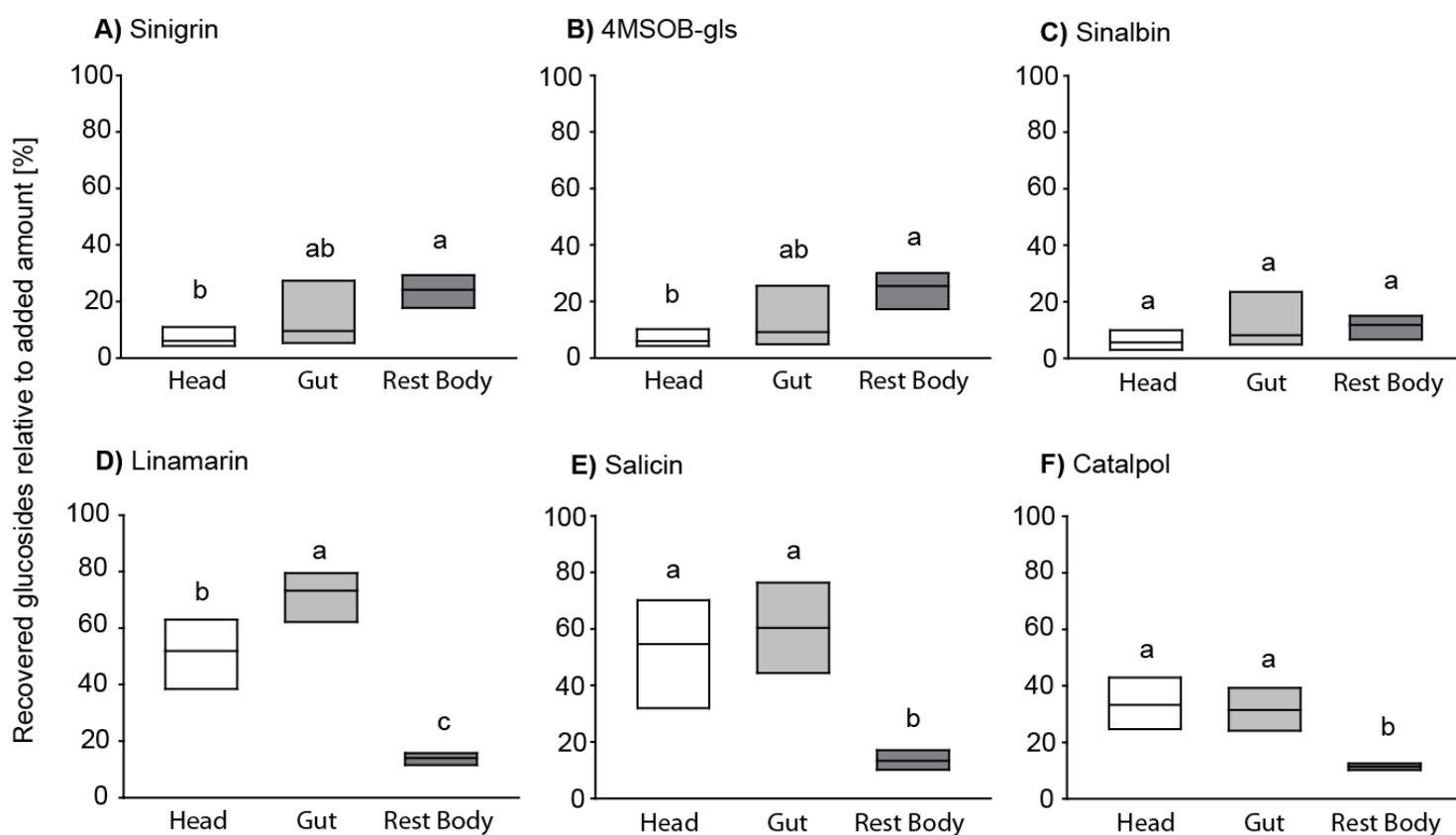


**Figure 7: Intact glucosides recovered after 5 min incubation from body part homogenates of *Phaedon cochleariae* relative to amount added to each homogenate (set to 100%).** Lower boxplot border shows 25th percentile of data, line in boxplot shows median and upper quartile border shows 75th percentile. Differences in recoveries between body parts were investigated with One Way ANOVAs. Different letters indicate significant differences in recovery between body parts. Gls = glucosinolate. N = 6. See Table S7 for details on statistics and data transformation.

### 3.4. Location of glucosinolate uptake in *P. armoraciae*

To determine if uptake of plant glucosinolate happens while the ingested plant material is only in the foregut of *P. armoraciae*, 15 s feeding experiments were performed. Beetles fed on *A. thaliana* in the Col-0 background. Wildtype (WT) plants and the mutant line *tgg1/tgg2*, which is devoid of myrosinase in above ground tissue (Barth and Jander, 2006) were used.

Initially this experiment was planned to be conducted with *P. armoraciae* as well as with *P. chrysocephala*. But plant material did not accumulated in the crop of *P. chrysocephala* as it did in *P. armoraciae* (*Data not shown*). Therefore, this experiment was only conducted with *P. armoraciae*. Also, the experiment was planned to distinguish between head, gut and 'Rest body'. But an error occurred during the experiment so that it could not be guaranteed that head and gut samples did not contaminate each other, wherefore these two body parts were combined in analysis (see Fig. S8).

Significantly more 4MSOB-glucosinolate was found in the 'Rest body' of *P. armoraciae* which have fed for 15 s on either *A. thaliana* Col-0 wildtype or the *tgg1/tgg2* mutant line, than in control beetles from the same batch which have not fed on *A. thaliana* (Fig. S6 A). Since only traces of 4MSOB-glucosinolate were found in the non-*A. thaliana* fed control samples (Fig. S6 A) and all leaves used for the experiment contained 4MSOB-glucosinolate (Fig. S7) it can be assumed that the 4MSOB-glucosinolate detected in the fed beetles came solely from the ingested *A. thaliana* plant material. Of the total detected, intact 4MSOB-glucosinolate, an average of 54% was found in the 'Rest body' of Col-0 WT fed beetles and an average of 50% in the 'Rest body' of *tgg1/tgg2* fed beetles (Fig. 8). The proportion of sequestered 4MSOB-glucosinolate did not differ significantly between the two treatments (Fig. 8). There was no significant difference in the amount of 4MSOB-glucosinolate detected in 'Rest body' between beetles which fed on the *A. thaliana* Col-0 wildtype and beetles fed on the *tgg1/tgg2* mutant (Fig. S6 B).
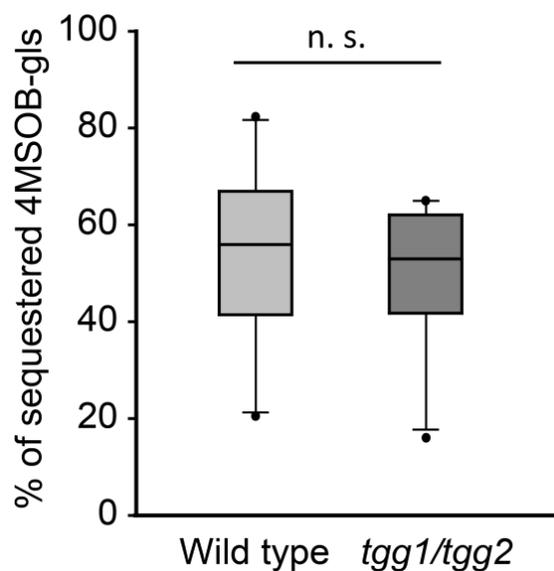
**Figure 8: Proportion of 4MSOB-glucosinolate sequestered by *Phyllotreta armoraciae* from *Arabidopsis thaliana* Col-0 WT and *tgg1/tgg2* mutant, relative to total detected amount (set to 100%).** N = 10 per treatment. Lower boxplots quartile border shows 25th percentile of data, line in boxplot shows median and upper quartile border shows 75th percentile. Whiskers show 10th respectively 90th percentile and filled black dots show outliers. Differences between treatments were investigated using a *t*-test, *t*=0.551, *p*=0.589

### 3.5. Comparative investigation of gut morphologies of the three leaf beetles

#### 3.5.1. Light microscopy

The alimentary canal of the three leaf beetles species was investigated with light microscopy and 3D reconstructions from µCT images. The three distinct gut parts were identifiable in all three species. The midgut of all three species performed a loop on its posterior end. *P. chrysocephala* was the only species, in which two midgut evaginations protruded laterally over the crop of (Fig.9B). *P. cochleariae* had the shortest hindgut of the three species (Fig. 9A). Whilst the crop of *P. cochleariae* and *P. armoraciae* were very extensible and flexible (Fig.9A, C; Fig.10A, C)), the crop of *P. chrysocephala* (Fig.9B; Fig.10B) was shorter and stiffer (*personal observation*). The crop had a length of around 300 µm in *P. chryscephala* whereas the slightly smaller species *P. armoraciae* had a crop roughly three times that length (Fig.10B I., CI.).

A parasite was found in the midgut of *P. cochleariae* (Fig.9 A, Fig. S9).

#### 3.5.2. Autofluorescence imaging of chitinous crop structure

Chitin autofluorescence was excited by a wavelength of 405 nm and visualised by confocal laser scanning microscopy.

The crops of all three species showed a clear fluorescence signal, which confirmed the presence of chitin in the crop intima (Fig.10). Structurally the crop differed between all three species investigated. The crop intima of *P. cochleariae* gave a continuous fluorescent response (Fig 10AII, III). The fluorescent response from the crop of *P. chrysocephala* showed a "brick like" structure (Fig. 10 BII, III) with a continuous signal stronger on the edges. The *P. armoraciae* chitinous crop structure gave a mesh like fluorescence response, which might indicate perforation (Fig. 10 C II, III).
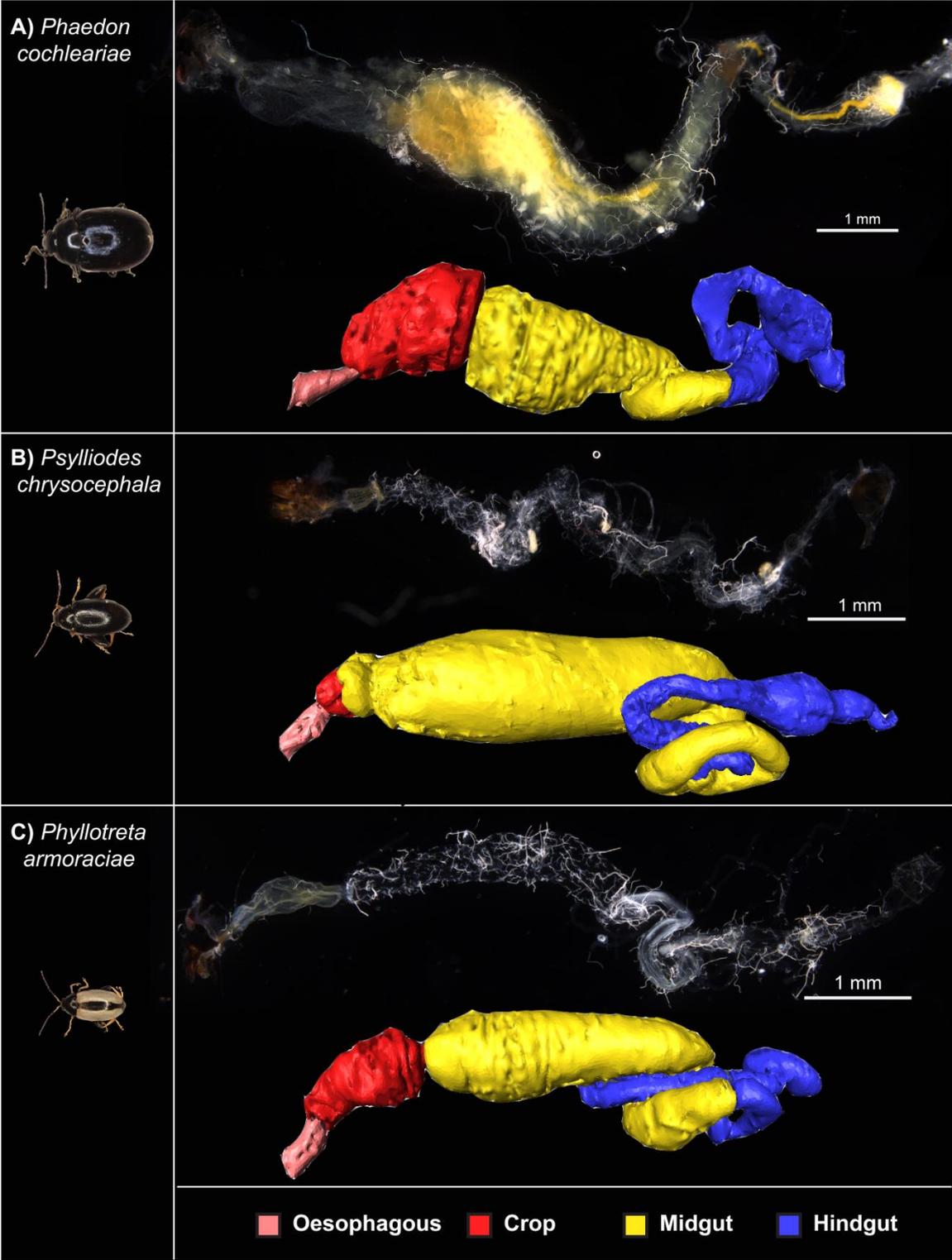
**Figure 9: Overview of the digestive system of A) *Phaedon cochleariae*, B) *Psylliodes chrysocephala* and C) *Phyllotreta armoraciae*.** Darkfield picture were taken on a "Axio Zoom.V16", Carl Zeiss with a Axiocam 506 color; 3D reconstructions from µCT images using Amira 6.0.1
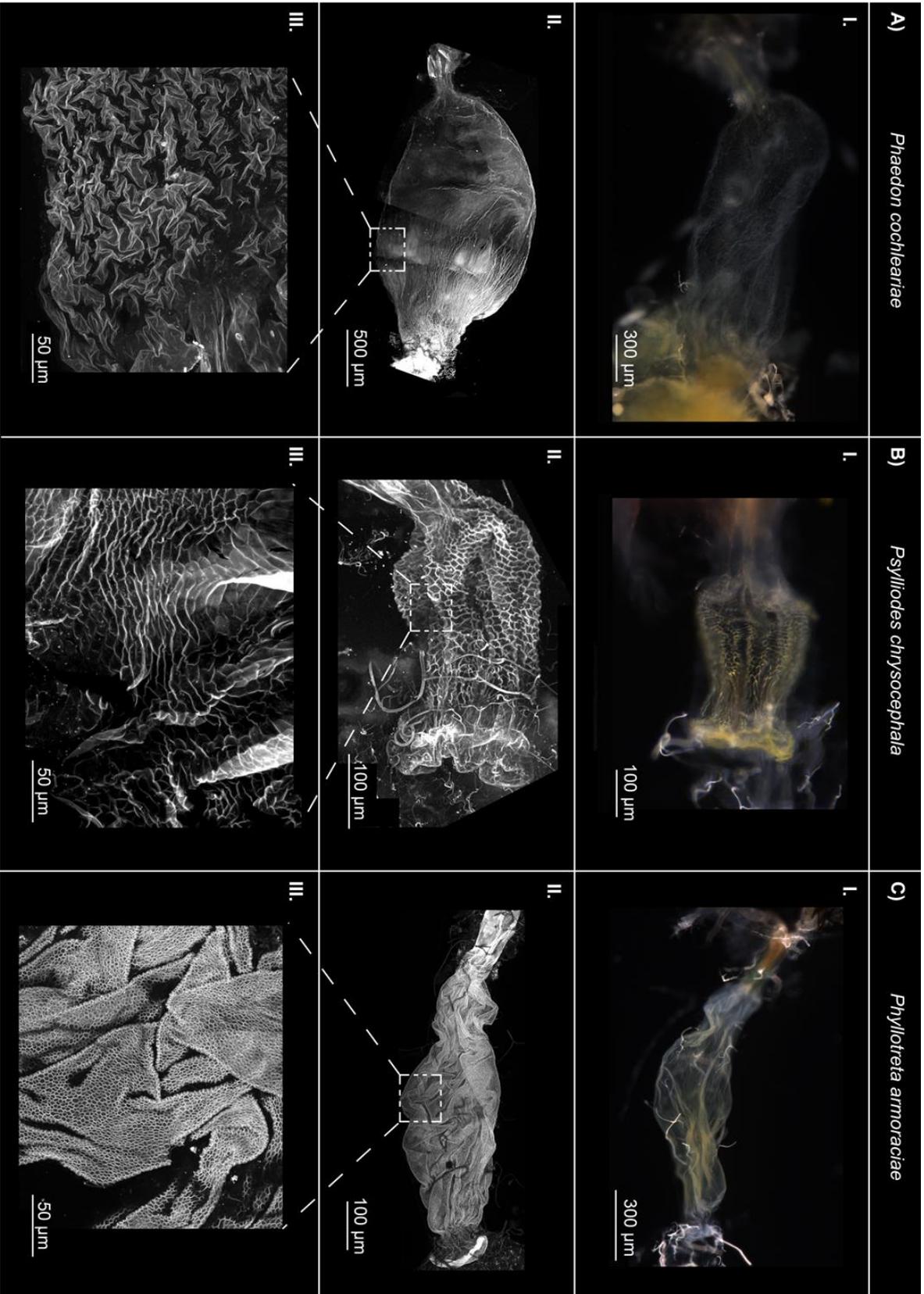
**Figure 10: Detailed crop structure of A)** *Phaedon cochleariae*, **B)** *Psylliodes chrysocephala* **and C)** *Phyllotreta armoraciae*. I. shows Darkfield images, II. shows the fluorescence response of chitin in the crop and III. shows a detail of II. I. were taken on an "Axio Zoom.V16", Carl Zeiss with a Axiocam 506 color, II. and III. were taken on a LSM 880 Axio Imager 2 (Carl Zeiss). Autofluorescence was excited by a wavelength of 405 nm (Diode 405-30) and detected at 575 nm. Dotted windows in II indicate approximate location of III. Note that C III. is taken from another specimen than portrayed in C II. and location was approximated. For detailed information on microscopy and image parameters see Tables S8

### 3.5.3. Crop ultrastructure with focus on intima

The differences in hardness between embedding resin and cuticle resulted in fissures and other artefacts (Nietzsche, *personal communication*).

The epithelium of all three species was folded, which indicated the extensibility of the crop as a storage organ (Fig.11). In all three species, three layers were distinguishable in the crop epithelium: Intima, cellular layer and muscular layer (Fig.11). The epithelia differed largely between the three species (Fig.11). *P. armoraciae* and *P. chrysocephala* were more similar to each other than to *P. chochleariae* (Fig.11). The epithelium of *P. chochleariae* was overall thicker than in the other two species with especially the cellular layer being more prominent (Fig.11). In *P. chrysocephala* a regular pattern of 8-10 µm wide "hills" was present (Fig.11 B). The ultrastructure of *P. armoraciae* revealed a regular pattern of around 2 µm wide invaginations of the intima in 2 µm intervals, which created teeth like appearance (Fig.11C; Fig.12). These invaginations almost reached down to the muscular layer and might compress the cellular layer (Fig.11C). *P. cochleariae* differed from the other two species in the dimension of the intima. All three species possessed an intima consisting of an epicuticle with a darker contrasted wax layer on the luminal side and a laminated procuticle (Fig.11). The intima of *P. cochleariae* had an overall thickness of 1-2.5 µm and a multi-layered procuticle with 6-10 distinct layers (Fig.11A). In contrast, the intima of *P. chrysocephala* had a total thickness of 0.5-0.7 µm and that of *P. armoraciae* 0.375-0.5 µm. This size difference between *P. cochleariae* and the other two species was due to a much thinner procuticle with only 3 distinct layers in *P. chrysocephala* and *P. armoraciae* (Fig.11, Fig.12) The cellular layer in *P. chrysocephala* was rich in mitochondria and showed multi-layered cell organelles at the luminal side (Fig.11B). Such folded cell organelles were not present in the *P. armoraciae* crop (Fig.11C) but mitochondria were also abundant in the cellular layer (Fig.11C). Cell organelles were not identifiable in *P. cochleariae*. The cellular layer was rich in granules (Fig. 11A). The nuclei in *P. chrysocephala* (Fig.11) and *P. armoraciae* (Fig.11C) crop epithelia were located on the base of the epithelium, neighbouring the muscle cells. The nuclei were not identifiable in *P. cochleariae*. The crop of two *P. armoraciae* specimens was investigated with TEM. In one specimen, the cellular layer appeared to be largely degenerated (Fig. 12). The cellular layer of the second specimen looked more alive and multiple mitochondria were present (Fig. 11). In both *P. armoraciae* specimens, an extracellular space between the living cell and the procuticle was observed (Fig. 11C; Fig. 12), which was not found in the other two species. It is unclear if this was an artefact caused by embedding and sectioning or an actual representation of the condition in the living specimen.
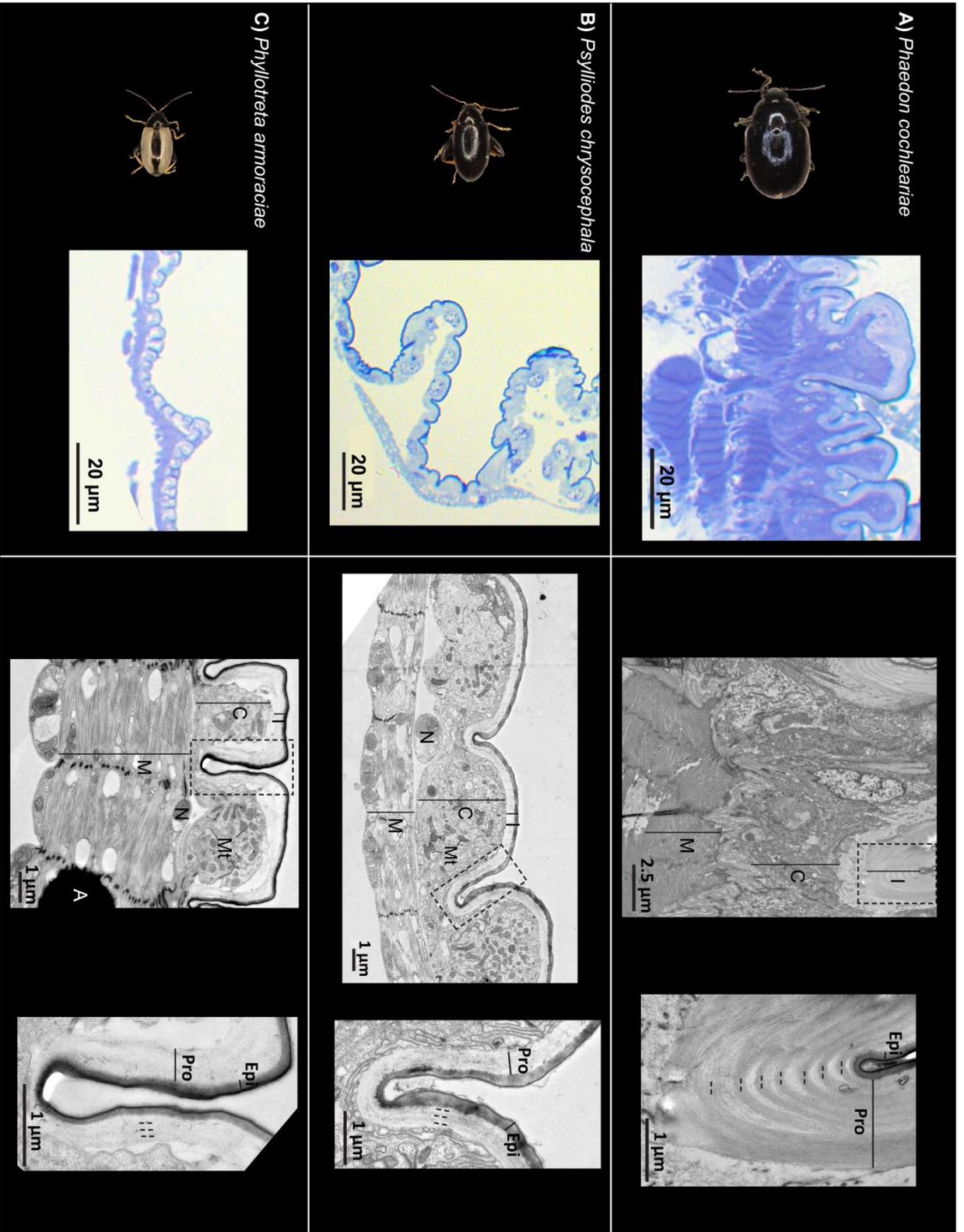
**Figure 11: Ultrastructure of the crop epithelium of A)** *Phaedon cochleariae,* **B)** *Psylliodes chrysocephala* **and C)** *Phyllotreta armoraciae.* Semi thin sections were stained with Richardson Blue. TEM images show the epithelium and the dashed frame indicates the location of the enlarged cuticle image. I = Intima, C = cellular layer, M = Muscle layer, N = Nucleus, Mt = Mitochondrium, A = Artefact, Epi = Epicuticle, Pro = Procuticle. Dashed lines mark the lamellae of the procuticle. All images taken by: Dr. S. Nietzsche
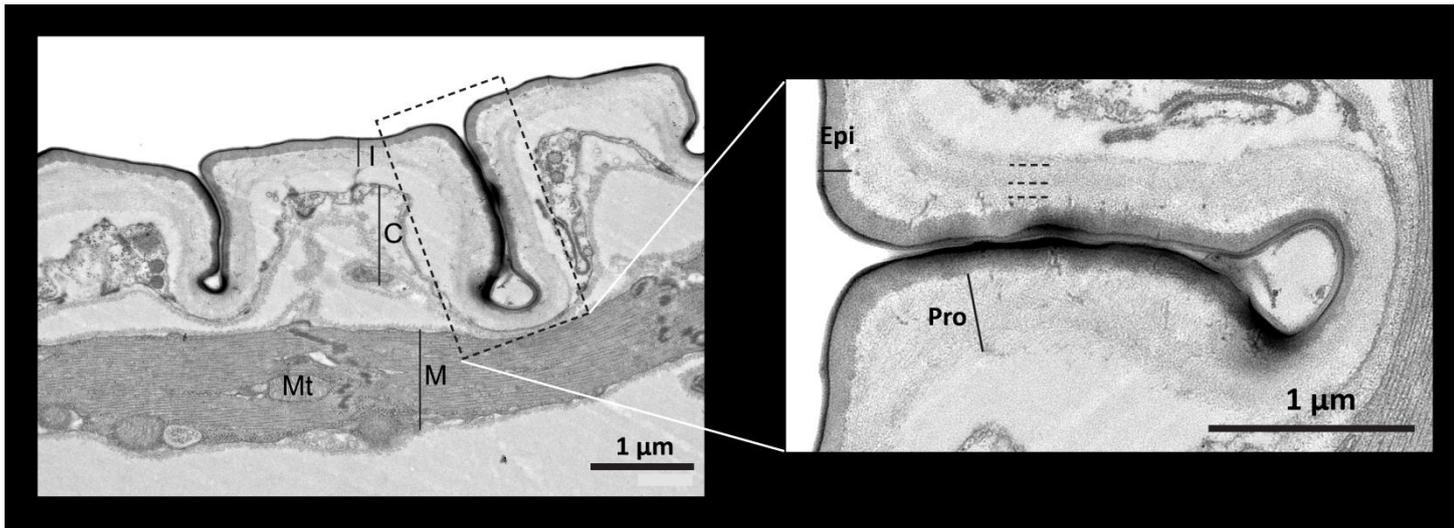
**Figure 12 TEM image of the crop epithelium of a second specimen of *Phyllotreta armoraciae* with a degenerated cellular layer**. I = Intima, C = cellular layer, M = Muscle layer, N = Nucleus, Mt = Mitochondrium Epi = Epicuticle, Pro = Procuticle. Dashed lines mark the lamellae of the procuticle. Image taken by: Dr. S. Nietzsche

# 4. Discussion

## 4.1. Composition of the *Phyllotreta armoraciae* haemolymph

It was already known, that sequestered glucosinolates are stored mainly in the haemolymph of *P. armoraciae* (Yang et al., 2020, Sporer et al., 2020). I could confirm this and found that the total concentration of glucosinolates in the haemolymph was very high (60.9 ± 17.2 mM). High glucosinolate concentrations are also reported from other sequestering species. In two *B. brassicae* specimen fed on a sinigrin containing host plant for one week, 102 and 148 ng sinigrin were detected (Kazana et al., 2007), which would correspond to roughly 5 nL of *P. armoraciae* haemolymph, which shows, that the glucosinolate amount in one *P.armoraciae* specimen is much higher than in the aphid. In larval *A. rosae,* fed on sinalbin containing host plants, a maximum of 0.15 µmol glucosinolate per larvae was detected (Müller and Wittstock, 2005), which would correspond to 2.5 µL of *P. armoraciae* haemolymph..

More than 92% of the total glucosinolates was found to be sinigrin. This was to be expected since sinigrin is the major glucosinolate in *B. juncea*, the plant the beetles were reared upon (Sporer et al., 2020, Beran et al., 2014). It furthermore is the major glucosinolate the natural host plant of *P. armoraciae,* horseradish, *Armoracia rusticana* (Vig and Verdyck, 2001, Popovic et al., 2020). The glucosinolate composition in the haemolymph was similar to that

of the whole beetle (Yang et al., 2020; Sporer et al., 2020), thus it can be assumed that different glucosinolate types are stored together in the haemolymph. The glucosinolate concentration in the haemolymph was much higher than that in the host plants (Bodnaryk and Palaniswamy, 1990, Beran et al., 2014, Bajpai et al., 2019). A transport from the gut lumen across the gut epithelium into the haemolymph has to happen against a very high concentration gradient. This indicates an active transport mechanism. Furthermore, maintaining such high concentrations of glucosinolates in the haemolymph requires specific mechanisms that prevent the excretory loss of glucosinolates by the Malpighian tubules. The excretion of intact glucosinolates from *P. armoraciae* haemolymph has been shown to be highly selective (Yang et al., 2020) and is at least partly mediated by glucosinolate specific transporters (Yang et al., *under review*).

It is unclear how the compartmentalization of glucosinolates and beetle myrosinase, which is mainly present in the haemolymph of larval *P. armoraciae* (Sporer et al., 2020), is maintained at a cellular level. One possibility is a spatial separation into haemocytes and haemoplasma as shown for the linamarin sequestering burnet moth *Zygaena filipendulae* (Pentzold et al., 2017).

Sugars are an essential part of the insect haemolymph. Surprisingly in *P. armoraciae* haemolymph, the monosaccharide glucose was found to account for 86% of total detected sugars. Its concentration was more than 600 times higher than that of trehalose, which is usually the major sugar in the insect haemolymph (Nation, 2016, Gillott, 2005, Bedford, 1977, Kanost, 2009, Wyatt, 1961). Similar cases of virtually absent trehalose in the insect haemolymph are very sparse. In *Anisolabis littorea* (WHITE, 1846) (Dermaptera) total carbohydrate levels were found to be unusually low and trehalose was virtually absent (Leader and Bedford, 1972). Bedford, 1977 found that in primitive insects and other terrestrial arthropods, trehalose is either absent or only present in small amounts, whereas in higher insects the amount of trehalose is high and the amount of glucose is moderate. The high glucose concentration may be advantageous for *P. armoraciae*. The beetle myrosinase is localised in the haemolymph (Sporer et al., 2020) like the glucosinolates. The reaction catalysed by myrosinase results in an aglucone and a free glucose moiety. The high glucose concentration might act as a form of product inhibition on the myrosinase. If myrosinase activity is indeed influenced by surrounding glucose concentration, could be tested in a simple experimental setup by incubating myrosinase with glucosinolates in different glucose concentration.

The high concentration of detected amino acids (66.1 ± 12.2 mM) was not surprising, since Insects are known to have a large amount of free amino acids in their haemolymph, which play a key role in osmoregulation (Nation, 2016, Woodring and Blakeney, 1980, Kanost, 2009). Proline and glutamine are usually among the highest concentrated amino acids in insect haemolymph (Wyatt, 1961, Kanost, 2009). Besides its role in osmoregulation and protein synthesis, proline can play a role as energy source for flight muscles (Gäde and Auerswald, 2002, Kanost, 2009) or resorption processes in the rectum, as was shown in the locust *S. gregaria* (Chamberlin and Phillips, 1983).

## 4.2. Selectivity of uptake

All six compounds presented to the beetles were recovered from the 'Rest body' of all three species. This confirms that an unselective uptake of different glucosides from the gut lumen into the haemolymph does happen, as proposed by (Discher et al., 2009). However, huge differences between compounds and species were found, indicating that selective uptake mechanisms do additionally exist and play a more important role in sequestration. Catalpol was identified as a feeding deterrent for *P. chrysocephala*, but was readily accepted by the other two species.

### 4.2.1. Glucosinolate sequestration

Sequestration by *P. armoraciae* was clearly selective. Glucosinolates were favoured over the non-host glucosides and sinigrin was significantly favoured over the other two tested glucosinolates (Fig. 6A). Sinigrin is the natural host plant glucosinolate of *P. armoraciae* (Vig, 1999, Popovic et al., 2020), so it is likely that an efficient uptake mechanism for this glucosinolate exists in this species.

*P. chrysocephala* also showed a high efficiency in sequestering glucosinolates. That sinalbin was found to be sequestered by *P. chrysocephala* is in conflict with prior reports that found only traces of sequestered sinalbin in this species (Körnig, 2015, Ahn et al., 2019 624). It is possible that sinalbin is sequestered and subsequently excreted by the Malpighian tubules. However, if this was the case, intact glucosinolates would be found in the faeces in considerable amount. But Ahn et al., 2019 found only traces of intact sinalbin in faeces. So this finding remains enigmatic and might require some follow up investigations.

### 4.2.2. Non-host glucoside sequestration

No significant sequestration of non-host glucosides was found in *P. armoraciae*. *P. chrysocephala* sequestered significantly more linamarin and salicin than the other two species (Fig.6 C, see also Fig. S1E). Salicin sequestration did not differ significantly from the sequestration of glucosinolates (Fig. S3). This is surprising, since *P. chrysocephala* is a brassicaceae specialist (Godan, 1951, Gikonyo et al., 2019) and thus never encounters salicin in nature. A scenario involving a host plant shift of a sequestering ancestral species as has been described for the genus *Athalia*, in which a switch from cyanogenic plants to plants containing glucosinolates occurred (Opitz et al., 2012), is unlikely. Only one species from the genus, *Psylliodes luteola* (MÜLLER, O. F., 1776), is known to feed on members of the salicaceae, among members from four other plant families (Gikonyo et al., 2019).

In *P. cochleariae* the recovery of salicin in the main experiment was low (Fig. 6 D) but it was stable in head and gut incubates (Fig. 7E). This suggests that salicin had been sequestered into the 'Rest body' and was metabolised there, leading to a low recovery of intact salicin. This is in line with a finding of Discher et al., 2009: They fed *P. cochleariae* larvae a glucoside mix which contained salicylaldehyde among other compounds. Salicylaldehyde was not found in the haemolymph but in the defence glands, so some of it must have been taken up from the gut into the haemolymph and from there imported into the defence gland.

### 4.2.3. Recovery

Glucosinolate recovery was lowest from *P. cochleariae*. Some of this loss occurred by dissection (Fig. S2). Recent findings indicate a plant myrosinase-independent activation of glucosinolates by larvae of *P. cochleariae* (Friedrichs et al., 2020). Such a hydrolysation of glucosinolates during the dissection process might have led to a loss of intact glucosinolates. To determine where exactly the intact glucosides were lost, the incubation experiment was performed. Recovery of glucosinolates was low from every body part and lowest from head incubates (Fig. 7 A-C). The low recovery of glucosinolates from head incubates might indicate a detoxification mechanism active in the head.

The total recovery of sinigrin was significantly less from *P. armoraciae* than from *P. chrysocephala* (Fig. 6 A). *P. armoraciae* possesses a beetle myrosinase enzyme (Sporer et al., 2020) which might have activated some ingested sinigrin during dissection. It is likely that this myrosinase has a high affinity to sinigrin as substrate, since sinigrin is the major host plant glucosinolate of *P. armoraciae* (Vig and Verdyck, 2001, Popovic et al., 2020) and the

beetle myrosinase of the closely related *P. striolata* has the highest activity towards sinigrin (Beran et al., 2014).

The recovery of sinalbin was significantly lower from *P. chrysocephala* than from *P. armoraciae* (Fig. 6A). Sequestration on the other hand, was significantly higher in *P. chrysocephala* (Fig. 10). *P .chrysocephala* possesses sulfatase activity in its gut (Beran et al., 2018). This gut membrane bound sulfatase has the highest affinity towards sinalbin (Ahn et al., 2019). The sulfatase converts sinalbin to desulfo-sinalbin, which is not detected by the applied method. The lower recovery and high sequestered proportion of sinalbin could be attributed to such a conversion of intact sinalbin present in the gut lumen (see also Fig. S1 C; Fig. S4 B).

Catalpol had a very low recovery from the two species it was fed to (Fig. 6 D). Catalpol degradation is promoted by the presence of amino acids (except proline)(Wei and Wen, 2014) so maybe the loss occurred after catalpol was taken up into the haemolymph and metabolized there promoted by the high concentration of free amino acids (Fig. 5C).

### 4.2.4. Outlook/ Possible follow up experiment

As a follow up experiment, a targeted qTOF (quantitive time of flight) analysis with the replicates of the experiment could be performed. Possible targets are glucosinolate degradation products in *P. cochleariae*, like glutathione- (Kawakishi and Kaneko, 1985) or aspartic acid conjugates of isothiocyanates (Friedrichs et al., 2020) and salicin breakdown products. If salicin breakdown products were found in the 'Rest body', it would confirm that an uptake happened prior to metabolisation. Catalpol degradation products should also be targeted to elucidate what caused the low recovery of this glucoside. Targeting desulfo-sinalbin in *P. chrysocephala* could confirm that sinalbin was indeed desulfated in the gut. It furthermore would be of interest, if the sulfatase is also active in the hindgut of this species. This could partly explain the discrepancy between my findings of sequestered sinalbin (Fig. 6A) and earlier reports, claiming no significant sinalbin sequestration (Körnig, 2015, Ahn et al., 2019 624) in *P. chrysocephala*. A possible scenario is that sinalbin is sequestered but subsequently excreted by the Malpighian tubules into the hindgut, where the desulfation takes place.

### 4.3.  Location of glucosinolate uptake in *P. armoraciae*

A short term feeding experiment was conducted with *P. armoraciae* adults, during which plant material was only allowed to be in the foregut. It revealed, that glucosinolate uptake happened rapidly in the foregut of this species. More than 50% of total detected glucosinolates were already in the 'Rest body' (Fig. 8, Fig. S6). No significant difference was found in sequestered 4MSOB-glucosinolate proportion between beetles which fed on wildtype and myrosinase-devoid mutant plants. This indicates that uptake happens faster than an activation of a significant amount of glucosinolates in the gut by plant myrosinase. Additionally to the rapid uptake, some form of myrosinase inhibition by an unknown mechanism might also occur. Rapid uptake of glucosinolates as a detoxification mechanism has been reported from the sawfly *A. rosae* in which the sequestration also happened in anterior gut parts (Abdalsamee et al., 2014). These findings conclusively point to an active uptake of glucosinolates in the foregut.

In an experiment with *P. chrysocephala*, only 26% of ingested 4MSOB-glucosinolate was recovered intact or as desulfo-glucosinolate (Beran et al., 2018). A large proportion was still activated by plant myrosinase, which raises the question if the uptake of intact glucosinolates happens as fast in *P. chrysocephala* as it did in *P. armoraciae.*

### 4.4.  Foregut structure

A parasite was found in the midgut of P. cochleariae (Fig.10, Fig. S7). It may have been a gregarine apicomplexan, which has been described from *P. cochleariae* before (Müller et al., 2017).

The crops of the three species differed from each other. All crops contained chitin, as was shown by exciting its autofluorescence using confocal laser scanning microscopy. The crop of *P. chrysocephala* was shorter (Fig. 11) and less flexible (*personal observation*) than that of the other two species. Transmission electron microscopy images revealed that all three species possessed a foregut intima composed of three distinct layers: an osmiophilic outer epicuticle (or wax layer) over an inner epicuticle followed by a laminated procuticle. The intima of *P. cochleariae* was distinctly different from that of the other two species. The biggest difference was in the thickness of the chitinous procuticle. The procuticle of *P. cochleariae* was prominent, showed 6-10 laminae and was 10-30 times thicker than the epicuticle. In contrast, the other two species had a thinner procuticle, which only showed 3 laminae in both species and was roughly 1.2-3 times the thickness of the epicuticle. The differences in thickness of the chitinous procuticle most likely account for the observed differences in the autofluorescence

signal between species. Due to the large procuticle, it can be assumed that the crop of *P. cochleariae* functions mainly as a storage organ and is impermeable for hydrophilic compounds. In the cellular layer of *P. armoraciae* and *P. chrysocephala*, mitochondria were present. Mitochondria play an important role in active uptake by providing energy (Markl et al., 2019). All TEM images taken from the *P. armoraciae* crop showed a continuous intima with wax layer. So the uptake has to happen through the intima. To fully exclude the possibility of an interrupted wax layer, the three dimensional structure of the crop needed to be investigated, since TEM sections only provide a two dimensional image. FiB-SEM (focussed ion beam scanning electron microscopy) (Holzer and Cantoni, 2012) or SBFSEM (serial block-face scanning electron microscopy) (Wipfler et al., 2016) are methods to provide such a three dimensional structure with high resolution. Two glucosinolate transporters (PaGTR3 and PaGTR2) which are able to transport a broad range of glucosinolates are expressed in the *P. armoraciae* foregut, as well as in the hindgut (Yang et al., *under review*). A fluorescence in situ hybridisation (FiSH) against the mRNA of these transporters could pinpoint areas of interest for further morphological analyses. An electron dense layer is clearly present on the luminal side of the crop intima in all three beetle species investigated. However, if this layer is composed of waxes and sclerotin/cuticuline like the integument cuticle (Wigglesworth, 1985, Wigglesworth, 1990, Moussian, 2013) is unclear. It might be composed differently and therefore be more permeable. A chitinous cuticle is not necessarily impermeable for hydrophilic compounds. The hindgut is of ectodermal origin like the foregut and possesses an intima, but despite that, the hindgut and especially the rectum was shown to be more permeable for water, ions (Maddrell and Gardiner, 1980, Phillips, 1980) and amino acids (Phillips et al., 1987b) then the foregut. Hindgut resorption is restricted to molecules with a size of less than 6-7 Å which led (Phillips and Dockrill, 1968) to speculate on the presence of channels in the rectum intima of 6.5 Å. Similar channels might also be present in the foregut intima of *P. armoraciae*.

The short term feeding experiment could not be conducted with *P. chrysocephala*, because plant material passed through the foregut very rapidly. Thus it is unclear, whether the glucosinolate uptake in this species also happens in the crop. Investigation of the crop of other Alticini (Flea beetles) and taxa from their sister clade, Galerucini, would be interesting. The thin crop procuticle could either be more widespread in this tribus or is an adaptation to sequestration of glucosinolates from Brassicaceaes. The morphological analyses focussed on the crop of all three species but it is possible that the esophagous and pharynx also play a role in glucosinolate uptake.

## 4.5. Conclusion

The uptake of plant defence compounds from the gut lumen into the haemocoel is the first step of the complex process known as sequestration. A general and unselective uptake of plant defence glucosides was found in all three investigated species. However, this unselective uptake was almost neglectable, compared to the selective uptake mechanism present in the investigated glucosinolate sequestering flea beetle species. At least in *P. armoraciae* the uptake of glucosinolates is happening in the foregut, which is remarkable since this gut part has so far been almost completely dismissed as an organ capable of hydrophilic uptake. The investigation of the crop structure of *P. armoraciae* revealed no direct evidence for uptake (eg. channel or areas devoid of wax layer or cuticle), but the procuticle in this species and in *P. chrysocephala* was much thinner compared to *P. cochleariae*.

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# 7. Supplementary Material

## 7.1. Introduction Supplement: Insect adaptations to the mustard oil bomb

Generalist feeding and performance is negatively influence by the glucosinolate myrosinase system (Kliebenstein et al., 2002, Müller et al., 2010). Whereas, for Brassicales specialists, glucosinolates and isothiocyanates can be attractants (Feeny et al., 1970, Pivnick et al., 1992, Bartlet et al., 1992), feeding stimulants (Tanton, 1977, Bartlet et al., 1994) or stimulants for oviposition (Mumm et al., 2008, Hopkins et al., 2009, Müller et al., 2010, Humphrey et al., 2016). Brassicales-specialist strategies to overcome the glucosinolate myrosinase system are plentiful (Fig.1). For example, aphids and other hemipterans are able to circumvent glucosinolate activation by plant myrosinase by a minimal invasive feeding technique (Barth and Jander, 2006), in which their highly mobile stylet takes an intercellular route and probes cells along the way (Powell et al., 2006). Larvae the diamondback moth, *Plutella xylostella* (Ratzka et al., 2002), the cabbage stem flea beetle *Psylliodes chrysocephala* (Beran et al., 2018) and the desert locust, *Schistocerca gregaria* (Falk and Gershenzon, 2007), express sulfatase in their gut, which desulfates glucosinolates to desulfo-glucosinolates which cannot be activated by myrosinase (Ettlinger et al., 1961). The butterfly *Pieris rapae* (Wittstock et al., 2004) and other brassicaceae feeding species from the family Pierinae (Wheat et al., 2007, Edger et al., 2015) possess NSP proteins, which lead to the formation of less toxic nitriles instead of isothiocyanates in the aglucone rearrangement process (Wittstock et al., 2004). The evolution of NSP in the ancestral species of the pierinae led to adaptative radiation in this group which resulted in significantly higher species numbers compared with related caldes (Wheat et al., 2007).

Formed isothiocyanates are generally detoxified by the conjugation with glutathione and subsquently the mercapturic acid pathway (Fig. 1) (Kawakishi and Kaneko, 1985, Wadleigh and Yu, 1988, Schramm et al., 2012, Jeschke et al., 2017, Winde and Wittstock, 2011, Beran et al., 2018, Gloss et al., 2014). But other detoxifiaction pathways also exist: Larvae of the mustard leaf beetle *Phaedon. cochleariae* detoxify glucosinolate-derived compounds via conjugation with aspartic acid (Friedrichs et al., 2020). In recent years the detoxification of isothiocyanates by microbial gut symbionts has become of increased interest (Welte et al., 2016a, Welte et al., 2016b, Shukla and Beran, 2020).

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Some insects are able to sequester glucosinolates for their own defence (Table 1): Larvae of the sawfly *Athalia rosae* (Hymenoptera) (Müller et al., 2001) and other *Athalia* species (Opitz et al., 2012) sequester glucosinolates into their haemolypmph. The glucosinolate containing haemolymph is emitted (reflex bleeding) to deter predators (Müller et al., 2002, Müller and Brakefield, 2003, Opitz et al., 2010). Glucosinolates are also sequestered by the harlequin bug *Murgantia histrionica* (Aliabadi et al., 2002) and *P. chrysocephala* (Beran et al., 2018). The two Brassicales specialist aphids *Brevicoryne brassicae* and *Lipaphis erysimi* both sequester intact glucosinolates in their haemolymph (Bridges et al., 2002, Kazana et al., 2007) and possess an aphid myrosinase enzyme which evolved convergent to plant myrosinase (Macgibbon and Beuzenberg, 1978, Jones et al., 2001, Pontoppidan et al., 2001, Francis et al., 2002). Myrosinase is stored in distinct microbodies in muscular tissue of non-flight muscles in both species, to constitute their own 'mustard oil bomb' (Bridges et al., 2002), which is a defence against predators (Pratt et al., 2008). The striped flea beetle *Phyllotreta striolata* and the horseradish flea beetle *Phyllotreta armoraciae* both selectively sequester glucosinolates from their host plants into their haemolymph and posses a beetle myrosinase enzyme (Beran et al., 2014, Sporer et al., 2020, Yang et al., 2020). In *P.armoraciae* larvae, the myrosinase is also localised in the haemolymph (Sporer et al., 2020)

## 7.2. Introduction: References of Table 1

[1] (Bak et al., 2006); [2] (Møller, 2010); [3] (Bjarnholt and Møller, 2008); [4] (Zagrobelny et al., 2008); [5] (Teuscher, 1994); [6] (Zagrobelny et al., 2004); [7] (Zagrobelny et al., 2007); [8] (Frerichs et al., 1927); [9] (Niedźwiedź-Siegień, 1998); [10] (Boeckler et al., 2011); [11] (Mahdi, 2014); [12] (Felton et al., 1992); [13] (Prudic et al., 2007); [14] (Rowell-Rahier and Pasteels, 1986); [15] (Pasteels et al., 1983); [16] (Kirsch et al., 2011); [17] (Maclagan, 1876); [18] (Albach et al., 2001); [19] (El-Naggar and Beal, 1980); [20] (Boros and Stermitz, 1990); [21] (Boros and Stermitz, 1991); [22] (Dobler et al., 2011); [23] (Kim et al., 2000); [24] (Bowers and Puttick, 1986); [25] (Lampert et al., 2011); [26] (Lampert, 2020); [27] (Opitz et al., 2010); [28] (Willinger and Dobler, 2001); [29] (Zhihua and Xuesen, 2013); [30] (Jurišić et al., 2004); [31] (Rodman et al., 1998); 32 (Agerbirk and Olsen, 2012); [33] (Blažević et al., 2020); [34] (Kawakishi and Kaneko, 1985); [35] (Kawakishi and Kaneko, 1987); [36] (Brown and Hampton, 2011); [37] (Müller et al., 2001); [38] (Opitz et al., 2012); [39] (Bridges et al., 2002); [40] (Kazana et al., 2007); [41] (Aliabadi et al., 2002); [42] (Beran et al., 2018); [43] (Beran et al., 2014); [44] (Sporer et al., 2020); [45] (Yang et al., 2020); [46] (Ettlinger and Lundeen, 1956); [47] (Li and Kushad, 2004)

## 7.3. Material and Methods: detailed dissection 2.7.

First, the head, the ring formed by the cuticle of the prothorax (consisting of the pronotum, the propleurites and the prosternum with the prothoracic coxae and legs) and the gut were pulled out from the remaining body, washed twice in PBS-buffer (pH 6.8) and immediately put onto a metal dissection dish on ice.

The "Rest body" was immediately put into liquid nitrogen. The head and prothoracic ring were dissected from the gut and collected in 70 µL 80% methanol. 70 µL 80% methanol was also added to the gut on the dissection dish. The gut in methanol was taken up with a pipette and immediately homogenized using a plastic pestle to stop all enzymatic activity. Following this method a total of 10 replicates per species were collected, each contained body parts of three beetles. After adding the third rest body to the replicate, 280 µL 80% methanol was added and it was immediately homogenized using a plastic pestle. After adding the third head to the replicate, it was also immediately homogenized with a plastic pestle.

## 7.4. Further results: Selectivity of uptake

### 7.4.1. Absolute detected glucoside amount
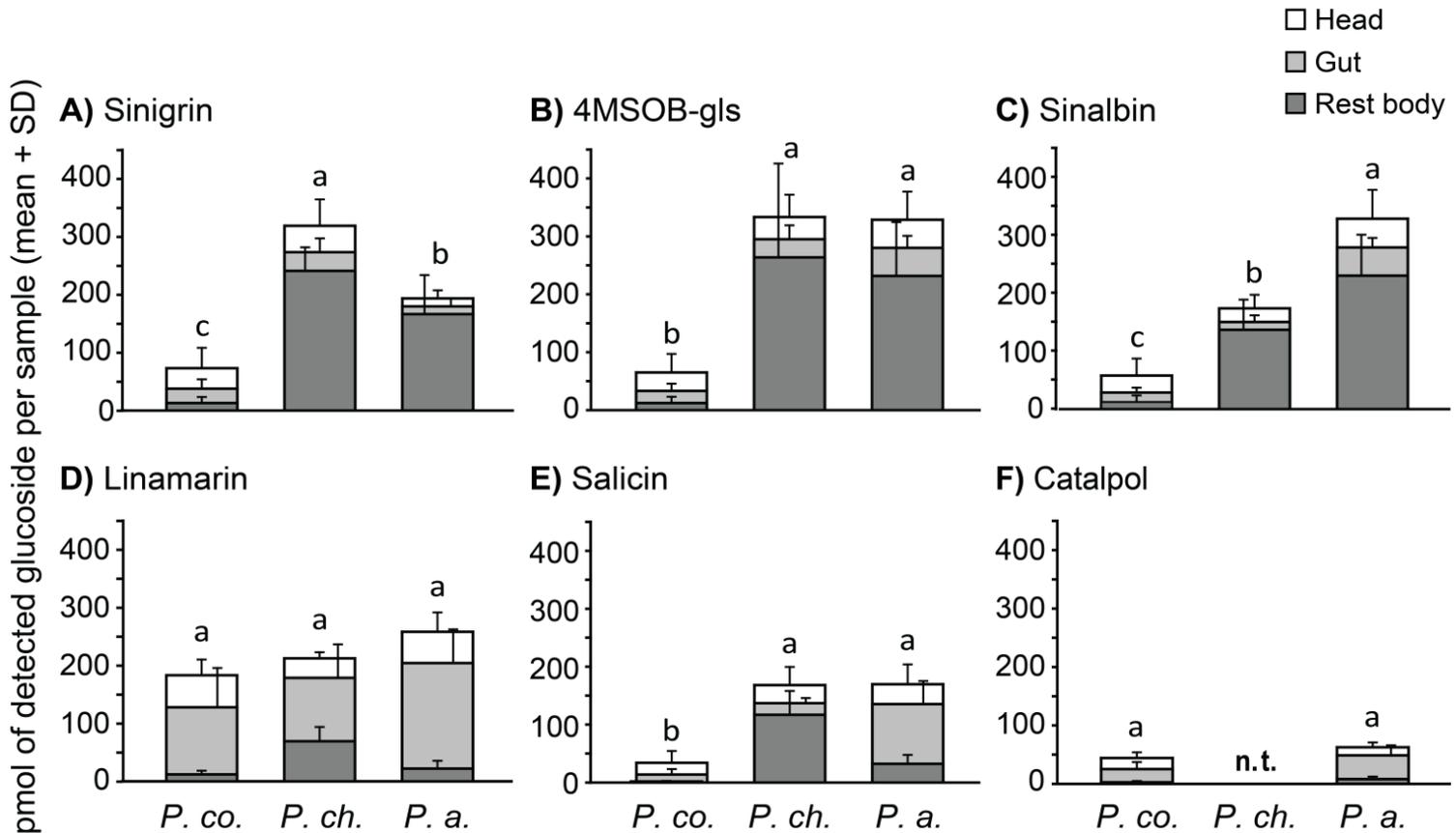


**Figure S1: Averages of absolute detected glucosides from body parts of dissected *Phaedon cochleariae* (*P. co*), *Psylliodes chrysocephala* (*P. ch*) and *Phyllotreta armoraciae* (*P. a*) in pmo**l. The three glucosinolates A) Sinigrin, B) 4MSOB-glucosinolate and C) Sinalbin and the three non-host glucosides D) Linamarin, E) Salicin and F) Catalpol are shown. Differences in total glucoside amount detected in dissected samples summed up between beetles were analysed using one way ANOVAs (A, C, D), Kruskal-Wallis one-way ANOVAs on ranks (B, E) and a *t*-test (F). Different letters indicate significant differences between species in absolute detected amount of respective glucoside. n. t. = not tested, gls = glucosinolate. N=10/species and body part. For details on statistics see Table S1.

**Table S1: Methods and results of the statistical analyses of the differences in total detected glucosides from summed up dissected samples between species.**

| Glucoside | Statistical method | Statistics | *p*-value | Post-hoc test |
|---|---|---|---|---|
| Sinigrin | One way ANOVA | $F = 36.937$ | < 0.001 | Holm-Sidak |
| 4MSOB-glucosinolate | Kruskal-Wallis One Way ANOVA on Ranks | $H = 19.56$ | < 0.001 | Tukey |
| Sinalbin | One way ANOVA | $F = 41.694$ | < 0.001 | Holm-Sidak |
| Linamarin | One way ANOVA | $F = 0.629$ | 0.541 | - |
| Salicin | Kruskal-Wallis One Way ANOVA on Ranks | $H = 19.357$ | < 0.001 | Tukey |
| Catalpol | *t*-test | $t = -1.825$ | 0.085 | - |

7.4.2. Loss by dissection
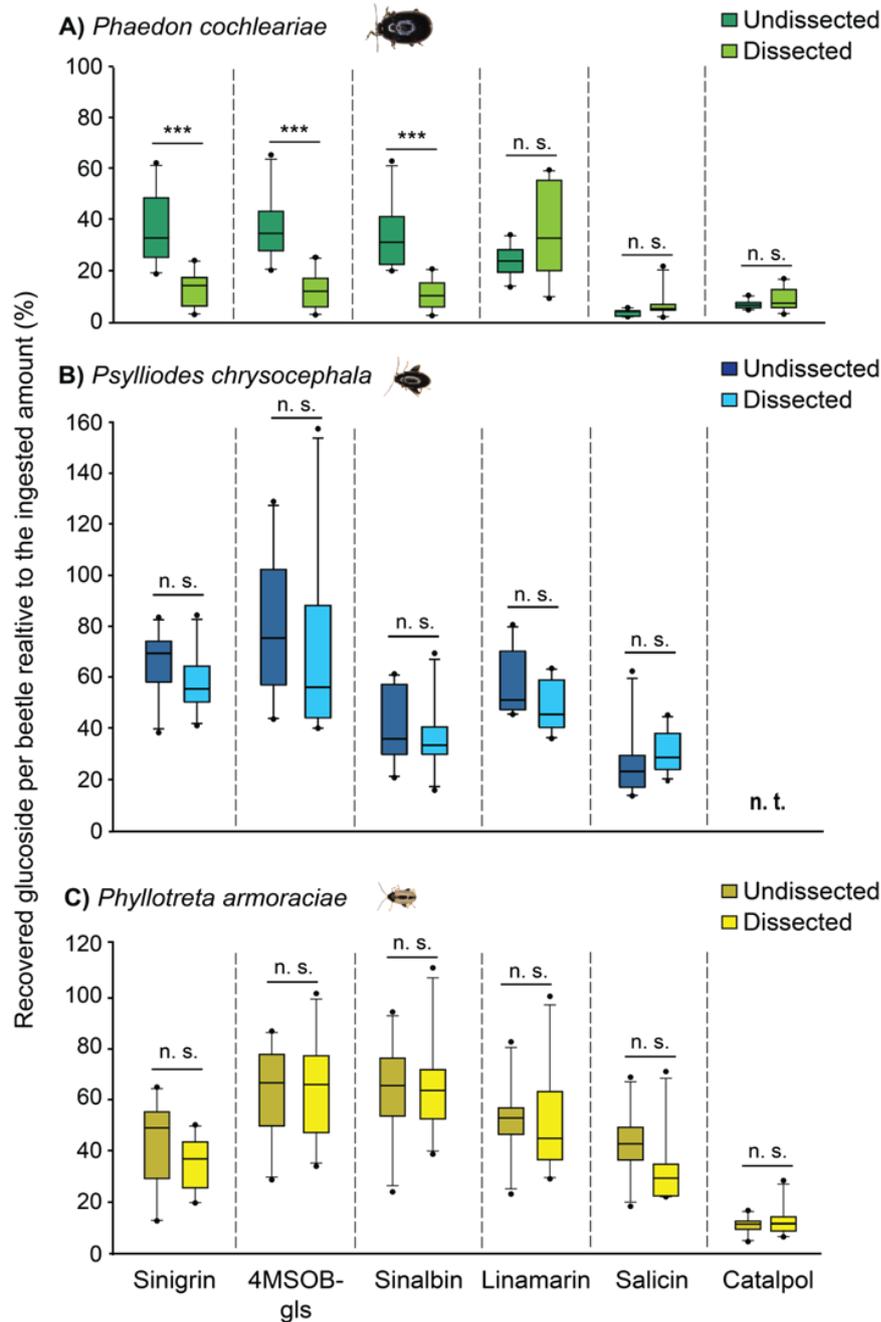


**Figure S2: Loss by dissection**. Comparison of plant glucoside recoveries between dissected and undissected samples, which ingested the same amount of glucosides. Recovery is shown relative to ingested amount (set to 100%) for A) *Phaedon cochleariae*, B) *Psylliodes chrysocephala* and C) *Phyllotreta armoraciae*. Dissected samples were summed up from "Head", "Gut" and "Rest Body" samples for each replicate. N=10 per species and treatment. Lower boxplots quartile border shows 25th percentile of data, line in boxplot shows median and upper quartile border shows 75th percentile. Whiskers show 10th respectively 90th percentile and filled black dots show outliers. Catalpol was not tested with *P. chrysocephala*. Differences in glucoside recovery between recovery samples and summed up samples were determined using *t*-tests. *** $p<0.001$; n. s. = not significant: $p>0.05$; n. t. = not tested; gls = glucosinolate. See table S2 for information on statistics and data transformation.

**Table S2: Methods, data transformation and results of the statistical analyses of loss by dissection**. [1]All transformations were performed on proportion data. gls= glucosinolate

| Species | Glucoside | Statistical method | Transformation[1] | Statistics | *P*- value |
|---------|-----------|--------------------|--------------------|------------|------------|
| *Phaedon cochleariae* | Sinigrin | *t*-test | - | $t = 4.722$ | < 0.001 |
| | 4MSOB- gls | *t*-test | - | $t = 5.262$ | < 0.001 |
| | Sinalbin | *t*-test | - | $t = 5.140$ | < 0.001 |
| | Linamarin | *t*-test | Arcsine square root | $t = -1.844$ | 0.098 |
| | Salicin | *t*-test | Arcsine square root | $t = -1.999$ | 0.077 |
| | Catalpol | *t*-test | Arcsine square root | $t = -1.463$ | 0.178 |
| *Psylliodes chrysocephala* | Sinigrin | *t*-test | - | $t = 1.406$ | 0.177 |
| | 4MSOB- gls | *t*-test | Arcsine square root | $t = 3.242$ | 0.014 |
| | Sinalbin | *t*-test | - | $t = 0.683$ | 0.503 |
| | Linamarin | *t*-test | Log(10) | $t = 5.995$ | < 0.001 |
| | Salicin | *t*-test | Log(10) | $t = -1.266$ | 0.222 |
| *Phyllotreta armoraciae* | Sinigrin | *t*-test | - | $t = 1.105$ | 0.284 |
| | 4MSOB- gls | *t*-test | - | $t = -0.119$ | 0.907 |
| | Sinalbin | *t*-test | - | $t = -0.151$ | 0.882 |
| | Linamarin | *t*-test | - | $t = 0.189$ | 0.852 |
| | Salicin | *t*-test | Log(10) | $t = 1.879$ | 0.077 |
| | Catalpol | *t*-test | Log(10) | $t = -0.67$ | 0.511 |

### 7.4.3. Statistics for Fig. 6: Proportion of glucosides in 'Rest body' between species and recovery of glucosides between species

**Table S3: Methods, data transformations and results of the statistical analyses of differences in proportion of glucoside in 'Rest body' relative to total detected amount between species**. (Fig. 6 A, B) and of differences in recovery of glucosinolates and non-host glucosides between species (Fig. 6 C, D). [1] all transformations were performed on proportion data. gls. = glucosinolate

| Analysis | Glucoside | Statistical method | Transfor-mation[1] | Statistics | P-value | Post Hoc test |
|---|---|---|---|---|---|---|
| Proportion of glucosides in "Rest body" between species | Sinigrin | One way ANOVA | - | $F = 100.532$ | < 0.001 | Holm-Sidak |
| | 4MSOB-gls | One way ANOVA | - | $F = 60.538$ | < 0.001 | Holm-Sidak |
| | Sinalbin | One way ANOVA | Arcsine square root | $F = 99.856$ | < 0.001 | Holm-Sidak |
| | Linamarin | One way ANOVA | Arcsine square root | $F = 25.755$ | < 0.001 | Holm-Sidak |
| | Salicin | One Way ANOVA | - | $F = 154.045$ | < 0.001 | Holm-Sidak |
| | Catalpol | t test | Arcsine square root | $t = -2.484$ | 0.023 | - |
| Recovery of glucosinolates between species | Sinigrin | One Way ANOVA | - | $F = 52.115$ | < 0.001 | Holm-Sidak |
| | 4MSOB-gls | One Way ANOVA | Arcsine square root | $F = 44.547$ | < 0.001 | Holm-Sidak |
| | Sinalbin | One Way ANOVA | Arcsine square root | $F = 50.354$ | < 0.001 | Holm-Sidak |
| | Linamarin | One Way ANOVA | Arcsine square root | $F = 3.038$ | 0.065 | |
| | Salicin | Kruskal-Wallis One Way ANOVA on Ranks | - | $H = 19.001$ | < 0.001 | Tukey |
| | Catalpol | t-test | Arcsine square root | $t = -1.842$ | 0.082 | |

### 7.4.4. Proportion of sequestered glucosides in 'Rest body' within species
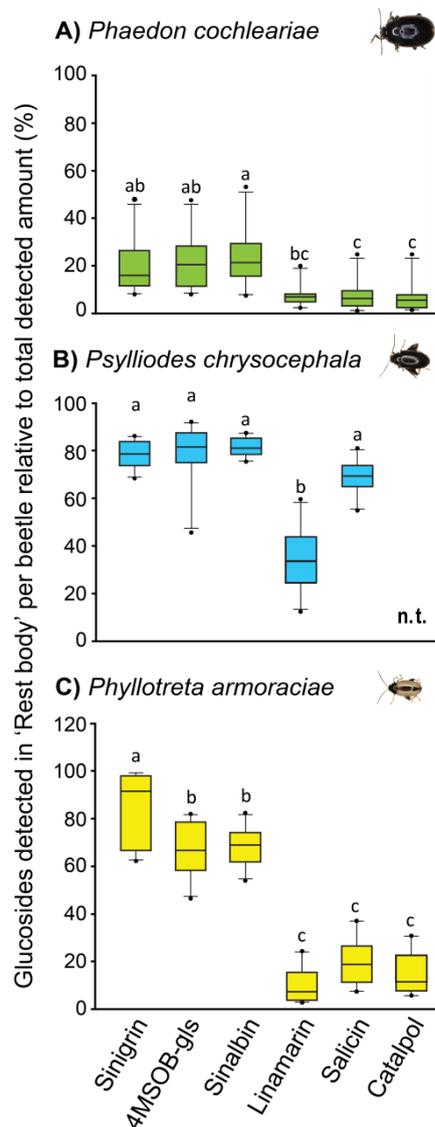


**Figure S3: Plant glucoside recovered from 'Rest body' relative to total recovered amount (set to 100%)**. Shown for A) *Phaedon cochleariae*, B) *Psylliodes chrysocephala* and C) *Phyllotreta armoraciae*. Lower boxplots border shows 25th percentile of data, line in boxplot shows median and upper quartile border shows 75th percentile. Whiskers show 10th respectively 90th percentile and filled black dots show outliers. Differences in recovery between plant glucosides from "Rest body" within species were analysed using one way ANOVAs. For further information on statistics and data transformations see table S4. Different letters indicate significant differences of recovery from rest body between glucosides within a species. n. t. = not tested; gls = glucosinolate. N=10/species

**Table S4: Methods, data transformations and results of statistical analysis of differences in glucoside recoveries from 'Rest body' relative total recovered amount within species**. [1]All transformations were performed on proportion data.

| Species | Glucoside | Statistical method | Transfor-mation[1] | Statistics | P-value | Post hoc test |
|---|---|---|---|---|---|---|
| *Phaedon cochleariae* | All six | One way ANOVA | Log(10) | $F = 6.812$ | <0.001 | Holm-Sidak |
| *Psylliodes chrysocephala* | Five | One way ANOVA | Arcsine square root | $F = 34.441$ | <0.001 | Holm-Sidak |
| *Phyllotreta armoraciae* | six | One way ANOVA | - | $F = 97.141$ | <0.001 | Holm-Sidak |

### 7.4.5. Relative distribution of recovered glucosides on the body parts
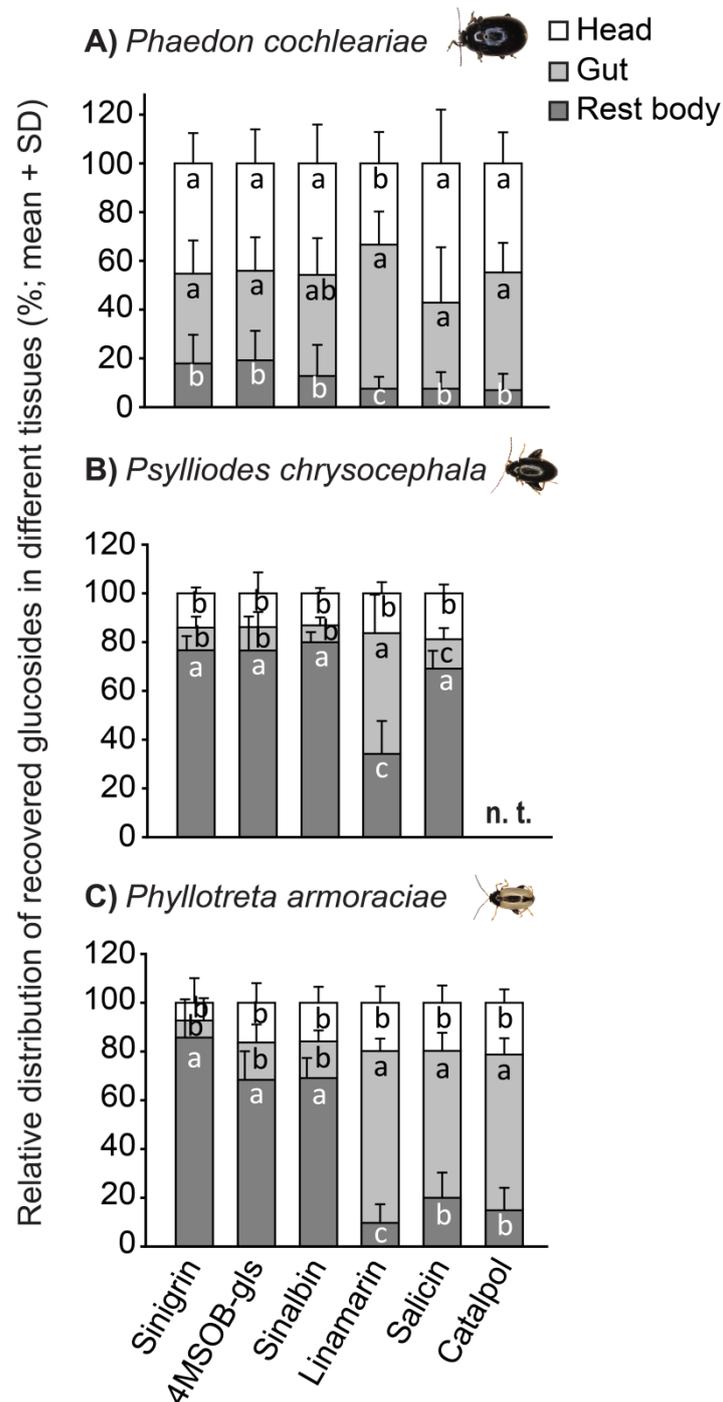


**Figure S4: Relative distribution of detected glucosides (set to 100%) between the body parts.** Shown for A) *Phaedon cochleariae*, B) *Psylliodes chrysocephala* and C) *Phyllotreta armoraciae*. Differences in relative distribution of detected glucoside between body parts within species were analysed using one Way ANOVAs and Kruskal Wallis one way ANOVAs on ranks (sinigrin and sinalbin in *P. chrysocephala*). Different letters indicate significant differences in distribution of glucoside between the body parts. For information on statistics and data transformations see Table S5. n. t. = not tested. 4MSOB-gls = 4MSOB-glucosinolate. N=10/species

**Table S5: Methods, data transformations and results of statistical analysis of distribution of glucoside between body parts within species, relative total recovered amount.** [1]All transformations were performed on proportion data.

| Species | Glucoside | Statistical method | Transformation[1] | Statistics | *P*- value | Post hoc test |
|---|---|---|---|---|---|---|
| *Phaedon cochleariae* | Sinigrin | One way ANOVA | - | $F = 12.246$ | < 0.001 | Holm-Sidak |
| | 4MSOB-glucosinolate | One way ANOVA | - | $F = 9.239$ | < 0.001 | Holm-Sidak |
| | Sinalbin | One way ANOVA | - | $F = 7.069$ | 0.003 | Holm-Sidak |
| | Linamarin | One way ANOVA | Arcsine square root | $F = 62.147$ | < 0.001 | Holm-Sidak |
| | Salicin | One way ANOVA | Log(10) | $F = 19.558$ | < 0.001 | Holm-Sidak |
| | Catalpol | One way ANOVA | - | $F = 44.359$ | < 0.001 | Holm-Sidak |
| *Psylliodes chrysocephala* | Sinigrin | Kruskal-Wallis One way ANOVA on ranks | - | $H = 22,165$ | < 0.001 | Tukey |
| | 4MSOB-glucosinolate | One way ANOVA | Arcsine square root | $F = 113.771$ | < 0.001 | Holm-Sidak |
| | Sinalbin | Kruskal-Wallis One way ANOVA on ranks | - | $H = 24.581$ | < 0.001 | Tukey |
| | Linamarin | One way ANOVA | - | $F = 18.325$ | < 0.001 | Holm-Sidak |
| | Salicin | One way ANOVA | - | $F = 333.027$ | < 0.001 | Holm-Sidak |
| *Phyllotreta armoraciae* | Sinigrin | One way ANOVA | - | $F = 150.947$ | < 0.001 | Holm-Sidak |
| | 4MSOB-glucosinolate | One way ANOVA | - | $F = 107.642$ | < 0.001 | Holm-Sidak |
| | Sinalbin | One way ANOVA | - | $F = 220.493$ | < 0.001 | Holm-Sidak |
| | Linamarin | One way ANOVA | - | $F = 245.877$ | < 0.001 | Holm-Sidak |
| | Salicin | One way ANOVA | - | $F = 76.910$ | < 0.001 | Holm-Sidak |
| | Catalpol | One way ANOVA | - | $F = 132.557$ | < 0.001 | Holm-Sidak |

7.4.6. Sequestration between species, head excluded

**A)** Glucosinolates



**B)** Other plant glucosides
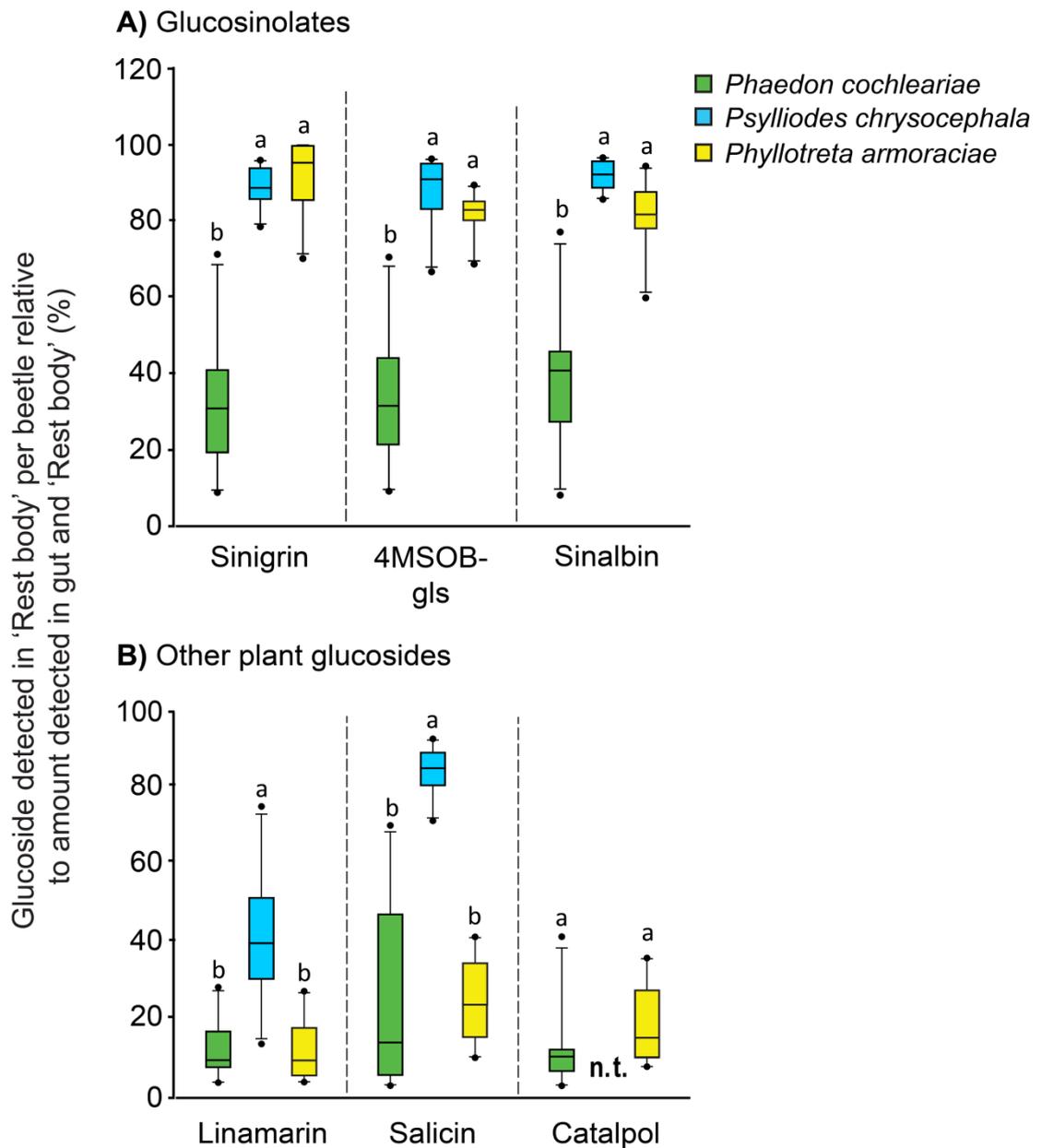


**Figure S5: Glucoside recovered from 'Rest body' relative to amount recovered from gut plus 'Rest body' (set to 100%) between species.** Lower boxplots border shows 25 percentile of data, line in boxplot shows median and upper quartile border shows 75 percentile. Whiskers show $10^{th}$ respectively $90^{th}$ percentile and filled black dots show outliers. N = 10/species. n. t. = not tested. 4MSOB-gls = 4MSOB-glucosinolate. Differences in proportion of glucosides in 'Rest body' between species were examined using one-way ANOVAs. Different letters indicate significant differences in proportion of respective glucoside in 'Rest body' between species. For further information on statistics and data transformation see table S6.

**Table S6: Methods, data transformations and results of the statistical analyses of differences in proportion of glucoside in 'Rest body' relative to amount recovered from gut plus 'Rest body' between species.** [1] all transformations were performed on proportion data.

| Glucoside | Statistical method | Transfor-mation[1] | Statistics | P-value | Post Hoc test |
|---|---|---|---|---|---|
| Sinigrin | One way ANOVA | Arcsine square root | $F = 59.318$ | <0.001 | Holm-Sidak |
| 4MSOB-glucosinolate | One way ANOVA | Arcsine square root | $F = 55.752$ | <0.001 | Holm-Sidak |
| Sinalbin | Kruskal-Wallis One Way ANOVA on Ranks | - | $H = 22.717$ | <0.001 | Tukey |
| Linamarin | One way ANOVA | Arcsine-square-root | $F = 20.917$ | <0.001 | Holm-Sidak |
| Salicin | Kruskal-Wallis One Way ANOVA on Ranks | - | $H = 19.520$ | <0.001 | Tukey |
| Catalpol | t test | Arcsine square root | $t = -1.510$ | 0.148 | |

## 7.5. Glucoside degradation by *P. cochleariae* body part homogenates – statistics table for Fig. 7

**Table S7: Methods, transformations and information on the statistical analysis of the comparison of glucoside recovery relative to ingested amount between incubates of the three body parts head, gut and 'Rest body'.** [1] all transformations were performed on proportion data.

| Glucoside | Statistical method | Transformation[1] | Statistics | P-value | Post-hoc test |
|---|---|---|---|---|---|
| Sinigrin | One way ANOVA | *Log*(10) | $F = 4.278$ | 0.034 | Holm-Sidak |
| 4MSOB-glucosinolate | One way ANOVA | *Log*(10) | $F = 4.623$ | 0.027 | Holm-Sidak |
| Sinalbin | One way ANOVA | *Log*(10) | $F = 1.285$ | 0.305 | - |
| Linamarin | One way ANOVA | Arcsine square root | $F = 29.776$ | <0.001 | Holm-Sidak |
| Salicin | One way ANOVA | *Log*(10) | $F = 19.933$ | <0.001 | Holm-Sidak |
| Catalpol | One way ANOVA | - | $F = 13.144$ | <0.001 | Holm-Sidak |

### 7.6. Location of glucosinolate uptake in *Phyllotreta armoraciae*

**A)** Total detected 4MSOB-gls

**B)** 4MSOB-gls detected in "Rest body"
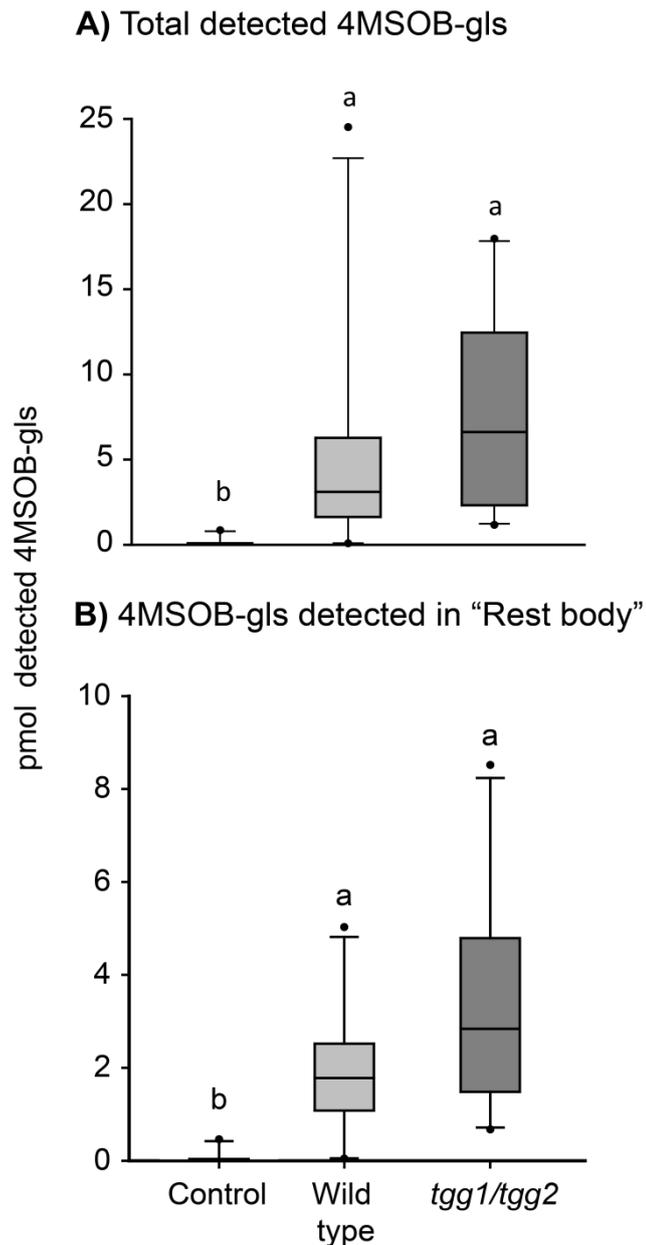
**Figure S6: Amount of 4MSOB-glucosinolate detected in *P. armoraciae* adults in pmol.** A) Total detected amount in whole beetle; B) Total amount detected in 'Rest body'. Control beetles did not feed; the other two treatments fed on *Arabidopsis thaliana* Col-0 wildtype (WT) and *tgg1/tgg2* mutant. N = 10 per treatment. Values were summed up from dissected samples. Lower boxplots quartile border shows 25th percentile of data, line in boxplot shows median and upper quartile border shows 75th percentile. Whiskers show 10th respectively 90th percentile and filled black dots show outliers. Differences in the amount of 4MSOB-glucosinolate were investigated using Kruskal-Wallis one Way ANOVA on ranks with post-hoc Tukey test A) $H$ = 18,980, $p$ <0,001 and B), H = 19,45, $p$ <0,001 Different letters indicate significant differences in 4MSOB-glucosinolate amount detected between treatments. gls = glucosinolate

### 7.6.1. Plant 4MSOB-glucosinolate concentration - figure

The concentrations of 4MSOB-glucosinolate in leaves used for the experiment 2.9. (Fig. S7) should be regarded as minimal concentrations: Due to a varying experimental time, the weight loss through drying during the experiment varied. *A. thaliana* possesses myrosinase {Wittstock, 2002 #996} which might have activated some glucosinolates due to drought damage, so that the displayed values might be lower than the concentration in leaf material actually ingested by the beetles. Furthermore, for the samples 6 and 7 of both treatments the continuous cooling chain after initial freezing cannot be guaranteed, therefore the displayed concentration might be lower than the actual concentration during feeding
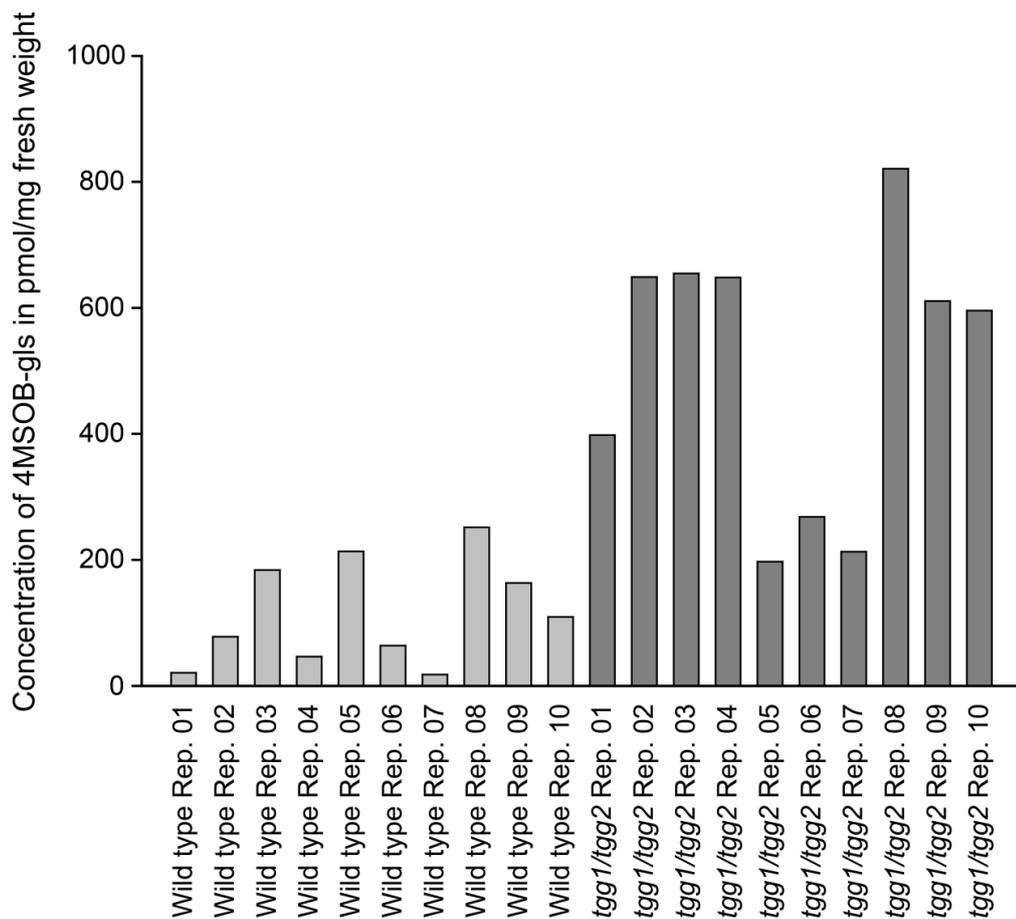


**Figure S7: Concentration of 4MSOB-glucosinolate in *Arabidopsis thaliana* Col-0 leaves used for the short term feeding experiment in pmol/mg fresh weight**. 4MSOB-glucosinolate was detected by HPLC-UV and quantified using an internal standard. For replicates Wild type Rep. 06 & 07 and *tgg1/tgg2* Rep. 06. & 07 the continual freezing before extraction was not maintained. 4MSOB-gls = 4MSOB-glucosinolate

## 7.6.2. Relative distribution of detected 4MSOB-glucosinolate

Due to an experimental error, the head sample might have contaminated the gut sample and vice versa So gut and head were analysed combined.The proportion of 4MSOB-glucosinolate in 'Rest body' did not differ significantly from the proportion in "Head + Gut" in both treatments (Fig S8A).. If "Head" is nonetheless treated as a separate sample, the proportion in "Rest body" resolved as significantly higher than the proportion in the other two body parts for both treatments (Fig. S8B).

**A)** Distibution of 4MSOB-gls between Head+Gut and Rest Body

**B)** Distibution of 4MSOB-gls between Head, Gut and Rest Body
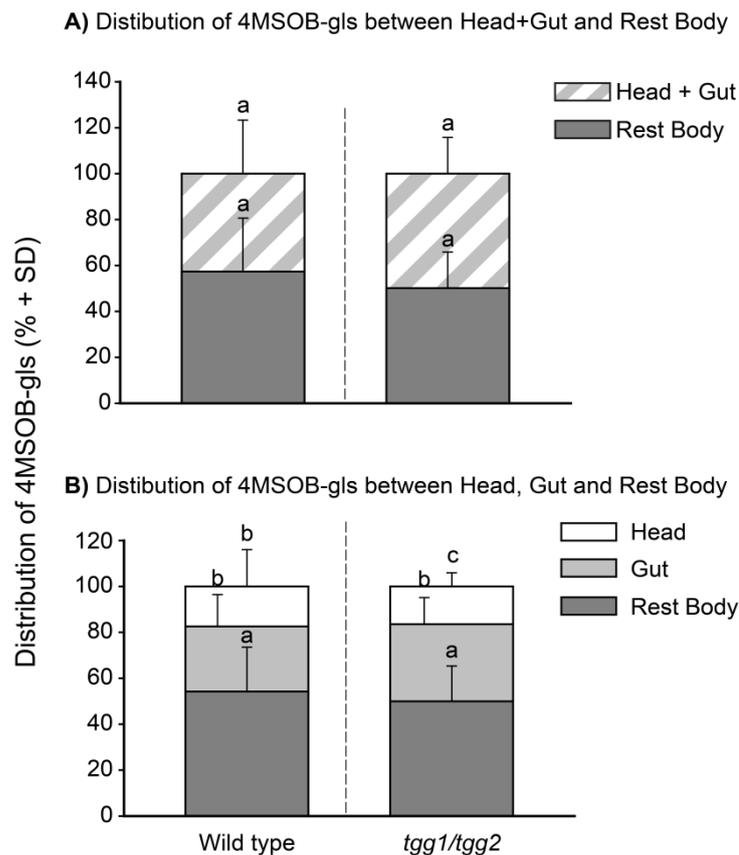
**Figure S8: Distribution of detected 4MSOB-glucosinolate relative to total detected amount (set to 100%) between the body parts** A) "Head + Gut" and "Rest body"; B) "Head", "Gut" and "Rest Body". *Phyllotreta armoraciae* fed either on *Arabidopsis thaliana* Col-0 wild type or *tgg1/tgg2* mutant. N = 10. Differences in proportion of 4MSOB-glucosinolate in body parts were investigated for both treatments using A) paired *t*-tests, WT: $t = -1.000$ $p = 0.344$, *tgg1/tgg2*: $t = -0.0301$ $p = 0.977$; B) One Way ANOVAs with post-hoc Holm-Sidak test, WT: $F = 13,097$, $p < 0.001$; *tgg1/tgg2*: $F = 20,726$, $p < 0.001$. Different letters indicate significant differences in the proportions of 4MSOB-glucosinolate between body parts. 4MSOB-gls = 4MSOB-glucosinolate

## 7.7. Crop morphology

### 7.7.1. *P. cochleariae* gut parasite

An organism was found in high numbers in the midgut of *P. cochleariae* (Fig.9, Fig. S9). It was not properly determined, but probably was a gregarine apicomplexan, which has been reported to be a gut parasite of *P. cochleariae* (Müller et al., 2017).
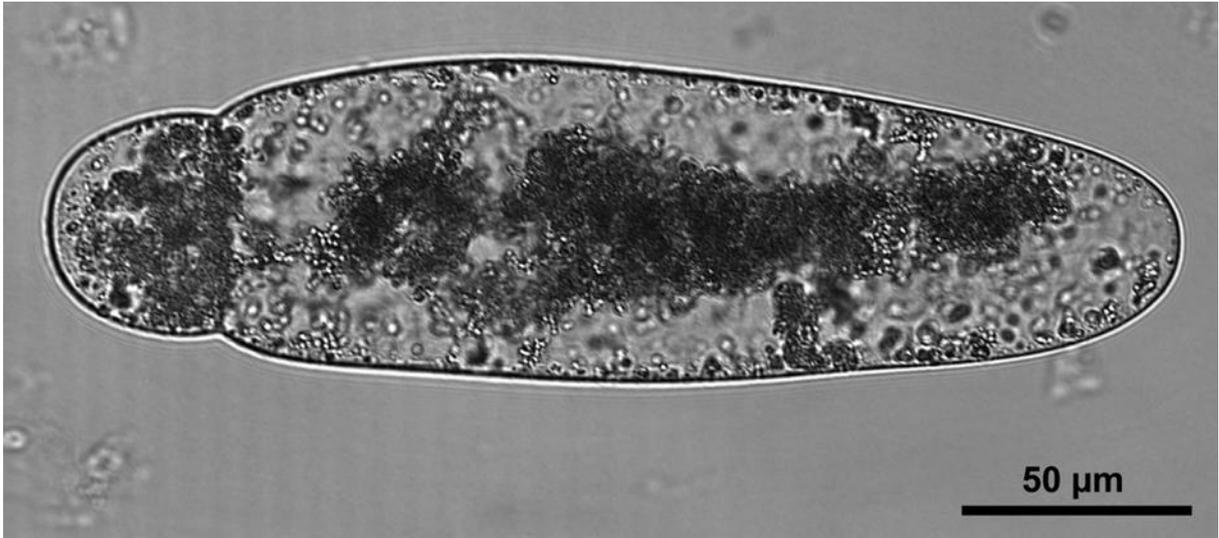


**Figure S9: Gut parasite found in *Phaedon cochleariae*.** Probably a gregarine apicomplexan. Stack of a time series, taken on a LSM 88, Axio Imager 2 (Zeiss), 40X magnified. Photographer: Dr. V. Grabe

7.7.2.  Confocal laser scanning microscopy – detailed parameters (Fig. 10)

**Table 8: Detailed information on confocal laser scanning microscopy images.**

| Species | *Phaedon cochleariae* | | *Psylliodes chrysocephala* | | *Phyllotreta armoraciae* | |
|---|---|---|---|---|---|---|
| Image | I.      crop | II.      detail | I.  crop | II.  detail | I.      crop | II.      detail |
| Microscope | Carl Zeiss, LSM 880 Axio Imager 2 | | | | | |
| Excitation Wavelength (nm) | 405 | | | | | |
| Laser (power) | Diode 405-30 (30%) | | | | | |
| Pinhole diameter (µm) | 38.8 | 39.97 | 31.55 | 41.8 | 31.5 | 40.96 |
| Dimensions (pixels) | 2867 x 1024 | 1024 x 1024 | 1024 x 1024 | 2048 x 2048 | 1944 x 1024 | 1024 x 1024 |
| Scaling (µm) | 1.38 x 1.38 x 6.08 | 0.35 x 0.35 x 0.48 | 0.69 x 0.69 x 0.87 | 0.17 x 0.17 x 1 | 0.69 x 0.69 x 0.87 | 0.35 x 0.35 x 0.49 |
| Image size | 2867 x 1024 | 354.25 x 354.25 | 708.8 x 708.49 | 354.25 x 354.25 | 1350 x 708.49 | 354.25 x 354.25 |
| Z-stack size | 31 (3-34 of 45) | 35 (10-45 of 109) | 80 (6-86 of 94) | 20 (7-27 of 29) | 28 (7-35 of 35) | 25 (10-35 of 68) |
| Magnification | 10 | 40 | 20 | 40 | 20 | 40 |
| Emission wavelength range (nm) | 410 – 694.8 | 416-735 | 415-735 | 415-735 | 415-735 | 416-735 |
| Emission wavelength (nm) | 552.45 | 575 | 575 | 575 | 575 | 575 |
| Gain #1 | 750 | 650 | 600 | 571 | 600 | 650 |
| Gain #2 | 175 | 270 | 190 | 200 | 230 | 230 |
| Objective | EC Plan-Neofluar 10x/ 0.30 M27 | C-Apochromat 40x/1.20 W Korr M27 | Plan-Apochromat 20x/0.8 M27 | C-Apochromat 40x/1.20 W Korr M27 | Plan-Apochromat 20x/0.8 M27 | C-Apochromat 40x/1.20 W Korr M27 |
| | Was taken in a slide with well | | | | | |

### 7.7.3. TEM – Midgut epithelium of *P. chrysocephala*
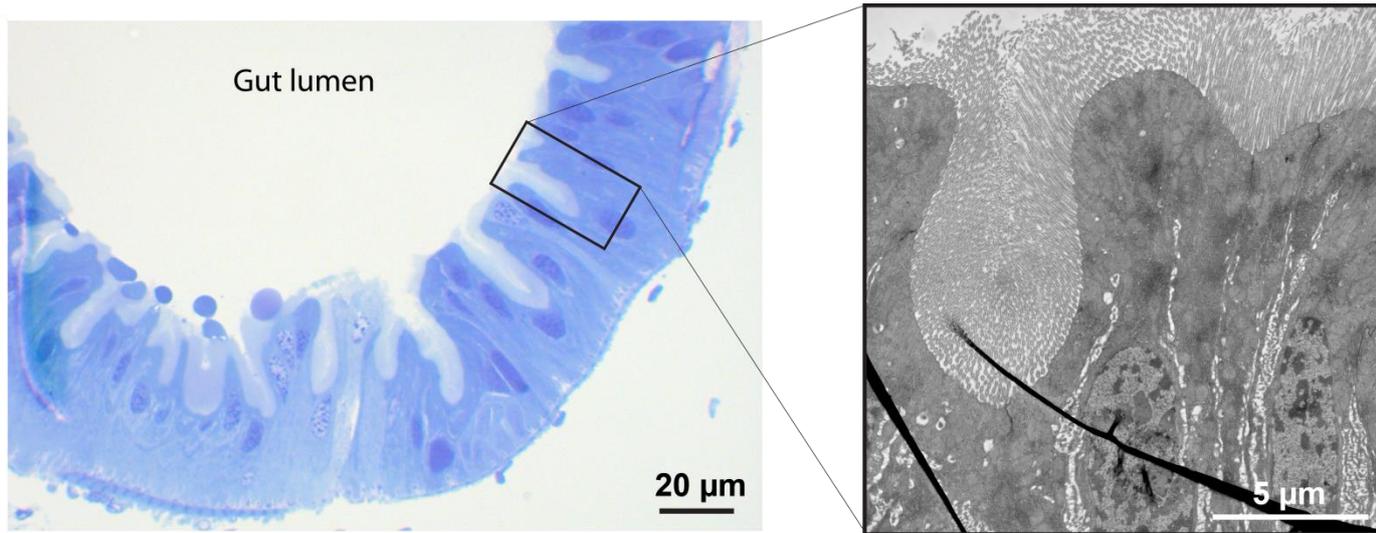


**Figure S10: Semi thin section and TEM picture of the midgut epithelium of *P.chrysocephala*.** Black rectangle indicates the location of the ultra-thin section. Photographer: S. Nietzsche

## 7.8. CD/DVD-Rom - digital appendix contents

1. Haemolymph Analysis (folder)
    Amino acids (folder)
        - AminoAcids_Quantification.xlsx
        - RawData_FMOC.xlsx
    Glucosinolates (folder)
        - Haemolymph_Glucosinolates.xlsx
    Sugars (folder)
        - RawData_Sugars.xlsx
    - Haemolymph Analysis.JNB (statistics file)

2. Selectivity of uptake
    - Linamarin.xlsx
    - Catalpol.xlsx
    - Salicin.xlsx
    - Sinigrin.xlsx
    - 4MSOB-glucosinolate.xlsx
    - Sinalbin.xlsx
    - All_data_Compiled.xlsx

    Loss by dissection
        - P.a.sum_vs_recov.JNB
        - P.ch.sum_ vs_recov.JNB
        - P.co.sum_ vs_recov.JNB

    Recovery between species
        - Other_glucosides.JNB
        - Glucosinolate.JNB

    Proportion in rest body between species
        - Glucosinolates.JNB
        - Other_glucosides.JNB

    Supplements
        Proportion in rest body within species
        - phaedon.JNB
        - phyllotreta.JNB
        - psylliode.JNB

        Prop. in rest body between species – head excluded
        - Glc.JNB
        - Gls.JNB

Proportional distribution between body parts
    - Phaedon_bodypart_distr.JNB
    - Phyllotreta_bodypart_distr.JNB
    - Psylliodes_bodypart_distr.JNB

2.1 Phaedon degradation Experiment
    - Phaedon_bodypart_degr.JNB
    - Phaedon_degr_exp_RawData.xlsx

3. Short term feeding Experiment
    - %_in_rb.JNB
    - FeedingExperiment_RawData.xlsx
    Supplement
        Distribution on body parts
            - Distribution_head_gut_restbody.JNB
            - Distribution_head+gut_restbody.JNB

        Plant Gls profile
            - PlantGlucosinolateProfile

        Total detected 4MSOB-gls
            - pmol_total_detected_4MSOB.JNB
            - Pmol_4MSOB_in_RB.JNB

4. Crop morphology
   µCT

   Phaedon cochleariae
   - Phaedon.am
   - Phaedon.Labels.obj
   - Phaedon.Labels.surf
   - Phaedon_dorsal.tif
   - Phaedon_lateral.tif
   - Phaedon_ventral.tif

   Psylliodes chrysocephala
   - Psylliodes_lateral.tif
   - Psylliodes_ventral.tif
   - Psylliodes_dorsal.tif
   - Psylliodes.am
   - Psylliodes.obj
   - Psylliodes.surf

   Phyllotreta armoraciae
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   - Phyllotreta_dorsal.tif
   - Phyllotreta_lateral.tif
   - Phyllotreta_lateral(2).tif
   - Phyllotreta.obj
   - Phyllotreta.am
   - Phyllotreta.surf.am
   - Phyllotreta.surf

   CLSM
   Phaedon cochleariae
   - Phaedon coch intact foregut AF 10x tile scan TL_Stitch.tif
   - 40x detail.czi
   - P.coFgZoom2.czi
   - 40x detail (2).tif
   - Pa_stack_Fg_Transversal.tif

   Psylliodes chrysocephala
   - Psylliodes_FgZoom.jpg
   - Psylliodes_FgZoom2.jpg
   - Transversal1.tif
   - Transversal2.tif
   - Foregut lateral view 20x.tif
   - Foregut lateral view 20x mit durchlicht.tif

   Phyllotreta armoraciae
   - 20x tile scan stitched.tif
   - 40x detail.czi
   - Image1_Stitch_MIP.tif
   - Transversal.jpg
   - Phyllotreta_foregut overview.tif
   - Paforegut overview.czi

   Light microscopy
   - FG_Phaedon.tif
   - FG_Phyllotreta.tif
   - FG_Psylliode.tif
   - Phaedon.tif
   - Phyllotreta.tif
   - Psylliodes.tif

   TEM

   Phaedon cochleariae
   - 6 .tif pictures
   Phyllotreta armoraciae
   Specimen 1
   - 11 .tif pictures
   Specimen 2
   - 23 .tif pictures
   Psylliodes chrysocephala
   - P_chrysoc_semi_1.tif
   - P_chryoc_TEM_1.tif
   - Seitz0920_29.TIF
   Midgut epithelium
   - 19 pictures

# 8. Declaration of Self-Dependence

Herewith I declare, that I prepared this thesis on my own, that I did not use any other sources and resources than those that are specified, that all arguments and ideas that were literally or analogously taken from other sources are sufficiently identified, and that the thesis in identical or similar form has not been use as part of an earlier course achievement or examination procedure.

.......................................................    .                    ...........................................................

Place, Date                                      Signature