Received: 29 April 2020 | Accepted: 13 August 2020

DOI: 10.1111/mec.15613

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Detoxification of plant defensive glucosinolates by an herbivorous caterpillar is beneficial to its endoparasitic wasp

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Funding information
Deutsche Forschungsgemeinschaft; China Scholarship Council, Grant/Award Number: 201508330285; Max Planck Society; Open access funding enabled and organized by Projekt DEAL

Abstract

Plant chemical defences impact not only herbivores, but also organisms in higher trophic levels that prey on or parasitize herbivores. While herbivorous insects can often detoxify plant chemicals ingested from suitable host plants, how such detoxification affects endoparasitoids that use these herbivores as hosts is largely unknown. Here, we used transformed plants to experimentally manipulate the major detoxification reaction used by *Plutella xylostella* (diamondback moth) to deactivate the glucosinolate defences of its Brassicaceae host plants. We then assessed the developmental, metabolic, immune, and reproductive consequences of this genetic manipulation on the herbivore as well as its hymenopteran endoparasitoid *Diadegma semiclausum*. Inhibition of *P. xylostella* glucosinolate metabolism by plant-mediated RNA interference increased the accumulation of the principal glucosinolate activation products, the toxic isothiocyanates, in the herbivore, with negative effects on its growth. Although the endoparasitoid manipulated the excretion of toxins by its insect host to its own advantage, the inhibition of herbivore glucosinolate detoxification slowed endoparasitoid development, impaired its reproduction, and suppressed the expression of genes of a parasitoid-symbiotic polydnavirus that aids parasitism. Therefore, the detoxification of plant glucosinolates by an herbivore lowers its toxicity as a host and benefits the parasitoid *D. semiclausum* at multiple levels.

KEYWORDS
detoxification, glucosinolate-myrosinase system, isothiocyanate, multitrophic interaction, parasitism

1 | INTRODUCTION

Multitrophic interactions involving plants, insect herbivores, and their antagonists are ubiquitous in terrestrial ecosystems and underpin our understanding of the structure and function of ecological communities (Stam et al., 2014). Most plants in nature are attacked by insect herbivores, and high infestations can severely damage plant tissues and thus reduce plant fitness (Johnson, Lajeunesse, & Agrawal, 2006). To prevent or reduce attack, plants employ an array of strategies to reduce herbivory, including the production of a wide assortment of toxic, repellent, antidigestive, and antinutritive chemical defences (Mithöfer & Boland, 2012). Plant chemical defences can also traverse trophic levels, moving up the food chain to affect not only the consuming herbivores but subsequently also herbivore predators (Hartmann, 2004;

Nishida, 2002; Petschenka & Agrawal, 2016) and parasitoids (Gols & Harvey, 2009; Harvey, 2005; Harvey, Van Dam, & Gols, 2003; Ode, 2019), and even the fourth trophic level (Harvey et al., 2003; Orr & Boethel, 1986). Nearly three decades ago, Gauld, Gaston, and Janzen (1992) formulated the "nasty host hypothesis", suggesting that herbivore feeding on toxic plants would be detrimental to endoparasitoid development and survival. In apparent support of this hypothesis, the fitness of the predatory wasp *Copidosoma sosares* was shown to be negatively influenced by the presence of toxic furanocoumarins in the haemolymph of its host, the specialized herbivore parsnip webworm *Depressaria pastinacella* (Lampert, Zangerl, Berenbaum, & Ode, 2008; McGovern, Zangerl, Ode, & Berenbaum, 2006; Ode, Berenbaum, Zangerl, & Hardy, 2004), while the nicotine content in the diet of the herbivore *Manduca sexta* affected not only the endoparasitoid *Cotesia congregata*, but also its hyperparasitoid *Lysibia nana* (Barbosa, Gross, & Kemper, 1991; Harvey, Van Dam, Witjes, Soler, & Gols, 2007; Thorpe & Barbosa, 1986). While specialized herbivores can sometimes detoxify ingested plant defensive chemicals efficiently, how these processes affect higher trophic levels and how these defences are in turn metabolized by predators and parasitoids is poorly understood.

Plants in the Brassicaceae, which include oilseed and vegetable crops, produce glucosinolates as their characteristic chemical defences. Glucosinolates are accompanied in plants by enzymes called myrosinases that hydrolyse the parent glucosinolates upon plant damage, forming isothiocyanates (ITCs) and other products (Halkier & Gershenzon, 2006). ITCs are toxic to a broad range of organisms, including the larvae of lepidopteran herbivores (Wittstock, Kliebenstein, Lambrix, Reichelt, & Gershenzon, 2003). Because of their amphiphilic properties and high reactivity (Hanschen et al., 2012), ITCs can directly modify proteins and interfere with intracellular redox homeostasis (Brown & Hampton, 2011). ITCs also typically react quickly with the intracellular nucleophile glutathione (GSH) leading to its depletion (Jeschke, Gershenzon, & Vassão, 2016a). The presence of glucosinolates in plants has fueled selection for a suite of mechanisms in herbivores that mitigate or circumvent exposure to toxic glucosinolate hydrolysis products (Jeschke et al., 2016b), including behavioural adjustments, detoxification, rapid excretion processes and sequestration (Winde & Wittstock, 2011). Larvae of *Plutella xylostella* (the diamondback moth, Lepidoptera: Plutellidae), a specialized herbivore that is a devastating pest of brassicaceous crops (Furlong, Wright, & Dosdall, 2013; Zalucki et al., 2012), can feed without ill effects on glucosinolate-containing plants due to the activity of glucosinolate sulphatases (GSS). These enzymes are abundant in the digestive system of this insect, and desulphate glucosinolates preventing myrosinase-catalysed hydrolysis and ITC formation (Ratzka, Vogel, Kliebenstein, Mitchell-Olds, & Kroymann, 2002). Sun et al. (2019) showed that genetic disruption of *P. xylostella* desulphation led to the increased formation of ITCs, which caused steep declines in larval growth, survival and reproduction, demonstrating desulphation to be a genuine detoxification strategy in this herbivore.

At higher trophic levels, plant defences can act indirectly by reducing the quality of herbivores available as prey or hosts. Alternatively, direct exposure to defensive chemicals ingested by the prey or host can negatively affect the growth and development of parasitoids and predators (Gols & Harvey, 2009; Kaplan, Carrillo, Garvey, & Ode, 2016; Lampert, Zangerl, Berenbaum, & Ode, 2011; Ode, 2019). When the common green lacewing *Chrysoperla carnea* fed on *P. xylostella* in which glucosinolate desulphation was blocked by RNA interference, its development was slowed by consuming these ITC-containing prey (Sun et al., 2019). Nevertheless, *C. carnea* suffered no reproductive effects, perhaps due to its ability to metabolize ITCs via a general detoxification pathway. However, it is not clear how other predators or parasitoids cope with plant defences such as ITCs in their prey or host.

The solitary endoparasitoid *Diadegma semiclausum* (Hymenoptera: Ichneumonidae) is an important natural enemy of *P. xylostella*, and is frequently used in biocontrol programmes against this pest (Furlong et al., 2013; Li, Eigenbrode, Stringam, & Thiagarajah, 2000). Young larvae of this parasitoid develop by feeding on the haemolymph of the caterpillar host until the parasitoid larva reaches its final instar. At this point, it starts to feed on all tissues indiscriminately and eats the host completely, until it pupates in the puparium made by the host caterpillar before death. During all phases of parasitoid growth, the host caterpillar keeps feeding on the plant, thereby exposing the parasitoid larvae continuously to glucosinolates and their metabolites. Previous research has shown that *D. semiclausum* development is influenced by the brassicaceous species on which their *P. xylostella* hosts were reared, possibly due to interspecific differences in glucosinolate profiles (Dosdall, Zalucki, Tansey, & Furlong, 2011; Gols & Harvey, 2009; Gols et al., 2008). However, whether glucosinolates, ITCs or other glucosinolate metabolites benefit or harm endoparasitoids of *P. xylostella* such as *D. semiclausum*, is unknown. Moreover, how parasitism by *D. semiclausum* influences *P. xylostella* glucosinolate metabolism and development is also not known.

Here, we manipulated the detoxification capacity of *P. xylostella* larvae in order to examine the effects of plant glucosinolate defences on the interactions between *P. xylostella* and *D. semiclausum*. We hypothesized that blocking the desulphation activity responsible for glucosinolate detoxification would lead to the accumulation of ITCs in host tissues, affecting in turn the development of the endoparasitoid. Additionally, we also examined the effects of blocking glucosinolate desulphation on the immune responses of the herbivore against parasitism.

2 | MATERIALS AND METHODS

2.1 | Experimental overview

Plant-mediated RNAi was used to silence detoxification-related genes in the herbivore *P. xylostella*. *Arabidopsis thaliana* Columbia-0 (Col-0) plants, which naturally contain glucosinolates, were engineered to produce dsRNA targeting the *Pxgss* genes encoding glucosinolate sulphatases (GSS) responsible for glucosinolate detoxification in the herbivore. While it is unclear whether *P. xylostella* is a natural herbivore of wild *A. thaliana*, this plant has been shown to serve as a suitable host for *P. xylostella*, supporting

similar growth as on cultivated *Brassica* crops (Barker, Poppy, & Payne, 2007). *Arabidopsis thaliana* mutants deficient in the production of glucosinolates were also engineered for RNAi and were used as toxin-free controls. Through the combination of these treatments (presence or absence of glucosinolate defences in the plant and presence or absence of glucosinolate detoxification in the herbivore), we investigated the effects of glucosinolate ingestion and its detoxification by the specialist herbivore both on the herbivore and on the parasitoid *D. semiclausum*. Insect parameters measured included growth and development, survival, protein and lipid contents, and herbivore immune phenoloxidase (PO) activity, while the movement of glucosinolate metabolites among trophic levels was quantified by targeted HPLC-MS/MS analyses. Finally, quantitative real-time PCR (qPCR) was conducted to examine the effects of glucosinolate-derived ITCs on the expression of selected herbivore and parasitoid genes. These procedures are described in detail in the following subsections.

2.2 | Plants and insects

Arabidopsis thaliana Col-0 accession plants with wild type-glucosinolate levels (obtained from the Arabidopsis stock center) and transgenic *myb28myb29* knockout mutants (without aliphatic glucosinolates, obtained from Daniel J. Kliebenstein, University of California Davis) (Sønderby et al., 2007) were used. *Arabidopsis thaliana* plants were grown in a climate-controlled short-day environmental chamber at 21°C, 60% relative humidity, and a 14:10 hr, light:dark photoperiod in a soil mixture (80% Fruhstorfer "Nullerde", 10% vermiculite, and 10% sand). *Brassica napus* used for rearing of insect cultures was grown in a greenhouse under the same conditions as for *A. thaliana*. Colonies of *Plutella xylostella*, obtained from Bayer (Monheim am Rhein, Germany), were fed on *B. napus* leaves and maintained in a controlled short-day environment chamber. Eggs of *P. xylostella* were collected for experiments by allowing females to lay eggs on a sheet of Parafilm placed on top of *B. napus* leaves for 2 days. *Diadegma semiclausum* were reared on *B. napus* infested with *P. xylostella* caterpillars in a climate-controlled long-day environmental chamber (21°C, 70% relative humidity, and a 16:8 hr light:dark photoperiod), in cages containing cotton balls imbibed with a 20% (w/v) sucrose solution in distilled water. Larvae of *P. xylostella* used for experiments were fed on *A. thaliana* plants in a similarly controlled long-day environmental chamber after hatching.

2.3 | *Plutella xylostella Pxgss* gene silencing by plant-mediated RNAi

Plant mediated RNAi was used to silence the expression of *Pxgss* genes in the midgut of *P. xylostella* larvae, as described in Sun et al. (2019). In short, we constructed a tobacco rattle virus-based dsRNA producing system (*Pxgss*-RNAi construct) targeting *Pxgss* in *P. xylostella* larvae. An empty vector construct was used as a negative control. Both the *Pxgss*-RNAi construct and empty vector construct were transformed into *A. thaliana* Col-0 wild-type and *myb28myb29* double knockout mutant plants via *Agrobacterium tumefaciens* (strain GV3101). We had previously determined that plant transformation with these constructs had no effects on plant morphology, glucosinolate profile, and levels of other defensive secondary metabolites (Sun et al., 2019).

2.4 | Measurements of *P. xylostella* growth and development

Plutella xylostella larvae were fed ad libitum on either empty vector-transformed or *Pxgss* RNAi-transformed *A. thaliana* plants (from both Col-0 and *myb28myb29* backgrounds) after hatching in a controlled environmental chamber under the conditions described above. The percentage of larval pupation, which occurred from 7–10 days post hatching, was recorded in the four treatment groups relative to the initial numbers of hatched larvae (approximately 120 larvae per treatment). In the late fourth-instar stage, *P. xylostella* larvae were collected in 1.5 ml Eppendorf tubes to measure larval weights (20 replicates per treatment) as well as soluble protein (five replicates per treatment, three larvae were pooled in one sample) and lipid content (five replicates per treatment, three larvae pooled) after freeze-drying (ALPHA 1–4 LD plus freeze dryer, Martin Christ, Osterode am Harz, Germany). Protein and lipid content were measured to assess the nutritional status of the insects after *Pxgss*-silencing and glucosinolate ingestion. Fresh and dry bodyweights were measured using a microbalance (XP26, Mettler Toledo, Gießen, Germany). Soluble protein and lipid content were measured as described below in 2.8.

2.5 | *Diadegma semiclausum* parasitism

Approximately three hundred *P. xylostella* second-instar larvae of each of the four treatments, *Pxgss*-silenced or unsilenced feeding on either *A. thaliana* Col-0 or *myb28myb29* plants, were individually exposed to *D. semiclausum* to be parasitized once. Female and male *D. semiclausum* adults were sexually mature and maintained together for a few days to promote mating before being exposed to *P. xylostella*. After parasitism, *P. xylostella* larvae were returned to their original food plant until they developed into prepupae. Before the parasitoid larva enters pupation, it excretes a meconium containing waste products, which is present as a black pellet inside its cocoon. The cocoons were kept individually in 5 ml amber glass vials with a cotton cover until emergence. Subsequently, newly emerged adults of *D. semiclausum* were sexed and transferred to 1.5 ml Eppendorf tubes, and immediately frozen in liquid nitrogen for further analyses. As part of these experiments, unparasitized *P. xylostella* fed on either *A. thaliana* Col-0 or *myb28myb29* plants were raised under the same conditions (21°C, 70% relative humidity, and a 16:8 hr light:dark photoperiod). The detailed experimental time line for *D. semiclausum* parasitism of *P. xylostella* is shown in Figure S1.

2.6 | *Diadegma semiclausum* larval development duration, adult emergence and adult bodyweight

To determine the physiological effects of glucosinolates ingested by host larvae on the parasitoid in the absence of RNAi (Figure 1), second-instar *P. xylostella* larvae were parasitized by female *D. semiclausum* as described above and allowed to feed ad libitum on either *A. thaliana* Col-0 or *myb28myb29* plants (one plant per treatment, approximately 50–100 larvae for each of the two treatments). Plants were changed every day to ensure that sufficient food was available. This experiment was repeated three times independently (see Supporting Information file). Then, the percentages of successful *D. semiclausum* adult emergence were calculated relative to the number of *P. xylostella* larvae parasitized. Emerged *D. semiclausum* adults were sexed, freeze-dried and weighed (approximately 20 males and 10 females were obtained per treatment).

To determine the physiological effects of silencing *Pxgss* on *D. semiclausum* (Figure 2g–j), a second experimental setup was used. Silenced and nonsilenced second-instar *P. xylostella* larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* plants (approximately 600 larvae per treatment, 2,400 larvae in total, with 100 larvae per plant in a single cage, plants exchanged daily to ensure food availability) were parasitized by female *D. semiclausum* adults. First, we recorded the emergence of *D. semiclausum* adults (12–16 days post parasitism) from only a subset of the insects (two cages per group, i.e., around 120–180 surviving parasitized *P. xylostella* per treatment, see Supporting Information file). Next, *D. semiclausum* adults emerging from all groups (all six cages per treatment) were sexed, immediately frozen in liquid nitrogen, freeze-dried and weighed (40 male replicates and 10 female replicates were collected per treatment). Soluble protein and lipid contents in these adults were measured as described below in 2.8. Only males were used for this chemical analysis, as the number of female wasps was insufficient for reliable measurement.

2.7 | 4MSOB-ITC complementation experiment

In leaves of *A. thaliana* Col-0 plants grown for these experiments, 4-methylsulphinylbutyl glucosinolate (4MSOB) represents over 70% of the aliphatic glucosinolates (Brown, Tokuhisa, Reichelt, & Gershenzon, 2003). To determine whether the elevated 4-methylsulphinylbutyl isothiocyanate (4MSOB-ITC) concentrations in *Pxgss*-silenced *P. xylostella* caused the *D. semiclausum* phenotypes observed, complementation experiments were conducted by infiltrating 4MSOB-ITC into leaves of *myb28myb29* plants lacking aliphatic glucosinolates as described. A 0.3 μl quantity of 800 μM 4MSOB-ITC per mg fresh leaf was injected using a needleless syringe (Katagiri, Thilmony, & He, 2002) to mimic the natural content of damaged leaves (Sun et al., 2019). Leaves infiltrated with solvent (0.4% aqueous ethanol) served as negative controls. *Diadegma semiclausum* female adults were allowed to parasitize *P. xylostella* larvae (approximately 600 per treatment) continuously feeding on these leaves, and *D. semiclausum* adult emergence percentage, body dry weight, soluble protein and lipid contents were measured. First, the percentage of *D. semiclausum* adult emergence (13–16 days post parasitization) from approximately 200 successfully parasitized *P. xylostella* per treatment was determined. Then, emerged *D. semiclausum* adults were sexed, immediately frozen in liquid nitrogen, freeze-dried and weighed (30 male replicates and 10 female replicates per treatment). Third, soluble protein and lipid content in adults was measured as described below in 2.8.

2.8 | Protein and lipid content

Soluble protein content was determined by the Bradford assay (Bradford, 1976). Samples were homogenized in 200 μl Tris-HCl buffer (100 mM, pH 7.5) with ceramic beads (Sigmund Lindner, Warmensteinach, Germany) in 1.5 ml Eppendorf tubes using a Skandex S-7 homogenizer (Grootec GmbH, Kirchheim, Germany) for 3 min. Homogenized samples were centrifuged at 13,000 ***g*** for 20 min at 4°C to separate undissolved particles. Clear supernatants were transferred to 1.5 ml Eppendorf tubes, and 20 μl of each sample were used to measure protein concentration (Serva Electrophoresis, Heidelberg, Germany) according to the manufacturer's instructions. Soluble protein content was normalized by insect dry weight.

Lipid content was determined following a previously published protocol (Jeschke et al., 2016a). Weighed and pulverized dried body samples (approximately 5 mg for *P. xylostella* larvae, and approximately 2.5 mg for *D. semiclausum* adults, five replicates per treatment) were extracted two times with 1 ml of 2:1 chloroform:methanol by vortexing for 30 s. After centrifugation at 13,000 ***g*** for 20 min at 4°C and careful removal of the lipid-containing solvent, the remaining powder was dried at 80°C for 48 hr and weighed to calculate the proportion of lipid present.

2.9 | Immune phenoloxidase (PO) activity

PO activity is part of a critical host immune defence reaction that promotes melanization during the encapsulation response against parasitoids (Strand & Pech, 1995). To compare PO activity in the haemolymph of nonsilenced and *Pxgss*-silenced *P. xylostella* fourth-instar larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* mutant plants, "spontaneous PO" activity assays were performed as described by Barthel et al. (2016), without a protease pretreatment to activate pro-PO in the samples. Haemolymph of eight larvae (1 μl from each larva) was pooled into one sample and 10 samples per treatment were assessed for their PO activities. Haemolymph was extracted by puncturing the larvae with a sterile hypodermic needle and immediately pipetting the "bleeding" haemolymph into 1.5 ml amber safe-lock Eppendorf tubes with 200 μl ice-cold sodium cacodylate solution (0.01 M Na-cacodylate, 0.005 M $CaCl_2$ in Milli-Q water). The haemolymph mixture was directly frozen in liquid nitrogen and stored at −80°C until measurement. To measure PO activity, frozen samples were thawed on ice then centrifuged at 4°C and 2,800 ***g*** for 15 min to remove cell debris. An aliquot (100 μl) of the resulting supernatant (10 replicates per treatment, one measurement per replicate) was transferred to a 96 well polystyrene plate (VWR International, Darmstadt, Germany) and subsequently mixed with 200 μl

of 3 mM L-DOPA (Sigma-Aldrich, Munich, Germany, freshly prepared and covered with silver foil) per single well, and 100 µl sodium cacodylate solution treated in the same way was measured as a negative control (eight replicates). Absorbance at 490 nm was measured once per minute for 45 min at 30°C in a Multiskan Spectrum multiplate reader (Thermo-Electron, Waltham, MA, USA). The changes in absorbance from 15–26 min of the 45 min measurements were confirmed to be linear and were used to calculate the PO activity (1U = 0.001 AU/min, V_{max} expressed as mU). Data were obtained with SkanIt Software for Multiskan Spectrum version 2.1 (Thermo-Electron).

2.10 | Metabolic analyses

To analyse how glucosinolates were metabolized by *D. semiclausum*, third-instar *D. semiclausum* larvae at 7–8 days post parasitism (three larvae pooled per sample) were collected from *P. xylostella* using a dissecting microscope and washed in TE buffer (Tris-EDTA buffer, pH 8) to remove residual *P. xylostella* haemolymph. The remaining carcass of *P. xylostella* was also collected (approximately 3 mg per sample). Meconium pellets (approximately 1.5 mg pooled per sample) from *D. semiclausum* cocoons were collected immediately after adult emergence. Eclosed adults of *D. semiclausum* (approximately 1.5 mg per sample) were sexed, weighed and frozen in liquid nitrogen for subsequent analyses. To compare how glucosinolates were metabolized by nonparasitized and parasitized *P. xylostella*, samples of approximately 5 mg of frass and 5 µl of haemolymph of *P. xylostella* fourth-instar larvae were collected as described by Sun et al. (2019). In addition, pupae of nonparasitized *P. xylostella* and synchronous prepupae of parasitized *P. xylostella* (approximately 4 mg each) were collected. Before tissues were collected from parasitized *P. xylostella*, the presence of a second-instar parasitoid was confirmed under the microscope. Prepupae parasitized by *D. semiclausum* or nonparasitized pupae were collected on the second day after *P. xylostella* stopped eating and moving. All tissues (five replicates per treatment) were collected (Figure S1), weighed, immediately frozen in liquid nitrogen, and stored at −80°C until analysis.

Diadegma semiclausum and *P. xylostella* tissues collected for quantification of 4MSOB metabolites were homogenized in 150 µl and 200 µl respectively of extraction solvent (60% aqueous methanol, pH 3.0), in 1.5 ml Eppendorf tubes with ceramic beads for 3 min in a Skandex S-7 homogenizer. Homogenized samples were centrifuged at 13,000 ***g*** for 20 min at room temperature to separate undissolved particles. Clear supernatants were transferred to 2 ml amber glass vials with 0.3 ml glass inserts and analysed by LC-MS/MS to determine the concentrations of the major glucosinolate in *A. thaliana* Col-0, 4MSOB, and its derivatives desulpho-4MSOB, 4MSOB-ITC, and ITC conjugates. Analyses were performed on an Agilent Technologies 1,200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 3,200 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, Darmstadt, Germany). 4MSOB and desulpho-4MSOB were analysed by loading samples onto a Nucleodur Sphinx RP column (250 × 4.6 mm × 5 µm, Macherey-Nagel, Düren, Germany) with mobile phase A (0.2% formic acid in milliQ water) and mobile phase B (acetonitrile). 4MSOB–ITC and its conjugates were analysed by loading samples onto a Agilent XDB-C18 column (50 × 4.6 mm × 1.8 µm, Agilent Technologies, Waldbronn, Germany) with mobile phase A (0.05% formic acid in milliQ water) and mobile phase B (acetonitrile). The elution gradient profiles were previously described in Sun et al. (2019); MS parameters for 4MSOB, desulpho-4MSOB, 4MSOB–ITC and its conjugates were also described before (Gloss et al., 2014; Malka et al., 2016); Analyst 1.5 software (Applied Biosystems Sciex, Germany) was used for data acquisition and processing. Quantification of individual compounds was achieved by external calibration curves (the external standards are given in Table S1).

2.11 | RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR)

We quantified *ecdysone receptor* (*EcR*) gene transcripts in nonparasitized and parasitized *P. xylostella* larvae (five replicates per treatment), and *D. semiclausum* symbiotic polydnavirus (PDV)-related gene transcripts, namely *vankyrin1*, *vankyrin2* and *viral innexin1*, in parasitized *P. xylostella* larvae (10 replicates per treatment). *EcR* is induced by the insect hormone ecdysone to control larval development and pupation (Israni & Rajam, 2017), while *vankyrin1*, *vankyrin2* and *viral innexin1* are three of the best-studied PDV-related gene transcripts (Etebari et al., 2011). For these measurements, fourth-instar larvae were individually pooled into TRIzol reagent (Invitrogen, Waltham, MA, USA) in 1.5 ml Eppendorf tubes, and then kept at 4°C before RNA isolation. Total RNA was isolated from stored larvae according to the manufacturer's protocol and then subjected to DNaseI (Thermo Fisher Scientific, Waltham, MA, USA) treatment to eliminate genomic DNA contamination. cDNA was synthesized from this RNA by SuperScript III Reverse transcriptase kits (Invitrogen). qPCR was performed to measure gene transcripts in the cDNA samples using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). The *P. xylostella* ubiquitin gene was used as an internal control to normalize the abundance of gene transcripts. All gene accession numbers and primer pairs (designed via Primer3 software version 4.0) are listed in Table S2.

2.12 | Statistical analyses

Data were analysed using the R statistics package version 3.6.1, including the "agricolae" (de Mendiburu, 2019), "car" (Fox & Weisberg, 2019), "dunn.test" (Dinno, 2017), "FSA" (Ogle, Wheeler, & Dinno, 2019), and "survival" (Therneau, 2015; Therneau & Grambsch, 2000) packages. Figures were created using Origin 2019 and Adobe Illustrator CS5. All data were checked for statistical prerequisites such as homogeneity of variances and normality. The statistical significance ($p \leq .05$) of differences in *P. xylostella* larval weights, *D. semiclausum* adult weights, soluble protein and lipid content, PO activity in the haemolymph of *P. xylostella*, metabolic analyses in comparisons between nonparasitized and parasitized *P. xylostella*, and *P. xylostella ecdysone receptor* gene transcripts in nonparasitized and parasitized *P. xylostella* larvae were all analysed with two-way or multifactor ANOVAs followed by Tukey HSD tests for post

hoc comparisons. The significance ($p \leq .05$) of differences in metabolic analyses among *D. semiclausum* tissues was analysed by nonparametric Mann-Whitney-Wilcoxon tests. The significance ($p \leq .05$) of the difference in the percentage of *D. semiclausum* adult emergence whose larvae parasitized on *P. xylostella* larvae feeding on *A. thaliana* plants containing or lacking aliphatic glucosinolates was evaluated using a two-sided proportions test. *D. semiclausum* adult protein and lipid content in ITC complementation experiments were expressed as independent means (±SE) and significant differences determined by two-tailed *t* tests. Significant differences ($p \leq .05$) in the results of *P. xylostella* pupation and *D. semiclausum* emergence percentages post hatching (parasitism) were evaluated using Cox-regression survival analyses, and differences in *D. semiclausum* emergence percentage in ITC complementation experiments were determined by Kaplan-Meier survival analysis. *D. semiclausum* symbiotic PDVs-related gene transcripts in *P. xylostella* larvae were analysed by Kruskal-Wallis tests with Dunn's post hoc tests. Letters in the graphs represent $p \leq .05$, and asterisks represent: n.s, $p \geq .05$; *, $p \leq .05$; **, $p \leq .01$; ***, $p \leq .001$.

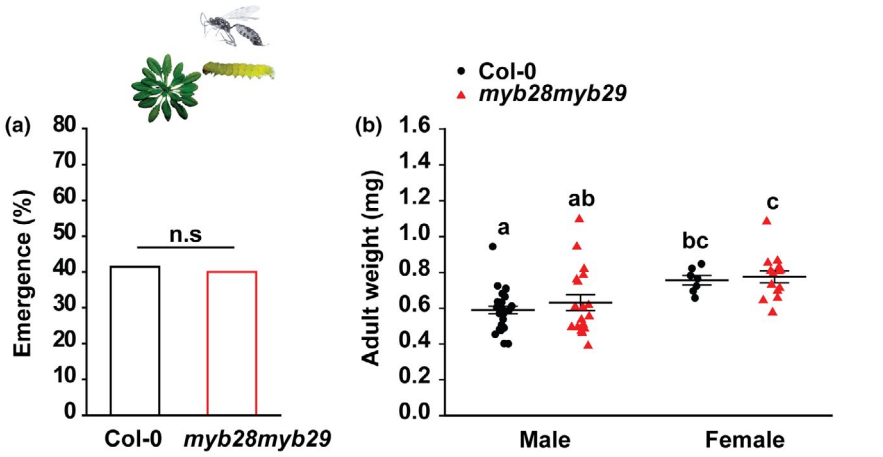
3 | RESULTS

3.1 | *Arabidopsis* glucosinolate content does not influence the development of the parasitoid *Diadegma semiclausum* in *Plutella xylostella* hosts with nonsilenced detoxification

To examine how the development of *D. semiclausum* is affected by the glucosinolate content in the diet of its *P. xylostella* host, *D. semiclausum* was allowed to parasitize *P. xylostella* second-instar larvae (Figure S1) that were fed ad libitum on *Arabidopsis thaliana* plants either containing (wild-type Col-0) or lacking (*myb28myb29* mutant) aliphatic glucosinolates. Aliphatic glucosinolates comprise 70%–80% of the total foliar glucosinolates in *A. thaliana* Col-0 plants (Brown et al., 2003), and are the only glucosinolate class in this plant that forms stable ITCs after hydrolysis by plant myrosinases. Adult emergence of *D. semiclausum* was similar irrespective of the glucosinolate content in the diet of its host, with approximately 40% emergence success in both groups (Figure 1a). The body masses of *D. semiclausum* male and female adults were also not affected by the glucosinolate content in the diet of their host (Figure 1b).

3.2 | Blocking glucosinolate detoxification affects the development and physiology of *Plutella xylostella* caterpillars

In order to explore how *D. semiclausum* is affected by plant toxins in its herbivorous host *P. xylostella*, we used RNAi targeting the *Pxgss* genes that encode glucosinolate sulphatases (GSSs) in the herbivore, to block glucosinolate detoxification. GSSs desulphate plant glucosinolates in the larval midgut, forming nontoxic desulpho-glucosinolates that are not capable of being activated by plant myrosinases to form toxic glucosinolate hydrolysis products (Ratzka et al., 2002). Suppression of *Pxgss* expression had previously been shown to cause reduced GSS activity and increased concentrations of the toxic ITCs resulting from hydrolysis of glucosinolates in *P. xylostella* larvae, causing negative



***D. semiclausum* developing in *P. xylostella* hosts feeding on *A. thaliana* plants with (Col-0) or without (*myb28myb29*) aliphatic glucosinolates**

FIGURE 1 Plant glucosinolate content has little impact on the development of *Diadegma semiclausum* in *Plutella xylostella* hosts. *D. semiclausum* females were allowed to parasitize *P. xylostella* larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* plants (with or without aliphatic glucosinolates, respectively), and the following variables were measured. (a) *Diadegma semiclausum* adult emergence percentage ($\chi^2 = 0.001$, $p = .976$, $n = 82$ and 80, respectively); and (b) *D. semiclausum* adult dry bodyweight (sex, $F_{1,62} = 20.356$, $p \leq .0001$; plant, $F_{1,62} = 1.020$, $p = .317$; sex × plant, $F_{1,62} = 0.084$, $p = .772$; $n = 27$, 18, 7 and 14 represent the respective numbers of replicates in each of the treatments presented in the graph, from left to right) were not affected by aliphatic glucosinolate content. Bars denote means and the interval is the SE. Significant differences ($p \leq .05$) were determined by a two proportions *z*-test in (a). and Tukey HSD tests in conjunction with two-way ANOVA in (b)

fitness effects (Sun et al., 2019). Here, we examined the effect of *Pxgss* silencing on *P. xylostella* growth, development and chemical composition in more detail. The pupation of *Pxgss*-silenced *P. xylostella* larvae was delayed in comparison to nonsilenced controls, but only on food plants containing aliphatic glucosinolates (Figure 2b). Silencing of *Pxgss* did not affect the growth of caterpillars in terms of late fourth-instar larval biomass, irrespective of whether the food plant contained glucosinolates or not (Figure 2c). Glucosinolates in leaf tissues did affect larval metabolism since feeding on glucosinolate-containing plants led to an approximately 40% reduction in soluble protein levels (Figure 2d), while lipid levels were not affected (Figure 2e). In order to assess how *Pxgss* silencing and exposure to ITCs affect general larval immune responses, we measured the activity of phenoloxidase (PO) in the larval haemolymph. PO activity is part of a critical host immune defence reaction that promotes melanization during the encapsulation response against parasitoids (Strand & Pech, 1995). However, PO activity was not affected by *Pxgss* silencing or dietary glucosinolate ingestion (Figure 2f), suggesting that exposure to ITCs does not impair this aspect of *P. xylostella* immunity.

3.3 | Blocking glucosinolate detoxification in the host caterpillar negatively affects development of the endoparasitoid *D. semiclausum*

We determined whether development of the endoparasitoid *D. semiclausum* was affected by the metabolism of glucosinolates in its herbivorous host *P. xylostella*. The emergence of *D. semiclausum* parasitizing *gss*-silenced *P. xylostella* larvae that had been fed on glucosinolate-containing Col-0 plants was delayed and less successful than the emergence of *D. semiclausum* parasitizing nonsilenced larvae or parasitizing larvae feeding on plants without aliphatic glucosinolates (Figure 2g). However, *D. semiclausum* adults from *Pxgss*-silenced hosts feeding on glucosinolate-containing plants were approximately 10% heavier than those from the other treatment groups (Figure 2h). Development of the parasitoid in *P. xylostella* larvae (both silenced and control) that had been fed on glucosinolate-containing Col-0 plants led to 40%–70% lower concentrations of soluble proteins in adults compared to conspecifics developing in hosts feeding on plants without aliphatic glucosinolates (Figure 2i). On the other hand, *D. semiclausum* parasitizing *Pxgss*-silenced *P. xylostella* larvae feeding on Col-0 plants contained approximately 25% more lipids than those in the other treatment groups (Figure 2j).

To confirm that the developmental effects described for *D. semiclausum* were indeed the result of glucosinolates and their derived ITCs in *Pxgss*-silenced *P. xylostella* larvae, a 4-methylsulphinylbutyl-ITC (4MSOB-ITC) complementation experiment was performed using *myb28myb29* leaves (lacking aliphatic glucosinolates) that were infiltrated with a natural ITC concentration mimicking ITCs found in damaged *A. thaliana* Col-0 leaves. 4MSOB, the glucosinolate precursor of 4MSOB-ITC, is the major glucosinolate in *A. thaliana* Col-0 aerial parts, comprising about 70% of the total aliphatic glucosinolate pool (Brown et al., 2003). *D. semiclausum* developing from *P. xylostella* larvae that had fed on 4MSOB-ITC-infiltrated plants had delayed development (Figure S2a), lower adult emergence success (Figure S2a), higher adult weights (Figure S2b) and higher adult lipid content (Figure S2d) compared to *D. semiclausum* parasitizing larvae that had fed on *myb28myb29* leaves infiltrated only with solvent (negative control).

3.4 | Glucosinolate metabolites are transferred from *P. xylostella* hosts to *D. semiclausum* larvae

Younger larvae of *D. semiclausum* primarily feed on the haemolymph of their hosts, but later instars consume almost all tissues just before they complete larval development and pupate (Figure S1). Therefore, these larvae will inevitably encounter glucosinolates or their metabolites while developing in a *P. xylostella* host that feeds on glucosinolate-containing plant tissues. *D. semiclausum* larvae developing in *gss*-silenced *P. xylostella* feeding on glucosinolate-containing plants encountered higher amounts of 4MSOB-ITC and reduced amounts of desulpho-4-methylsulphinylbutyl glucosinolate (desulpho-4MSOB) in host tissues than those developing on nonsilenced *P. xylostella* larvae (Figure 3a,b). In addition, higher amounts of the known 4MSOB-ITC mercapturic acid pathway conjugates (ITC-GSH, ITC-CG and ITC-Cys; Figure S3a,b) were detected in *D. semiclausum* larvae developing in *Pxgss*-silenced *P. xylostella* larvae feeding on Col-0 plants, compared to nonsilenced larvae (Figure 3c). Before pupation, *D. semiclausum* excreted nearly all of the 4MSOB-derived metabolites ingested from *P. xylostella* larvae into the meconium pellet, resulting in nearly no glucosinolate metabolites remaining in the bodies of adult parasitoids (Figure 3).

3.5 | Parasitism by *D. semiclausum* alters glucosinolate metabolism and excretion in the host *P. xylostella*

The increases in levels of 4MSOB-ITC and its conjugates in *Pxgss*-silenced *P. xylostella* carcasses after *D. semiclausum* parasitism were much lower in magnitude than the decreases in desulpho-4MSOB (Figure 3). To learn more about the efflux of 4MSOB metabolites, we directly compared the quantities of 4MSOB metabolites present in haemolymph, frass, and pupae of parasitized and nonparasitized *P. xylostella* larvae. Although the concentrations of 4MSOB-ITC were significantly higher in the haemolymph of *gss*-silenced than in nonsilenced *P. xylostella* larvae, parasitism reduced 4MSOB-ITC haemolymph levels in *Pxgss*-silenced larvae by 87% (Figure 4a). Conversely, parasitism significantly increased 4MSOB-ITC levels in the frass of *Pxgss*-silenced larvae compared to unparasitized silenced controls (Figure 4b). At the prepupal stage, parasitism decreased 4MSOB-ITC in *P. xylostella* to nearly undetectable levels, while the pupae of unparasitized larvae still had measurable 4MSOB-ITC content (Figure 4c). Accordingly, 4MSOB-ITC conjugates were more abundant in the frass and prepupae of parasitized

P. xylostella, compared to nonparasitized larvae, although these conjugates were found in lower amounts than 4MSOB-ITC itself (Figure S3).

Although *Pxgss* silencing successfully reduced the formation of the 4MSOB detoxification product desulpho-4MSOB in the host, the haemolymph of parasitized *P. xylostella* larvae contained approximately 75% less desulpho-4MSOB than that of nonparasitized larvae, for both *Pxgss*-silenced and nonsilenced larvae (Figure 4d). Similarly, the frass from parasitized *Pxgss*-silenced *P. xylostella* larvae contained about 85% less desulpho-4MSOB than frass from nonparasitized silenced larvae, whereas desulpho-4MSOB in nonsilenced larvae was not affected by parasitism (Figure 4e). However, the prepupae of both *Pxgss*-silenced and unsilenced larvae parasitized by *D. semiclausum* contained 17.3- and 47.9-fold higher concentrations of desulpho-4MSOB, respectively, than nonparasitized *P. xylostella* pupae (Figure 4f). Therefore, larvae of *D. semiclausum* appear to influence the excretion of 4MSOB-ITC by the host and to absorb desulpho-glucosinolates when parasitizing *P. xylostella*, and these compounds are retained in the prepupae (Figure 4g).

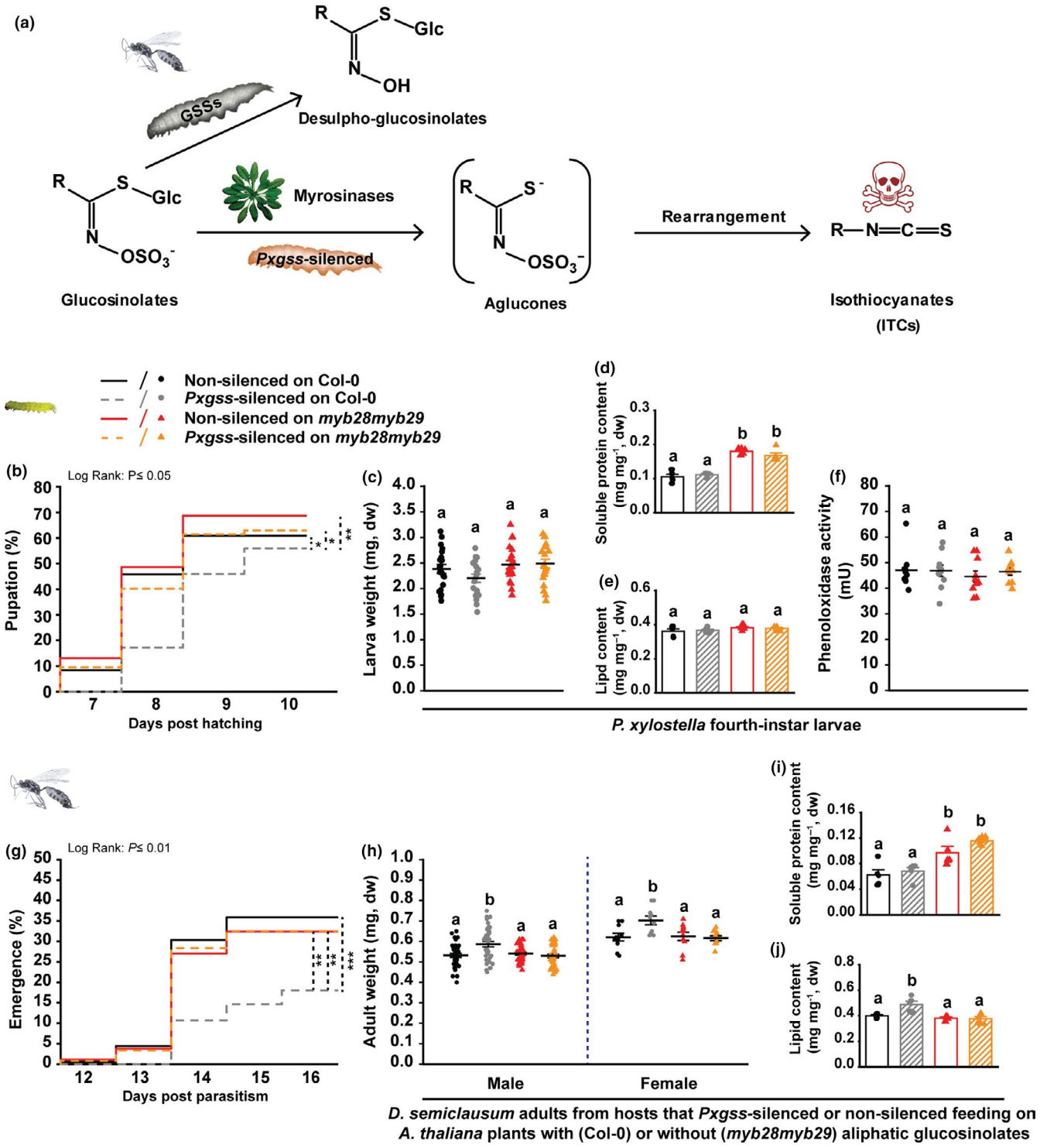


FIGURE 2 The endoparasitoid *Diadegma semiclausum* and its host *Plutella xylostella* are negatively affected by silencing of *Pxgss* in the presence of plant glucosinolates. (a) Plant glucosinolates are normally rapidly desulphated by *P. xylostella* glucosinolate sulphatases (GSSs) to form nontoxic desulpho-glucosinolates. Silencing of *Pxgss* increases glucosinolate hydrolysis by plant myrosinases, resulting in formation of toxic isothiocyanates (ITCs). (b–f) Nonsilenced and *Pxgss*-silenced *P. xylostella* larvae fed on *A. thaliana* Col-0 (with aliphatic glucosinolates) and *myb28myb29* (without aliphatic glucosinolates) plants and the following variables were measured: (b) *Plutella xylostella* egg-to-pupation percentage between 7 and 10 d post hatching; Log rank, $df = 3$, $p \leq .05$; *Pxgss*-silenced on Col-0 compared with nonsilenced on Col-0, $Z = 2.025$, $p \leq .05$, with nonsilenced on *myb28myb29*, $Z = 3.252$, $p \leq .01$, with *Pxgss*-silenced on *myb28myb29*, $Z = 2.089$, $p \leq .05$; egg numbers were 120, 111, 115 and 127, respectively; (c) larval dry weight (plant, $F_{1,76} = 4.834$, $p \leq .05$; *gss*-silencing, $F_{1,76} = 0.936$, $p = .336$; plant × *gss*-silencing, $F_{1,76} = 1.380$, $p = .244$; $n = 20$ in all treatments); (d) soluble protein content (plant, $F_{1,16} = 125.027$, $p \leq .0001$; *gss*-silencing, $F_{1,16} = 0.267$, $p = .612$; plant × *gss*-silencing, $F_{1,16} = 2.539$, $p = .131$; $n = 5$ in all treatments); (e) lipid content (plant, $F_{1,16} = 3.299$, $p = .088$; *gss*-silencing, $F_{1,16} = 0.008$, $p = .930$; plant × *gss*-silencing, $F_{1,16} = 0.222$, $p = .643$; $n = 5$ in all treatments) of *P. xylostella* fourth-instar larvae; and (f) immune phenoloxidase (PO) activity in fourth-instar *P. xylostella* larval haemolymph (plant, $F_{1,36} = 0.479$, $p = .493$; *gss*-silencing, $F_{1,36} = 0.185$, $p = .670$; plant × *gss*-silencing, $F_{1,36} = 0.280$, $p = .600$; $n = 10$ in all treatments). (g–j) *Diadegma semiclausum* larvae were allowed to parasitize nonsilenced and *Pxgss*-silenced *P. xylostella* larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* plants, and the following parameters were measured: (g) cumulative *D. semiclausum* adult emergence percentage between 12 and 16 d post parasitism; Log Rank, $df = 3$, $p \leq .01$; *Pxgss*-silenced on Col-0 compared with nonsilenced on Col-0, $Z = 3.685$, $p \leq .001$, with nonsilenced on *myb28myb29*, $Z = 3.086$, $p \leq .01$, with *Pxgss*-silenced on *myb28myb29*, $Z = 2.845$, $p \leq .01$; parasitized numbers were 181, 150, 185 and 120, respectively; (h) *D. semiclausum* adult dry bodyweight (male, plant, $F_{1,156} = 6.284$, $p \leq .05$; *gss*-silencing, $F_{1,156} = 5.067$, $p \leq .05$; plant × *gss*-silencing, $F_{1,156} = 11.229$, $p \leq .01$; $n = 40$; female, plant, $F_{1,36} = 5.133$, $p \leq .05$; *gss*-silencing, $F_{1,36} = 3.971$, $p = .054$; plant × *gss*-silencing, $F_{1,36} = 6.170$, $p \leq .05$; $n = 10$); and *D. semiclausum* male adult; (i) soluble protein content (plant, $F_{1,16} = 33.186$, $p \leq .0001$; *gss*-silencing, $F_{1,16} = 2.852$, $p = .111$; plant × *gss*-silencing, $F_{1,16} = 0.820$, $p = .378$; $n = 5$ in all bars); and (j) lipid content (plant, $F_{1,16} = 15.192$, $p \leq .01$; *gss*-silencing, $F_{1,16} = 6.785$, $p \leq .05$; plant × *gss*-silencing, $F_{1,16} = 7.886$, $p \leq .05$; $n = 5$ in all bars). Significant differences ($p \leq .05$) were determined by Cox regression survival analysis tests in (b) and (g), significant differences between means (±SE) were determined by Tukey HSD tests in conjunction with two-way ANOVA in (c–f) and (h–j).

3.6 | Blocking glucosinolate detoxification in the host caterpillar reduces expression of immune suppression genes by a symbiotic virus of the parasitoid

Symbiotic polydnaviruses (PDVs), a family of dsDNA viruses, are injected by female parasitoids in several subfamilies of the Ichneumonidae and Braconidae (e.g., Campopleginae, Microgastrinae) into the haemolymph of their hosts and cause immune suppression, resulting in lower rates of encapsulation of the developing parasitoids (Beckage, 2011; Webb et al., 2006) as well as changes in the phenology of the host (Harvey, 2005). PDVs associated with ichneumonid parasitoids like *D. semiclausum* are called ichnoviruses, and produce proteins important in infection, such as vankyrins and viral annexins (Tanaka et al., 2007), to reduce the rates of encapsulation of the developing parasitoids in host haemolymph. To determine if higher ITC levels might affect the expression of symbiotic PDV-related genes upon parasitism, the expression of three well-studied viral-related gene transcripts (Etebari et al., 2011), *vankyrin1*, *vankyrin2* and *viral innexin1*, was measured by qRT-PCR. Expression of these three genes was reduced by 80%, 70% and 62%, respectively, upon *D. semiclausum* parasitism in *Pxgss*-silenced hosts feeding on Col-0 plants (with aliphatic glucosinolates) compared to nonsilenced hosts feeding on Col-0 plants or either silenced or nonsilenced hosts feeding on *myb28myb29* plants (without aliphatic glucosinolates) (Figure 5).

3.7 | Parasitism by *D. semiclausum* reduces *P. xylostella ecdysone receptor* (*EcR*) expression

To further explore how *gss* silencing influences parasitism of *P. xylostella* by *D. semiclausum*, we measured the expression of a *P. xylostella* ecdysone-related gene (*EcR*), which is induced by ecdysone to control larval development and pupation (Israni & Rajam, 2017). *EcR* transcripts were reduced 30%–70% in *P. xylostella* fourth-instar larvae upon successful parasitism by *D. semiclausum* (Figure S4). However, *Pxgss* silencing and the glucosinolate content of the *P. xylostella* diet had no significant effect on expression.

4 | DISCUSSION

4.1 | Detoxification of plant defences by an herbivore improves the performance of an endoparasitoid

Plant defensive chemicals consumed by herbivores can affect higher trophic levels, impacting predators and parasitoids both directly and indirectly (Gols & Harvey, 2009; Harvey et al., 2003; Nishida, 2002; Ode, 2019; Petschenka & Agrawal, 2016). As a corollary of the "nasty host hypothesis" (Gauld et al., 1992), herbivores with efficient mechanisms to detoxify plant defences might be expected to serve as more suitable hosts or prey for their enemies. Our results agree with this assertion demonstrating that the glucosinolate detoxification pathway in the crucifer-feeding host *P. xylostella* benefits not only the herbivore, but also maximizes the performance of *D. semiclausum* parasitoids developing inside this herbivore. When the gene encoding glucosinolate-detoxifying sulphatase in *P. xylostella* was silenced, this herbivore suffered a 50% decline in growth and a 4-fold increase in pupal mortality (Sun et al., 2019). Moreover, the emergence of adult *D. semiclausum* from silenced hosts decreased by about 50% (Figure 2g). These effects could be directly attributed to the accumulation in the parasitoid of ITCs, the toxic hydrolysis products of glucosinolates (Figure 3a). When *D. semiclausum* was

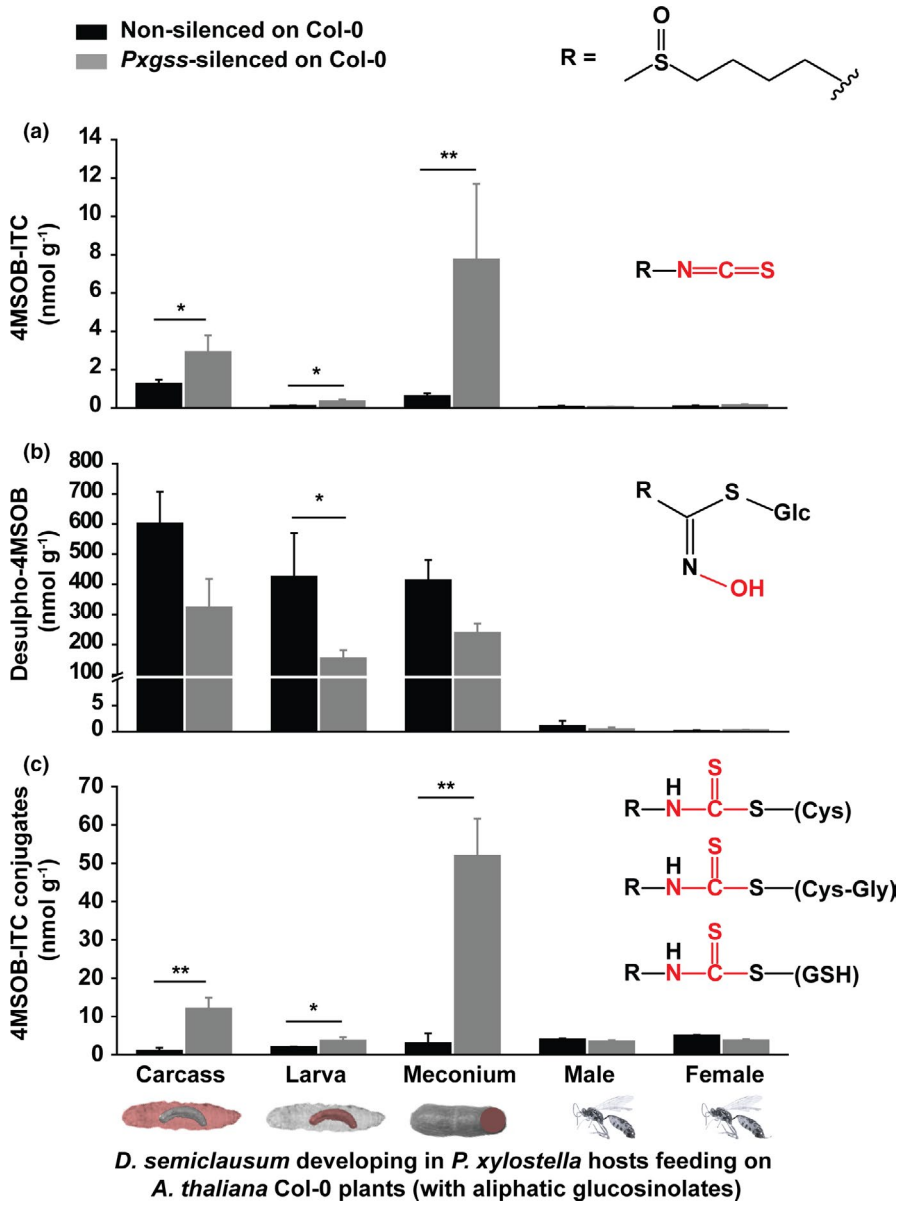


FIGURE 3 Metabolites of 4MSOB are present in *Diadegma semiclausum* parasitizing *P. xylostella* larvae fed on *A. thaliana* Col-0 plants. *D. semiclausum* was allowed to parasitize nonsilenced and *Pxgss*-silenced *P. xylostella* larvae feeding on either *A. thaliana* Col-0 (with aliphatic glucosinolates) or *myb28myb29* (without aliphatic glucosinolates) plants. (a) 4MSOB-ITC (host carcass, $p \leq .05$; parasitoid larva, $p \leq .05$; meconium, $p \leq .01$; $n = 5$); (b) Desulpho-4MSOB (host carcass, $p = .151$; parasitoid larva, $p \leq .05$; meconium, $p = .056$; $n = 5$ in all bars): and (c) 4MSOB-ITC conjugates (host carcass, $p \leq .01$; parasitoid larva, $p \leq .05$; meconium, $p \leq .01$; $n = 5$) were quantified in the carcass of *P. xylostella* prepupae, third-instar larvae of *D. semiclausum*, meconium left in the cocoon and adults of *D. semiclausum*, in which *D. semiclausum* parasitized either nonsilenced (black bars) or *Pxgss*-silenced (grey bars) *P. xylostella*. The general mercapturic acid pathway is shown in Figure S3a. 4MSOB-ITC-GSH: 4MSOB-ITC-glutathione conjugate; 4MSOB-ITC-CG: 4MSOB-ITC-cysteinylglycine conjugate; and 4MSOB-ITC-Cys: 4MSOB-ITC-cysteine conjugate; concentrations are shown in Figure S3b as stacked bars. 4MSOB and its metabolites were nearly undetectable in *P. xylostella* larvae fed on *myb28myb29* plants and are not shown in the graphs. Coloured objects depict the parts being analysed. Significant differences ($p \leq .05$) between means (±SE) were determined by Mann-Whitney Wilcoxon tests in a–c. separately conducted for each tissue

reared on *P. xylostella* larvae developing on ITC-infiltrated foliage, the same declines in parasitoid emergence were noted (Figure S2a). In untransformed *P. xylostella* where the glucosinolate detoxification system was functional, *D. semiclausum* tolerated a range of glucosinolate content in the herbivore diet (Figure 1). This suggests that previous reports on changes in *D. semiclausum* developmental variables, such as cocoon and adult weight and duration of development (Dosdall et al., 2011; Kahuthia-Gathu, Löhr, & Poehling, 2008), are probably not caused by alterations in the glucosinolate content of the food plant of the host herbivore. Although we employed genetically

modified plant lines in this study to block a specialized detoxification reaction of an herbivore, which increased the accumulation of toxic isothiocyanate products in its body, this is also reflective of natural situations. Brassicaceae plants are also subject to herbivory by numerous species of generalist herbivores without specialized detoxification pathways. Several lepidopteran species are known to produce and accumulate large quantities of isothiocyanates when feeding on glucosinolate-containing plants (Jeschke et al., 2017), and so infesting parasitoids would encounter a similar situation to that in sulphatase-silenced *P. xylostella*.

How plant defences affect endoparasitoid performance is poorly understood despite the fact that endoparasitoids constitute a very abundant group of enemies of insect herbivores (Gols & Harvey, 2009; Harvey, van Dam, Raaijmakers, Bullock, & Gols, 2011; Ode, 2006). Since the physiology of endoparasitoids is very tightly coupled with that of their hosts, these insects could be very susceptible to plant toxin content in the host diet and the extent of detoxification by the host. Accordingly, a few studies have shown that increased toxin levels in the host diet can actually lead to reduced parasitoid performance (Barbosa et al., 1991; Garvey, Creighton, & Kaplan, 2020). The tolerance of natural enemies of insect herbivores to allelochemicals in host or prey tissues can also depend on the degree of specialization of the natural enemy on their host or prey. For example, while low concentrations of the furanocoumarin xanthotoxin did not affect the specialist parasitoid *Copidosoma sosares* in its interaction with its specialist herbivore host *Depressaria pastinacella*, exposure of the parasitoid *Copidosoma floridanum* (which has a much broader host range) to this compound in the haemolymph of one of its polyphagous hosts reduced survival and offspring production (Lampert et al., 2011). Plant chemical defences can also affect predators. For instance, the wolf spider *Camptocosa parallela* shows lower preference for tobacco hornworm (*Manduca sexta*) prey containing higher nicotine levels (Kumar, Pandit, Steppuhn, & Baldwin, 2014), and this predator prefers prey that detoxify nicotine (Kumar, Rathi, Schöttner, Baldwin, & Pandit, 2014). Likewise, predators such as the ladybug *Adalia bipunctata* are deterred by the glucosinolate-sequestering cabbage aphid *Brevicoryne brassicae* (Kazana et al., 2007). The larval development of the lacewing *C. carnea* is reduced when preying on *Pxgss*-silenced *P. xylostella* (Sun et al., 2019). The greater physiological intimacy between parasitoids and their hosts compared to predators and their prey may have resulted in parasitoids of insect herbivores being better adapted to the presence of plant defence metabolites in their hosts than predators. Furthermore, parasitoids developing in hosts that contain plants toxins can themselves coopt these plant defences against their own antagonists such as hyperparasitoids (Bowers, 2003; van Nouhuys, Reudler, Biere, & Harvey, 2012) offering them the opportunity to exploit enemy free space, a concept that is usually restricted to insect herbivores (Murphy, Lill, Bowers, & Singer, 2014). Thus, the presence of toxins in their host may not only help protect parasitoids indirectly (avoiding predation of their host), but also directly against their own enemies in the fourth-trophic level (Murphy et al., 2014).

4.2 | Herbivore detoxification can modulate the immune response to endoparasitoids

Numerous factors can influence the outcome of insect herbivore-endoparasitoid interactions. We hypothesized that plant defences could influence the immune response of the herbivore host against the parasitoid. Adult female parasitoids in several subfamilies of the Ichneumonidae and Braconidae (e.g., Campopleginae, Microgastrinae) inject symbiotic polydnaviruses (PDVs) into the host haemolymph, which produce proteins that alter host growth (Harvey, 2005) and disrupt cellular and humoral immune responses leading to overall immune suppression (Hasegawa, Erickson, Hersh, & Turnbull, 2017). PDV vankyrins are homologues of the *Drosophila melanogaster* NF-κβ transcription factor inhibitor Iκβ (Kroemer & Webb, 2005, 2006). These proteins are thought to protect parasitoids from the cellular immune system of the host by suppressing NF-κβ signaling cascades (Tian, Zhang, & Wang, 2007), which blocks blood cell formation and the cellular encapsulation response against parasitoids (Gueguen, Kalamarz, Ramroop, Uribe, & Govind, 2013). PDV innexins are homologues of insect innexins, which form gap junctions between the cytoplasm of insect cells and so play crucial roles in cellular immune responses (Hasegawa & Turnbull, 2014; Turnbull, Volkoff, Webb, & Phelan, 2005). Viral innexins can perturb the physiological functions of native insect innexins (Hasegawa et al., 2017). Here, we observed that the expression of the PDV genes *vankyrin1*, *vankyrin2* and *viral innexin1* was suppressed when glucosinolate detoxification was blocked in *P. xylostella* hosts feeding on glucosinolate-containing plants (Figure 5). This may have contributed to the lower emergence rate of *D. semiclausum* adults from these hosts (Figure 2g). Interestingly, *D. semiclausum* adults that emerged successfully (but belatedly; Figure 2g) from *Pxgss*-silenced *P. xylostella* larvae feeding on Col-0 plants were slightly heavier than the *D. semiclausum* adults from the other treatments (Figure 2h). These wasps also had a higher lipid content (Figure 2j) than those developing from *P. xylostella* control hosts, but did not differ in protein content (Figure 2i). At the herbivore level, dietary ITCs are known to increase larval lipid content while decreasing protein content, due to the imbalance in amino acid metabolism caused by ITC detoxification, which depletes cysteine levels (Jeschke et al., 2016a).

Plant defence compounds could also act directly on the herbivore's immune response against parasitism (Kaplan et al., 2016). The enzyme phenoloxidase (PO) has an important function in melanization against foreign agents by producing quinone groups that are polymerized to promote encapsulation (González-Santoyo & Córdoba-Aguilar, 2011). PO activity in the haemolymph of *Heliothis subflexa* was shown to benefit from the withanolide defences of its *Physalis* host plant (Barthel et al., 2016). However, here we found that PO enzyme activity in the haemolymph of *P. xylostella* was not influenced by manipulating its glucosinolate metabolism and the resulting exposure to ITCs (Figure 2f). Karimzadeh and Wright (2008) compared the

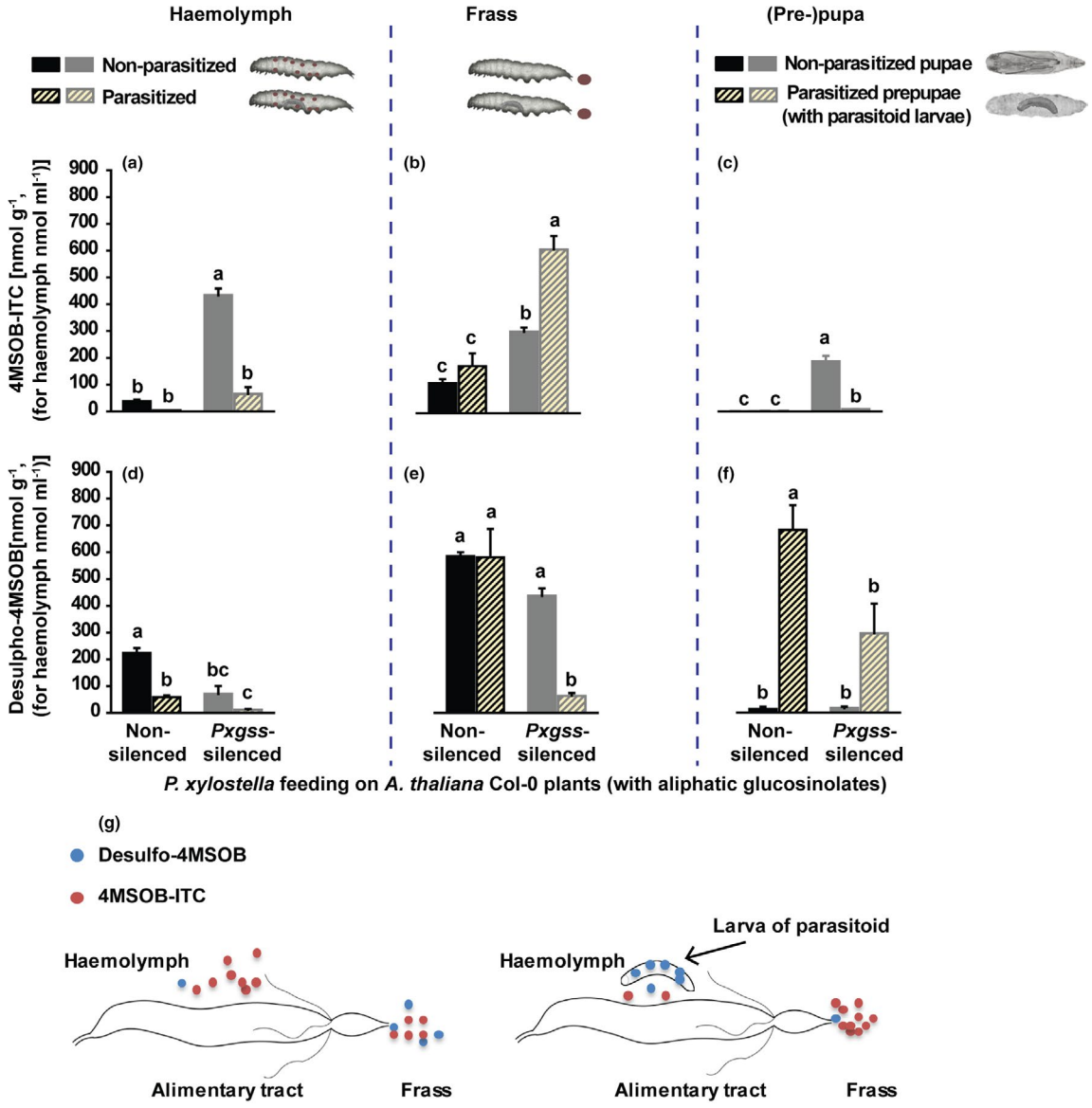


FIGURE 4 Parasitism by *D. semiclausum* affects the concentrations and distribution of 4MSOB metabolites in *gss*-silenced and nonsilenced *P. xylostella* larvae feeding on *A. thaliana* Col-0 plants. Comparison of 4msob-ITC in (a) haemolymph (*gss*-silencing, $F_{1,16} = 162.62$, $p \leq .0001$; parasitism, $F_{1,16} = 125.73$, $p \leq .0001$; *gss*-silencing × parasitism, $F_{1,16} = 86.04$, $p \leq .0001$; $n = 5$ in all bars); and (b) frass (*gss*-silencing, $F_{1,16} = 74.87$, $p \leq .0001$; parasitism, $F_{1,16} = 26.06$, $p \leq .001$; *gss*-silencing × parasitism, $F_{1,16} = 11.18$, $p \leq .01$; $n = 5$ in all bars) of nonparasitized and parasitized *P. xylostella* fourth-instar larvae. Comparison of 4MSOB-ITC concentrations in (c) pupae of nonparasitized *P. xylostella* and prepupae of parasitized *P. xylostella* with parasitoids inside (*gss*-silencing, $F_{1,16} = 125.623$, $p \leq .0001$; parasitism, $F_{1,16} = 22.565$, $p \leq .001$; *gss*-silencing × parasitism, $F_{1,16} = 6.882$, $p \leq .05$; $n = 5$ in all bars). Comparison of desulpho-4MSOB concentrations in (d) haemolymph (*gss*-silencing, $F_{1,16} = 115.72$, $p \leq .0001$; parasitism, $F_{1,16} = 92.58$, $p \leq .0001$; *gss*-silencing × parasitism, $F_{1,16} = 39.05$, $p \leq .0001$; $n = 5$ in all bars); and (e) frass (*gss*-silencing, $F_{1,16} = 35.62$, $p \leq .0001$; parasitism, $F_{1,16} = 11.80$, $p \leq .01$; *gss*-silencing × parasitism, $F_{1,16} = 10.96$, $p \leq .01$; $n = 5$ in all bars) of nonparasitized and parasitized *P. xylostella* fourth-instar larvae. Comparison of desulpho-4MSOB concentrations in (f) pupae of nonparasitized *P. xylostella* and prepupae of parasitized *P. xylostella* with parasitoids inside (*gss*-silencing, $F_{1,16} = 7.034$, $p \leq .05$; parasitism, $F_{1,16} = 42.909$, $p \leq .0001$; *gss*-silencing × parasitism, $F_{1,16} = 7.245$, $p \leq .05$; $n = 5$ in all bars). (g) A schematic representation of the alimentary tract of nonparasitized (left) and parasitized (right) *Pxgss*-silenced *P. xylostella* larvae feeding on *A. thaliana* Col-0 (with aliphatic glucosinolates) plants, with coloured dots representing relative quantities of desulpho-4MSOB and 4MSOB-ITC and where they might accumulate. Significant differences ($p \leq .05$) between means (±SE) were determined by Tukey's HSD tests in conjunction with two-way ANOVA in (a–f)

effect of variation in plant quality on the immune response in parasitized and unparasitized *P. xylostella* hosts and found that these responses were only transient or were negated by the effect of parasitism by *Cotesia plutellae* itself. In contrast, Bukovinszky et al. (2009) reported that the induction of plant defences can impair immune functionality of the host suggesting a detoxification-immunity tradeoff. Thus, the effect of plant toxins on the host immune response depends on the players involved in the tritrophic interaction.

4.3 | Endoparasitoid performance is enhanced by modification of host gene expression and alteration of ingestion and distribution of plant defences

Parasitism can influence the physiology and behaviour of herbivores in a multitude of ways. We demonstrated here that *D. semiclausum* parasitism alters *P. xylostella* ecdysone receptor (*EcR*) expression in a way that inhibits host pupation. However, this inhibition happened independently of the glucosinolate content of the host diet (Figure S4). Such manipulation of host development is crucial for completion of the endoparasitoid life cycle. Another example involving *EcR* concerns a symbiotic bracovirus of the endoparasitoid *Cotesia vestalis*, which produces a miRNA that arrests host growth by altering the expression of the host *EcR* gene (Wang et al., 2018).

Parasitism can sometimes induce herbivores to ingest plant toxins to improve survival of the host (Bruce, 2014) or that of the parasitoid (Pashalidou et al., 2015). For instance, parasitized *Grammia incorrupta* (woolly bear caterpillars) engage in self-medication, increasing their ingestion of plant pyrrolizidine alkaloid toxins in response to endoparasitic tachinid flies resulting in improved herbivore survival (Singer, Mace, & Bernays, 2009). Parasitization can also affect the feeding habits of *Pieris rapae* larvae (Van Der Meijden & Klinkhamer, 2000), and *M. sexta* larvae parasitized by *Cotesia congregata* decrease their feeding in the last stages of parasitoid development by induced anorexia, which actually seems to benefit the parasitoid (Adamo, Linn, & Beckage, 1997). Moreover, parasitism can also affect the metabolism of plant toxins by the herbivorous host. For example, parasitism of webworm larvae (*D. pastinacella*) by *C. sosares* lowers furanocoumarin detoxification rates (per unit of larval weight) in the host, potentially increasing the haemolymph concentrations of these toxins (McGovern et al., 2006). In the present study, parasitism by *D. semiclausum* did not increase glucosinolate ingestion by its host, but did result in increased excretion of ITCs. When desulphation was blocked by *Pxgss* gene silencing, the hydrolysis of glucosinolates by plant myrosinases resulted in increased concentrations of toxic ITCs in haemolymph and frass (Figure 4a,b), but these were lower in parasitized hosts than in nonparasitized ones. Therefore, *D. semiclausum* appears to alter the distribution of toxic plant defence metabolites in *P. xylostella* to limit its exposure while it feeds on herbivore tissues. The mechanisms used by this parasitoid to manipulate ITC distribution remain to be determined.

4.4 | Herbivores that detoxify defences and parasitoids that benefit from detoxification: Consequences for plant protection

The benefits of *P. xylostella* glucosinolate detoxification for both the herbivore and its parasitoid *D. semiclausum* are of considerable relevance to the plant as well. Plants that are attacked by herbivores with the ability to detoxify their major defence compounds face a dilemma, since producing increased concentrations of defences will probably not be an effective countermeasure. One strategy is to switch resources to the production of greater amounts of other defence compounds in its arsenal (Koricheva, Nykanen, & Gianoli, 2004), a tactic employed by *A. thaliana* (Burow et al., 2009) and other Brassicaceae (Kuchernig, Burow, & Wittstock, 2012), especially when fed upon by *Pieris rapae*, a herbivore that also

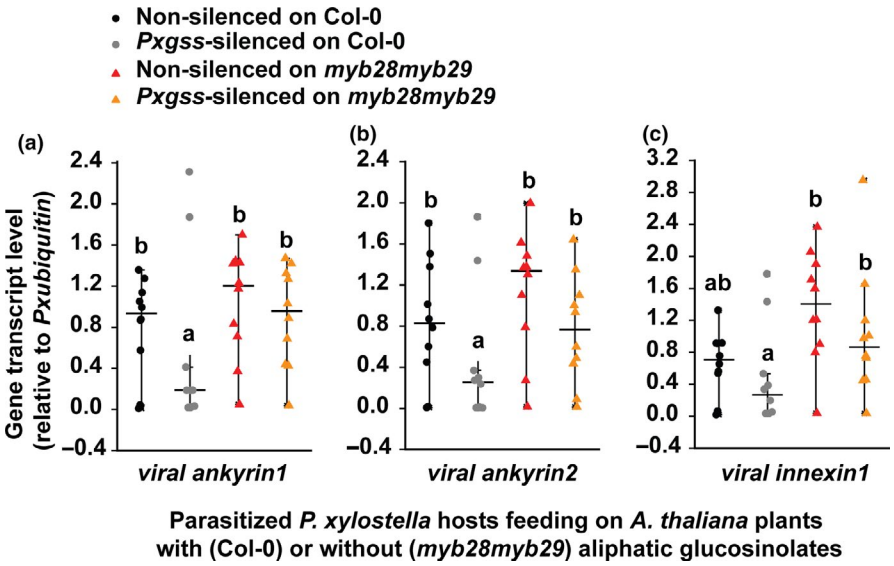


FIGURE 5 Expression of *D. semiclausum* symbiotic polydnavirus (PDV)-related genes is suppressed in *Pxgss*-silenced *P. xylostella* larvae. (a) *vankyrin1* ($X^2 = 13.704$, $df = 3$, $p \leq .01$; $n = 10, 8, 10$ and 10, respectively); (b) *vankyrin2* ($X^2 = 13.299$, $df = 3$, $p \leq .01$; $n = 10, 8, 10$ and 10, respectively); and (c) *viral innexin1* ($X^2 = 16.105$, $df = 3$, $p \leq .01$; $n = 10, 8, 10$ and 10, respectively) gene transcripts in *D. semiclausum*-parasitized *P. xylostella* fourth-instar larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* plants. Significant differences ($p \leq .05$) between medians were determined by Kruskal-Wallis tests with Dunn's post hoc tests in a–c

detoxifies glucosinolates (Wittstock et al., 2004). Herbivory by *P. rapae* alters the major route of glucosinolate activation in *A. thaliana* so that instead of isothiocyanates, nitriles are formed (Burow et al., 2009), with these compounds deterring *P. rapae* oviposition (Mumm et al., 2008). Such diversion of defensive resources to other products of the same pathway, or even the activation of separate biosynthetic pathways can therefore help plants protect themselves from attack by a well-adapted herbivore.

Another plant strategy to combat an herbivore with a strong capacity to detoxify chemical defences is to recruit natural enemies of herbivores (Gols et al., 2015). Plant volatiles released in response to herbivore damage have been demonstrated to attract herbivore enemies (Clavijo McCormick, Unsicker, & Gershenzon, 2012; Hare, 2011). While this can increase the fitness of plants attacked by herbivores (Gols et al., 2015; van Loon, de Boer, & Dicke, 2000), evidence showing that this happens under natural conditions is scarce (Clavijo McCormick et al., 2012). In the Brassicaceae, *A. thaliana* and other species emit volatile glucosinolate hydrolysis products and other volatile metabolites that attract herbivore predators and parasitoids (Bruce, 2014; Gols & Harvey, 2009; Hopkins, van Dam, & van Loon, 2009; Mumm et al., 2008). For example, the predator *C. carnea* and the parasitoid *C. plutellae* are both significantly attracted by allyl isothiocyanate present in the frass of *P. xylostella* larvae feeding on cabbage (Reddy, Holopainen, & Guerrero, 2002). 3-Butenyl-ITC is attractive to *Diaeretiella rapae*, a parasitoid that predominantly attacks Brassicaceae-feeding aphids (Blande, Pickett, & Poppy, 2007). As one of the world's most destructive pests of Brassicaceae plants (Talekar & Shelton, 1993), *P. xylostella* has developed significant resistance to most synthetic pesticides, as well as to modern biological pesticides like *Bacillus thuringiensis* (Bt) toxins (Li, Feng, Liu, You, & Furlong, 2016). Thus increased use of natural enemies is being explored to reduce *P. xylostella* damage (Furlong et al., 2013; Sarfraz, Keddie, & Dosdall, 2005). Our current results suggest that the manipulation of herbivore metabolism could be useful in such an effort. On the one hand, plants that inhibit herbivore detoxification should suffer less damage due to the decreased performance of herbivores. On the other hand, in the case of the *P. xylostella* – crucifer interaction, the higher ITC levels in the frass of silenced *P. xylostella* caterpillars may attract more *P. xylostella* (Pivnick, Jarvis, & Slater, 1994; Renwick, Haribal, Gouinguené, & Städler, 2006) as well as predators and parasitoids, potentially making these plants useful as "dead-end trap crops". However, the effects of such modifications on the populations of herbivore enemies are nuanced and species-specific, and the ecological ramifications of such an approach in both natural and agricultural settings need further research. In this study, we demonstrated that *P. xylostella* detoxification of glucosinolates enhances the performance of *D. semiclausum*. Enhanced parasitoid performance may positively impact parasitoid population dynamics and enhance their recruitment by plants, which may ultimately have a positive effect on plant fitness.

In conclusion, the desulphation of plant glucosinolates by the specialist herbivore *P. xylostella* prevents the formation of toxic ITCs and thus increases insect growth, survival and reproductive success. Here we show that this detoxification reaction also benefits a representative of the next trophic level, *D. semiclausum*, a widespread endoparasitoid of *P. xylostella* caterpillars, by increasing adult emergence and decreasing development time. Future research on other endoparasitoids is needed to determine if the overall susceptibility of *D. semiclausum* to plant defences in its herbivore host is a general trait of this group. Glucosinolate desulphation also appears to facilitate the action of symbiotic polydnaviruses of *D. semiclausum* that suppress host immunity, but more work is needed to understand the mechanisms responsible for improved parasitoid performance.

ACKNOWLEDGEMENTS

We thank Marie Pauline Sell for helping with the measurements of immune phenoloxidase activities and helpful discussions, Bettina Raguschke for technical assistance, Dr Sascha Eilmus from the Bayer Corporation for supplying *Plutella xylostella*, student assistants for helping with insect rearing and parasitism work, and the MPICE greenhouse team for plant rearing. This study was funded by the China Scholarship Council (201508330285), the Deutsche Forschungsgemeinschaft (DFG Collaborative Research Center 1127 ChemBioSys), the Max Planck Society and Open access funding enabled and organized by Projekt DEAL.

AUTHOR CONTRIBUTIONS

R.S., D.G.V., J.G., S.S.P., R.G., and J.A.H. planned and designed the research; R.S. performed experiments and collected data; R.S., and M.R. analysed the data; and R.S., D.G.V., R.G., J.A.H., and J.G. drafted the manuscript with input from all coauthors.

DATA AVAILABILITY STATEMENT

Supporting Information figures and tables are available in the Supporting Information document. Raw data and statistical analyses are available as a separate Supporting Information file.

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SUPPORTING INFORMATION

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

How to cite this article: Sun R, Gols R, Harvey JA, et al. Detoxification of plant defensive glucosinolates by an herbivorous caterpillar is beneficial to its endoparasitic wasp. *Mol Ecol*. 2020;29:4014–4031. https://doi.org/10.1111/mec.15613