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ORIGINAL ARTICLE

Balancing nutrients in a toxic environment: the challenge of eating

Peng Wang[1], Daniel G. Vassão[2], Bettina Raguschke[2], Michael J. Furlong[1] and Myron P. Zalucki[1]

[1]*School of Biological Sciences, The University of Queensland, St Lucia, Queensland, Australia and* [2]*Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany*

Abstract Insect herbivores can regulate their food intake by mixing food sources with different nutrient content, but face the resulting challenge of ingesting various plant secondary metabolites. How insects deal with toxins in a complex nutrient environment is unclear. Here we investigated the influence of a classic plant secondary metabolite, allyl glucosinolate (sinigrin), and its hydrolyzed product allyl isothiocyanate (AITC), on the development of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) when fed on diets with different protein-to-carbohydrate (p : c) ratios. We also examined the effects of these toxins on larval biochemistry, by chemically analyzing the frass produced by insects feeding on the different diets. As expected, AITC had a greater negative effect than sinigrin on *H. armigera* life-history traits. However, AITC at low concentration appeared to have a positive effect on some traits. Both sinigrin and AITC-induced detoxification activity in the gut, and the reaction was related to diet protein concentration. High-protein diets can provide the required free amino acid, especially cysteine, needed for the detoxification process. The nutrient content of the diet influences how plant secondary metabolites are handled, and the use of artificial diets in experiments investigating the metabolic fate of plant secondary compounds needs to be carefully evaluated.

Key words diet protein; glucosinolate; gut metabolism; *Helicoverpa armigera*; isothiocyanate

Introduction

The presence of plant secondary metabolites poses a dilemma for insect herbivores: to eat more to gain nutrition and ingest a greater dose of toxin that may kill them, or to eat less to reduce toxin effects, but consequently to retard development due to the lack of nutrients. Many insects can select from a range of food sources to acquire different macronutrients to fulfill their requirements; they consume different amounts of macronutrients that are optimal for their growth, development and reproduction (Simpson *et al*., 2004; Behmer, 2009; Simpson *et al*., 2015). The extent to which plant secondary metabolites influence insect feeding behavior in different nutritional environments and how insects cope with the toxins is not well understood.

The glucosinolate–myrosinase system found in the Brassicales is a classic example of plant chemical defense (Mithöfer & Boland, 2012). Glucosinolates and their hydrolytic enzymes, myrosinases, are stored in separate compartments in intact plant tissue. Upon tissue disruption, myrosinases come into contact with glucosinolate substrates, and glucosinolate hydrolysis results in the formation of toxic isothiocyanates and other biologically active products (Winde & Wittstock, 2011). Isothiocyanates

Correspondence: Myron P. Zalucki, School of Biological Sciences, The University of Queensland, St Lucia, Queensland 4072, Australia. Email: m.zalucki@uq.edu.au

[Correction added on 24 Dec 2021, after first online publication: The copyright line was changed.]

(ITCs) have a characteristic pungent odor and are toxic to a broad range of organisms (Winde & Wittstock, 2011). More than 130 glucosinolates with different side-chains have been characterized (Blažević *et al*., 2020), and the aliphatic allyl glucosinolate sinigrin and the corresponding allyl-ITC are widespread and abundant in many cultivated crucifers including black mustard, *Brassica nigra* (L.), wasabi, *Eutrema japonicum* (Miq.) and common cabbage, *Brassica oleracea* (L.). ITCs reduce herbivore survival and growth and increase developmental time in a dose-dependent manner (Li *et al*., 2000; Agrawal & Kurashige, 2003). Intact glucosinolates alone are normally considered nonactive; however, they can influence insect development at high doses, suggesting that hydrolysis may occur in the gut (Li *et al*., 2000).

The generalist herbivore *Helicoverpa armigera* (Hübner) can feed on plants from many different families, including cruciferae (Zalucki *et al*., 1986; Zalucki *et al*., 1994; Cunningham & Zalucki, 2014), and it is a serious agricultural pest globally (Tay *et al*., 2013; Kriticos *et al*., 2015). However, unlike insects such as the diamondback moth, *Plutella xylostella* (L.) (Ratzka *et al*., 2002), which specializes on cruciferae, *H. armigera* does not have a specialized glucosinolate detoxification system, although they can survive (albeit poorly) on members of the Cruciferae such as common cabbage (Zalucki *et al*., unpublished data) and *Arabidopsis thaliana* (L.) Heynh (Zalucki *et al*., 2017). Adult female *H. armigera* tend to lay few eggs on common cabbage (Firempong & Zalucki, 1989; Zalucki *et al*., 2012), and larvae typically avoid tissues high in glucosinolates when foraging (Shroff *et al*., 2008). The ability to feed on glucosinolate containing plants has been attributed to the movement of insects between leaves (or leaf parts) minimizing the effects of induced plant defenses (Shroff *et al*., 2008; Perkins *et al*., 2013; Zalucki *et al*., 2017), and the formation of glutathione-conjugates of ITCs in the gut of larvae, enabling the toxin to be excreted (Schramm *et al*., 2012).

Detoxification processes in insects are costly metabolically (Lindroth *et al*., 1990; Despres *et al*., 2007; Petschenka & Agrawal, 2016), and insects cope better when fed on certain diets. Simpson and Raubenheimer (2001) showed that with increasing amounts of tannic acid in diet, mortality of fifth-instar *Locusta migratoria* (L.) increased in all five diets with different protein to carbohydrate (p : c) ratios, but that survival was highest when the p : c ratio was a balanced p21 : c21. Bt toxin susceptibility may also be influenced by diet in *H. armigera* and *Helicoverpa zea* (Boddie); larvae that fed on diet with protein-to-carbohydrate ratios corresponding to self-selected diets were less susceptible to Bt toxin than larvae fed on diet with different nutritional composition (Deans *et al*., 2016; Tessnow *et al*., 2018; Luong *et al*., 2019). Similarly, the detoxification of glucosinolate-derived ITCs can compromise larval nutrition, depleting certain amino acids and leading to reduced growth, but these effects can be mitigated by changes in the amino acid composition of the diet (Jeschke *et al*., 2016).

Here we investigated the performance of *H. armigera* larvae on three diets of different p : c ratios containing both sinigrin (allyl glucosinolate) and its hydrolyzed product allyl isothiocyanate (AITC). Although AITC is volatile, we encapsulated it into β-cyclodextrin (β-CD), and unlike previous studies (Li *et al*., 2000; Agrawal & Kurashige, 2003), we carefully monitored its release over time to ensure that a known concentration was delivered to feeding larvae. Life-history traits of larvae reared on different diets with and without toxins were recorded and frass was collected to determine potential detoxification activity during digestion by caterpillars. In diets used concentrations of sinigrin and insect toxic by-products (AITC) that approximated levels expected in plants. We expected larvae to perform better on high protein diets, but there may be processing costs evident in frass analysis not detected in crude life-history assays.

Materials and methods

Insect

The *H. armigera* was reared at The University of Queensland, and sourced from the Australian Cotton Research Institute, Narrabri, NSW, Australia. The rearing method is described in Wang *et al*. (2019)

Diet

The standard artificial rearing diet (Appendix A) and nutritionally defined artificial diets containing high carbohydrate (HC), optimal (OP) and high protein (HP) (Appendix B) were made as described in Wang *et al*. (2019). The HC diet had a protein: carbohydrate ratio of p12 : c30, and the OP and HP diets had ratios of p24 : c18 and p30 : c12, respectively. The OP diet was developed using final-instar larvae of *H. armigera* (Tessnow *et al*., 2018).

AITC encapsulation

The method used to encapsulate AITC (Sigma, catalog no. w203408, ≥ 95%) was as described by Li *et al*. (2007) with minor modification: β-CD (5 g) was dissolved in 150 mL of distilled water at 60 °C on a hot plate. After cooling the β-CD solution to 40 °C, AITC in ethanol (1 : 1, v : v) was slowly added to the solution with continuous stirring. The vessel was sealed, and the solution continuously stirred for 3 hours with a magnetic stirrer; the resulting slurry was refrigerated overnight at 4 °C. The cold precipitate was recovered by vacuum-filtration and dried in an oven at 70 °C for 24 hours. The final dry encapsulated powder was stored in an airtight tube at room temperature.

Total AITC determination

The total content of AITC in the powder was measured as described by Li *et al*. (2007). Encapsulated powder (0.10 g) was weighed into a 50 mL flask and mixed with 5 mL of distilled water and 7 mL of n-hexane. The flask was connected to an upright glass condenser cooled by tap water. Then the mixture was heated in a water bath at 85 °C for 20 minutes with intermittent shaking. On heating, a glass lid was attached to the top of the condenser, to avoid the loss of AITC. After the first extraction, the flask was cooled to room temperature and the inner wall of the condenser was washed with 3 mL of hexane in order to collect the maximum amount of AITC. The upper hexane layer, containing AITC, was then separated by decantation. Finally, the volume (V, mL) and the absorbance at 248 nm of the combined hexane extracts were measured. The concentration of AITC (C, g/mL) of the extracts was assessed against a calibration curve of AITC standards. The final amount of AITC complexed in the powder was calculated using the product of V and C:

$$\text{AITC content} = V \times C/0.1.$$

Release characteristics of AITC from diet

Diet was microwaved and cooled down to about 40 °C, then mixed with encapsulated AITC with a pellet pestle motor (Kontes, Vineland, NJ).

In order to determine how much AITC was left in the diet over time, three concentrations of encapsulated AITC (1.79, 3.37, and 4.96 μmol/g) were mixed with all three diets. Once solid, 3 g of diet was placed in a 9 cm diameter Petri dish covered with a lid at room temperature. At 0, 12, 36, and 60 hours, 300 mg of diet from all three concentrations was removed and put into separate 50 mL flasks and processed using the same procedure for determining the AITC in the complex above. The weights of the leftover diets at each sampling time were recorded.

Samples were analyzed by GC-MS (GCMS-QP2010 Plus; Shimadzu, Japan) and the GC was equipped with a ZB5-MS column (30 m × 0.25 mm id, 0.25 μm film thickness; Phenomenex, USA). The oven temperature was programmed from 50°C to hold for 5 minutes, to 110°C with a rate increase of 5°C/min, and to 300°C with a rate increase of 20°C/min, and hold for a final 3.5 minutes. The temperature of the ion source was set to 200°C, for the interface at 250°C. The injection was split, and the injector temperature was set to 250°C. The volume of the injected sample was 1 μL. The flow rate of the helium carrier gas in the column was 3.14 mL/min. Quantification was made by selective ion monitoring of the 99 m/z fragment for AITC, 119 m/z for internal standard tert-Butylbenzene.

A series of concentrations of pure AITC in n-hexane were prepared to make a standard curve. The internal standard concentration was 0.00025 μL/mL in all samples.

Diet preparation and feeding larvae with sinigrin and encapsulated AITC

Sinigrin (Sigma, catalog no. 85440, ≥ 99%) concentrations of 0, 0.25, 5, 10, and 20 μmol/g, and AITC concentrations of 0, 0.14, 0.3, and 0.48 μmol/g (concentration at 12 hours) were prepared for all three diets (concentrations determined from preliminary experiment).

As sinigrin is stable, diets containing it were freshly made at the start of the feeding experiments and then stored in a fridge prior to use. All diets containing encapsulated AITC were made 12 hours before feeding experiments were conducted and fresh diet was supplied to larvae every day until they pupated. The control diet in AITC feeding assay contained the same amount of β-CD as in the diet containing the highest concentration of AITC.

All treatments investigated feeding from the first instar, except for the highest AITC concentration test treatment, which started from the third instar, as preliminary experiment showed that all first instar larvae died within 12 hours when tested at this concentration. All sinigrin treatments started with 20 larvae, and all AITC treatments started with 30 larvae. Larval frass was collected at the fourth instar for further analysis. Larval developmental time, pupal weight, and pupal duration were recorded.

Insects were checked daily. Pupae were weighed one day after pupation. All insects were reared in a Contherm Phytotron Climate Simulator (Contherm, Wellington, New Zealand) at 25 ± 1 °C, L : D = 12 : 12.

Frass analysis

Sinigrin in frass was measured using an Agilent HP1200 (Agilent Technologies, Böblingen, Germany) instrument equipped with a Nucleodur Sphinx RP column (250 × 4.6 mm, 5 μm; Macherey-Nagel, Duren, Germany), the method was as described by Burow *et al*. (2007)

Extracts for uric acid and amino acid measurements were prepared from freeze-dried frass samples. Approximately 10 mg of each frass sample was extracted with 400 μL of aqueous tris buffer (50 mmol/L, pH 7.5) in 2 mL Eppendorf tubes under vigorous shaking (2 × 4 minutes) at room temperature. After centrifugation (20 minutes under 4300 r/min at 4 °C), the clear supernatants were transferred to new vials, and aliquots separated for further analyses as described below.

Amino acids were measured as their fluorenylmethyloxycarbonyl (FMOC) derivatives using a LC-MS/MS system (Jeschke *et al*., 2016). The FMOC-derivatization was carried out as follows: 10 μL of the aqueous extract was mixed with 90 μL ^{13}C- and ^{15}N-labeled amino acid standard solution (Isotec (Miamisburg, Ohio, USA), 20 μg/mL and 100 μL borate buffer (0.8 mol/L, pH 10). FMOC-reagent (200 μL of 30 mmol/L FMOC-Cl in acetonitrile) was added and the reaction was gently mixed and incubated for 5 minutes. Excess FMOC-Cl was removed by extraction with hexane (800 μL). After phase separation, 200 μL of the bottom aqueous phase were carefully collected and transferred to a glass vial. FMOC-derivatized amino acids were analyzed by employing an Agilent 1260 HPLC (Agilent Technologies, Böblingen, Germany) coupled to an API5000 tandem mass spectrometer (Applied Biosciences, Darmstadt, Germany). The HPLC was equipped with a C18 reversed phase column (XDB C18, 1.8 mm, 4.6 × 50 mm; Agilent Technologies, Böblingen, Germany) and the separation was achieved with a gradient of water/0.05% formic acid (solvent A) – acetonitrile (solvent B) at a flow rate of 1.1 mL/min at 25 °C with the following gradient: 10 % B (0.5 minutes), 10%–90% B (4 minutes), 90%–100% B (1.5 minutes), 100% B (0.5 minutes), 100%–10% (0.1 minutes), 10% (2.5 minutes). The ionspray voltage was maintained at −4.5 keV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. MS parameters for detection were as in Jeschke *et al*. (2016). Quantification relied on the isotopically labelled amino acids added to individual samples. Labeled cysteine is not present in the internal standard, so it is not possible to quantify it in absolute concentrations, only to compare peak areas in different groups.

Uric acid was measured directly using an Agilent HP1200 (Agilent Technologies, Böblingen, Germany) Series instrument coupled to an API3200 tandem mass spectrometer (Applied Biosciences, Darmstadt, Germany) (Jeschke *et al*., 2016). The HPLC was equipped with a C18 reversed phase column (XDB C18, 1.8 mm, 4.6×50 mm; Agilent Technologies, Böblingen, Germany) and the separation was achieved with a gradient of water/0.05% formic acid (solvent A) - acetonitrile (solvent B) at a flow rate of 1.1 mL/min at 25 °C with the following gradient: 3% B (1 minute), 3%–100% B (1.7 minutes), 100% B (0.3 minutes), 100%–3% B (0.1 minutes), 3% B (2.9 minutes). The ionspray voltage was maintained at −4.2 keV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. Quantification relied on an external calibration curve using an authentic standard.

Statistical analyses

All data analyses were conducted in R, version number 3.2.5 (R Core Team, 2016) using the the car, ggplot2, survival and survminer packages (Therneau & Grambsch, 2000; Wickham, 2016; Fox & Weisberg, 2019; Kassambara *et al*., 2020; Therneau, 2020). Frass from fourth instar larvae was analyzed to examine free amino acids and uric acid levels via LC-MS/MS. Comparisons of amino acid and uric acid in frass of sinigrin treatments were undertaken for control groups and the highest sinigrin concentration, as this was the only treatment that showed developmental effects. The highest AITC concentration groups were not included in life-history trait results because these treatments were only applied from third instar. Comparisons of amino acid and uric acid in frass following AITC treatment were undertaken for all concentrations. Levene's test and Shapiro-Wilk test were used to check the data for normality and homogenous variances. Two-way ANOVA was used to detect differences between main effects (diet, concentration), and interactions. One-way ANOVA was used to analyze the simple effects of one factor between groups. When appropriate, multiple comparisons were made using Tukey's HSD post hoc test following ANOVA. The Kruskal–Wallis test was used to analyze the effect of AITC concentration on pupal

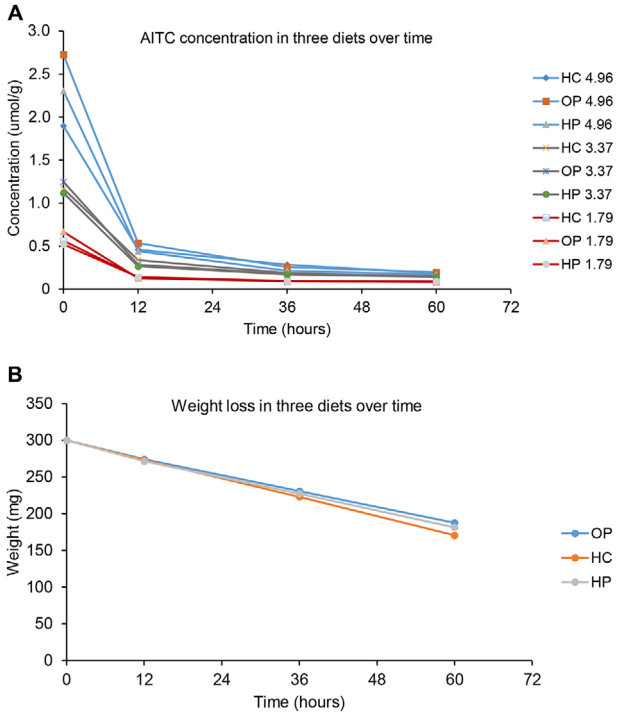

Fig. 1 The change of AITC concentration over time in three diets, with the initial concentrations at 4.96, 3.37, 1.79 μmol/g (A), and the change in weight (±SE) of 300 mg fresh diet for three diet types over time (B). HC, OP, and HP represent high carbohydrate, optimal, and high protein diet, respectively.

developmental time as data were nonnormal. Kaplan–Meier method was used to estimate survival probability.

Results

Levels of encapsulated AITC in diets decreased fast in the first 12 hours along with water loss

Several batches of encapsulated AITC were made during the experiment and all were tested before use to determine the total amount of AITC. Although small differences were detected between batches, all contained AITC in the range of 470–550 μmol/g.

When mixed in diet, AITC content declined rapidly in the first 12 hours at all concentrations for all three diets, but concentrations then remained relatively stable (Fig. 1A). However, the weight of 300 mg fresh diets dropped steadily after calibration, indicating that water was lost from the diet (Fig. 1B); weight losses were consistent between different diets.

Diet mixed with sinigrin showed low toxicity to Helicoverpa armigera in life-history treats

Most larvae survived even at the highest sinigrin concentration (≤3 died in each group, ca1-5%), but some differences in larval developmental time were found between concentrations and diets. Two-way ANOVA showed a significant interaction between diet and sinigrin concentration (ANOVA: $F_{8,251} = 3.413$, $P < 0.001$). All diets showed a significant effect on developmental time of larvae (ANOVA: HC, $F_{4,78} = 5.715$, $P < 0.001$; OP, $F_{4,85} = 9.671$, $P < 0.001$; HP, $F_{4,88} = 2.920$, $P = 0.026$). When fed on HC diet, larvae took significantly longer to complete the larval stage when fed diet containing 20 μmol/g and 10 μmol/g sinigrin than when fed diet containing 0.25 μmol/g and 5 μmol/g sinigrin (Table S1). When fed on OP diet, larvae took significantly less time to complete the larval stage when fed diet containing 20 μmol/g sinigrin than in all other treatments (Table S1). When fed on HP diet, no significant differences were found between treatments (Table S1). Within control and 0.25 μmol/g sinigrin-treated groups, larval developmental time was significantly longer when fed on HC diet than when fed on OP or HP diet (Table S2). At 10 μmol/g sinigrin, larvae fed on OP diet took significantly less time to develop than larvae fed on HC and HP diet (Table S2) (Figs. 2A and 2C).

Two-way ANOVA showed significant effects of diet and sinigrin concentration on pupal weight (ANOVA: Diet, $F_{2,259} = 5.919$, $P = 0.003$; Sinigrin concentration, $F_{4,259} = 2.880$, $P = 0.023$), but there was no significant interaction (ANOVA: $F_{8,251} = 1.620$, $P = 0.119$). When fed on OP diet, larvae treated with 5 μmol/g sinigrin resulted in significantly heavier pupae than larvae treated with 0.25 μmol/g sinigrin (Table S3). At 0.25 μmol/g and 10 μmol/g, larvae fed on HC diet resulted in significantly heavier pupae than larvae fed on OP diet (Table S4) (Figs. 2B and 2D).

No significant differences between pupal developmental times (the time between pupation and eclosion) were found within concentration or diet treatments (Fig. S1). The average pupal times were 11.8 (± 0.9), 11.6 (± 0.9), and 11.7 (± 0.8) days for HC, OP, and HP diet, respectively.

Free amino acid and uric acid levels in frass changed dramatically when larvae treated with sinigrin

Most of the ingested sinigrin was not hydrolyzed during digestion and was excreted intact in the frass. The average amounts of sinigrin present in frass were 120.0

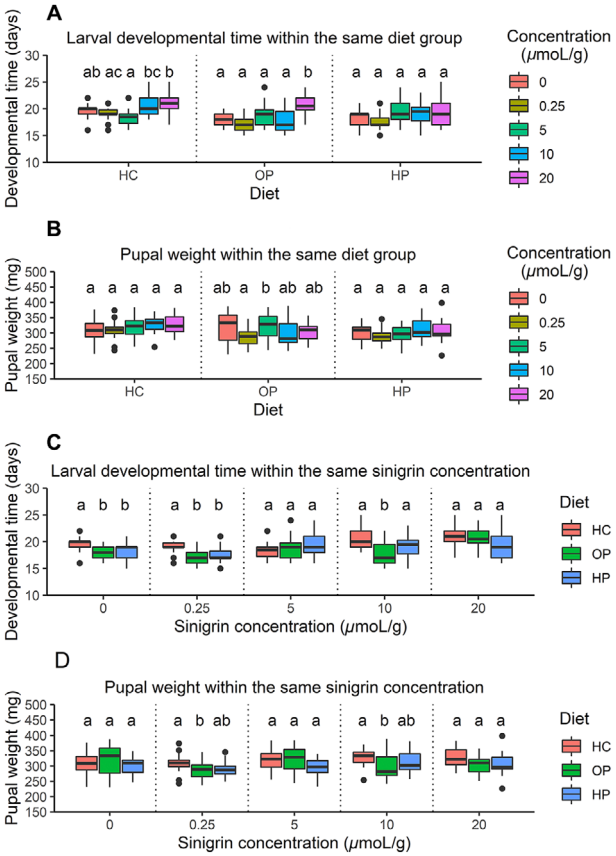

Fig. 2 Box and whiskers plot of larval developmental time (days) (A, C) and pupal weight (mg) (B, D) when treated with HC (high carbohydrate), OP (optimal) and HP (high protein) diet in different concentrations of sinigrin (0–20 μmol/g). Different letters represent significant difference between treatments.

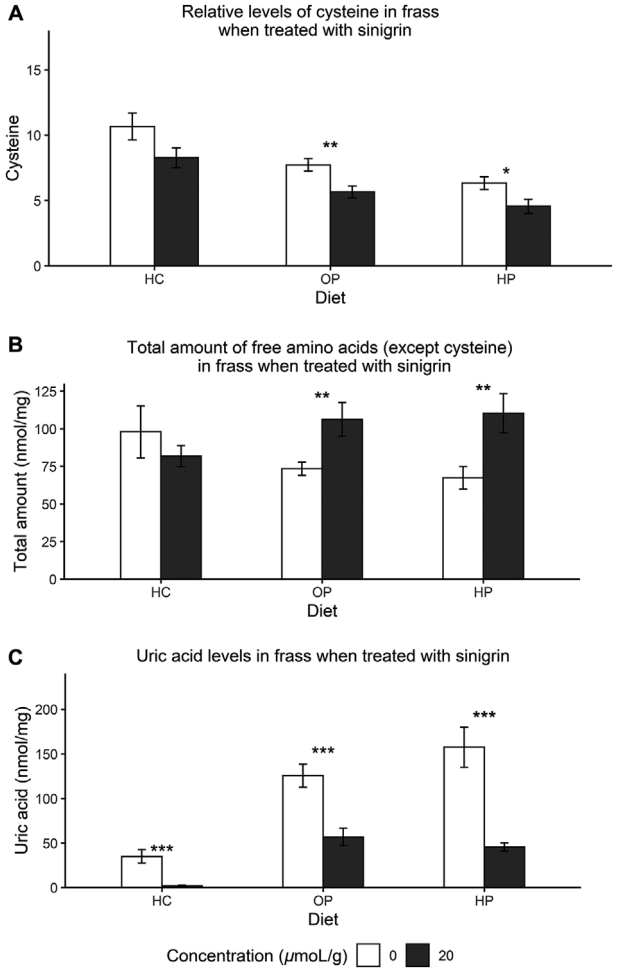

Fig. 3 The levels of free cysteine (A), total amount of free amino acids (except cysteine) (B), and uric acid (C) in frass of larvae fed on different diets with (20 μmol/g) or without sinigrin. HC, OP, and HP represent high carbohydrate, optimal, and high protein diet, respectively. Asterisks represent significance between groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

(± 8.5), 123.7 (± 6.8), 108.6 (± 8.7) μmol/g dry weight (DW) for HC-, OP-, and HP-treated groups, respectively. Sinigrin concentrations in the three diets were 109.2, 107.7, and 107.0 μmol/g DW, respectively. The observed differences between sinigrin concentrations in diet and frass are likely due to nutrient assimilation during digestion and water losses in the diet making process. There was no significant effect of diet on sinigrin concentration in frass (ANOVA: $F_{2,40} = 1.148$, $P = 0.327$).

Levels of free individual amino acids excreted in the frass varied greatly within the same diet and between diets for each amino acid. In most cases, larvae fed on OP and HP diet excreted greater amounts of free amino acids (alanine, glycine, proline, threonine, glutamate, arginine, lysine, histidine) when treated with sinigrin than larvae treated with control diet (Fig. S2). However, the amount of cysteine in sinigrin-treated groups were significantly less than in control groups in both OP and HP diet treatments (Fig. 3A). When fed on HC diet, most free amino acids were excreted at lower levels in sinigrin-treated groups than controls (Fig. S2).

There was a significant interaction between diet and sinigrin concentration on the levels of total free amino acids (excluding cysteine) in frass (ANOVA: $F_{2,89} = 3.224$, $P = 0.044$). When treated with sinigrin, total free amino acid in frass only increased when insects fed on protein biased diets (OP and HP diet), but decreased when insects fed on low protein HC diet (Fig. 3B).

Uric acid is one of the main products of protein digestion (Chapman & Chapman, 1998). The levels of uric

acid in frass were significantly higher in control groups than 20 μmol/g sinigrin-treated groups in all three diet treatments. There was a significant interaction between diet and sinigrin concentration on uric acid levels in larval frass (ANOVA: $F_{2,82} = 15.919$, $P < 0.001$). Uric acid was correlated with the protein concentration in control diets (HP > OP > HC), but not in diets containing 20 μmol/g sinigrin (Fig. 3C).

Diet mixed with AITC showed diverse effects on Helicoverpa armigera life-history traits

The hydrolysis product of sinigrin, AITC, is toxic to insects and it affected larval performance much more dramatically than sinigrin, inducing high mortality during larval development (Fig. 4). Survival probability was analyzed by the Kaplan–Meier (KM) method in each diet treatment. AITC concentration had a significant influence on larval survival probability in all diet treatments (log-rank test: HC, $P = 0.007$; OP, $P = 0.011$; HP, $P = 0.027$). In pairwise comparisons, larvae fed on all three diets with 0.14 μmol/g of AITC had significantly higher survival probability than larvae fed on control diets (Table S5). In HC and HP diet treatments, survival probability was higher when larvae fed on diet with 0.14 μmol/g of AITC than fed on diet with 0.3 μmol/g of AITC (Table S5) (Fig. 4).

Two-way ANOVA showed significant effects of diet and AITC concentration to larval developmental time (ANOVA: Diet, $F_{2,151} = 14.839$, $P < 0.001$; AITC concentration, $F_{2,151} = 183.367$, $P < 0.001$), but no significant interaction was detected between these two factors (ANOVA: $F_{4,147} = 1.781$, $P = 0.136$). In all three diet treatments, AITC concentration showed a significant impact on larval developmental time (ANOVA: HC, $F_{2,52} = 62.757$, $P < 0.001$; OP, $F_{2,42} = 92.776$, $P < 0.001$; HP, $F_{2,53} = 46.682$, $P < 0.001$), larvae took significantly less time to complete the larval stage when diet contained 0.14 μmol/g AITC than all other treatments (Table S6), but no significant differences were found between control and 0.3 μmol/g AITC diet groups (Fig. 5A). When comparing different diets at the same AITC concentration, larvae treated with 0 or 0.3 μmol/g AITC took significantly less time to complete development when fed on HC control diet than when fed on OP diet, and larvae treated with HC control diet took significantly less time to complete development than larvae treated with HP control diet (Table S7) (Fig. 5C).

A significant interaction between diet and AITC concentration on pupal weight was detected (ANOVA: $F_{4,147}$

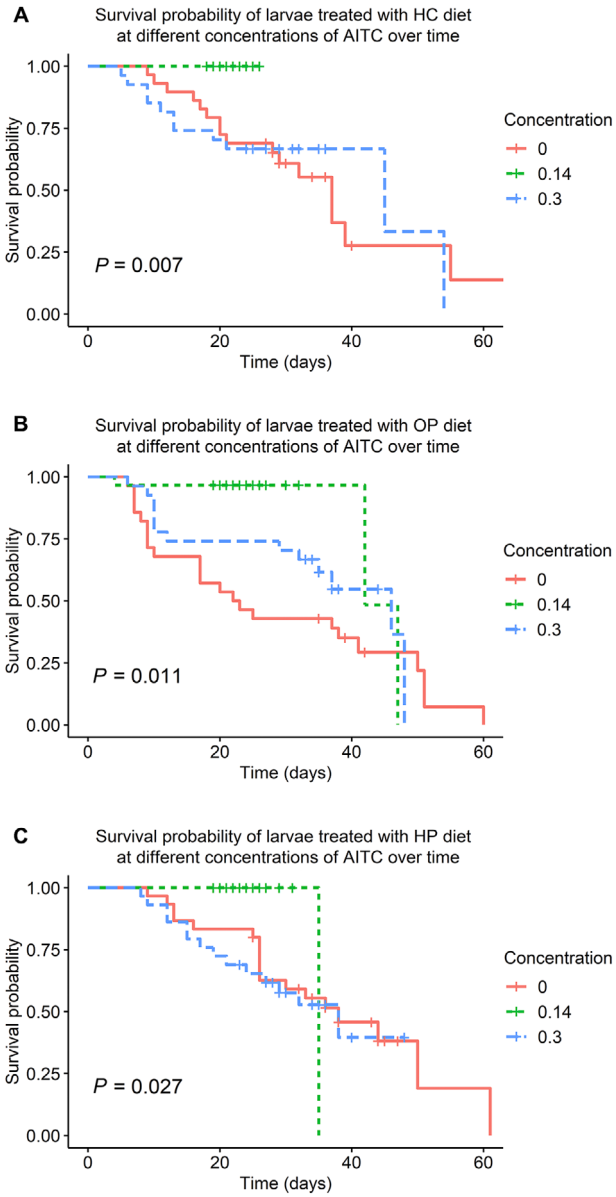

Fig. 4 Survival curve of larvae fed on HC (high carbohydrate) (A), OP (optimal) (B), and HP (high protein) (C) diets with different AITC concentrations. *P* value on each plot indicate overall significance of influence of different concentrations of AITC on survival. "+" means a survival till the end of larval stage.

$= 5.509$, $P < 0.001$). In all three diet treatments, AITC concentration showed significant impact on larval developmental time (ANOVA: HC, $F_{2,52} = 9.206$, $P < 0.001$; OP, $F_{2,42} = 40.212$, $P < 0.001$; HP, $F_{2,53} = 22.17$, $P < 0.001$). All larvae fed on diets treated with 0.14 μmol/g AITC developed into significantly heavier pupae than

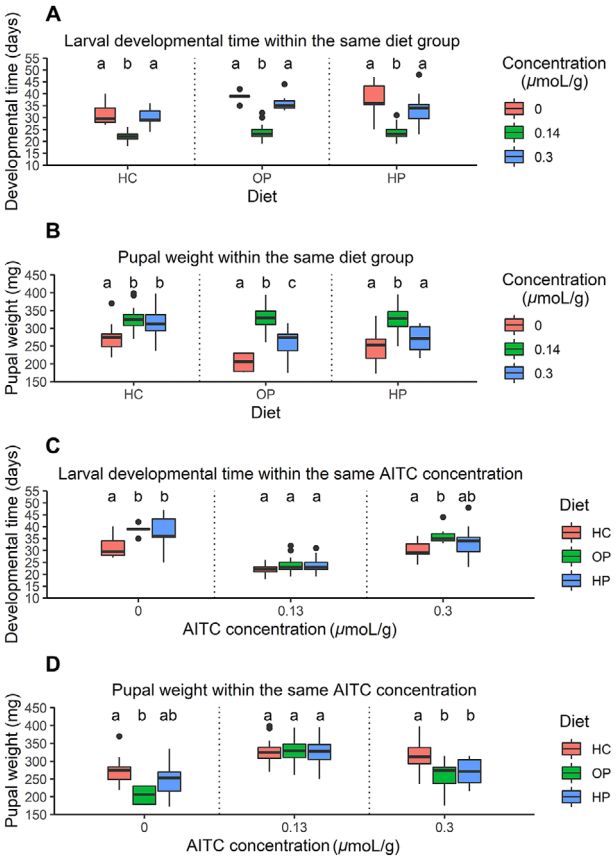

Fig. 5 Box and whiskers plot of larval developmental time (days) (A, C) and pupal weight (mg) (B, D) when treated with HC (high carbohydrate), OP (optimal) and HP (high protein) diet in different concentrations of AITC. Different letters represent significant difference between treatments.

larvae treated with control diets without AITC. Larvae fed on HC and OP diets developed into significantly heavier pupae when treated with 0.3 μmol/g AITC than without AITC. Larvae fed on OP and HP diets developed into significantly heavier pupae when treated with 0.14 μmol/g AITC than treated with 0.3 μmol/g AITC (Table S8) (Fig. 5B). In the absence of AITC, larvae fed on OP diet developed into significantly lighter pupae than larvae fed on HC diet, and when treated with 0.3 μmol/g AITC, larvae fed on HC diet developed into significantly heavier pupae than larvae fed on OP or HP diet (Table S9) (Fig. 5D).

Pupal durations in all treatment groups were similar, except in OP diet treatment where insects treated with 0.3 μmol/g AITC took significantly longer to complete development than insects treated with 0.14 μmol/g AITC (Kruskal–Wallis test: $P = 0.011$) (Fig. S3).

Free amino acids and uric acid levels in frass showed complex response to different diets and AITC concentrations

Levels of free amino acids excreted in frass were influenced by AITC concentrations. Excretion of almost all amino acids was affected by the diet treatments with different concentrations of AITC (Fig. S4). There was a significant interaction between diet and AITC concentration on cysteine levels in larval frass (ANOVA: $F_{6,155} = 7.500$, $P < 0.001$). Larvae fed on HC diet normally excreted less cysteine in frass when treated with AITC compared with the control treatment. However, larvae fed on OP or HP diet excreted more cysteine in frass when treated with AITC, compared with the control treatment (Table S10) (Fig. 6A).

When pooled together, both diet and AITC concentration had a significant influence on total amino acid levels in the frass (excluding cysteine) (ANOVA: diet, $F_{2,161} = 25.093$, $P < 0.001$; concentration, $F_{3,161} = 16.597$, $P < 0.001$), however there was no interaction between the two factors (ANOVA: $F_{6,155} = 2.044$, $P = 0.063$). When fed on protein biased diets (OP and HP), total amino acid values showed similar changes with increased AITC concentration; values decreased at 0.14 and 0.3 μmol/g AITC, then jumped higher than control at the highest concentration. In all three diet treatments, the smallest total free amino acids values occurred when larvae were fed diet treated with 0.3 μmol/g AITC (Table S11) (Fig. 6B).

There was a significant interaction between diet and concentration of AITC on the amount of uric acid excreted in larval frass (ANOVA: $F_{6,155} = 2.551$, $P = 0.022$). In simple effect tests, only diet had a significant effect on uric acid levels in frass (ANOVA: $F_{2,164} = 339.740$, $P < 0.001$). The abundance of uric acid in frass was correlated with diet protein concentration, being highest in HP diet and lowest in HC diet. Protein biased diets (OP and HP) had a similar pattern of uric acid levels, with the highest uric acid levels when larvae fed on control diet, and the lowest uric acid levels when fed on diet with 0.14 μmol/g AITC (Table S12). Conversely, uric acid levels in frass showed no significant difference between concentrations when larvae fed on low protein diet (HC) (Fig. 6C).

Discussion

The adverse effect of isothiocyanates (ITCs) on insects has been noted in previous research (Li *et al.*, 2000; Ratzka *et al.*, 2002; Burow *et al.*, 2006; Mumm *et al.*, 2008; Rohr *et al.*, 2011). However, ITCs are mostly

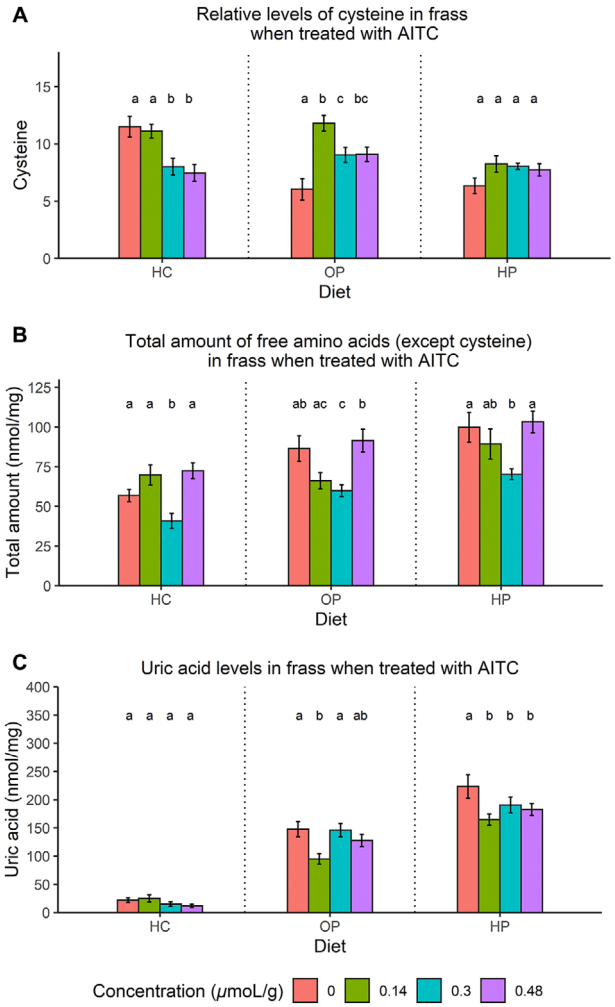

Fig. 6 The levels of free cysteine (A), total amount of free amino acids (except cysteine) (B), and uric acid (C) in frass of larvae fed on different diets with (0.14, 0.3, or 0.48 μmol/g) or without AITC. HC, OP, and HP represent high carbohydrate, optimal, and high protein diet, respectively. Different letters represent significant difference within the same diet between doses.

volatile, which makes it difficult to address this topic directly with the use of controlled diets. The use of microencapsulated AITC was first introduced by Agrawal and Kurashige (2003) to investigate the interaction of *Pieris rapae* (L.) with ITCs. We found this is not a definitive approach, because AITC can still be lost albeit at a slower rate. When added to the diet, AITC levels were reduced by more than 1/2 during the mixing process, and the concentration continued to decline during the first 12 hours. However, in the following 24 hours, AITC concentration remained relatively stable (Fig. 1A), partly because the drying process concentrated the remaining AITC in the diet, but the loss does not stop (Fig. 1B). That is why the diet mixed with AITC was only used after 12 hours for 24 hours. Agrawal and Kurashige (2003) did not report on this problem.

As a substrate that reacts with myrosinases, sinigrin alone is considered nonactive (Donkin *et al*., 1995; Jeschke *et al*., 2016), and our results suggest that most ingested sinigrin is excreted in frass. However, Li *et al*. (2000) recorded a $LC_{50} \pm 95\%$ CI at 6.73 ± 1.39 μmol/g for sinigrin in the generalist *Spodoptera eridania* (Stoll). In a 4-day feeding assay, *H. armigera* neonates suffered 50% mortality when fed on diet with 50 μmol/g sinigrin concentration (Wang P., unpublished data). These results suggest the occurrence of sinigrin hydrolysis in the insect gut even in the absence of plant myrosinases, supporting a previous report (Agnihotri *et al*., 2018). In rats and humans, gut bacteria can hydrolyze glucosinolates but in a very inefficient way (Krul *et al*., 2002; Angelino *et al*., 2015), and such an effect may also be present in herbivorous insects in spite of its negative consequences to the herbivore., The influence of 20 μmol/g sinigrin on larval development, pupal weight and pupal duration was not dramatic when compared with control, except for developmental time in OP diet group, which might be due to a few abnormal values (outliers) in this group (Fig. 2A). The tolerance to toxins was associated with increasing larval instars; larger larvae can tolerate higher doses (Yu, 1983; Jeschke *et al*., 2017). Larvae were given a constant concentration of sinigrin throughout all instars, so even though a small influence appeared at early instars, it is possible they can overcome and catch up with control group in later instars.

On the other hand, addition of encapsulated AITC into the diets had a significant impact on larval survival and development. In most cases, 0.14 μmol/g AITC-treated groups had the highest survival probability (Fig. 4). Larval developmental time and pupal weight both varied with increased concentration of AITC, with the shortest developmental time and biggest pupal weight in 0.14 μmol/g AITC-treated groups across diets (Figs. 5A and 5B). The shorter developmental time and increased pupal weight at a low concentration of AITC might be attributed to a hormesis effect, a dose–response relationship characterized by a reversal in response between low and high doses of a stressor (insecticide for example) (Guedes & Cutler, 2014). The hormesis phenomenon is widely recognized in various organisms, and insecticide-induced hormesis is reported for arthropods (Knutson, 1955; Ouye & Knutson, 1957; Luckey, 1968; Morse & Zareh, 1991; Guedes *et al*., 2009; Rabhi *et al*., 2014). The prevalent explanation for hormesis is resource allocation theory: the stress-exposed individual shifts the balance between potentially

energy-conflicting physiological trade-offs, favoring one trait (e.g., reproduction) at the expense of another (e.g., longevity) (Guedes & Cutler, 2014). At the transcript level, hormesis was observed in *H. armigera* at low dose of gossypol; genes involved in energy acquisition such as β-fructofuranosidases were upregulated in the gut, and genes involved in cell adhesion were downregulated in the body (de la Paz Celorio-Mancera *et al*., 2011).

Diets with different p : c ratios had a profound influence on larvae when exposed to potential toxins. Some HC diet-treated groups showed small but significant shorter developmental time and heavier pupal weights compared with other diet groups treated with sinigrin (Figs. 2C and 2D). It has been reported that some species of caterpillar change their feeding habit as they grow, switching to foods with higher sugar content (Scheltes, 1978; Cohen *et al*., 1987; Kantiki & Ampofo, 1989). Our previous study showed an elevated relative growth rate when larger instar *H. armigera* larvae fed on HC diet (Wang *et al*., 2019). Large caterpillars need energy to maintain respiration, and store energy for pupation and eclosion. High carbohydrate diet may help shorten the ingestion process and increase the efficiency to accumulate fat in certain conditions, but no differences were detected in developmental time and pupal weight between diet treatments at 0.14 μmol/g of AITC (Figs. 5C and 5D).

Larval excretion of free amino acids in frass increased with higher concentration of sinigrin and showed clear differences between diet treatments. The total free amino acids levels in frass were significantly elevated following feeding on OP and HP diets and decreased (though not significantly) following feeding on HC diet, as would be expected from protein composition in diets (Fig. 3B). Larvae break down more protein to cope with toxin challenge, but less free amino acids were left in frass when protein in food was below a certain amount. Cysteine decreased in all groups, as expected given its role in detoxification (Fig. 3A). When treated with AITC, total free amino acid levels in OP and HP diet groups shared similar response to AITC concentrations, but not HC diet groups. Normally total free amino acid levels were positively correlated with diet protein concentrations (Fig. 6B).

According to Schramm *et al*. (2012) and Jeschke *et al*. (2016), generalist herbivores detoxify ITCs by conjugation with GSH, and further hydrolysis via the mercapturic acid pathway leads to the corresponding cysteinylglycine (CysGly) and cysteine (Cys) conjugates. The balance between conjugation and dissociation reactions is likely to be a dynamic process, which can eventually release free AITC that can once again conjugate with GSH, leading to depletion of the intracellular GSH pool (Equation 1). The protein in diets can help replenish the shortage of cysteine, the limiting substrate in GSH biosynthesis, and other amino acids that can assist to overcome stress caused by AITC (Figs. 3A, 3B, 6A, 6B).

$$\text{AITC} \xrightarrow{+\text{GSH}} \text{Allyl-GSH} \xrightarrow{-\text{Glu}} \text{Allyl-CysGly} \xrightarrow{-\text{Gly}} \text{Allyl-Cys} \quad (1)$$

Equation 1 describes the larval detoxification process in gut after ingestion AITC, where Allyl-GSH, Allyl-CysGly, Allyl-Cys represent conjugates of AITC with glutathione, cysteinylglycine, and cysteine, respectively.

Uric acid is the final product of protein metabolism in most terrestrial insects and is related to the level of protein catabolism. Excretion of ITC-conjugates increased the demand for cysteine in GSH biosynthesis, therefore increased protein catabolism (Jeschke *et al*., 2016). Excess amino acids will go through an amino acid deamination process to form uric acid (Weihrauch *et al*., 2012). It is logical to conclude that elevated free amino acid in frass stimulated by sinigrin will be accompanied by higher levels of uric acid, which have been reported in a few studies (Horie & Inokuchi, 1978; Horie & Watanabe, 1983; Jeschke *et al*., 2016). Interestingly in the sinigrin feeding experiment, uric acid levels in all diet treatments were significantly lower in sinigrin-fed groups than in controls (Fig. 3C). In the AITC feeding experiment, uric acid levels in frass were significantly lower when larvae fed on protein biased diets (OP and HP) at 0.14 μmol/g AITC compared with controls, but the levels increased in higher concentrations (Fig. 6C). These results indicate a complicated relationship between uric acid level, sinigrin/ AITC dose, and diet type. Uric acid level in frass does not always relate to total amino acid, and different toxin doses can lead to completely different results. Further investigation involving measurements of total body nitrogen, hemolymph protein concentration, and deamination related enzymes, are needed to understand this phenomenon.

Even though the effect of sinigrin in the gut was presumably a result of its hydrolysis product–AITC, diet treatment still showed different effects in frass analysis. In control groups, most HC diet treatments showed the highest levels of free amino acid in sinigrin feeding assays, but lowest levels of amino acid in AITC feeding assays amongst the three diets (Figs. 3A, 3B, 6A, 6B). The only difference of the control diets was additional β-CD in diets of AITC feeding assay, but logically, HC diet should have the lowest amino acid concentration amongst the three diets. Higher mortality in controls of AITC feeding assay than in sinigrin feeding assay also showed this inconsistency (Fig. 4). We suggest it is not appropriate to

compare the results of the two assays, but this should not influence comparisons within an assay.

In conclusion, the influence of sinigrin and AITC on the development of the generalist herbivore *H. armigera*, and its detoxification capability when fed diets containing different p : c ratios were investigated. Although AITC caused higher toxicity than sinigrin, as expected, they both triggered active detoxification responses, highlighted with a hormesis effect at low concentration of AITC treatment across all diets. Diets with different p : c ratios were for the first time investigated in relation to the AITC detoxification process, and we demonstrate that protein could be a crucial factor in the process. Further research involving the analysis of gene expression, enzyme activity, and amino acid measurements inside larvae, may help to shed more light on the interaction of diet, toxins and insect physiology.

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Disclosure

The authors declare that they have no conflict of interests.

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Appendix A

General diet

Soybean flour was microwaved with water for 3 minutes and stirred between each minute, then mixed with all other solid ingredients except agar. Agar was microwaved with water before added into the mixture. Antifungal

Table A1 Standard diet.

Component	Weight (g)
Soybean flour	86 g
Water for soy flour (mL)	500
Wheatgerm	60
Yeast	50
methyl paraben	3
L-ascorbic acid	3
Sorbic acid	1
Antifungal solution (mL)	2.6
Agar	10.5
Water for agar (mL)	300

Table A2 Antifungal solution.

Component	Volume (mL)
Propionic acid	42
Phosphoric acid	4
Water	54

Table A3 Adult diet.

Component	Weight (g)
Water (mL)	1000
Sucrose	100
Ascorbic acid	3

solution was added at last and then mixed thoroughly all together. Adult diet was prepared by mixing sucrose and ascorbic acid with water (Table A3).

Appendix B

Diet with different protein : carbohydrate ratio

Prepare vitamin mix and vitamin solution ahead of time (Tables B1 and B2). Premix casein, cellulose, and cholesterol in a 500 mL beaker. The cholesterol and chloroform mixed in a small (30 mL) beaker in a fume hood and then pour into the mixture. Cholesterol will dissolve in chloroform and the beaker should be washed three times with chloroform. The mixture should be left in a fume hood overnight.

Table B1 Vitamin solution.

Component	Weight (g)
Nicotinic acid amide	0.5
D-pantothenic acid hemicalcium salt	0.5
Thiamine HCl	0.0125
Riboflavin	0.025
Pyridoxine HCl	0.0125
Folic acid	0.0125
Biotin	0.001
Vitamin B12	0.0001
Zinc acetat	0.025
Cobalt chloride	0.0125
Sodium molybdate	0.0125
Distilled water (mL)	300

Note: Store in a flask with parafilm on top to prevent evaporation in the refrigerator 4 °C.

Table B2 Vitamin mix.

Vitamin	Weight (mg)
Thiamine	75
Riboflavin	75
Nicotinic acid	300
Pyridoxine	75
Folic acid	75
Myoinositol	750
Calcium pantothenate	150
p-Aminobenzoic acid	75
Choline	3750
Biotin	3
Total (mg)	5328

Note: Seal with parafilm in a tube in −20 °C.

The next day, add sucrose, Wesson's salt, cysteine HCl, myoinositol, choline chloride, torula yeast, dry milk, vitamin mix, sorbic acid, ascorbic acid, methyl paraben, chlorotetracycline, and streptomycin to the 500 mL beaker. Cholecalciferol, menadione, linoleic linolenic acid, and tocopherol were first dissolved in 5 mL absolute ethanol before pouring into the mixture. Add 125 mL distilled water, 365 μL formaldehyde, and 30 mL vitamin solution to the mixture and mix thoroughly with stirring rod. At last, boil the mixture of 10 g agar and 250 mL distilled water in microwave, add it to the rest of the ingredients in the 500 mL beaker, and stir thoroughly. Once

Table B3 Diet with different protein: carbohydrate ratio.

	Protein : carbohydrate ratio		
Component	30 : 12 (g)	12 : 30 (g)	24 :18 (g)
Cellulose	30	30	30
Vitamin-free casein	26.63	7.58	20.18
Cholesterol	0.1	0.1	0.1
Chloroform (mL)	35	35	35
Sucrose	9.82	26.57	15.5
Wesson's salt	5	5	5
Cysteine HCl	0.557	0.557	0.557
Myoinositol	0.2	0.2	0.2
Choline chloride	0.5	0.5	0.5
Torula yeast	5	5	5
Dry milk (whole)	3.75	3.75	3.75
Vitamin mix	0.8125	0.8125	0.8125
Sorbic acid	0.5	0.5	0.5
L-ascorbic acid	1	1	1
Methyl 4-hydroxybenzoate	0.4875	0.4875	0.4875
Chlorotetracycline hcl	0.008675	0.008675	0.008675
Streptomycin sulphate	0.008675	0.008675	0.008675
37% formaldehyde (μL)	365	365	365
100% ethanol (mL)	5	5	5
Cholecalciferol	0.0025	0.0025	0.0025
Menadione (vit K)	0.0025	0.0025	0.0025
Linoleic acid (μL)	275	275	275
Alpha-linolenic acid (μL)	193	193	193
DL-alpha-tocopherol acetate	0.05	0.05	0.05
Vitamin solution (mL)	30	30	30
Distilled water (mL)	375	375	375
Agar	10	10	10

stirred, pour into the molds. The diet will cool and set-up in about 30 minutes.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Box and whiskers plot of pupal developmental time of larvae fed on HC (high carbohydrate), OP (optimal), and HP (high protein) diet with different concentrations of sinigrin.

Fig. S2. The levels of free amino acid (except cysteine) in frass of larvae fed on different diets with (20 μmol/g) or without sinigrin. HC, OP, and HP represent high carbohydrate, optimal, and high protein diet, respectively.

Fig. S3. Box and whiskers plot of pupal developmental time of larvae fed on HC (high carbohydrate), OP (optimal), and HP (high protein) diet with different concentrations of AITC.

Fig. S4. The levels of free amino acid (except cysteine) in frass of larvae fed on different diets with (0.14, 0.3, or 0.48 μmol/g) or without AITC (A and B).

Table S1 Tukey's HSD test for developmental time of larvae on diets with different concentrations of sinigrin.

Table S2 Tukey's HSD test for developmental time of larvae on different diets with same sinigrin concentrations.

Table S3 Tukey's HSD test for pupal weight of larvae on OP diet with different concentrations of sinigrin.

Table S4 Tukey's HSD test for pupal weight of larvae on different diets with same sinigrin concentrations

Table S5 Log-rank test of pairwise comparison of survival probability between diets with different AITC concentration.

Table S6 Tukey's HSD test for developmental time of larvae on diets with different concentrations of AITC.

Table S7 Tukey's HSD test for developmental time of larvae on different diets with same AITC concentrations.

Table S8 Tukey's HSD test for pupal weight of larvae on diets with different concentrations of AITC.

Table S9 Tukey's HSD test for pupal weight of larvae on different diets with same AITC concentrations

Table S10 Tukey's HSD test for cysteine levels in frass when larvae on diet with different AITC concentrations.

Table S11 Tukey's HSD test for total free amino acid (except cysteine) levels in frass when larvae on diet with different AITC concentrations.

Table S12 Tukey's HSD test for uric acid levels of larvae on diet with different AITC concentrations.