

# Consumption of gossypol increases fatty acid-amino acid conjugates in the cotton pests *Helicoverpa armigera* and *Heliothis virescens*

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## Abstract

Gossypol is a toxic sesquiterpene dimer produced by cotton plants which deters herbivory by insects and vertebrates. Two highly reactive aldehyde groups contribute to gossypol toxicity by cross-linking herbivore proteins. We identified another consequence of consuming gossypol in two insect pests of cotton: increased amounts of fatty acid-amino acid conjugates (FACs). Eight different FACs in the feces of larval *Helicoverpa armigera* and *Heliothis virescens* increased when larvae consumed artificial diet containing gossypol, but not a gossypol derivative lacking free aldehyde groups (SB-gossypol). FACs are produced by joining plant-derived fatty acids with amino acids of insect origin in the larval midgut tissue by an unknown conjugase, and translocated into the gut lumen by an unknown transporter. FACs are hydrolyzed back into fatty acids and amino acids by an aminoacylase (L-ACY-1) in the gut lumen. The equilibrium level of FACs in the lumen is determined by a balance between conjugation and hydrolysis, which may differ among species. When heterologously expressed, L-ACY-1 of *H. armigera* but not *H. virescens* was inhibited by gossypol; consistent with the

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excretion of more FACs in the feces by *H. armigera*. FACs are known to benefit the plant host by inducing anti-herbivore defensive responses, and have been hypothesized to benefit the herbivore by acting as a surfactant and increasing nitrogen uptake efficiency. Thus in addition to its direct toxic effects, gossypol may negatively impact insect nitrogen uptake efficiency and amplify the signal used by the plant to elicit release of volatile compounds that attract parasitoids.

#### KEYWORDS

amidase, aminoacylase, *Chloridea virescens*, elicitor, fatty acid amide, gossypol

## 1 | INTRODUCTION

Fatty acid-amino acid conjugates (FACs, also fatty acid amides, *N*-acyl amino acids) are produced in the gut cells of several lepidopteran species, and are present in the oral secretions of the larvae feeding on plant hosts (Alborn et al., 1997; C. M. De Moraes & Mescher, 2004; Mori et al., 2001, 2003; Pohnert et al., 1999; Yoshinaga et al., 2010). They are well-known elicitors of the indirect defense system of different plants, such as cotton, maize, tobacco, and corn (C. De Moraes et al., 1998; Kessler & Baldwin, 2001; Turlings et al., 1993). FACs stimulate the *de novo* synthesis of volatile compounds by the plants which attract natural enemies of the herbivore, such as parasitoid wasps (Paré & Tumlinson, 1997; Turlings et al., 1990). Since the discovery of FACs and the isolation of the most-studied FAC, volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] from oral secretions of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Alborn et al., 1997) some progress has been made on the biosynthesis and potential physiological functions of FACs, but the ecological role in lepidopteran larvae is still not well understood (Tumlinson & Lait, 2005).

The fatty acid component of FACs derives from the ingested plant (Paré et al., 1998) while the conjugated amino acid, preferably glutamine or glutamic acid, comes from the insect (Pohnert et al., 1999). This conjugation step takes place in the insect midgut via an unknown enzyme in microsomes of the anterior midgut epithelial cells (Lait et al., 2003; Tumlinson & Lait, 2005; Yoshinaga et al., 2005). The hydrolyzing enzyme, an aminoacylase (L-ACY-1), is present in the midgut lumen and can be found in its active form in the feces of several lepidopteran larvae (Kuhns et al., 2012a; Mori et al., 2001). L-ACY-1 from *Heliothis virescens* has been cloned, heterologously expressed in *Escherichia coli* and functionally characterized (Kuhns et al., 2012b).

Despite the fitness cost due to the induction of indirect plant defenses, there may be a benefit for the insect of producing FACs. FACs have been suggested to play important roles in larval development and survival (Kuhns et al., 2012b) or to be used as biosurfactants (Collatz & Mommsen, 1974; Halitschke et al., 2001; Spiteller et al., 2000). Yoshinaga et al. (2008) investigated the fate of the FACs in the larval body by using <sup>14</sup>C-labeled glutamine, glutamic acid and linolenic acid in feeding studies with larvae of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). The authors suggested that FACs might act as a sink for glutamine by depleting glutamine in the midgut cells and further to be involved in nitrogen assimilation and to serve as a primary storage of glutamine (Yoshinaga, 2016; Yoshinaga et al., 2008). The relative contribution of these potential benefits to insect fitness has not yet been experimentally quantified.

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), and the tobacco budworm, *H. virescens* (Fabricius) (Lepidoptera: Noctuidae), are two generalist noctuid moth species belonging to the subfamily Heliothinae and are major agricultural pests on many different crop plants. (*H. virescens* has been renamed as *Chloridea virescens* Duncan and (Westwood) (Pogue, 2013) but *Heliothis* will be used here for continuity with the earlier literature.) One favorite host plant of *H. armigera* and *H. virescens* is cotton (*Gossypium* spp.), which produces gossypol, a sesquiterpene dimer that is toxic to many organisms (Gershenson & Dudareva, 2007). Previous studies have shown that gossypol toxicity is mainly due to two very reactive aldehyde groups and that negative effects of gossypol on larval growth can be eliminated when larvae were fed on a SB-gossypol, a gossypol derivative with blocked aldehyde groups (Krempl, Heidel-Fischer, et al., 2016). Further effects of gossypol on larval physiology are not known and knowledge of the molecular mechanisms underlying the biological activities of gossypol is still limited.

In this study, we detected increased FAC amounts in *H. armigera* and *H. virescens* larvae and feces after gossypol ingestion, more so in *H. armigera*. *H. armigera* larvae are more sensitive to gossypol and grow more slowly when feeding on gossypol. Ingestion of SB-gossypol did not increase FAC amounts in either species. Gossypol inhibits hydrolysis of FACs by the aminoacylase L-ACY-1 of *H. armigera*. Our results indicate a correlation between gossypol toxicity and increased FAC amounts in *H. armigera* and *H. virescens* larvae, providing new insights into the impact of ingested gossypol on heliothine larvae.

## 2 | MATERIAL AND METHODS

### 2.1 | Insects, diet preparation, and bioassay

*H. armigera* (TWB strain) were collected from Toowoomba, Queensland, Australia, in 2003. *H. virescens* (JEN strain) were collected in Clayton, North Carolina, USA, in 1988 and from North Carolina State University (NCSU) laboratory colonies. *H. armigera* was reared on artificial diet F9772 (Frontier Agricultural Services) and *H. virescens* on pinto bean diet (Barthel et al., 2016) under laboratory conditions (26°C, 55% relative humidity, 16:8 h light:dark photoperiod) in Jena, Germany. Pinto bean diet was used for feeding assays with both species.

Gossypol-supplemented diets (mixture of isomers; TimTec) were prepared as described previously (Krempl, Sporer, et al., 2016). Gossypol was dissolved in ethyl acetate (35 ml) and added to alphacel (15 g; MP Biomedicals), a nonnutritive cellulose bulk, and evaporated to dryness for 24 h. Hexane (50 ml) was added and evaporated for 36 h. Freshly prepared pinto bean diet (490 g) was cooled down to about 47°C and the gossypol-alphacel mixture was added to the diet to get final gossypol concentrations of: 3 nmol g<sup>-1</sup> (800 mg, 1.5 mmol in 490 g diet) and 6 nmol g<sup>-1</sup> (1600 mg, 3 mmol in 490 g diet), respectively.

Newly molted fifth instar *H. armigera* and *H. virescens* larvae were reared individually in 24-well plates for 2–3 days on 1 g fresh weight of pinto bean diet, each, supplemented with different gossypol concentrations (3 and 6 nmol g<sup>-1</sup>) and of control diet lacking gossypol, respectively. Forty larvae were used per treatment. Larval net weight gain was calculated by weighing the larvae before the treatment and after consuming 1 g of diet. Only larvae that consumed the complete 1 g of diet within 3 days were collected as well as their feces and further analyzed, except for *H. armigera* larvae feeding on 6 nmol g<sup>-1</sup> gossypol diet, as larvae failed to consume the entire diet within 3 days. In this case, feces and larvae were collected after 3 days. Statistical analyses were performed using R (RStudio Team 2020).

For each species, three larvae or their feces were pooled for one biological replicate. The feces and larvae were freeze-dried separately for 3 days, homogenized, and stored at -20°C. Regurgitant was collected from larvae after feeding for 3 days on the different diets using an Eppendorf pipette. The regurgitant of three larvae was pooled for one biological replicate and stored at -80°C.

In an additional feeding experiment, a subset of forty 4th instar *H. armigera* and *H. virescens* larvae was fed for 3 days on diets supplemented with gossypol or SB-gossypol (Figure S1) or on control diet. *H. armigera* larvae were fed on diets supplemented with gossypol (3 nmol g<sup>-1</sup>) or SB-gossypol (3 and 6 nmol g<sup>-1</sup>, respectively), or on control diet. *H. virescens* larvae showed higher tolerance toward gossypol in artificial diet, thus larvae were fed on diets supplemented with higher doses of gossypol (6 nmol g<sup>-1</sup>) or SB-gossypol (6 and 12 nmol g<sup>-1</sup>, respectively), or on control diet. Feces of three larvae were pooled for one biological replicate, freeze-dried for 3 days, homogenized, and stored at -20°C.

## 2.2 | Extraction and quantification of FACs in larvae, feces, and regurgitant

For the analysis of feces 10 biological replicates and for the analysis of larvae four biological replicates of gossypol-fed and control-fed larvae were used for each species. Freeze-dried material of feces or larvae, 25 mg each, were vortexed with 4 µg of the internal standard *N*-arachidonoyl-L-serine (Enzo Life Science) and with 500 µl of acetonitrile:water [50:50 (v:v)] for 5 min, followed by a centrifugation step at 3000g for 5 min at room temperature. Supernatants of feces were analyzed by LC-MS/MS via an Agilent 1200 HPLC Series coupled to an API 3200 tandem mass spectrometer (Applied Biosystems). Larval supernatants were analyzed via an Agilent 1200 HPLC Series coupled to an API 5000 tandem mass spectrometer (Supporting Information S1.1 and Table S1). Injection volumes of feces samples were 10 µl and of larval samples 2 µl.

For the analysis of regurgitant four to eight biological replicates of gossypol-fed and control-fed larvae were used for both species. Regurgitant was centrifuged at 3000g for 3 min at room temperature. The supernatant (15 µl) was added to 4 µg of the internal standard *N*-arachidonoyl-L-serine dissolved in 10 µl of DMSO and to 45 µl of methanol and was analyzed via LC-MS/MS (API 5000; Supporting Information S1.1). Injection volumes of regurgitant samples were 2 µl.

For feces analysis of the feeding experiment with SB-gossypol, eight biological replicates of SB-gossypol-fed larvae, four biological replicates of control-fed larvae, and four biological replicates of gossypol-fed larvae were used. Feces, 25 mg each, was extracted with acetonitrile:water [50:50 (v:v)] as described above and measured via an Agilent 1200 HPLC Series coupled to an API 3200 tandem mass spectrometer (Supporting Information S1.1). Injection volumes of feces samples were 10 µl.

Total FAC intensity values were calculated by summing up the intensity values of all detected FACs per sample. Statistical analyses were performed using R (RStudio Team 2020) and SigmaPlot 12.0. High-resolution MS (HRMS) was used to estimate the accurate masses of FACs (Supporting Information S1.2).

## 2.3 | Heterologous expression of the aminoacylase (L-ACY-1) of *H. armigera*, *H. virescens*, and *Spodoptera frugiperda* in insect cells

The species-specific L-ACY-1 coding sequences of *H. armigera*, *H. virescens*, and *S. frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) were purchased from GenScript and individually ligated into the pIZT/V5-His expression vector (Thermo Fisher Scientific). Each L-ACY-1 protein was transiently expressed in *S. frugiperda* cells (Sf9, Thermo Fisher Scientific) using the transfection reagent FuGENE HD (Promega). After 72 h, cells were harvested and centrifuged at 500g for 5 min at 4°C. The supernatant (culture medium) was collected and centrifuged (4000g) for 5 min at 4°C.

The culture medium of transiently transfected cells was first dialyzed against distilled water at 4°C for 24 h and then against 0.1 M potassium phosphate buffer at pH 8 using Slide-A-Lyzer Dialysis Cassettes with a 10 kDa cut-off, before being desalted with Zeba Desalt Spin Columns 7 kDa cut-off (both Thermo Scientific), according to the manufacturer's guidelines. Samples were stored at -20°C until used. Successful heterologous expression was verified via western blot using the V5-HRP antibody (1:10,000; Thermo Fisher Scientific).

## 2.4 | Enzyme activity and inhibition assay with heterologous L-ACY-1 of *H. armigera*, *H. virescens*, and *S. frugiperda*

Enzyme activity of heterologously expressed L-ACY-1 was tested using the substrate *N*-linolenoyl-L-glutamine (NLLG) by analyzing the hydrolysis product glutamine via LC/MS/MS. Either 2.5  $\mu$ l protein of L-ACY-1 of *H. armigera* or *S. frugiperda*, or 5  $\mu$ l protein of L-ACY-1 of *H. virescens* or non-transfected control cells (NTC) were incubated with 250  $\mu$ l 0.1 M sodium phosphate buffer (pH 8) and with 16  $\mu$ g of the substrate NLLG at 37°C for 3 h. The reaction was stopped with 250  $\mu$ l methanol. Inhibition of L-ACY-1 was investigated using a subset of samples that were treated as described above, with addition of 4  $\mu$ g gossypol to each reaction. All enzyme assays were performed with four replicates each. Enzyme assays were analyzed by measuring the glutamine produced by hydrolysis (in  $\mu$ M) via LC/MS/MS (QTRAP6500, tandem mass spectrometer equipped with a turbospray ion source in positive ionization mode, Supporting Information S1.3).

For quantification of the glutamine, 50  $\mu$ l of the enzyme extract was diluted with 450  $\mu$ l H<sub>2</sub>O (1:10) containing the U-<sup>13</sup>C, <sup>15</sup>N labeled algal amino acid mix (Isotec).

## 2.5 | Synthesis of NLLG

Following the protocol of Koch et al. (1999) with some modifications, triethylamine (192  $\mu$ l) and ethyl chloroformate (132  $\mu$ l) were added to a solution of linolenic acid (500 mg, 1.8 mmol) in tetrahydrofuran (14 ml) stirred at 0°C under argon atmosphere. After 5 min L-glutamine (550 mg, 3.9 mmol) dissolved in aqueous sodium hydroxide (14 ml, 0.3 N) was rapidly added and after 5 min the stirred solution was allowed to heat up to room temperature. After 30 min the reaction mixture was acidified with 2 N hydrochloric acid and extracted with ethyl acetate (3  $\times$  20 ml). The combined organic layers were dried (Na<sub>2</sub>SO<sub>4</sub>) yielding 432 mg (84.5%) light yellow crystals which were recrystallized from ethyl acetate. For details of <sup>1</sup>H NMR see Supporting Information S1.4.

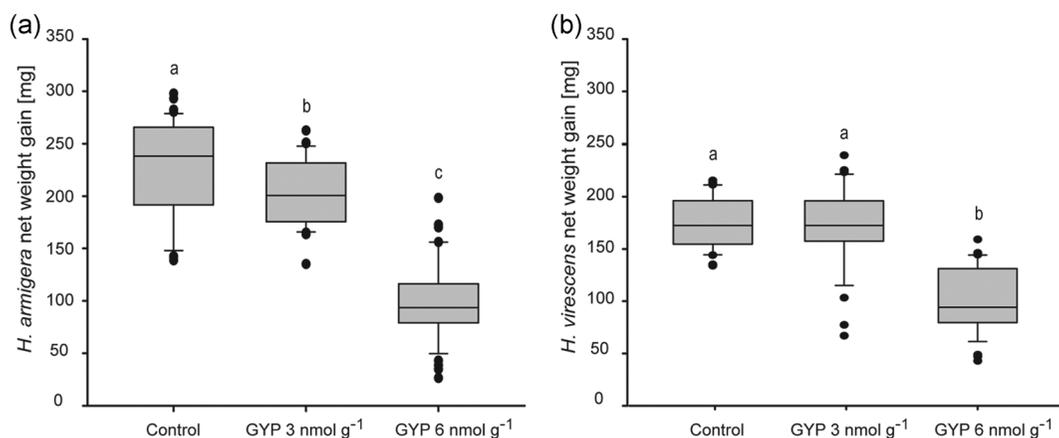
# 3 | RESULTS

## 3.1 | Growth of *H. armigera* and *H. virescens* larvae after gossypol ingestion

Newly molted fifth instar *H. armigera* larvae that fed for 3 days on diet supplemented with 3 nmol g<sup>-1</sup> gossypol gained less weight than larvae that fed on control diet (Figure 1a). This effect was even stronger when larvae fed on 6 nmol g<sup>-1</sup> gossypol supplemented diet. *H. virescens* larval growth was not inhibited by 3 nmol g<sup>-1</sup> gossypol diet, but larval net weight gain was significantly lower when larvae fed on 6 nmol g<sup>-1</sup> gossypol diet compared with larvae that fed on control diet (Figure 1b).

## 3.2 | FACs amounts in larvae, feces, and regurgitant of *H. armigera* and *H. virescens* after gossypol ingestion

Feces analysis of *H. armigera* and *H. virescens* larvae via LC-MS revealed specific compounds that were present in higher amounts when larvae fed on gossypol diet compared to control diet. These compounds were identified via high-resolution mass spectrometry as FACs. In total, eight different FACs were detected in feces and larval extracts of both species (Table 1). The relative amounts of the FACs determined in the feces of *H. armigera* and *H. virescens* are shown in Figure 2. In general, all FACs were present in the feces in higher amounts when larvae fed on the highest gossypol concentration (6 nmol g<sup>-1</sup>). *N*-linoleoyl-L-glutamine was the most abundant FAC in both species



**FIGURE 1** Net weight gain of (a) *Helicoverpa armigera* larvae and (b) *Heliiothis virescens* larvae after feeding on control diet or gossypol supplemented diet (GYP 3 and 6 nmol g<sup>-1</sup>, respectively).  $N = 40$  per treatment; ANOVA, Tukey HSD;  $p = 0.001$ . Different letters represent significant differences between treatments. ANOVA, analysis of variance; HSD, honestly significant difference

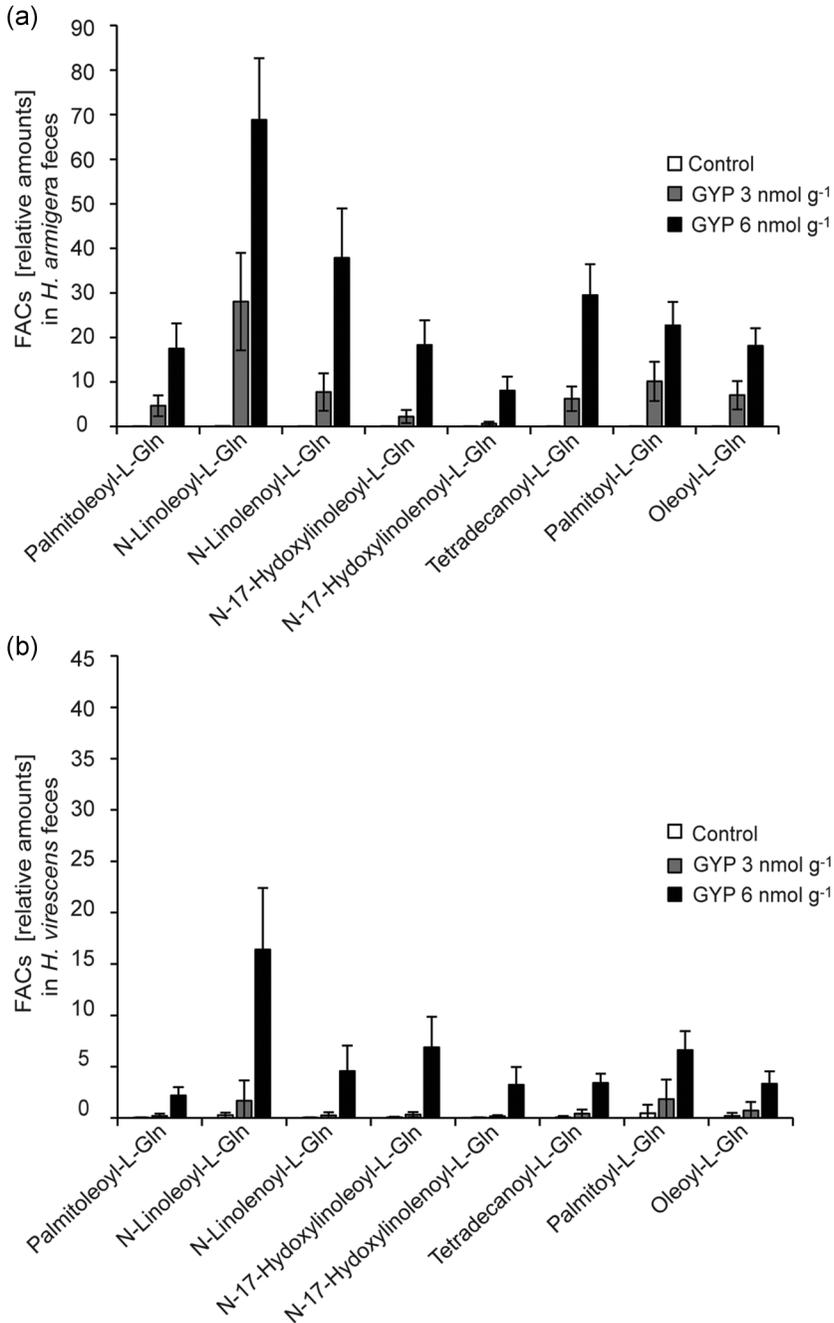
**TABLE 1** FACs identified in the feces of *Helicoverpa armigera* and *Heliiothis virescens* larvae after gossypol ingestion

FAC name	Chemical formula	M	$m/z$ M [M-H] <sup>-</sup>	calcd. $m/z$ M [M-H] <sup>-</sup>	$\Delta$ ppm
Tetradecanoyl-L-Gln	C <sub>19</sub> H <sub>36</sub> O <sub>4</sub> N <sub>2</sub>	356	355.2603	355.2602	0.32
Palmitoleoyl-L-Gln	C <sub>21</sub> H <sub>38</sub> O <sub>4</sub> N <sub>2</sub>	382	381.2763	381.2759	1.04
Palmitoyl-L-Gln	C <sub>21</sub> H <sub>40</sub> O <sub>4</sub> N <sub>2</sub>	384	383.2914	383.2915	-0.47
N-Linolenoyl-L-Gln	C <sub>23</sub> H <sub>38</sub> O <sub>4</sub> N <sub>2</sub>	406	405.2763	405.2759	0.97
N-Linoleoyl-L-Gln	C <sub>23</sub> H <sub>40</sub> O <sub>4</sub> N <sub>2</sub>	408	407.2916	407.2915	-0.2
N-Oleoyl-L-Gln	C <sub>23</sub> H <sub>42</sub> O <sub>4</sub> N <sub>2</sub>	410	409.3073	409.3072	0.32
N-17-Hydroxylinolenoyl-L-Gln	C <sub>23</sub> H <sub>37</sub> O <sub>5</sub> N <sub>2</sub>	422	421.2712	421.2708	-1.0
N-17-Hydroxylinoleoyl-L-Gln	C <sub>23</sub> H <sub>40</sub> O <sub>5</sub> N <sub>2</sub>	424	423.2865	423.2864	0.05

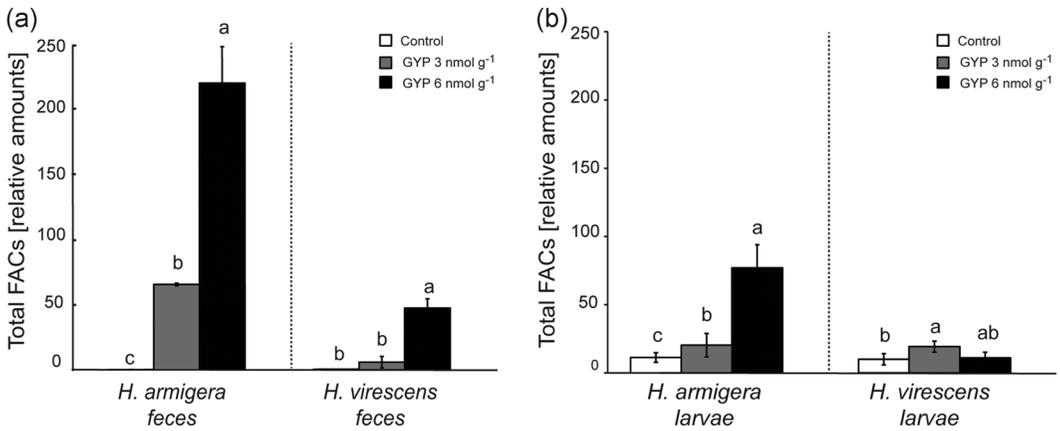
Abbreviations: calcd, calculated; FAC, fatty acid-amino acid conjugate; Gln, glutamine;  $\Delta$ ppm, difference between observed and calculated  $m/z$ .

after feeding on high gossypol concentrations (6 nmol g<sup>-1</sup>), whereas N-17-hydroxylinolenoyl-L-glutamine (volicitin) was least abundant in the feces.

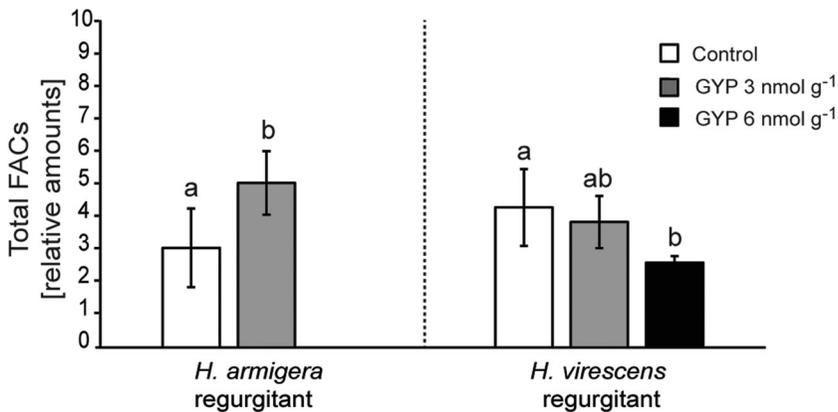
The total FAC amount was estimated by summing up relative intensity values of all eight FACs. *H. armigera* and *H. virescens* larvae showed a significant increase in the fecal FAC amount, when feeding on 6 nmol g<sup>-1</sup> gossypol diet compared to larvae fed on control diet, where both species exhibited similar low FAC amounts (Figure 3a). In general, *H. armigera* larvae excreted higher FAC amounts in feces than *H. virescens* larvae after feeding on 3 or 6 nmol g<sup>-1</sup> gossypol supplemented diet. Raising the gossypol concentration in diet from 3 to 6 nmol g<sup>-1</sup> led to an increase of the FAC amount in the feces of both species. Feces of *H. virescens* larvae showed no significant difference in FAC amounts when larvae fed on 3 nmol g<sup>-1</sup> gossypol diet compared to control diet. FACs were also detected in whole larvae of both species; the highest amounts were observed in *H. armigera* larvae, which fed on 6 nmol g<sup>-1</sup> gossypol diet (Figure 3b).



**FIGURE 2** FACs detected in (a) *Helicoverpa armigera* feces and (b) *Heliiothis virescens* feces after feeding on gossypol supplemented diet (GYP 3 nmol g<sup>-1</sup> and GYP 6 nmol g<sup>-1</sup>, respectively) or on control diet (Control). Four to twelve biological replicates, each, were used for feces analysis. Standard deviation is represented by error bars. FAC, fatty acid-amino acid conjugate



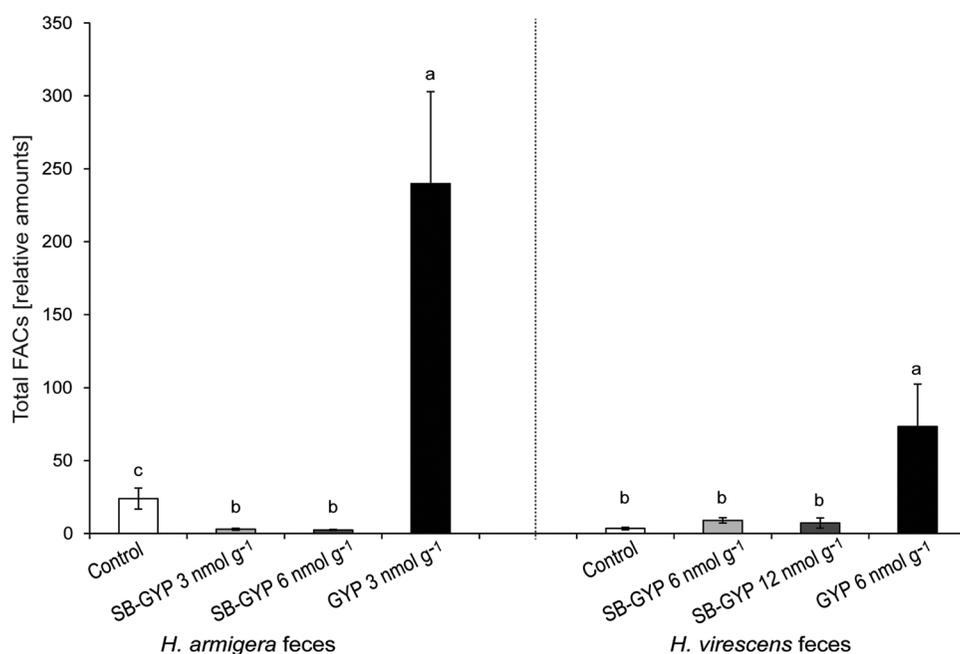
**FIGURE 3** Species-specific differences in total FAC amounts in (a) feces and (b) larvae of *Helicoverpa armigera* and *Heliiothis virescens* after feeding on gossypol diet (GYP 3 and 6 nmol g<sup>-1</sup>, respectively). Ten biological replicates, each, were used for feces analysis and four biological replicates, each, for larvae; standard deviation is represented by error bars; different letters represent significant differences between treatments. ANOVA, Tukey HSD;  $p = 0.005$ . ANOVA, analysis of variance; FAC, fatty acid-amino acid conjugate; HSD, honestly significant difference



**FIGURE 4** Total FAC amounts in regurgitant of *Helicoverpa armigera* and *Heliiothis virescens* larvae after feeding on gossypol supplemented diet (GYP 3 and 6 nmol g<sup>-1</sup>, respectively) or control diet (Control). As *H. armigera* larvae feeding on 6 nmol g<sup>-1</sup> gossypol diet failed to consume the whole amount, no regurgitant was collected from these larvae. Four to eight biological replicates each were used; ANOVA, Tukey HSD;  $p = 0.05$ . Different letters represent significant differences between treatments. Standard deviation is represented by error bars. ANOVA, analysis of variance; FAC, fatty acid-amino acid conjugate; HSD, honestly significant difference

Besides larvae and feces, the regurgitant of larvae was also analyzed for the presence of FACs. Significantly higher FAC amounts were detected in regurgitant of *H. armigera* larvae that fed on gossypol diet (3 nmol g<sup>-1</sup>) compared with larvae fed on control diet. In contrast, *H. virescens* larvae showed similar FAC amounts when fed on 3 nmol g<sup>-1</sup> gossypol diet or on control diet, while FAC amounts were lower in larvae that fed on 6 nmol g<sup>-1</sup> gossypol diet compared with control diet (Figure 4).

*H. armigera* as well as *H. virescens* larvae that fed on SB-gossypol diet did not show elevated FAC amounts in feces compared with larvae on gossypol diet (Figure 5).



**FIGURE 5** Total FAC amounts in *Helicoverpa armigera* and *Heliothis virescens* feces after feeding on SB-gossypol (SB-GYP 3, 6, and 12 nmol g<sup>-1</sup>, respectively), gossypol supplemented diet (GYP 3 and 6 nmol g<sup>-1</sup>, respectively) or control diet. Four biological replicates, each, were used for feces analysis; data were log-transformed; ANOVA, Tukey HSD;  $p = 0.05$ . Different letters represent significant differences between treatments. Standard deviation is represented by error bars. ANOVA, analysis of variance; FAC, fatty acid-amino acid conjugate; HSD, honestly significant difference

### 3.3 | Activity and inhibition assay of heterologous aminoacylase (L-ACY-1)

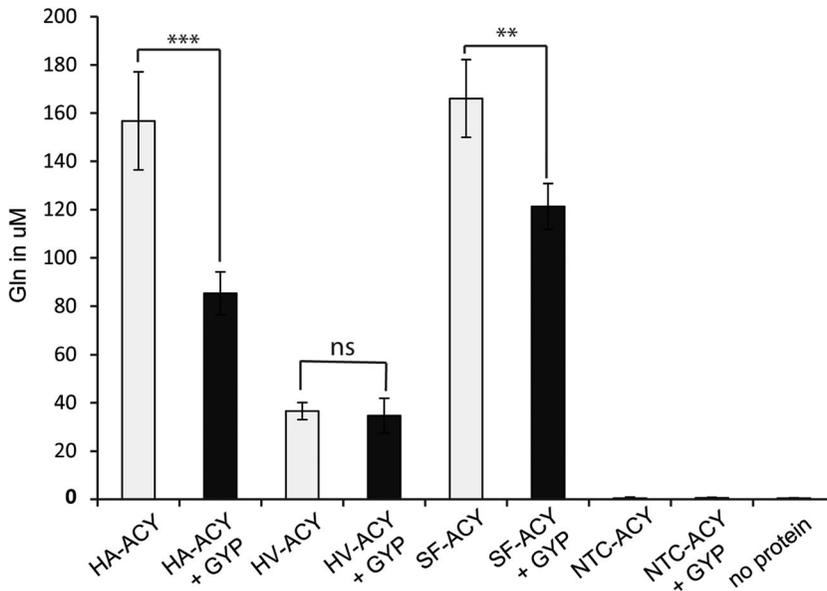
The candidate proteins for FAC hydrolysis, L-ACY-1, of *H. armigera* (HA-ACY), *H. virescens* (HV-ACY) and a third noctuid moth species, *S. frugiperda* (SF-ACY) were transiently expressed in Sf9 cells with a carboxyl-terminal tag allowing their detection by western blot. HA-ACY and SF-ACY showed stronger band intensities on the western blot than heterologous HV-ACY (Figure S2).

Enzyme activity was analyzed by measuring the hydrolysis of glutamine after a 3-h incubation time. All three heterologously expressed HA-ACY, HV-ACY, and SF-ACY were shown to be active enzymes, hydrolyzing glutamine from the substrate *N*-linolenoyl-L glutamine. In assays with HV-ACY lower glutamine amounts were measured compared with HA-ACY and SF-ACY, likely due to the different protein amounts seen in the western blot.

To test whether L-ACY-1 can be inhibited by gossypol, protein samples were incubated with 4 μg gossypol. The hydrolysis activity was significantly inhibited by gossypol in the case of HA-ACY (45%) and SF-ACY (20%), while HV-ACY was not inhibited at the gossypol concentration used and showed no significant reduction of FAC hydrolysis to glutamine (Figure 6).

## 4 | DISCUSSION

The presence of FACs in the oral secretions and midgut of lepidopteran larvae is puzzling at many levels. Since the fatty acid originates from the plant food and the amino acid from the insect (Paré et al., 1998), the FACs would appear to be the ideal signal to distinguish herbivory from other insults to the plant tissue. However, the insect's



**FIGURE 6** Activity and inhibition of heterologously expressed *Helicoverpa armigera* ACY (HA-ACY), *Heliothis virescens* (HV-ACY), *Spodoptera frugiperda* (SF-ACY) aminoacylase and non-transfected control cells (NTC). The hydrolysis of *N*-linolenoyl-L-glutamine (16  $\mu$ g) to glutamine (Gln) is shown after a 3-h incubation without (white bars) or with gossypol (GYP, 4  $\mu$ g; black bars). Error bars represent the standard deviation among four replicates, each. Significant differences are shown by asterisks, \*\* $p < 0.01$  \*\*\* $p < 0.001$ , *t* test; not significant (ns)

strategy of making a conjugate that decreases its own fitness defies evolutionary interpretation. Although FACs can specifically upregulate direct and indirect plant defenses (Hettenhausen et al., 2014), the mechanism is still unknown. The conjugating enzyme in the insect is still unknown (Lait et al., 2003), although one active hydrolase has been identified (Kuhns et al., 2012b). A microbial source for the conjugating and hydrolyzing activities has been suggested (Ping et al., 2007), but its contribution relative to that of the insect's own contribution has not been measured. The balance between conjugation and hydrolysis in the insect seems to be important, and an interesting hypothesis has been advanced concerning nitrogen assimilation (Yoshinaga et al., 2008) but the fitness benefit to the insect has not yet been experimentally measured. Yet the fitness cost to the insect in terms of increased parasitism as a result of FAC-stimulated volatile release by the plant has been experimentally verified (Turlings et al., 1993). Our finding that gossypol increases FACs amounts by inhibiting the aminoacylase adds another level of complexity to the story.

Gossypol is well-known to inhibit insect growth, yet certain specialists can tolerate low amounts and even grow faster with very low amounts, a nonlinear response known as hormesis (Celorio-Mancera et al., 2011). *H. virescens* appears to tolerate gossypol better than *H. armigera*; possibly by higher excretion as previously shown (Krempl, Heidel-Fischer, et al., 2016). In addition, the present study showed that the aminoacylase of *H. virescens* was significantly less inhibited by gossypol, which could explain the decrease of FACs in the regurgitant with increasing gossypol concentration in contrast to the opposite response by *H. armigera* (Figure 4). Previous feeding experiments with SB-gossypol in which the aldehyde groups are blocked by Schiff bases with *n*-butylamine produced no growth inhibition; Our feeding study indicates that there is no aminoacylase inhibition by SB-gossypol as well, implicating the aldehyde groups and not the phenolic groups of gossypol as involved in both growth inhibition and aminoacylase enzyme inhibition.

Gossypol caused a striking increase of several FACs in the feces, but not the regurgitant. Therefore if most of the induction of plant secondary defenses occurs through reflux of gut contents contacting the leaf surface (Peiffer & Felton, 2009; Truitt & Paré, 2004; Vadassery et al., 2012), the insect may be mitigating the effect of

increased FACs by sequestering them in the peritrophic matrix and excreting them (Barbehenn, 2001). This assumes that contact of feces to other plant parts has a lesser or negligible effect on induction. As the food bolus passes through the midgut, the increased FACs amounts could be available to participate in the proposed nitrogen-assimilation cycle (Yoshinaga et al., 2008), which might benefit the insect when nitrogen was limiting. To our knowledge, the affinity of the peritrophic matrix for FACs or gossypol has not been measured.

If there is an optimal level of FACs it may vary for different insect species, and be controlled by several different factors. A comparison of the hostplant generalist *H. armigera* and the related Solanaceae specialist *H. assulta* showed generally higher mRNA levels of L-ACY-1 in the latter species, with divergent transcriptional responses to the plant allelochemicals gossypol, capsaicin, or nicotine, although aminoacylase activity and FACs were not directly measured in this study (Cheng et al., 2017). A comparison of three other heliothines showed that L-ACY-1 transcript abundance was highest in the *Physalis* specialist *Heliothis subflexa*, intermediate in the generalist *H. virescens* and lowest in the generalist *Helicoverpa zea* (Kuhns et al., 2012a). This study also measured L-ACY-1 protein abundance with antibodies and aminoacylase activity with an in-gel assay. However, direct measurement of FACs in *H. virescens* and *H. zea* showed that they were nearly the same (Mori et al., 2001). There are probably too few species for a convincing demonstration of a correlation between whether FACs are used for nitrogen assimilation by specialists, versus removal of excess nitrogen in generalists as has been suggested (Cheng et al., 2017). Conjugase activity by an insect enzyme that is still unknown will also play a role in the balance, as well as a possible contribution from the microbiota in the midgut. Discovery of the unknown conjugase would be an important step in understanding why the insect synthesizes a potent elicitor of plant defenses against it.

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**Corinna Krempf:** conceptualization (lead); formal analysis (lead); investigation (lead); methodology (lead); writing original draft (lead). **Nicole Joussem:** Conceptualization (supporting); review and editing (equal). **Michael Reichelt:** data curation (supporting); formal analysis (supporting); methodology (supporting); writing review & editing (supporting). **Marco Kai:** data curation (supporting); methodology (supporting); writing review & editing (supporting). **Heiko Vogel:** conceptualization (supporting); resources (supporting); writing review & editing (equal). **David G. Heckel:** conceptualization (supporting); funding acquisition (lead); resources (lead); writing review & editing (equal).

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