

## RESEARCH ARTICLE

# Effects of class-specific, synthetic, and natural proteinase inhibitors on life-history traits of the cotton bollworm *Helicoverpa armigera*

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**Abstract**

Herbivorous insects have more difficulty obtaining proteins from their food than do predators and parasites. The scarcity of proteins in their diet requires herbivores to feed voraciously, thus heavily damaging their host plants. Plants respond to herbivory by producing defense compounds, which reduce insect growth, retard development, and increase mortality. Herbivores use both pre- and postdigestive response mechanisms to detect and avoid plant defense compounds. Proteinase inhibitors (PIs) are one example of plant compounds produced as a direct defense against herbivory. Many insects can adapt to PIs when these are incorporated into artificial diets. However, little is known about the effect of PIs on diet choice and feeding behavior. We monitored the diet choice, life-history traits, and gut proteinase activity of *Helicoverpa armigera* larvae using diets supplemented with synthetic and natural PIs. In choice experiments, both neonates and fourth-instar larvae preferred the control diet over PI-supplemented diets, to varying degrees. Larvae that fed on PI-supplemented diets weighed less than those that fed on the control diet and produced smaller pupae. Trypsin-specific PIs had a stronger effect on mean larval weight than did other PIs. A reduction of trypsin activity but not of

**Abbreviations:** LBTI, lima bean trypsin inhibitor; PI, proteinase inhibitor; PMSF, phenyl methyl sulfonyl fluoride; SKTI, soybean Kunitz trypsin inhibitor; TLCK, N-alpha-Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, N-Tosyl-L-phenylalanine chloromethyl ketone.

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chymotrypsin activity was observed in larvae fed on PI-supplemented diets. Therefore, behavioral avoidance of feeding on plant parts high in PIs could be an adaptation to minimize the impact of this plant's defensive strategy.

#### KEYWORDS

feeding choice, *Helicoverpa armigera*, protease inhibitor, proteinase activity, proteinase inhibitor

## 1 | INTRODUCTION

Polyphagous insects, such as the cotton bollworm *Helicoverpa armigera*, are responsible for the destruction of up to 14% of crops worldwide, causing billions of dollars of economic losses (Czepak, Albernaz, Vivan, Oliveira, & Carvalhais, 2013; Kriticos et al., 2015; Nicholson, 2007; Zalucki et al., 1994). Several measures have been established to try to control *H. armigera*, either by using chemical and biological pesticides or by generating transgenic crop cultivars that produce high levels of plant defense compounds (Downes et al., 2017). Plant-derived proteinase inhibitors (PIs) represent potential candidates for deployment in such transgenic crops because plants naturally produce them when attacked by herbivorous insects (Gatehouse et al., 1994; Gatehouse et al., 1997; Green & Ryan, 1972; Zhu, Abel, & Chen, 2007).

When ingested by insects, PIs competitively inhibit digestive proteinases present in the insect gut, thus preventing dietary proteins from being broken down into amino acids; this delays the insects' development and may eventually cause mortality (Duffey & Stout, 1996; Johnston, Gatehouse, & Anstee, 1993; Ryan, 1990). In the laboratory, insects reared on PI-supplemented artificial diets were found to grow slowly, their development was delayed and their mortality was increased (Gatehouse & Boulter, 1983; Green & Ryan, 1972). However, several studies have found that the growth of insects fed on PI-supplemented diets was not reduced (Bolter & Jongsma, 1995; Bown, Wilkinson, & Gatehouse, 2004; Brioschi et al., 2007; Jongsma & Beekwilder, 2011; Jongsma & Bolter, 1997; Jongsma, Bakker, Peters, Bosch, & Stiekema, 1995; Yang, Fang, Dicke, van Loon, & Jongsma, 2009; Zhu-Salzman & Zeng, 2008). In addition, these studies suggested the possibility that insects could produce PI-insensitive proteinases (Ahn, Salzman, Braunagel, Koiwa, & Zhu-Salzman, 2004; Bown et al., 2004; Zhu-Salzman, Koiwa, Salzman, Shade, & Ahn, 2003). Despite these observations, there is little information on whether insects can discriminate between diets that contain PIs and those that do not. Such knowledge will become important in the context of the development of transgenic crops that over-express PIs. Insects can be selective at the microscale and perhaps can feed selectively on plant parts with reduced PI content.

Herbivorous insects use physiological as well as behavioral mechanisms to cope with plant defense compounds. Before ingestion occurs, insects use olfactory and gustatory receptors which respond selectively and immediately to noxious secondary compounds; after compounds have been ingested, it can take up to several minutes for the insects to detect such noxious compounds. For example, when fall armyworm caterpillars (*Spodoptera frugiperda*) were given a diet containing indole 3-carbinol, at first they fed vigorously, then after 2–3 min they stopped feeding and remained motionless: a postingestive effect rather than a preingestive one (Glendinning, 2002; Glendinning & Slansky, 1995).

The ability of larvae to avoid toxic diets is well known, especially diets containing Cry toxins derived from the bacterium *Bacillus thuringiensis* (Bt). *H. armigera*, *Helicoverpa zea*, and *Heliothis virescens* are known to selectively feed on nontreated diet over Bt toxin-treated diets (Gore, Adamczyk, & Blanco, 2005; Luong, Zalucki, Perkins, & Downes, 2018). *H. virescens* larvae avoided feeding on diets containing Bt-formulation and Cry toxin when they had the choice between these and a control diet (Gould, Anderson, Landis, & Van Mellaert, 1991). Similarly, *Danaus plexippus* was able to detect and avoid Cry1Ab-expressing corn anthers and pollen in a petri dish (Anderson,

Hellmich, Prasifka, & Lewis, 2005). Such selective feeding behaviors allow insects to avoid noxious compounds, and ultimately these behaviors contribute to insects' survival.

Previous studies have addressed many aspects of diet choice and the feeding behavior of lepidopteran larvae in response to insecticides, Bt-incorporated artificial diets or transgenic crops. However, little is known about the food preferences of caterpillars or their responses to PIs, especially class-specific synthetic and natural PIs. Here, we conducted assays to determine the effect of PIs on life-history traits and proteinase activity of *H. armigera* larvae. The two main classes of digestive proteinases in the larval midgut are trypsins, which cleave peptide chains at the carboxyl side of lysine or arginine, and chymotrypsins, which cleave at the large hydrophobic amino acids tyrosine, tryptophan, or phenylalanine. Two proteinaceous PIs studied here were derived from plants: SKTI (soybean Kunitz trypsin inhibitor) and LBTI (lima bean trypsin inhibitor). Despite the name, LBTI has both trypsin inhibitor and chymotrypsin inhibitor domains. A third trypsin inhibitor is TLCK (N-alpha-Tosyl-L-lysine chloromethyl ketone hydrochloride). TPCK (N-Tosyl-L-phenylalanine chloromethyl ketone) specifically inhibits chymotrypsins due to its phenylalanine. PMSF (phenylmethylsulfonyl fluoride) is a broad-spectrum inhibitor of serine hydrolases, including trypsins and chymotrypsin but also acetylcholinesterase and even cysteine proteases. We found that although trypsin activity was inhibited more than chymotrypsin activity, patterns of avoidance and growth inhibition depended on the type of inhibitor. Consumption rates decreased for some PIs and increased for others, indicating that larval behavioral responses to the diet may modulate exposure to harmful substances.

## 2 | MATERIALS AND METHOD

### 2.1 | Insect culture and diet

*H. armigera* was collected from Toowoomba, Australia in 2003 and supplemented with fresh field-collected insects every 2 or 3 years. To reduce inbreeding, the colony was maintained via 48 single-pair matings per discrete generation, with equal contribution of each fertile couple to the next generation, and avoidance of brother-sister matings. Larvae were individually reared to avoid cannibalism. Neonates were assigned to treatments immediately after hatching. Dietary constituents were (per liter of diet): 15 g agar (Carl Roth GmbH, Germany), 0.5 g ascorbic acid, 1.25 g sorbic acid, 1.75 g methyl-4-hydroxybenzoate, 14.4 g vitamin mix (Vanderzant vitamin mixture; Sigma), 1 g tetracycline (Sigma), 70 g wheat germ powder, 54 g sucrose, 10 g cellulose, 54 g casein, 14.4 g Wesson salt mixture, 1.13 ml linseed oil, 0.5 ml formaldehyde (37%), and distilled water. Insects were reared at 28–30°C with 60% relative humidity and 16 hr daylight in a walk-in chamber.

### 2.2 | Diet choice and larval growth

Neonates and fourth-instar larvae were used for choice assays. Fourth-instar larvae were raised on the control diet before the assays. Individual 20 ml plastic cups contained one 5 × 5 mm cube of the control diet and one equal-sized cube of the diet supplemented with 0.25% wt/vol inhibitor, placed 10 mm apart. Larvae were placed in the middle of the cup, between the two diet cubes. Inhibitors used were SKTI (T9128; Sigma), LBTI (T9378; Sigma), TPCK (T4376; Sigma), TLCK (T7254; Sigma), and PMSF (P7626; Sigma). SKTI was further purified following Kuwar, Pauchet, Vogel, and Heckel (2015). Fourth-instar larvae were observed after 12 and 24 hr, when fresh diet cubes were added, whereas neonates were observed after 12, 24, 48, and 72 hr. Contingency tables reporting the distribution of larvae on the control diet, PI diet, or off the diet were analyzed using G-tests with continuity correction (Sokal & Rohlf, 1969) with formulas implemented in Excel.

In parallel, fourth-instar larvae (N = 30) were reared in individual cups on either PI-containing diet or the control diet to measure their growth. Larvae were weighed every 24 hr and at pupation. Weights were plotted against time, and a one-way analysis of variance (ANOVA) was conducted to compare the effect of PIs on larval weight gain and

pupal weight, followed by post hoc comparisons of means using the Tukey honest significant difference (HSD) test in SPSS version 17.0 (SPSS, Inc., Chicago, IL).

## 2.3 | Effect of PIs on growth and diet consumption

Larvae were reared to the fourth instar on wheat germ diet and then transferred to individual plastic cups containing fresh diet (control) or a diet supplemented with PIs (treatments). Larvae ( $N = 12$ ) were weighed before being transferred to the diets and after 12 and 24 hr. The growth rate and percentage of diet consumed were calculated. The larval growth rate was calculated using the formula “growth rate =  $(\log_{10} [\text{final weight}] - \log_{10} [\text{initial weight}]) / \text{time (days)}$ ”. The amount of diet consumed was measured by weighing the diet cube before and after feeding and using the formula “% diet consumption =  $([\text{initial weight} - \text{final weight}] / \text{initial weight}) \times 100$ ”. Mean growth rate and percentage of diet consumed were plotted against time, and a one-way ANOVA was conducted to compare the effect of PIs on the growth rate, and percentage of diet consumed; followed by post hoc comparisons of means using the Tukey HSD test using SPSS.

## 2.4 | Preparation of gut lumen samples

Larvae were chilled on ice and dissected longitudinally on the dorsal side; midgut lumen contents with the peritrophic matrix were removed and stored in 500  $\mu\text{l}$  of ice-cold 0.1 M Tris-HCl, pH 7.8. Lumen samples were then homogenized with a hand-held pestle and centrifuged at 13,000g for 30 min at 4°C, and the supernatant was carefully removed and saved for enzyme assays. Four biological replicates were prepared by pooling gut lumen from three individuals per replicate.

## 2.5 | Proteinase activity assays

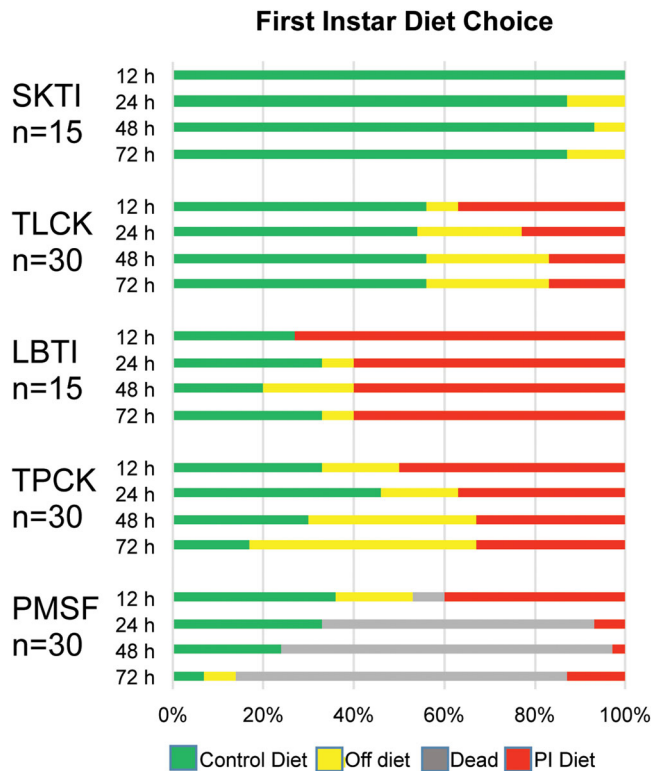
Trypsin and chymotrypsin activities were assayed using a modified protocol (Bian, Shaw, Han, & Christeller, 1996) in a total volume of 100  $\mu\text{l}$  in wells of a microtiter plate. Assays contained 85  $\mu\text{l}$  of 0.1 M Tris-HCl pH 7.8, 5  $\mu\text{l}$  of lumen sample and 10  $\mu\text{l}$  of *N*-benzoyl-DL-arginine-*p*-nitroanilide and *N*-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide as specific substrates for trypsin and chymotrypsin, respectively. Substrates were prepared in dimethyl sulfoxide; final concentrations in the reactions were 2 and 0.2 mM, respectively. When required, PIs (1  $\mu\text{M}$ ) were preincubated with the gut extract at 30°C for 10 min before the addition of the substrate. The optical density (OD) at 410 nm was monitored on a TECAN infinite M200 plate reader (Tecan Group Ltd.) to follow the release of *p*-nitroanilide. The microtiter plate was incubated at 37°C, and absorbance was measured every 5 min for trypsin activity and every 2 min for chymotrypsin activity. Appropriate blanks were used, and care was taken to ensure that the substrate concentration was not limiting. Enzymatic activities were expressed in units per microgram of total protein measured by the Bradford assay. One proteinase unit was defined as the amount of enzyme that liberates 1 mmol of *p*-nitroanilide from the substrate per minute in the given assay conditions.

Overall proteinase activity was assayed using a modified protocol (Parde, Sharma, & Kachole, 2010) in a total volume of 300  $\mu\text{l}$  in a microfuge tube. Assays were performed in 90  $\mu\text{l}$  of 200 mM glycine-NaOH buffer pH 10, and 200  $\mu\text{l}$  of 1% azocasein was used as a substrate. Ten microliters of lumen sample was used. Reactions were incubated at 37°C for 30 min in a thermomixer, stopped by adding 5% trichloroacetic acid, allowed to stand at room temperature for 30 min, then centrifuged at 9,300g for 10 min. Aliquots of 100  $\mu\text{l}$  were taken from the supernatant, transferred in a microtiter plate and mixed with an equal volume of 1 N sodium hydroxide. OD was measured at 450 nm. Enzyme activities were expressed in units per microgram of proteins measured by the Bradford assay. One proteinase unit was defined as the amount of enzyme that increases the absorbance by one OD per minute in the given assay conditions. One-way ANOVAs followed by post hoc comparisons of means using the Tukey HSD test in SPSS were used to compare the treatments.

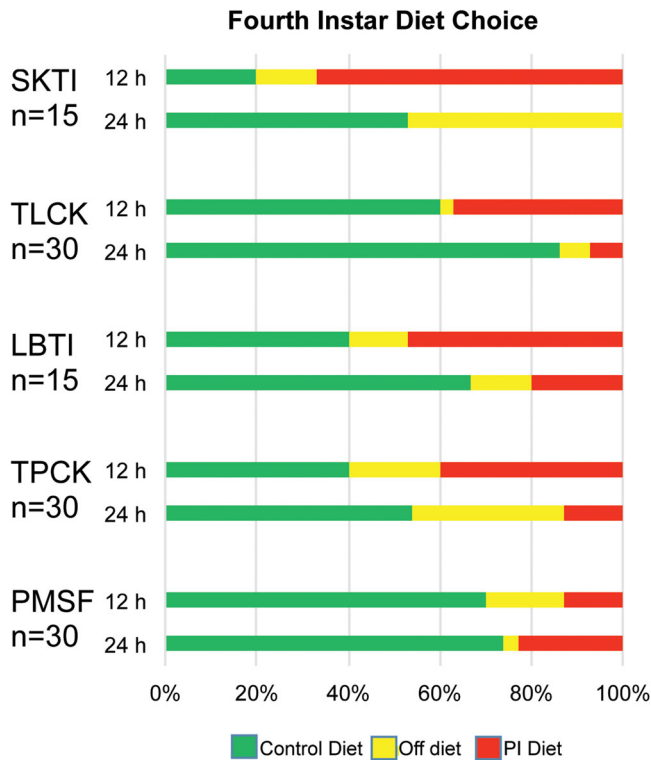
### 3 | RESULTS

#### 3.1 | Diet choice

To investigate postingestive effects of PIs, we allowed *H. armigera* larvae to choose among diet cubes with or without PIs (Figure 1). In this figure and the following, treatments appear in the following order: the proteinaceous trypsin inhibitor SKTI and the chemical trypsin inhibitor TLCK, the bifunctional LBTI and the chemical chymotrypsin inhibitor TPCK, then the general serine hydrolase inhibitor PMSF. Neonate mortality on PMSF was significantly higher than in all the other treatments, (G adj = 74.04;  $df = 1$ ;  $p < .0001$ ), with 60% already dead by 24 hr. The high potency of PMSF rendered preference comparisons irrelevant, as a lethal dose could be ingested even with a short exposure to the toxin-treated diet. Neonates avoided SKTI significantly more than the other PIs (G adj = 61.48;  $df = 16$ ;  $p < .0001$ ), no larvae were seen resting on the SKTI diet. Neonate responses to LBTI, TLCK, and TPCK were not homogeneous (G adj = 37.93;  $df = 24$ ;  $p < .035$ ); however, no pairwise comparison of these three was significantly different at  $p = .05$ . Neonates showed a slight preference for LBTI over control diet, and LBTI and TLCK were slightly more different (G adj = 23.12;  $df = 16$ ;  $p > .11$ ) than TLCK and TPCK (G adj = 16.99;  $df = 16$ ;  $p > .18$ ) or LBTI and TPCK (G adj = 17.09;  $df = 16$ ;  $p > .38$ ). Preference was correlated with the type of inhibitor: trypsin-specific PIs (SKTI and TLCK) were chosen less, but the chymotrypsin-inhibiting PI (TPCK) and the two-domain trypsin/chymotrypsin PI (LBTI) were chosen more.



**FIGURE 1** Effect of PIs on the diet choice of *Helicoverpa armigera* neonates. Bars represent the occurrence of neonates feeding on either PI-supplemented (red) or PI-free diet (green) or off-diet (yellow) at the postrelease intervals. Mortality (gray bars) was only seen in the PMSF treatment. LBTI, lima bean trypsin inhibitor; PI, proteinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; SKTI, soybean Kunitz trypsin inhibitor; TLCK, *N*-alpha-Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, *N*-Tosyl-L-phenylalanine chloromethyl ketone

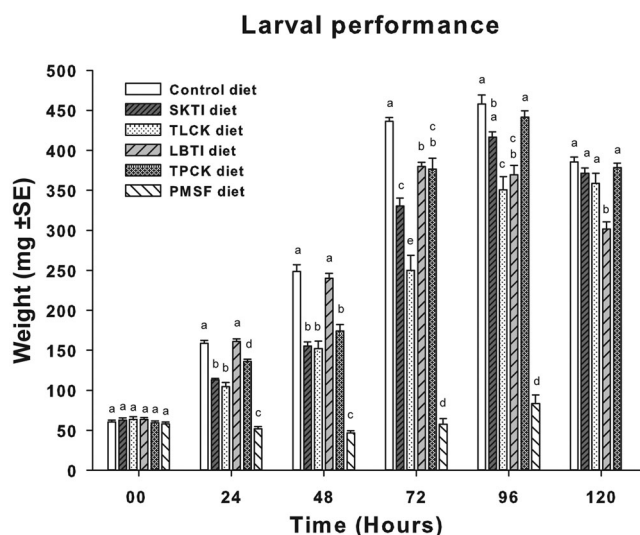


**FIGURE 2** Effect of PIs on the diet choice of fourth-instar *H. armigera* larvae. Bars represent the occurrence of fourth-instar larvae either on PI-supplemented (red) or PI-free diet (green) or off-diet (yellow) at 12 and 24 hr postrelease. LBTI, lima bean trypsin inhibitor; PI, proteinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; SKTI, soybean Kunitz trypsin inhibitor; TLCK, N-alpha-Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, N-Tosyl-L-phenylalanine chloromethyl ketone

Fourth-instar larval responses to the five PIs (Figure 2) were not homogeneous ( $G_{adj} = 42.09$ ;  $df = 20$ ;  $p < .0026$ ). PMSF diet was significantly less preferred than the SKTI diet ( $G_{adj} = 26.40$ ;  $df = 8$ ;  $p < .0009$ ) but unlike neonates, there was no mortality on PMSF. Fourth-instar larvae generally consumed the cube of PI-supplemented diet after consuming the cube of the control diet, but the numbers resting on one or the other were not significantly different for TPCK, TLCK, and LBTI ( $G_{adj} = 14.26$ ;  $df = 12$ ;  $p > .28$ ).

### 3.2 | Effect of PIs on larval growth

To compare the effect of class-specific synthetic and natural PIs on larval growth, we fed fourth-instar *H. armigera* larvae wheat germ diet with or without PIs (Figure 3). PMSF immediately and completely arrested larval growth, and 29 out of 30 larvae had died by 96 hr. The other PIs inhibited growth to a lesser degree and did not cause mortality. SKTI, TLCK, and TPCK inhibited growth at earlier times, but accelerated growth rates caused larval weights to equal control weights by 96 hr (SKTI, TPCK) or 120 hr (TLCK). LBTI did not inhibit growth initially, but by 72 hr and afterward, LBTI weights remained significantly less than control weights. Compared to the chymotrypsin-specific TPCK and to the trypsin/chymotrypsin inhibitor LBTI, the trypsin-specific SKTI and TLCK initially had a stronger inhibitory effect on growth, but this inhibition was reversed and completely compensated for after 48 hr.



**FIGURE 3** Effect of PI-supplemented diets on the growth of fourth-instar *H. armigera* larvae. Net weights of larvae fed either on a control diet or PI-supplemented diets are plotted against time. The bars represent the mean  $\pm$  SE. Values followed by the different small letters were significantly different across diets within the same time point by the Tukey honest significant difference test ( $p < .05$ ). LBTI, lima bean trypsin inhibitor; PI, proteinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; SE, standard error; SKTI, soybean Kunitz trypsin inhibitor; TLCK, N-alpha-Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, N-Tosyl-L-phenylalanine chloromethyl ketone

### 3.3 | Effect of PIs on growth rate and diet consumption

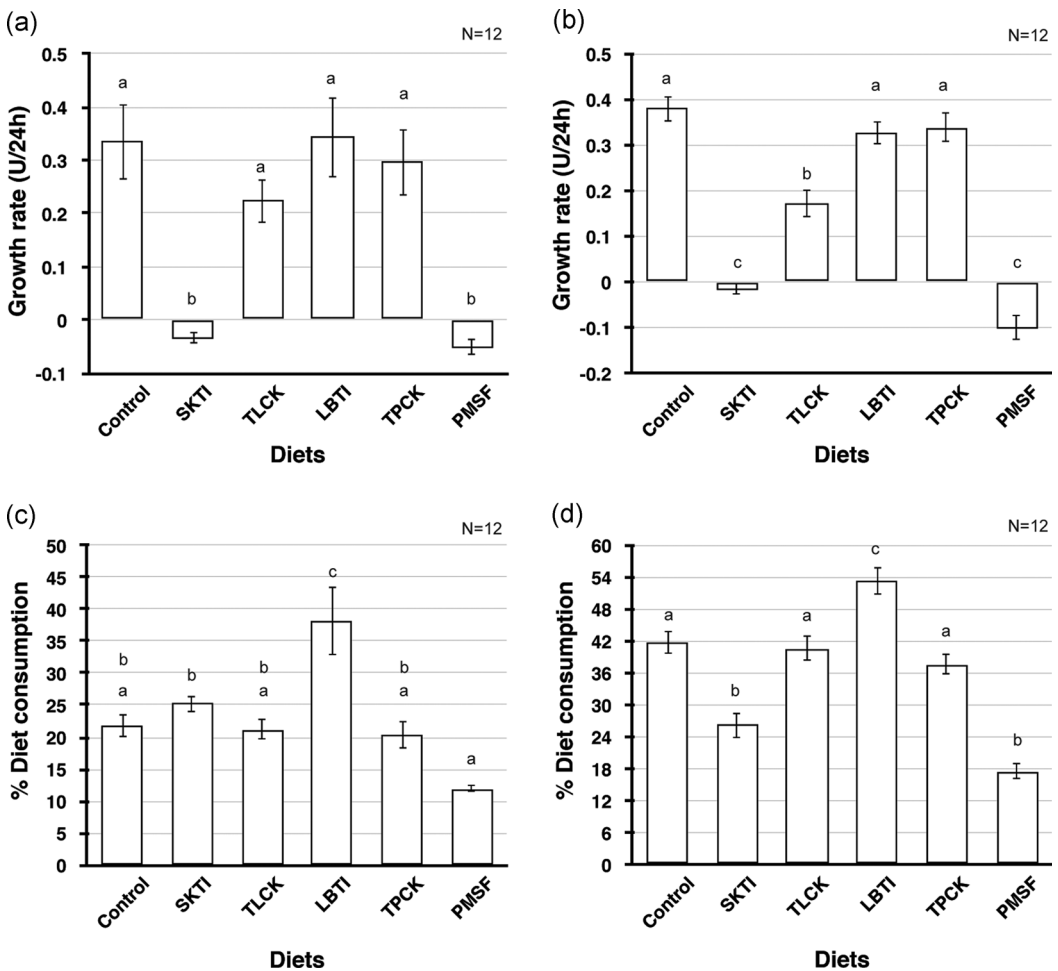
To obtain insight into the effect of PIs on assimilation efficiency, we measured diet consumption and larval growth rates of fourth-instar larvae feeding on wheat germ diets, with or without PIs (Figure 4). Larvae exposed to PMSF in diet ate very little and had greatly depressed growth. LBTI caused insignificant decreases in larval growth rate but significant increases in diet consumption. Larvae feeding on TPCK had insignificant decreases in both growth rate and diet consumption. TLCK gave the same response but the growth rate had significantly declined by 24 hr. SKTI halted larval growth despite maintaining consumption at the same (12 hr) or slightly reduced (24 hr) amounts. In general, the presence of PIs in the diet reduced larval growth rates but increased or decreased the amount of diet consumed, depending on the PI. Only for LBTI did an increase in consumption compensate for the inhibition, resulting in a steady growth rate.

### 3.4 | Effect of PIs on pupal weights

To analyze how feeding on PI-supplemented diets as larvae affected pupal weight, pupae from the larval growth experiments were weighed after every 24 hr. No larvae survived to pupation in the PMSF treatment. We observed significantly reduced pupal weights for larvae that fed on LBTI- and TLCK-supplemented diets compared to larvae that fed on the control diet at all time points (Figure 5). We also observed a significant reduction of pupal weight for larvae fed on the SKTI-supplemented diet after the fourth day of pupae formation.

### 3.5 | Effect of PIs on gut proteinase activity

To investigate potential postingestion effects of PIs on proteinase activity and the sensitivity of such effects to class-specific proteinases, we measured trypsin, chymotrypsin, and total proteinase activity in the gut extract of

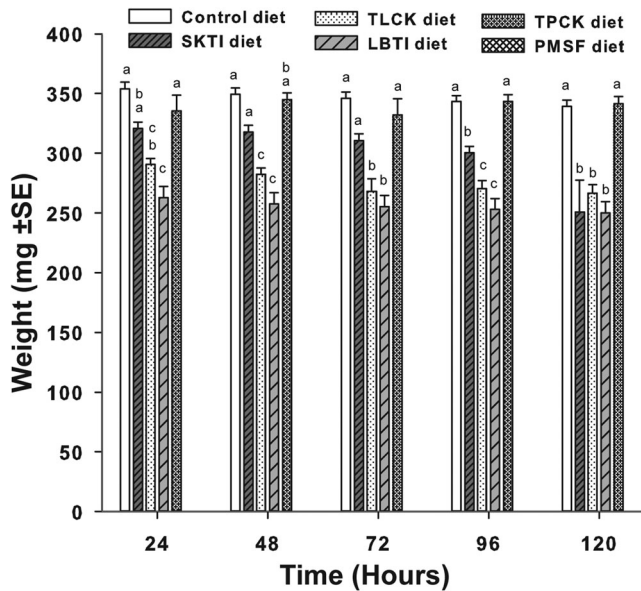


**FIGURE 4** Effect of PI-supplemented diets on the growth rate and diet consumption of fourth-instar *H. armigera* larvae. Bar graph represents the mean growth rate and means percentage diet consumption for larvae feeding either on a control diet or PI-supplemented diets. (a) 12-hr growth rate; (b) 24-hr growth rate; (c) 12-hr diet consumption; (d) 24-hr diet consumption. The bars represent the mean  $\pm$  SE. Values followed by the different small letters (at least one) were significantly different by Tukey honest significant difference test ( $p < .05$ ). For growth rate, one unit is the difference in the  $\log_{10}$  of (final weight)  $-\log_{10}$  (initial weight). LBTI, lima bean trypsin inhibitor; PI, proteinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; SE, standard error; SKTI, soybean Kunitz trypsin inhibitor; TLCK, *N*-alpha-Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, *N*-Tosyl-L-phenylalanine chloromethyl ketone

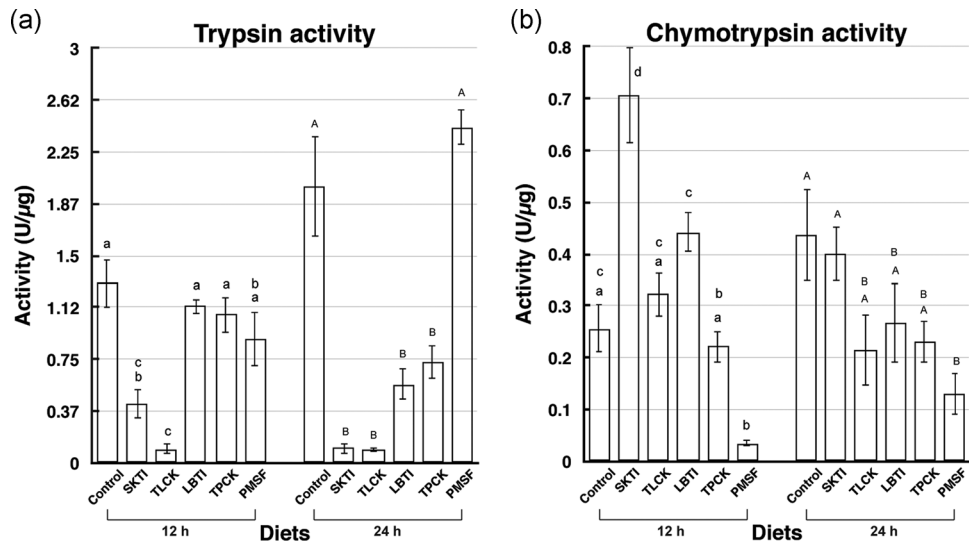
larvae fed on PI-free (control) and PI-supplemented diets. We measured proteinase activities at 12 and 24 hr postfeeding, first without (Figure 6) and then with PI preincubation for 10 min in the assay (Figure 7).

Trypsin activity was significantly reduced for larvae fed on diets supplemented with the known trypsin inhibitors SKTI and TLCK after 12 hr or 24 hr of feeding (Figure 6a). In contrast, no significant differences in trypsin activity could be observed for larvae fed on LBTI-, TPCK- and PMSF-supplemented diets compared to those fed on the control diet after 12 hr feeding (Figure 6a). At 24 hr, LBTI and TPCK but not PMSF inhibited trypsin activity. Chymotrypsin activity was significantly reduced only for larvae fed on the PMSF-supplemented diet and was transiently elevated by SKTI after 12 hr (Figure 6b).

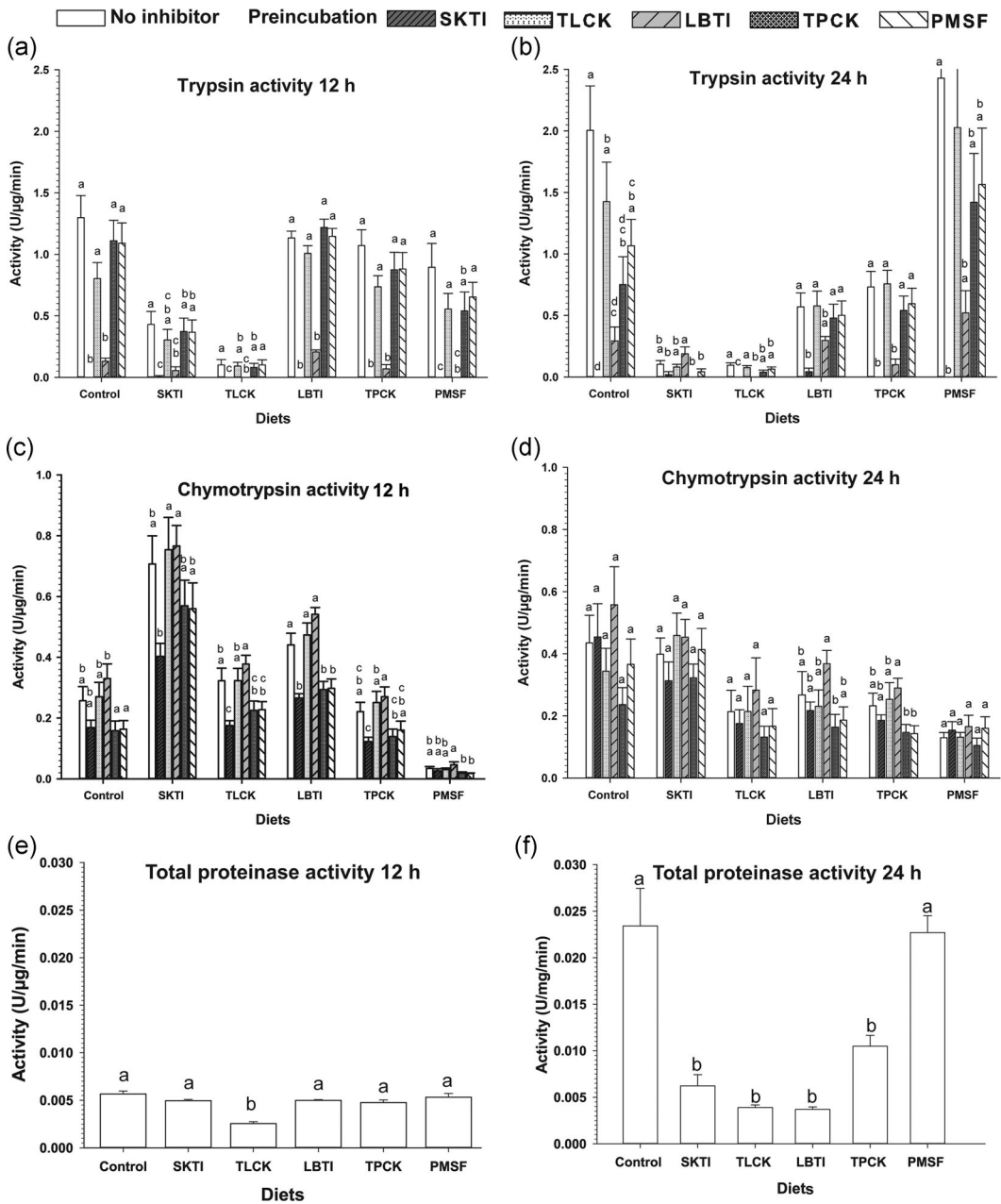




**FIGURE 5** Effect of larvae having fed on PI-supplemented diet on pupal weight. The bar graph represents the net pupal weight of insects that previously fed on either control or PI-supplemented diets. The bars represent the mean  $\pm$  SE. Values followed by the different small letters were significantly different across diets within the same time point by the Tukey honest significant difference test ( $p < .05$ ). LBTI, lima bean trypsin inhibitor; PI, proteinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; SE, standard error; SKTI, soybean Kunitz trypsin inhibitor; TLCK, *N*-alpha-Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, *N*-Tosyl-L-phenylalanine chloromethyl ketone



**FIGURE 6** Midgut proteinase activity of fourth-instar *H. armigera* larvae fed either on a control diet or on PI-supplemented diets for 12 and 24 hr. (a) Trypsin activity (using BApNA); (b) chymotrypsin activity (using SAAPFpNA). Bars represent means  $\pm$  SE. Means followed by the different small letters (at least one) were significantly different (post hoc Tukey HSD test:  $p < .001$ ). BApNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; HSD, honest significant difference; PI, proteinase inhibitor; SE, standard error; SAAPFpNA, *N*-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide



**FIGURE 7** Midgut proteinase activity of fourth-instar *H. armigera* larvae fed either on a control diet or on PI-supplemented diets with the presence of PIs in the assays. (a) Trypsin activity at 12 hr; (b) trypsin activity at 24 hr; (c) chymotrypsin activity at 12 hr; (d) chymotrypsin activity at 24 hr; (e) total proteinase activity at 12 hr; (f) total proteinase activity at 24 hr feeding. Values followed by the different small letters were significantly different by Tukey honest significant difference test ( $p < .05$ ). LBTI, lima bean trypsin inhibitor; PI, proteinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; SKTI, soybean Kunitz trypsin inhibitor; TLCK, N-alpha-Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, N-Tosyl-L-phenylalanine chloromethyl ketone

The preincubation of gut extracts with the proteinaceous inhibitors SKTI and LBTI significantly reduced trypsin activity in larvae fed on the control diet as well as on PI-supplemented diets at 12 hr (Figure 7a) and 24 hr (Figure 7b). However, preincubating gut extracts with TLCK, TPCK, and PMSF had no significant effect on trypsin activity. The transient elevation of chymotrypsin activity on the SKTI diet at 12 hr was seen for all preincubation treatments, but pretreatment with SKTI generally depressed chymotrypsin activity relative to the other pretreatments (Figure 7c). At 24 hr chymotrypsin activity had increased on the control diet but generally decreased on the other diets, regardless of the preincubation treatment (Figure 7d).

Total proteinase activity was significantly reduced only in larvae fed on the TLCK-supplemented diet compared to those fed the control diet at 12 hr (Figure 7e). Total proteinase activity was reduced significantly in larvae fed on SKTI-, TLCK-, LBTI-, and TPCK-supplemented diets compared to larvae fed on the control diet at 24 hr (Figure 7f). Overall, the longer *H. armigera* larvae were exposed to PIs, the greater was the effect on their proteinase activity.

## 4 | DISCUSSION

The presence of class-specific synthetic and natural PIs in the diet negatively alters the life-history traits of *H. armigera* caterpillars. The presence of PIs in the diet decreases (a) the amount of diet consumed except LBTI; (b) larval growth; (c) gut proteinase activity and, eventually; (d) pupal weight. To the best of our knowledge, this study represents the first example illustrating that PIs have an effect on both larval feeding choice and digestive physiology in *H. armigera*.

We observed that, when given the choice, *H. armigera* larvae were more likely to be found feeding on a PI-free diet over the PI-supplemented diet. Similar behavior was previously observed when *Frankliniella occidentalis* (Western flower thrips) was given the choice between feeding on *Solanum tuberosum* transgenic plants expressing cysteine PIs (stefin and equistatin) or on control plants (Outchkourov, de Kogel, Schuurman-de Bruin, Abrahamson, & Jongsma, 2004). In these experiments, thrips selectively fed on plants exhibiting a low level of cysteine PIs. In a different study, host plant-derived chymotrypsin inhibitors were found to induce restlessness in the pea aphid, *Acyrtosiphon pisum* (Rahbe, Ferrasson, Rabesona, & Quillien, 2003). Compared to neonates, fourth-instar larvae showed a weaker preference for PI-free diet, as they could better cope with the concentration of PIs used in our assays.

Neonates preferred the control diet over SKTI- and TLCK-supplemented diets but chose to feed more on LBTI- and TPCK-supplemented diets compared to the control diet. These observations suggest that the effects on proteinase activity and class-specificity of PIs are likely to play a role in the choice of diet a caterpillar will make. LBTI is a Bowman-Birk PI with trypsin- and chymotrypsin-specific PI-domains (Birk, 1976), and TPCK is a chymotrypsin-specific inhibitor. SKTI and TLCK, on the other hand, are strong inhibitors of trypsin, the predominant class of digestive proteinases in these insects (Bown, Wilkinson, & Gatehouse, 1997; Johnston, Lee, Gatehouse, & Anstee, 1991). PMSF did not deter neonate consumption; most neonates consumed a lethal dose by 24 hr. PMSF is a potent inhibitor of most serine hydrolases, not only trypsin. Yet the concentration employed here did not kill fourth-instar larvae, which consumed the PMSF-supplemented diet after finishing the control diet cube. Neonates choose diet after feeding, that is, postprandial over preprandial, implicating that there is a gut feedback mechanism involved in the diet choice.

The effects on larval growth were strongly dependent on the type of PI, as previously observed (Broadway & Duffey, 1986; Johnston et al., 1993). Trypsin-specific PIs (SKTI and TLCK) altered larval growth much more than a chymotrypsin-specific PI (TPCK) and a two-domain trypsin/chymotrypsin PI (LBTI). However, with time, larvae seemed to reverse the effects of ingesting PIs, strongly for SKTI and to a lesser extent for the other PIs (except PMSF; Figure 3); this ability suggests the possibility that larvae are able to produce PI-insensitive proteinases, as has been previously reported for *H. armigera* larvae that were fed on SKTI-supplemented diet (Bown et al., 1997; Bown et al., 2004).

The relationship between PI consumption and growth rate was complex. SKTI diet was consumed somewhat less, but growth was stopped, suggesting an inability to induce PI-insensitive trypsin. The reduced growth rate

following the steady consumption of TLCK diet supports this suggestion. LBTI diet was consumed more, resulting in a normal growth rate, suggesting that the increased food intake without induction was able to compensate for the inhibition of chymotrypsins. We did not observe a reduced growth rate coupled with an excessive diet consumption as seen in *Plutella xylostella* feeding on transgenic plants expressing potato Type II PI (Winterer & Bergelson, 2001) or on PI-containing artificial diet (Broadway, 1995).

Enzyme assays for fourth-instar *H. armigera* larvae fed on PI-supplemented diets indicated that trypsin activity was inhibited by SKTI and TLCK after 12 hr, as we saw earlier (Kuwar et al., 2015), and to a lesser degree by LBTI and TPCK after 24 hr. Surprisingly, no trypsin inhibition by PMSF was observed, perhaps due to PMSF irreversibly inhibiting other serine hydrolases, such as chymotrypsin which was strongly inhibited.

The preincubation experiments were done to determine whether feeding on a particular PI caused a shift in the sensitivity of proteinases to it and to other PIs. Because SKTI preincubation completely abolished trypsin activity irrespective of the diet, we conclude that trypsin maintained their sensitivity to SKTI throughout the feeding time; that is, induction of new trypsin that are insensitive to SKTI is not a response to feeding on SKTI or any other PI tested here. The same conclusion pertains to the response to the trypsin-inhibitor domain of LBTI. However, preincubation of lumen extract with the dual-function LBTI did not suppress chymotrypsin activity; preincubation with the chymotrypsin inhibitor TPCK did suppress chymotrypsin activity but only at 12 hr. We take this as evidence for the constitutive production of some chymotrypsins that are poorly inhibited by the chymotrypsin domain of LBTI. Total proteinase activity for which the major portion corresponds to the activity of trypsin was reduced after 12 and 24 hr of feeding, which correlates well with the reduced trypsin-specific activity we observed. These results also confirm that the longer caterpillars feed on PI-supplemented diets the more the total proteinase activity is inhibited.

In conclusion, we investigated the effect of class-specific PIs on the life history traits of *H. armigera*. We found that *H. armigera* larvae, when given the choice, can discriminate among some but not all PIs. The choice is more important for neonates, which are more sensitive to PIs than fourth-instar larvae. Trypsin-specific PIs had a stronger effect on larval growth, growth rate, and diet consumption compared to chymotrypsin-specific PIs. There was reduced trypsin—but not chymotrypsin-specific activity in larvae fed on PI-supplemented diets. Understanding the basis of these overall trends will ultimately depend on determining the identity and expression level of individual trypsin and chymotrypsin genes, which will be the subject of a future report.

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