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Plant Defense Inhibitors Affect the Structures of Midgut Cells in *Drosophila melanogaster* and *Callosobruchus maculatus*

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ABSTRACT: Plants produce proteins such as protease inhibitors and lectins as defenses against herbivorous insects and pathogens. However, no systematic studies have explored the structural responses in the midguts of insects when challenged with plant defensive proteins and lectins across different species. In this study, we fed two kinds of protease inhibitors and lectins to the fruit fly *Drosophila melanogaster* and alpha-amylase inhibitors and lectins to the cowpea bruchid *Callosobruchus maculatus*. We assessed the changes in midgut cell structures by comparing them with such structures in insects receiving normal diets or subjected to food deprivation. Using light and transmission electron microscopy in both species, we observed structural changes in the midgut peritrophic matrix as well as shortened microvilli on the surfaces of midgut epithelial cells in *D. melanogaster*. Dietary inhibitors and lectins caused similar lesions in the epithelial cells but not much change in the peritrophic matrix in both species. We also noted structural damages in the *Drosophila* midgut after six hours of starvation and changes were still present after 12 hours. Our study provided the first evidence of key structural changes of midguts using a comparative approach between a dipteran and a coleopteran. Our particular observation and discussion on plant–insect interaction and dietary stress are relevant for future mode of action studies of plant defensive protein in insect physiology.

KEYWORDS: microvillus, peritrophic matrix, starvation, insect

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Introduction

Insects use digestive enzymes in their midguts to break down proteins, lipids, and complex carbohydrates for the nutrients they contain and thereby obtain the nutrition needed for their growth and development. Plant protease inhibitors are important natural plant defenses targeting insect proteases.[1–4] Naturally occurring protein protease inhibitors inhibit different classes of proteases, including serine, cysteine, aspartic protease, or metalloprotease. Bowman–Birk inhibitors (BBIs) occur naturally in soybeans and inhibit serine proteases such as trypsins and chymotrypsins.[5,6] The BBI from cowpea (*Vigna unguiculata* Walp) causes increased mortality, weight loss, and developmental delay in a variety of insects.[7,8] BBI from soybeans (*Glycine max* (L.) Merr.) causes retardation of growth in the Sugarcane Borer (*Diatraea saccharalis* Fabricius) (Lepidoptera: Crambidae).[9]

In addition, other defense proteins such as lectins and amylase inhibitors (AIs) also interfere with digestive activity in the insect midgut. Wheat germ agglutinin (WGA) is a lectin that binds *N*-acetyl-D-glucosamine, the building block of chitin found in insect cuticle and peritrophic matrix (PM).[6,10] Ingestion of WGA can cause structural damage in the midguts of coleopterans,[11] lepidopterans,[12–14] and dipterans.[6] αAIs block starch digestion by complexing with alpha-amylase,[10,15] and delay larval development and maturation of several coleopterans and two lepidopterans.[16–19]

Our understanding of how these enzyme inhibitors and lectins act remains incomplete. Additional knowledge about the vulnerability and plasticity of the insect digestive system in response to plant defense proteins is needed. Because insects cannot obtain amino acids and nutrition they need during the defense protein treatment, the midgut cells are most likely to undergo a similar effect to starvation.[20]

Therefore, we also compared midgut cells undergoing starvation to understand the morphological changes.

Peritrophic matrix and midgut cells are the major physiological barriers for plant defensive proteins and compounds and pathogen invasion.[21] One example is the *Bacillus thuringiensis* toxin that directly affects the midgut cell structure of insects by lysing midgut epithelial cells.[22] Microvilli (Mv) in the epithelial cells are also important for understanding the function of midgut, digestion, and related physiological questions.[6,23,24] Disruption of Mv in midgut cells resulted in a delay of development in *Drosophila melanogaster*.[5]

We studied two insect systems, the fruit fly (*D. melanogaster* Meigen) and the cowpea bruchid (*Callosobruchus maculatus* Fabricius). The *D. melanogaster* larval midgut was investigated from a developmental biology perspective. Even though information on larval cross-section through the proventriculus has been recorded earlier as part of the research on the digestive system,[25] we found no study on the microstructure of midgut cells in *D. melanogaster*. *C. maculatus* is a coleopteran pest of stored cowpea seeds and those of other grain legumes.[26] The ultrastructure of midguts of several other insects has been described.[27] Various studies have been conducted on the insect larval digestion system and on the effects of lectins on larval development.[28,29] However, a more comprehensive understanding of changes in midgut ultrastructure after feeding protease inhibitors, lectins, or αAI is still needed to shed light on the effects of these plant defensive proteins.

Here, we explored the structural responses in the midguts when *D. melanogaster* and *C. maculatus* larvae species are challenged with BBI, WGA, and αAIs in the diet. Since some plant defense inhibitors may mimic starvation,[6] we included studies with *D. melanogaster* deprived of food as a basis for comparison. We focused on PM and Mv structural changes using light and transmission electron microscopy (TEM), and compared these with changes observed following starvation.

Materials and Methods

Insect strains and bioassays. The w^{1118} strain of *D. melanogaster* was obtained from Misha Ludwig (University of Chicago). The larvae were reared to the third instar on a Formula 24 *Drosophila* diet (Carolina Biological Supply) at room temperature (22–23°C and 60–70% relative humidity). The *C. maculatus* population (CmNnC-0) was originally collected in Niamey, Niger, and the insects were reared on cowpea seeds in our laboratory at 25°C and 40–60% relative humidity.

Experimental design. Three experiments were conducted in the following manner: In Experiment I, the *D. melanogaster* larvae were subjected to one of four treatments—(i) no chemicals to the diet (control), (ii) 0.3% BBI in the diet (Sigma-Aldrich), (iii) 1% wheat germ agglutinin (WGA; Vector Labs), and (iv) starved but provided water as in the other treatments. Dosages were determined based on mortality and developmental times determined in preliminary experiments.[5,6] All larvae were 108 to 110 hours of age (recorded from the time the eggs were laid) at the time of transfer. After transfer, the larvae were allowed to feed on the test media for various periods of time. At the end of the feeding period, the larvae were removed from the media, and samples from each treatment were chosen for light and TEM analysis.

In Experiment II, the *D. melanogaster* larvae were subjected to either control (normal diet) or starved for three hours, six hours, or 12 hours. Larval growing conditions were the same as for Experiment I.

In Experiment III, the artificial seed pellets (79 mg) for *C. maculatus* were made with either 1% (w/w) WGA or 0.5% (w/w) alpha-amylase inhibitor (*Phaseolus vulgaris* αAI).[26] The control pellets were made using a standard protocol.[26] The dose was chosen based on preliminary experiments. Three and in some cases four larvae from each treatment were examined by TEM. The *C. maculatus* larvae were allowed to continue feeding until they reached the early fourth-instar stage. They were then transferred to artificial seeds (1 larva/seed) and kept there for 24 hours before removal and dissection for TEM sample preparation. Larvae fed on cowpea seeds were used as controls. WGA was purchased from Vector Laboratories (Burlingame) and aAI was donated by Dr. Maarten Chrispeels.

Tissue preparation for microscopy. Three *D. melanogaster* third-instar larval midguts were used for each replicate, with two replicates per treatment. Larval midguts were observed with an Olympus SZX12 light microscope (Olympus Corporation). Images were taken with an Olympus U-TV1X-2 digital camera with Olympus MicroSuite-B3 software and were processed in Adobe Photoshop CS-2 (Adobe Systems). The larvae were dissected in 214 mM NaCl saline immediately before the images of the whole midgut were taken.

For TEM analysis of midgut sections, *D. melanogaster* third-instar larval midguts or *C. maculatus* fourth-instar larval midguts were dissected in 0.2 M Na-cacodylate buffer (pH 7.4). The midguts were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer containing 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1 M sucrose; postfixed with 1% OsO_4 and 1.5% $K_3Fe(CN)_6$; and then dehydrated with a series of 10, 30, 50, 70, 90, and 100% ethanol (2 ×) and propylene oxide. Samples were embedded in a mold with partially polymerized EPON resin (containing LX-112) in the bottom overlain by additional resin. The samples were then polymerized at 60°C for 48 hours. Ultra-thin sections were stained with 2% uranyl acetate in 70% methanol for 10 minutes and lead citrate for 5 minutes. Images were taken on an FEI/Philips CM-10 transmission electron microscope (FEI Company) using an accelerating voltage of 80 kV, with varying magnifications.

Mv examined were along the anterior axis of the midgut. The length (h) and diameter (d or $2r$) of each intact midgut microvillus (baseline to the apical Mv) were measured. Intact microvilli were identified by visualization on the TEM film. The surface area was calculated by adding the single microvillus surface area and surface areas of the sphere with radius r, using the formula given below (shape of each Mv was assumed to be cylinder-like):

$$A = 2\pi^* r^* h + 2\pi^* r2 \quad (1)$$

Statistical analyses. For Experiment I, one-way analysis of variance (ANOVA) for independent samples and a post hoc Tukey honestly significant difference (HSD) test was applied. For Experiments II and III, two sample t-tests for independent samples were applied. Analyses were performed in R software (R3.0.3 for Windows, http://cran.r-project.org/).

The normality of each data set was also checked and confirmed by quantile–quantile plots (data not shown).

Results

To understand the morphological changes the *D. melanogaster* midgut undergoes in response to the different challenges, we first examined the shape of the midgut using light microscopy in Experiment I. The midguts of *D. melanogaster* from the standard diet group (control) exhibited a uniform distribution of food contents (Fig. 1A). Those feeding on the 0.3% BBI diet for 12 hours (Fig. 1B) exhibited greater food accumulation in the central region of the digestive tract as compared with insects receiving the control diet. Midguts from insects fed the 0.1% WGA diet for 12 hours had a morphology similar to that of the control group (Fig. 1C). Starvation for 12 hours resulted in marked changes. Midgut lengths were shorter than those fed the control diet, and more food was found in the central region of the tract (Fig. 1D).

Cross sections of these midguts revealed marked differences under the four different dietary conditions. In insects feeding on a normal diet, the PM was complete and the epithelial Mv appeared normal (Fig. 2A). In midguts of BBI-fed larvae, food content had accumulated in the central region of the midgut. Fewer Mv covered the apical surfaces of enterocytes, the epithelial monolayer of cells (Fig. 2B). Gaps were observed between the brush border and the PM (Fig. 2B). In midguts of WGA-fed larvae, the enterocytes were dramatically smaller and the brush border formed by Mv was not smooth (Fig. 2C). As expected, the midguts of starved larvae contained little food. Numerous folded areas in the brush border were observed, possibly indicating damage to the Mv. Only fragments of the PM were visible (Fig. 2D).

TEM was carried out using the same four treatments. Compared to the straight, long, and parallel Mv in the control treatment (Fig. 3A), the Mv in the midguts of larvae fed BBI were shorter (Fig. 3B). The midgut cells of larvae fed WGA displayed more severe structural damage when compared to the control, including branched, swollen Mv on the apical surface of enterocytes (Fig. 3C). The surfaces of the enterocytes were also not as smooth as they were in the control group, indicating abnormal physical structures (Fig. 3C). The length of Mv in starved larvae was even shorter than the control and the BBI-fed larvae (Fig. 3D).

To better evaluate the structural changes in Mv, we measured the length (h) and diameter ($2r$ or d), and calculated the surface area of Mv (A) in the four treatments. One-way ANOVA analyses showed a significant difference among the four treatments when length was measured. Mv lengths in

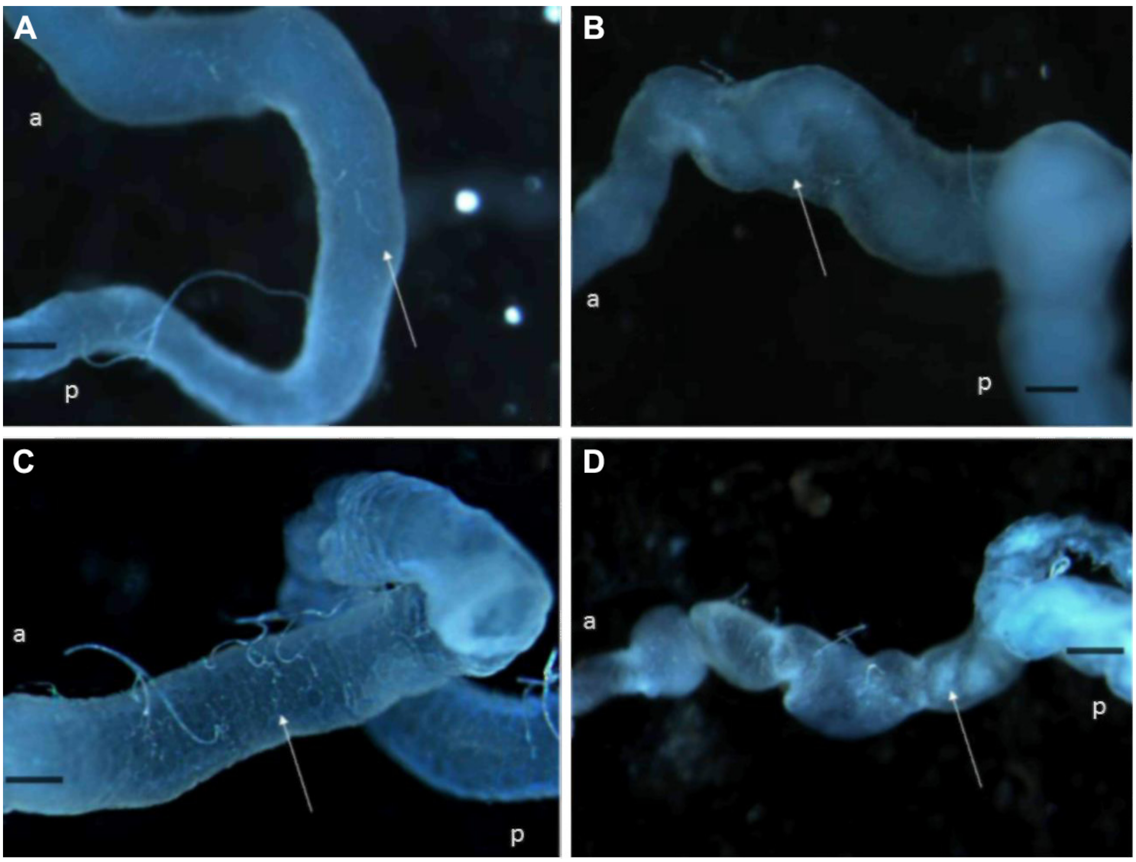

Figure 1. Midguts of third-instar *Drosophila melanogaster* larvae (**A**) fed a normal diet, (**B**) fed 0.3% of BBI for 12 hours, (**C**) fed 0.1% of wheat germ agglutinin for 12 hours, or (**D**) starved for 12 hours. Scale bar: 0.1 mm. Letters a and p indicate the anterior and the posterior end of the midgut. Arrows point to the central region.

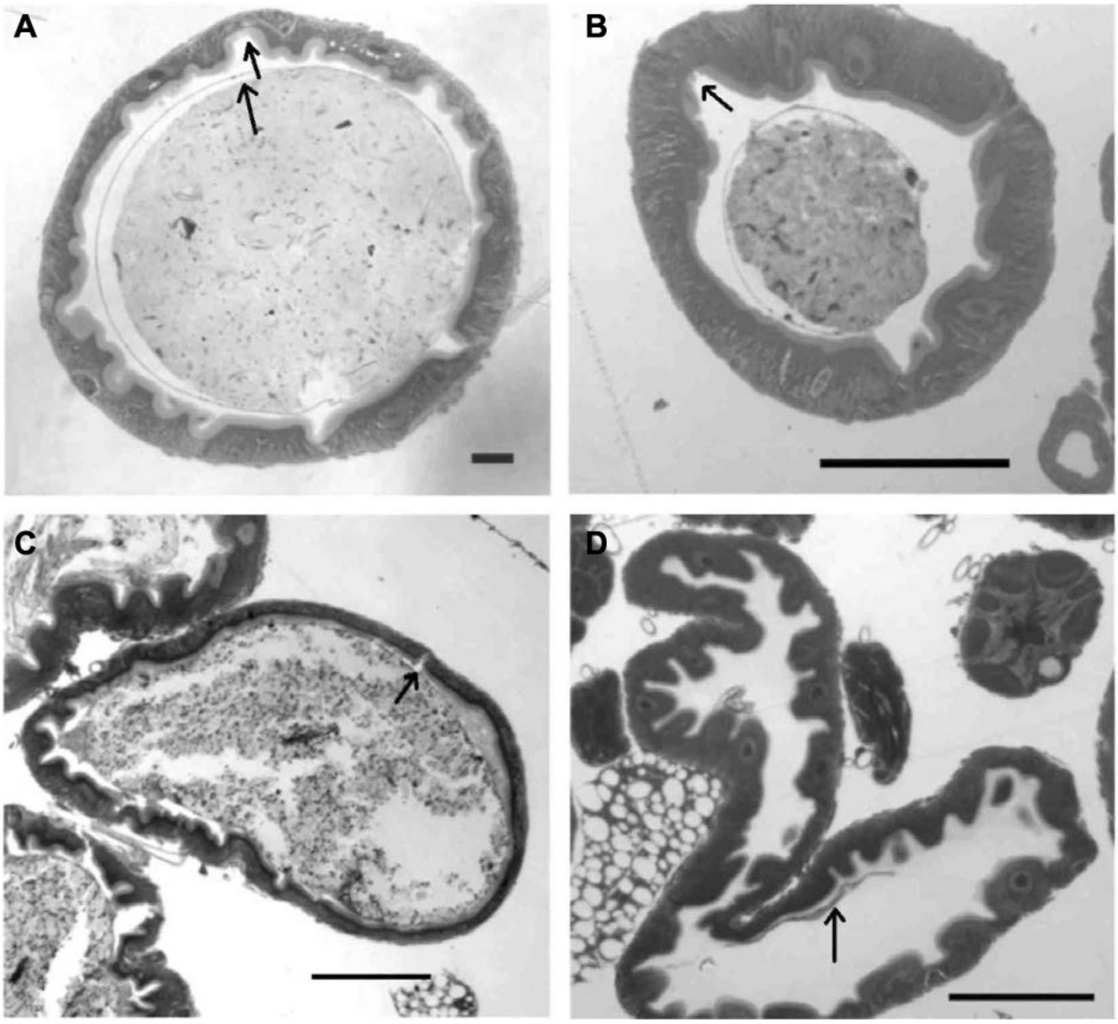

Figure 2. Cross sections through the midguts of third-instar *Drosophila melanogaster* larvae fed (**A**) a normal diet, (**B**) 0.3% of Bowman–Birk inhibitor for 12 hours, (**C**) 0.1% of wheat germ agglutinin for 12 hours, or (**D**) starved for 12 hours. Scale bar: 0.1 mm. Arrows indicate the Mv distribution and PM shapes.

the 12-hour, BBI-fed, WGA-fed, and starved larvae were significantly shorter than the control larvae ($F_{3, 219} = 158.59$, $P < 0.001$, HSD[.05] = 2.59; Fig. 4A) with decreases of 34, 36, and 42% respectively. The diameters of Mv in the WGA-fed insects were the greatest among the Mv of all four treatments (Fig. 4B). Significant differences in diameter were observed among groups ($F_{3, 131} = 10.21$, $P < 0.001$, HSD[.05] = 2.6; Fig. 4B). No difference in the number of Mv was identified among all groups (Fig. 4C) even though larvae-fed WGA showed a strong trend toward low numbers of Mv. The surface areas in control samples showed the highest value (Fig. 4D). Significant decreases in surface area were also observed among treatments (15% in BBI-fed, 47% in WGA-fed, 63% in starvation 12 h) ($F_{3, 131} = 61.52$, $P < 0.001$, HSD[.05] = 2.6; Fig. 4D). Starved larvae had the smallest surface area (Fig. 4D).

To determine the time course of changes in Mv during food deprivation, we examined starved larvae at three- and six-hour intervals (Experiment II). Results showed no significant difference in Mv length in *D. melanogaster* larvae after 3 hours of starvation ($F_{1, 23} = 0.01$; Fig. 5A & B). However, after six hours of starvation, Mv length was significantly decreased ($F_{1, 24} = 33.30$, $P < 0.001$; Fig. 5C & D). Experiment I demonstrated that after 12 hours of starvation, the average length of Mv was significantly shortened (Figs. 3A & D, 4A). Note that the distal termini of the Mv may break off into the lumen after six hours of starvation (Fig. 5D). PM was also observed in starved and control individuals (Fig. 5A, B, & D), and no difference was detected regarding shape or thickness (data not shown).

Because BBI is a serine protease inhibitor while the major protease in the digestive system of *C. maculatus* is a cysteine protease, we did not test BBI with *C. maculatus*. Instead, we tested WGA and αAI in Experiment III, and carried out TEM analyses of the midgut. Due to the technical problems, we did not obtain enough replicates to make an assessment of changes in the PM. WGA-fed bruchid larvae had shortened Mv, exhibited a decrease of 24% in the length when compared to controls (mean for control = 5.80 ± 0.09 μm, mean for WGA = 4.42 ± 0.08 μm, $F_{1, 190} = 132.14$, $P < 0.001$; Fig. 6A

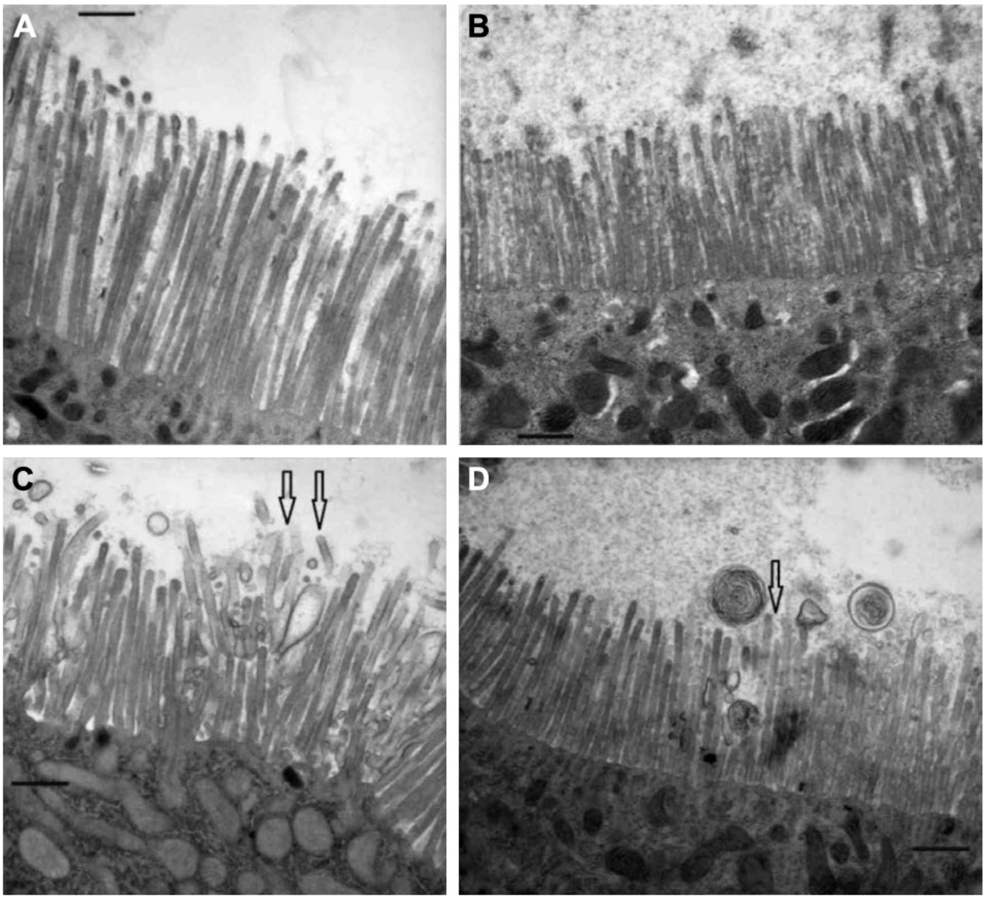

Figure 3. TEM images of third-instar *Drosophila melanogaster* larval midguts fed different diets for 12 hours: (**A**) control; (**B**) Bowman–Birk inhibitor; (**C**) wheat germ agglutinin; (**D**) starvation. Arrows indicate the cellular damage. Scale bar: 1 µm; magnification 15,000 ×.

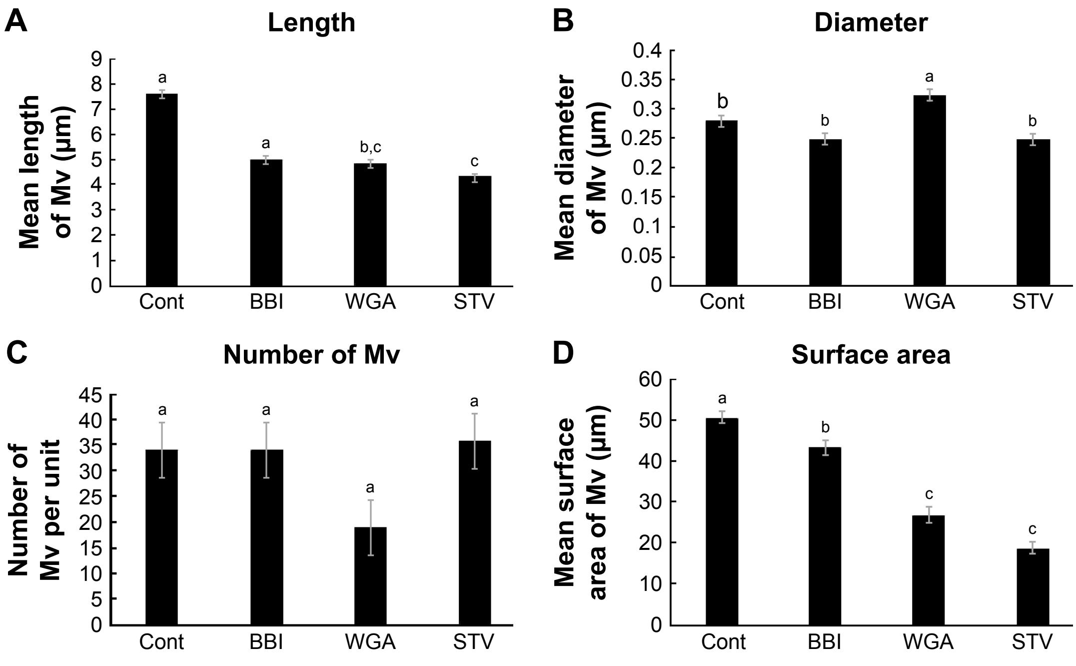

Figure 4. Bar graph of the height, diameter, number, and surface area of microvilli in four treatments (Control [Cont], Bowman–Birk inhibitor [BBI], wheat germ agglutinin [WGA], and starvation [STV]; mean ± SE). One-way ANOVA analysis was conducted in JMP (Pro11.0.0) with a post hoc Tukey HSD test. (**A**) Length (*h*) of microvilli (Mv) measured. (**B**) Diameter (*d*) of Mv measured. (**C**) Number of Mv per unit area. (**D**) Surface area calculated based on the length and diameter. Treatment sharing the same letter are not significantly different.

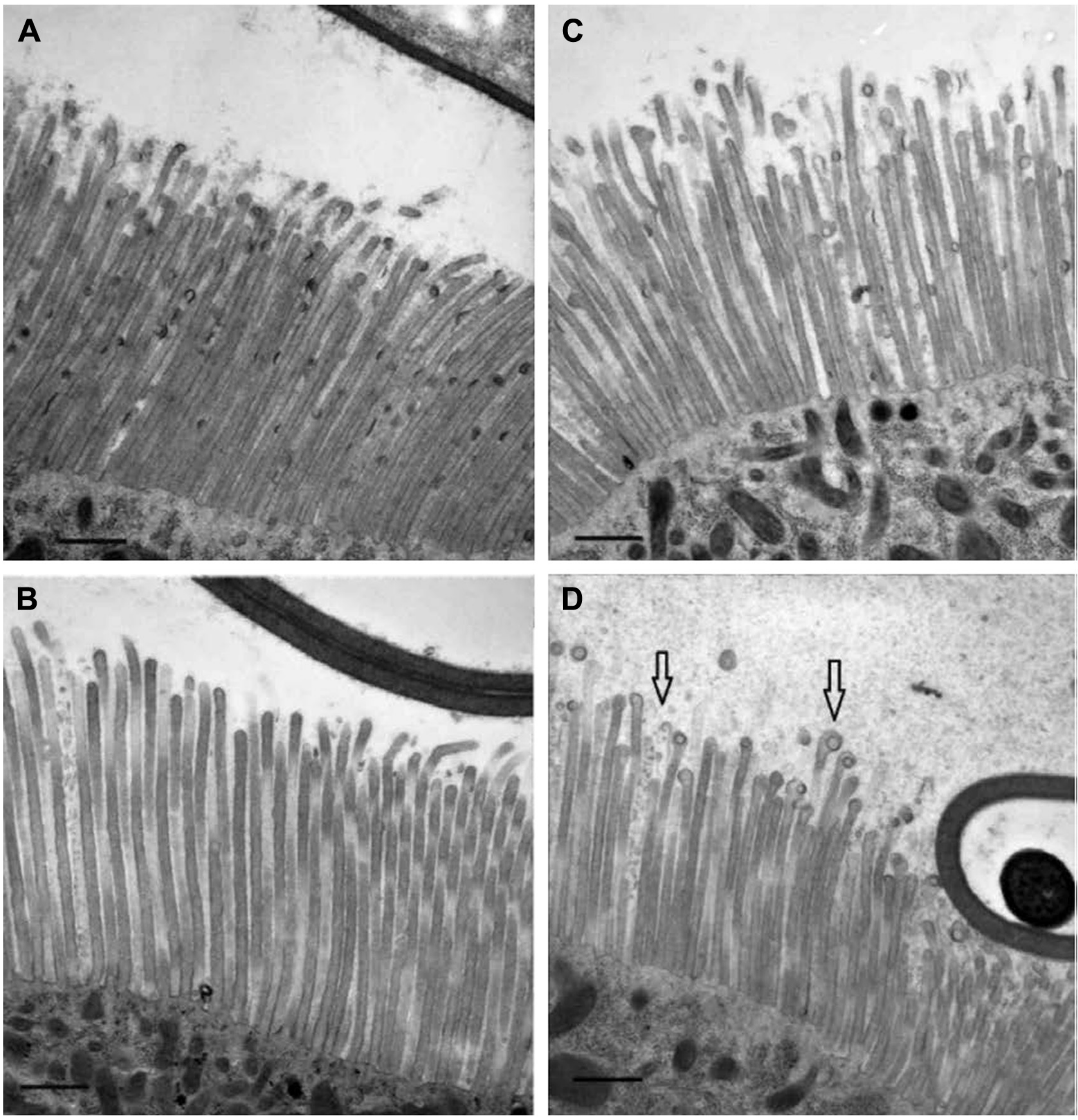

Figure 5. TEM images of midguts of third-instar *Drosophila melanogaster* larvae starved for three or six hours: (**A**) control, three hours; (**B**) starvation, three hours; (**C**) control, six hours; (**D**) starvation, six hours. Arrows indicate the cellular damage. Scale bar: 1 μm; magnification: 15,000 ×. PM appeared in A, B, & D.

and B). Fewer and less-dense Mv and gaps between Mv were observed in WGA-fed larvae (Fig. 6B).

Mv of αAI-fed larvae of *C. maculatus* exhibited a significant shortening (7% decrease) compared to the control ($\text{Mean}_{con} = 5.61 \pm 0.10$ μm, $\text{Mean}_{\alpha AI} = 5.21 \pm 0.08$ μm, $F_{1,\ 228} = 10.76$, $P < 0.01$; Fig. 7). Gaps between Mv were also detected in αAI-fed larvae, a change similar to that we observed in WGA-fed larvae (see arrows in Fig. 7B).

Discussion

Insect digestion involves the breakdown of food and absorption of nutrients, processes critical for development and survival.[29,30] In the present study, we show that plant defense proteins affect the cellular structure of insect midguts. Ours is the first report dealing with how key structures in the guts of a dipteran and a coleopteran change when subjected to the different dietary stresses imposed by different kinds of plant defense proteins.

These results are consistent with previous reports of insect midgut response.[2] The larval response to plant protein damage to midgut cells was proliferation of the epithelial cells. It will be interesting to see whether the larvae will have an ability to recover if the treatment of plant-defense proteins stops.

Light microscopy indicated that the epithelial cell Mv were damaged after exposure to the plant defense protein in the diet. This physical change was most obvious in the TEM. The damage of the absorptive surface and the epithelial cell layer may interfere with the uptake of nutrients by the larvae.

Nutrient uptake may also be reduced by lesions to or changes in the peritrophic matrix, a key element in food and nutrient digestion.[31] Previous research has shown chitin,

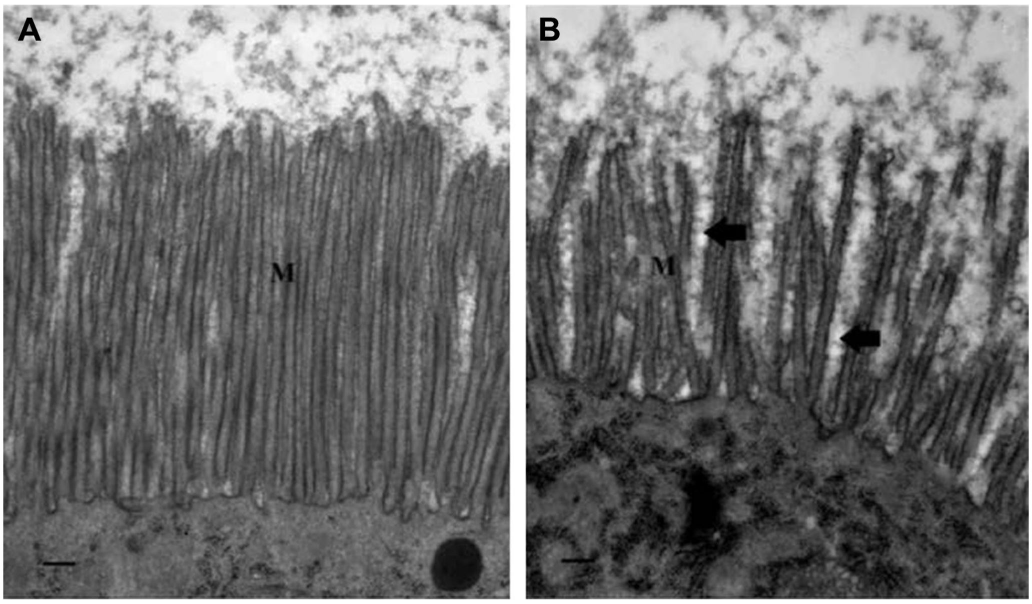

Figure 6. Images of fourth-instar *Callosobruchus maculatus* larval midguts after consuming the wheat germ agglutinin (WGA) diet: (**A**) normal diet for 24 hours; (**B**) 1% WGA (w/w) for 24 hours. Scale bar: 1 μm; magnification: 20,000 ×.

proteases, lipase, chitin deacetylase, chitinase, proteoglycans, and peritrophin proteins to be associated with the peritrophic matrix.[32,33] The peritrophic matrix encloses the food bolus and is involved in the compartmentalization of digestion.[34] The peritrophic matrix was observed in starved larvae in our study, and no damage but only shrinkage was exhibited. However, we cannot rule out the possibility of negative effects on peritrophic by plant defense proteins. Future work is needed to elucidate the potential changes on the peritrophic matrix under plant defense proteins.

We also found evidence that dramatic changes in midgut structures begin as soon as six hours after the onset of food deprivation. Our results indicate that plant defense proteins can cause structural dysfunction similar to that caused by food deprivation or starvation possibly because the reduced uptake of nutrients.

Among the plant defense proteins tested, WGA affected the midgut the most severely, its impact on the Mv being similar to that of starvation. Starved larval midgut contained very little food residue in the lumen, and the midgut appeared to be leaner than with the other treatments. Carbohydrate-binding proteins or lectins such as WGA serve as plant defensive protein against phytophagous insects. It is known that they can cause severe disruption of Mv and PM in insects.[13,14,35] Furthermore, lectins in the diet may also target other tissues in insect bodies such as fat body, hemolymph, ovarioles, or Malpighian tubules.[36,37] Furthermore, previous reports demonstrated WGA-induced changes in gene expression at

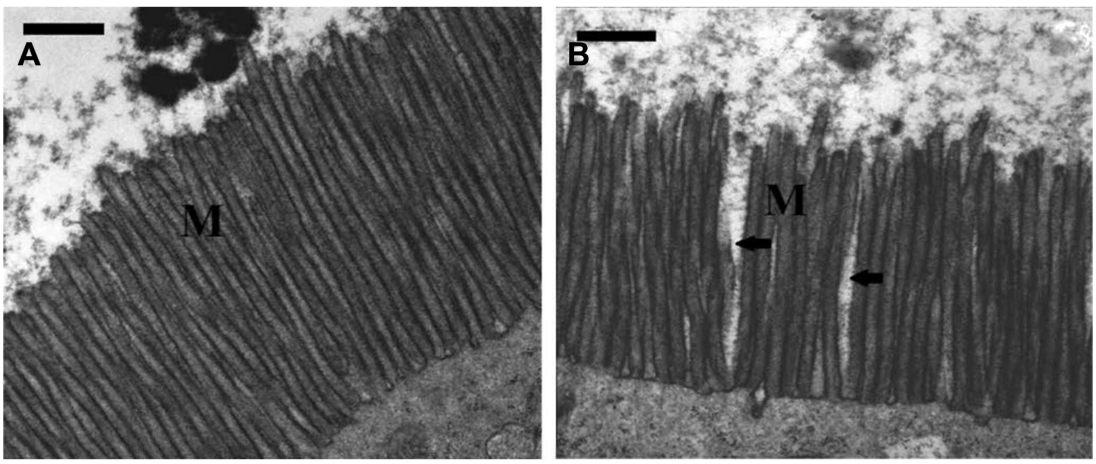

Figure 7. TEM images of fourth-instar *Callosobruchus maculatus* larval midguts from the alpha-amylase inhibitor (αAI) treatment: (**A**) normal diet for 24 hours; (**B**) 0.5% αAI for 24 hours. M: microvilli. Arrows indicate the gaps between M. Scale bar: 1 μm; magnification: 20,000 ×.

transcriptomic levels, affecting genes involved in several key processes including cellular structural organization, digestion, energy metabolism, and detoxification.[6] Although BBI and αAI showed certain levels of structural damage, WGA and starvation induced the greatest changes. Starvation affects the development of microvillar membranes in other insects such as cotton stainer *Dysdercus peruvianus* (Guerin-Meneville) (Hemiptera: Pyrrhocoridae),[38] and midgut cell death was observed in starved spiders.[20]

Our results indicated that abnormal droplet or small vesicles formed at the top of the Mv, and then broke off (Fig. 5D); this may be the main mechanism leading to the observed shortening of the Mv we observed during starvation. In rats, Mv may generate vesicles that release digestive enzymes to the lumen.[39] The shortening of Mv of the gut epithelial cells may have a dramatic impact on the nutrient absorption and nutrition.

Our findings are consistent with similar reports about other insects such as lygus bugs, aphids, and moths.[1,40–42] Other treatments, including radiation, can also lead to alterations in midgut structures of other beetles such as *Tribolium*, *Trogoderma,* and *Plodia*.[43]

Abbreviations

αAI, alpha-amylase inhibitor; ANOVA, analysis of variance; BBI, Bowman-Birk inhibitor; HSD, honestly significant difference; Mv, microvilli; PM, peritrophic matrix; TEM, transmission electron microscopy; WGA, wheat germ agglutinin.

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Author Contributions

Conceived and designed the experiments: HL-B, BRP, LLM. Performed experiments and analyzed the data: HL-B. Wrote the first draft of the manuscript: HL-B. Agree with manuscript results and conclusions: BRP, LLM. Made critical revisions and approved final version: HL-B, BRP, LLM. All authors reviewed and approved of the final manuscript.

REFERENCES

1. War AR, Paulraj MG, Ahmad T, et al. Mechanisms of plant defense against insect herbivores. *Plant Signal Behav*. 2012;7(10):1306–1320.
2. Barbeta BL, Marshall AT, Gillon AD, Craiks DJ, Anderson MA. Plant cyclotides disrupt epithelial cells in the midgut of lepidopteran larvae. *Proc Natl Acad Sci U S A*. 2008;105(4):1221–1225.
3. Jongsma MA, Bolter C. The adaptation of insects to plant protease inhibitors. *J Insect Physiol*. 1997;43(10):885–895.
4. Ortego F. Physiological adaptations of the insect gut to herbivory. In: Smagghe G, Diaz I, eds. *Arthropod-Plant Interactions*. Berlin: Springer; 2012:75–88.
5. Li HM, Margam V, Muir WM, Murdock LL, Pittendrigh BR. Changes in *Drosophila melanogaster* midgut proteins in response to dietary Bowman-Birk inhibitor. *Insect Mol Biol*. 2007;16(5):539–549.
6. Li HM, Sun L, Mittapalli O, et al. Transcriptional signatures in response to wheat germ agglutinin and starvation in *Drosophila melanogaster* larval midgut. *Insect Mol Biol*. 2009;18(1):21–31.
7. Bell HA, Fitches EC, Down RE, et al. Effect of dietary cowpea trypsin inhibitor (CpTI) on the growth and development of the tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae) and on the success of the gregarious ectoparasitoid *Eulophus pennicornis* (Hymenoptera: Eulophidae). *Pest Manag Sci*. 2001;57(1): 57–65.
8. Xu D, Xue Q, McElroy D, Mawal Y, Hilder VA, Wu R. Constitutive expression of a cowpea trypsin inhibitor gene, CpTi, in transgenic rice plants confers resistance to two major rice insect pests. *Mol Breed*. 1996;2(2):167–173.
9. Falco MC, Silva-Filho MC. Expression of soybean proteinase inhibitors in transgenic sugarcane plants: effects on natural defense against *Diatraea saccharalis*. *Plant Physiol Biochem*. 2003;41(8):761–766.
10. Carlini CR, Grossi-de-Sá MF. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicon*. 2002;40(11): 1515–1539.
11. Wang L-H, Chi YH, Guo F-G, et al. Transcriptomic response of cowpea bruchids to N-acetylglucosamine-specific lectins. *Insect Sci*. 2015;22(1):83–94.
12. Czapla TH, Lang BA. Effect of plant lectins on the larval development of European corn borer (Lepidoptera: Pyralidae) and southern corn rootworm (Coleoptera: Chrysomelidae). *J Econ Entomol*. 1990;83(6):2480–2485.
13. Harper MS, Hopkins TL, Czapla TH. Effect of wheat germ agglutinin on formation and structure of the peritrophic membrane in European corn borer (*Ostrinia nubilalis*) larvae. *Tissue Cell*. 1998;30(2):166–176.
14. Hopkins TL, Harper MS. Lepidopteran peritrophic membranes and effects of dietary wheat germ agglutinin on their formation and structure. *Arch Insect Biochem Physiol*. 2001;47(2):100–109.
15. Franco OL, Rigden DJ, Melo FR, Grossi-de-Sá MF. Plant a-amylase inhibitors and their interaction with insect a-amylases. *Eur J Biochem*. 2002;269(2):397–412.
16. Ishimoto M, Chrispeels MJ. Protective mechanism of the Mexican bean weevil against high levels of alpha-amylase inhibitor in the common bean. *Plant Physiol*. 1996;111(2):393–401.
17. Morton RL, Schroeder HE, Bateman KS, Chrispeels MJ, Armstrong E, Higgins TJ. Bean alpha-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. *Proc Natl Acad Sci U S A*. 2000;97(8):3820–3825.
18. Schroeder HE, Gollasch S, Moore A, et al. Bean [alpha]-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum* L.). *Plant Physiol*. 1995;107(4):1233–1239.
19. Valencia-Jiménez A, Arboleda VJW, Lopez Avila A, Grossi-de-Sá MdF. Digestive a-amylases from Tecia solanivora larvae (Lepidoptera: Gelechiidae): response to pH, temperature and plant amylase inhibitors. *Bull Entomol Res*. 2008; 98(06):575–579.
20. Wilczek G, Rost-Roszkowska M, Wilczek P, et al. Apoptotic and necrotic changes in the midgut glands of the wolf spider *Xerolycosa nemoralis* (Lycosidae) in response to starvation and dimethoate exposure. *Ecotoxicol Environ Saf*. 2014; 101:157–167.
21. Zhou YJ, Xue B, Li YY, et al. Construction of Silkworm Midgut cDNA Library for screen and sequence analysis of peritrophic membrane protein genes. *Arch Insect Biochem Physiol*. 2016;91(1):3–16.
22. Bravo A, Gill SS, Soberón M. Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control. *Toxicon*. 2007;49(4):423–435.
23. Li HM, Buczkowski G, Mittapalli O, et al. Transcriptomic profiles of *Drosophila melanogaster* third instar larval midgut and responses to oxidative stress. *Insect Mol Biol*. 2008;17(4):325–339.
24. Chen Q, Wei T. Viral receptors of the gut: insect-borne propagative plant viruses of agricultural importance. *Curr Opin Insect Sci*. 2016;16:9–13.
25. Campos-Ortega JA, Hartenstein V. *The Embryonic Development of Drosophila melanogaster*. Berlin: Springer Science & Business Media; 2013.
26. Chi YH, Salzman RA, Balfe S, et al. Cowpea bruchid midgut transcriptome response to a soybean cystatin—costs and benefits of counter-defence. *Insect Mol Biol*. 2009;18(1):97–110.
27. Billingsley P, Lehane M. Structure and ultrastructure of the insect midgut. *Biol Insect Midgut*. 1996:3–30.
28. Macedo MLR, Freire MdGM, da Silva MBR. Coelho LCBB. Insecticidal action of *Bauhinia monandra* leaf lectin (BmoLL) against *Anagasta kuehniella* (Lepidoptera: Pyralidae), *Zabrotes subfasciatus* and *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Comp Biochem Physiol A Mol Integr Physiol*. 2007;146(4):486–498.
29. Zhu-Salzman K, Zeng R. Insect response to plant defensive protease inhibitors. *Annu Rev Entomol*. 2015;60:233–252.
30. Nation JL. *Insect Physiology and Biochemistry*. Boca Raton: CRC Press; 2001.
31. Hegedus D, Erlandson M, Gillott C, Toprak U. New insights into peritrophic matrix synthesis, architecture, and function. *Annu Rev Entomol*. 2009;54:285–302.
32. Hu X, Chen L, Xiang X, Yang R, Yu S, Wu X. Proteomic analysis of peritrophic membrane (PM) from the midgut of fifth-instar larvae, *Bombyx mori*. *Mol Biol Rep*. 2012;39(4):3427–3434.

33. Ramos MV, Pereira DA, Souza DP, et al. Peptidases and peptidase inhibitors in gut of caterpillars and in the latex of their host plants. *Planta*. 2015;241(1): 167–178.
34. Hegedus DD, Toprak U, Erlandson M. Lepidopteran peritrophic matrix composition, function, and formation. *Short Views Insect Genomics Proteomics*. 2016;4:63–87.
35. Vandenborre G, Smagghe G, Van Damme EJM. Plant lectins as defense proteins against phytophagous insects. *Phytochemistry*. 2011;72(13):1538–1550.
36. Powell KS, Spence J, Bharathi M, Gatehouse JA, Gatehouse AMR. Immunohistochemical and developmental studies to elucidate the mechanism of action of the snowdrop lectin on the rice brown planthopper, *Nilaparvata lugens* (Stal). *J Insect Physiol*. 1998;44(7–8):529–539.
37. Fitches E, Woodhouse SD, Edwards JP, Gatehouse JA. In vitro and in vivo binding of snowdrop (Galanthus nivalis agglutinin; GNA) and jackbean (*Canavalia ensiformis*; Con A) lectins within tomato moth (*Lacanobia oleracea*) larvae; mechanisms of insecticidal action. *J Insect Physiol*. 2001;47(7):777–787.
38. Damasceno-Sa JC, Carneiro CNB, Da Matta RA, Samuels RI, Terra WR, Silva CP. Biphasic perimicrovillar membrane production following feeding by previously starved *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). *J Insect Physiol*. 2007;53(6):592–600.
39. McConnell RE, Higginbotham JN, Shifrin DA Jr, Tabb DL, Coffey RJ, Tyska MJ. The enterocyte microvillus is a vesicle-generating organelle. *J Cell Biol*. 2009; 185(7):1285–1298.
40. Coelho MB, Marangoni S, Macedo MLR. Insecticidal action of *Annona coriacea* lectin against the flour moth *Anagasta kuehniella* and the rice moth *Corcyra cephalonica* (Lepidoptera: Pyralidae). *Comp Biochem Physiol C Toxicol Pharmacol*. 2007; 146(3):406–414.
41. Habibi J, Backus EA, Huesing JE. Effects of phytohemagglutinin (PHA) on the structure of midgut epithelial cells and localization of its binding sites in western tarnished plant bug, Lygus hesperus Knight. *J Insect Physiol*. 2000;46(5):611–619.
42. Sauvion N, Nardon C, Febvay G, Gatehouse AMR, Rahbe Y. Binding of the insecticidal lectin Concanavalin A in pea aphid, *Acyrthosiphon pisum* (Harris) and induced effects on the structure of midgut epithelial cells. *J Insect Physiol*. 2004; 50(12):1137–1150.
43. Ignatowicz S. Detection methods for irradiated mites and insects. *Quarantine Treat Arthropod Pests*. 1999:141.