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Effects of Carbaryl and γ-BHC on the Histology of Midgut and Digestive Enzyme Profiles in the Third Instar Larvae of Fruit-sucking Moth, *Othreis materna* (Linn.) (Lepidoptera: Noctuidae)

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Abstract: The effects of LC50 dose of carbaryl and gamma benzene hexachloride (γ-BHC) on midgut histopathology and midgut enzyme activities in the third instar larvae of fruit-sucking moth, *Othreis materna* (L.) were studied. The sub-lethal doses of carbaryl and γ-BHC caused initially hypersecretory activity in the midgut epithelial cells leading to deposition of numerous secretory vesicles in the extraperitrophic space, clumping of chromatin matter, and regression and vacuolization of the columnar cells, but later on they were recovered within 24 h. Activities of the midgut digestive enzymes (amylase, invertase, lipase, and protease) were significantly affected. Carbaryl enhanced ($P < 0.05$) the amylase, invertase, lipase, and protease activities initially up to 4 h and 6 h of treatment, but after 24 h of treatment, amylase and invertase activities resumed while an inhibitory effect was observed on lipase and protease activities. γ-BHC did not affect the amylase and protease activities up to 4 h but later on it significantly ($P < 0.05$) enhanced their activities up to 6 h and 12 h, respectively. Thereafter it fell without showing any sign of recovery. γ-BHC enhanced ($P < 0.05$) invertase and lipase activities but they fell after 4 h treatment.

Key Words: *Othreis materna*, midgut histopathology, digestive enzymes, pesticides

Introduction

Synthetic organic pesticides have been reported to make a revolution in the agriculture production in India by controlling pests of various crops (Matsumura, 1985). Pesticides used in pest control programs resulted in damage to the environment, resistance to insecticide, and adverse effects on humans and animals (Abudulai et al., 2001). Histopathological effects of synthetic organic insecticides (Toppozada et al., 1968; Rizvi and Khan, 1973) and bacterial toxins (Kinsinger and McGaughey, 1979; Endo and Nishiitsutsuji-Uwo, 1980; Blackburn et al., 1998) were studied on different organs including the midgut. Semi-lethal doses of organophosphates induced initially hypersecretory activity in the midgut epithelial cells and stimulated amylase, invertase, lipase and protease activities (Deshmukh and Tembhare, 1998). Sub-lethal concentration of thuringiensin and abamectin decreased invertase, amylase and trehalase activities of *Spodoptera littoralis* (Boisd) larvae (Abo El-Ghar et al., 1995). Fenvalerate treatment induced a reduction in the midgut amylase, sucrase, and protease activity of *Bombyx mori* L. (Vyjayanthi and Subramanyam, 2002). *Bacillus*

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thuringiensis toxin caused damage to the epithelial cells of the midgut. The damage to the midgut decreased digestive enzyme activities (Eguchi et al., 1972; Mathavan et al., 1989; Abo El-Ghar et al., 1995; Smirle et al., 1996; Huang et al., 2008). Exposure of *Cnaphalocrocis medinalis* (Guenée) larvae to sub-lethal doses of *Bacillus thuringiensis* (Kurstaki) in the laboratory reduced digestive enzyme activities (Senthil Nathan et al., 2005 and 2006). In spite of extensive work on the effects of pesticides in insects, little information is available on the effects of classical insecticides, such as carbaryl and γ-BHC, in the larvae of lepidopteran insects. The present study aimed to investigate the effect of carbaryl and γ-BHC on the midgut histopathology and digestive enzyme activities in the third instar larvae of fruit-sucking moth, *O. materna,* adults of which are the major pest of orange crop in the Vidarbha region of Maharashtra, India (Mohite et al., 2006).

Materials and Methods

Laboratory culture of *Othreis materna*

Adult fruit-sucking moths of *O. materna* were collected at night from the citrus orchards of different places of Vidarbha region of Maharashtra state with hand nets. They were reared at 70 ± 5% RH, 27 ± 1 °C with a photoperiod of L:D 14:10. Adults were kept in specially prepared cages covered with fine wire nets on sides. Adult moths were fed with ripen oranges (when available) or a mixture of 10% sucrose solution and honey (3:1). The larvae were reared in plastic trays. The larvae were fed on fresh leaves of *Tinospora cardifolia* (Mohite et al., 2004a). After pupation, emerged adults were kept in cages. To maintain the stock culture 10 males and 10 females were placed in an oviposition cage.

Bioassay and treatments

The lethal concentration (LC_{50}) of carbaryl (Sevin 50 WP 10D, Bayer India Ltd., Bombay) and γ-BHC (Lindane, M/s Yawalkar Pesticides Pvt. Ltd., Nagpur) was determined according to Finney (1971) and Verma and Khurana (1977). The LC_{50} values for carbaryl and γ-BHC were 0.0140 ml l^{-1} and 0.0440 ml l^{-1}, respectively (Mohite et al., 2004b). In each petri dish (7.5 cm diameter) 10 third instar larvae were placed and sprayed under Potter's tower at 40 ± 2 lbs/sq inch pressure with 1 ml aqueous suspension containing half of the LC_{50} dose of each insecticide. The control larvae were sprayed with water. Each treatment was replicated 3 times. Fifty third instar larvae were sprayed in batches of 10 for each experiment. The control and treated larvae were then transferred to clean plastic jar containing fresh leaves of *Tinospora cardifolia.* Control and treated larvae were sacrificed after 1, 4, 6, 12, and 24 h for histological preparations and midgut digestive enzymes activities determination.

Histological techniques

Five third instar larvae from each group were sacrificed after 1, 2, 4, 6, 12, and 24 h since the commencement of experiment and the midgut was dissected out, fixed in aqueous Bouin's fluid for 18-24 h, washed, dehydrated in ethanol, cleared in xylene, and embedded in paraffin wax (58-60 °C). Sections of anterior part of midgut were cut at 4 µm thickness and stained with Haematoxylin-Eosin or Heidenhain's Iron-Haematoxylin-Orange-G staining techniques (Humason, 1962). Histological sections were examined by Labomed Digi-3 compound microscope.

Preparation of enzyme extract

Five third instars of treated *O. materna* were used to estimate the enzyme activities. Enzyme extracts were prepared by the method of Applebaum et al. (1961). Larvae were anaesthetized with cotton pads soaked in ether and entire digestive tract was dissected out in ice-cold insect Ringer's physiological saline solution. The Malpighian tubules, adhering tissues, and gut contents were removed. The gut was split into regions (foregut, midgut, and hindgut). Entire midgut was weighed and homogenized for 3 min at 4 °C in ice-cold citrate-phosphate buffer (pH 6.8) using hand operated tissue homogenizer and made up to 1 ml. The homogenate was centrifuged at 1000 rpm for 15 min and the supernatant was used as the enzyme source. Absorbance was measured on a Spectronic-20 spectrophotometer (Milton Roy Co.)

Amylase activity

Amylase activity was determined based on the method as described by Ishaaya and Swirski (1970). The reaction mixture consisted of 2 ml of 2.5% freshly prepared starch solution, 1 ml of 0.01 M phosphate buffer (pH 7.5), and 0.25 ml of enzyme extract. After incubating for 1 h at 37 °C, the enzyme activity was terminated by adding 0.4 ml of 3, 5-dinitro salicylic acid reagent. The reaction mixture was boiled for 2 min. Absorbance of the sample was measured at 550 nm against a blank in which

the enzyme extract was replaced with distilled water. The enzyme activity was expressed in terms of mg of glucose liberated/midgut/ml enzyme solution/min.

Invertase activity

Invertase activity was determined based on the method as described by Ishaaya and Swirski (1970). The reaction mixture includes 2 ml of 1.2% sucrose solution, 1 ml of 0.01 M phosphate buffer (pH 6.2), and 0.15 ml of enzyme extract. After incubating for 1 h at 37 °C, the enzyme activity was terminated by adding 0.4 ml of 3, 5-dinitro salicylic acid reagent. The reaction mixture was boiled for 2 min. The differences in absorbance were measured at 550 nm. The enzyme activity was expressed in terms of mg of glucose liberated/midgut/ml enzyme solution/min.

Lipase activity

The enzyme assays were carried out as described by Cherry and Crandall (1932). First, 1 ml enzyme extract (the control tube was placed in a boiling water bath for 15 min to destroy the enzyme activity and then cooled), 1 ml of phosphate buffer solution (pH 6.5), and 2 ml of olive oil emulsion were added into a tube. Then, the tube was shaken well and incubated at 37 °C for 24 h. 3 ml of 95% ethyl alcohol and 2 drops of 2% phenolphthalein indicator were added to each tube. The tubes were titrated separately with 0.05 N NaOH, and the end point of titration was marked by the appearance of pink color. The enzyme activity was expressed in terms of mg of oleic acid liberated/midgut/ml enzyme solution/min.

Protease activity

The enzyme assays were carried out as described by Snell and Snell (1971). The reaction mixture consisted of 1 ml of 50 ppm bovine serum albumin, 1 ml of enzyme extract (the control tube contain heat-treated extract), and 1 ml pf phosphate buffer (pH 8.5). After incubating for 1 h at 37 °C the reaction was terminated by adding 1 ml of 50% trichloroacetic acid. The differences in absorbance were measured at 660 nm. The enzyme activity expressed in terms of mg of protein liberated/midgut/ml enzyme solution/min.

Statistical analysis

Data from enzyme activity were subjected to one way analysis of variance (ANOVA). Differences between the treatments were determined by Tukey's multiple range test ($P \leq 0.05$) (Snedecor and Cochran, 1989).

Results

Histopathological changes in the midgut

In the control insects sacrificed at the onset of experiment (0 h) and after 1, 6, 12 and 24 h, the structure of the midgut was not significantly altered. The midgut epithelium is thin, slightly folded in the anterior region while uniformly folded in the posterior region. It consists of tall columnar cells and is interspersed apically with the goblet and basally with regenerative cells. The columnar cells contain large spherical nuclei in the middle of apical region and the cytoplasm is granular. The columnar cell possesses a fine brush border facing towards lumen and large number of vesicles discharging into extra peritrophic region of the lumen. The goblet cells are flask shaped with oval nuclei and a bulk of cytoplasm in the basal region. The regenerative cells are small spherical or elongated, and are basally located. They contain large spherical nuclei at the center and a granular cytoplasm. The peritrophic membrane is well evident in the lumen of the midgut (Figure 1).

After 1 h treatment of carbaryl, the nucleus was condensed at the basal region of the goblet cells while it was granular in the columnar cells. The peritrophic membrane was not closely lying to the epithelial cells and the space in between the epithelium and peritrophic membrane was filled with few cytoplasmic vesicles (Figure 2a). After 6 h, clusters of small round regenerative cells were prominent adjacent to the basal

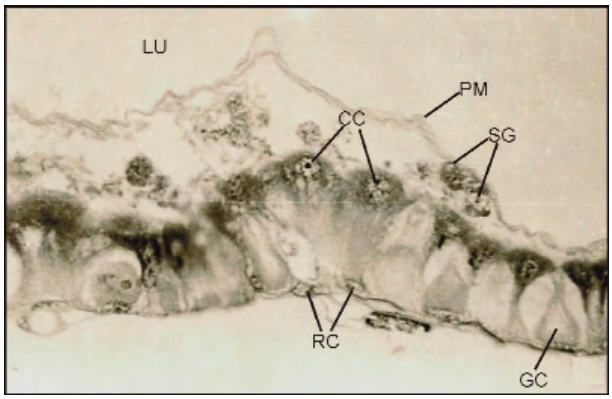

Figure 1. Section showing the anterior midgut epithelium of control third instar larvae of *O. materna.* Sections stained with Haematoxylin-Eosin. Note the columnar cells (CC), goblet cells (GC), lumen (L), peritrophic membrane (PM), regenerative cells (RC), and secretory vesicles (SV) ×200.

membrane. The goblet cells were empty and vacuolated. The membranous vesicles present in the space lying in between the epithelium and peritrophic membrane were empty. After 12 h of treatment, the nuclei were condensed in some columnar and goblet cells while remaining cells were filled with cytoplasmic inclusions. After 24 h, the columnar cells reproduce membranous vesicles filled with secretory material indicating resumption of the secretory activity by the midgut epithelia (Figure 2b).

One hour after treatment of γ-BHC, the columnar epithelial cells showed little regression. The nuclei were well evident in the apical region of the columnar and goblet cells. Cytoplasmic granules and globules from the columnar cells into the space lying in between peritrophic membrane and epithelium were observed (Figure 3a). After 6 h, the columnar cells of the anterior midgut swell apically and began to extrude large cytoplasmic vesicles into the gut lumen. The goblet cells were vacuolated. The membranous vesicles were absent. The regenerative cells remain unaffected. After 12 h of treatment, destruction of the gut epithelium was complete and only disorganized layer of shrunken columnar cells remain, along with condensed nuclei. Vacuolated goblet cells can be observed extending to the basal membrane itself (Figure 3b). After 24 h, the goblet and columnar cells discharged membranous vesicles indicating normal functioning of the midgut after the due recovery from the histopathological injury.

Effects on digestive enzymes

There were significant differences in enzyme activities after the treatments of carbaryl and γ-BHC (Table).

Carbaryl caused significant ($P < 0.05$) enhancement of the amylase activity initially; however, after 4 h of treatment, it fell gradually without undergoing the recovery. γ-BHC caused non-significant changes in the amylase activity up to 4 h but later on it significantly enhanced up to 6 h. Thereafter, the activity constantly fell without showing any sign of recovery.

Invertase activity enhanced initially and then it fell rapidly up to 12 h in carbaryl treated larvae. The invertase activity was again significantly elevated thereafter. γ-BHC enhanced the invertase activity up to 4 h but thereafter it fell and showed considerable recovery.

Carbaryl caused extreme enhancement of the lipase activity up to 4 h then there was a significant reduction in the activity up to 6 h. Later on it again enhanced up to 12 h and thereafter dropped down. γ-BHC also caused extreme enhancement of the lipase activity up to 4 h, but there was significant reduction till 12 h. The lipase activity was elevated thereafter.

Gradual depletion in the protease activity was recorded initially up to 4 h in carbaryl treated larvae. Later on it significantly enhanced up to 6 h and thereafter dropped down. γ-BHC did not stimulate the protease activity up to 4 h. Later on it enhanced up to 12 h but thereafter it showed steep depletion.

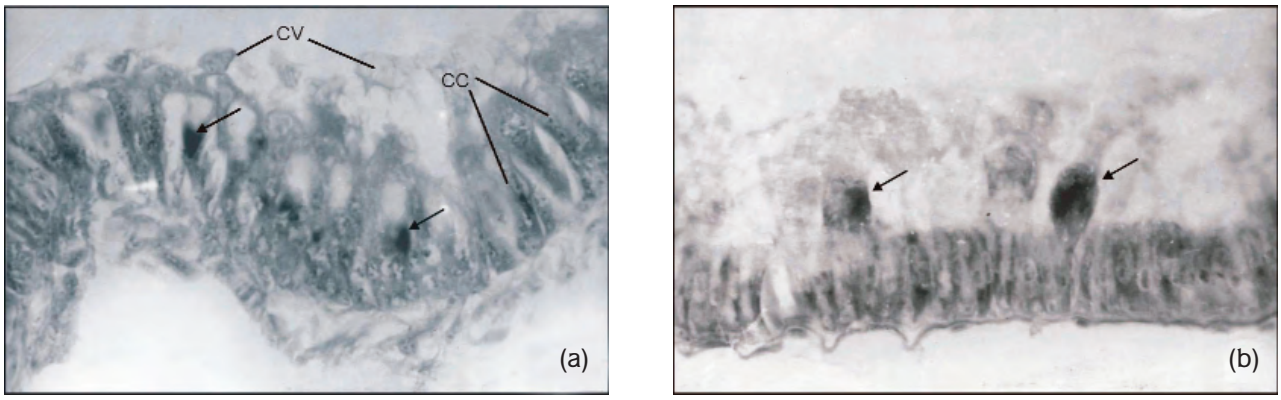

Figure 2. Carbaryl induced changes in histological structure of midgut in third instar larvae of *O. materna*. Sections stained with Haematoxylin-Eosin. (a) Carbaryl treated larva at 1 h showing condensed nuclei (arrows) of the goblet cells and apical cytoplasmic vesicles (CV), which are shed into the gut lumen. Note the basal-apical elongation of the epithelial cells, granular nuclei in the columnar cells (CC) ×320, (b) Carbaryl treated larva at 24 h showing membranous vesicles extruding into the gut lumen (arrows) ×320.

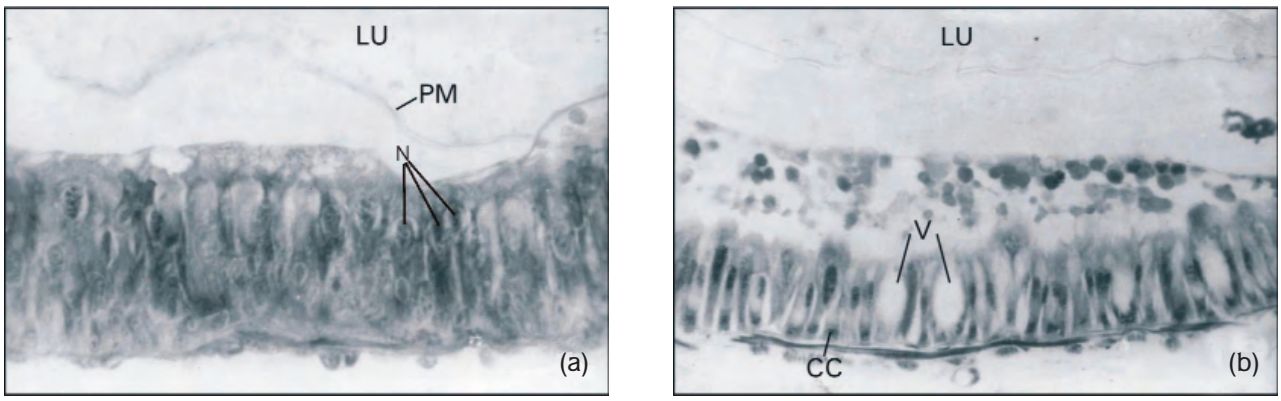

Figure 3. γ-BHC induced changes in histological structure of midgut in third instar larvae of *O. materna*. Sections stained with Haematoxylin-Eosin. (a) γ-BHC treated larva at 1 h showing regressed columnar epithelial cells with apically located nucleus (N) ×320, (b) γ-BHC treated larva at 12 h showing complete destruction of columnar cells. Note the shrunken columnar cells with condensed nuclei and vacuolated goblet cells (V) ×320. CC-columnar cells, LU-lumen, PM-peritrophic membrane, N-nucleus, V-vacuole.

Table. Midgut digestive enzyme activities of third instar larvae of *O. materna* after the treatment with carbaryl and γ-BHC.

Experimental Period	Amylase			Invertase			Lipase			Protease		
	Control	Carbaryl	γ-BHC	Control	Carbaryl	γ-BHC	Control	Carbaryl	γ-BHC	Control	Carbaryl	γ-BHC
0 h	0.223 ± 0.007a	0.223 ± 0.007a	0.223 ± 0.007a	0.125 ± 0.009a	0.125 ± 0.009a	0.125 ± 0.009a	0.067 ± 0.008a	0.067 ± 0.008a	0.067 ± 0.008a	0.043 ± 0.008a	0.043 ± 0.008a	0.043 ± 0.008a
1 h	0.227 ± 0.025a	0.408 ± 0.019b	0.196 ± 0.009a	0.124 ± 0.009a	0.173 ± 0.007a	0.310 ± 0.011b	0.064 ± 0.012a	0.650 ± 0.035b	0.177 ± 0.014	0.044 ± 0.008a	0.033 ± 0.005a	0.040 ± 0.007a
4 h	0.225 ± 0.019a	0.577 ± 0.014b	0.213 ± 0.007a	0.136 ± 0.017a	0.578 ± 0.010b	0.380 ± 0.011b	0.065 ± 0.005a	0.840 ± 0.008b	0.950 ± 0.180c	0.044 ± 0.007a	0.017 ± 0.004c	0.037 ± 0.004a
6 h	0.236 ± 0.036a	0.300 ± 0.016a	0.410 ± 0.008b	0.142 ± 0.012a	0.281 ± 0.006b	0.210 ± 0.012b	0.080 ± 0.026a	0.057 ± 0.018a	0.169 ± 0.012b	0.049 ± 0.010a	0.082 ± 0.008b	0.065 ± 0.005b
12 h	0.272 ± 0.012a	0.195 ± 0.009a	0.319 ± 0.013b	0.161 ± 0.016a	0.093 ± 0.004c	0.113 ± 0.010a	0.086 ± 0.007a	0.295 ± 0.011c	0.030 ± 0.007d	0.041 ± 0.009a	0.017 ± 0.004c	0.078 ± 0.004b
24 h	0.289 ± 0.017a	0.146 ± 0.009c	0.140 ± 0.010c	0.168 ± 0.007a	0.290 ± 0.110b	0.169 ± 0.010a	0.104 ± 0.021a	0.100 ± 0.007a	0.102 ± 0.012a	0.046 ± 0.007a	0.025 ± 0.005a	0.012 ± 0.004c

Means ± SE followed by the same letter within columns are not significantly different $P \leq 0.05$ (ANOVA followed by LSD post test), n = 5.

Discussion

The histological organization of the midgut in larvae of *O. materna* represents a typical structure of to that lepidopteran larvae (Chapman, 1985; Deshmukh, 2006). Carbaryl and γ-BHC caused the structural damage to the midgut epithelium of *O. materna*. The vacuolization was induced after 1 and 2 h of treatment in the larvae. Vacuolization of the midgut epithelium was also reported after the treatment of arsenides (Rizvi and Khan, 1973) and organophosphates (Deshmukh and Tembhare, 1998) in different insect groups.

Treatment of carbaryl and γ-BHC on third instar larvae of *O. materna* demonstrated that some cytological changes and hypersecretory activity. Several workers reported the elongation and regression of the epithelial cells and deposition of numerous vesicles in the extra peritrophic space mostly due to hypersecretory activity, after the treatment of insecticides (Toppozada et al., 1968; Rizvi and Khan, 1973; Deshmukh and Tembhare, 1998). Apical swelling and blebbing of large cytoplasmic vesicles by the columnar cells leads to the eventual extrusion of cell nuclei in vesicles into the gut lumen and lysis of the gut epithelium (Blackburn et al., 1998). Condensation of the

nuclei of midgut epithelium of the larvae of *O. materna* was prominently evident similar to that in *Spodoptera littoralis* (Boisd) (Toppozada et al., 1968). In the present study the histopathological changes induced by the carbaryl and γ-BHC are almost recovered within 24 h. These results were confirmed by findings of previous studies (Ishaaya and cassida, 1981; Deshmukh and Tembhare, 1998). There seems to be little effect on undifferentiated regenerative cells. Thus, clusters of small round cells can still be observed intact adjacent to the basal membrane.

In the present study, after carbaryl and γ-BHC treatment, the enzymatic profiles were markedly affected. Significant increase in the activities of amylase, invertase, and lipase after the treatment of carbaryl and γ-BHC was initially well evident. The insecticides may cause rapid secretion of the stored enzymes in the midgut tissue (Eguchi et al., 1972; Chanda and Roy, 1986; Deshmukh and Tembhare, 1998). Insecticides interact with a variety of neurochemical processes and are likely to be involved in the disruption of nerve function (Soderlund and Bloomquist, 1989), which might have caused the initial hypersecretory activity and cytological changes. Later on inhibitory effects of insecticides on enzyme activity were observed. The hydrocarbons are reported to have a long-term inhibitory effect on a variety of enzymes (Barnes, 1976; Matsumura, 1985; Osborne, 1985). Fenvalerate treatment induced a reduction in the activity of amylase, sucrase, and protease when midgut was used as the source of enzyme (Vyjayanthi and Subramanyam, 2002). Imbalance in enzyme-substrate complex and inhibition of peristaltic movement of the gut might have inhibited the enzyme activity in the treated insects (Senthil Nathan et al., 2006). Insecticides may interfere with the production of certain types of proteins (Smirle et al., 1996; Senthil Nathan et al., 2006). Carbaryl and γ-BHC caused damage to the epithelial cells of the midgut of *O. materna*. The damage to the midgut caused a decrease in the digestive enzyme activity (Mathavan et al., 1989; Abo El-Ghar et al., 1995; Smirle et al., 1996; Senthil Nathan et al., 2005; Huang et al., 2008).

The results obtained in the present study suggest that Carbaryl and γ-BHC disturb the digestive enzymes secretion, which may affect the physiology of digestion to a great extent. This supports O'Brien (1967) in that the impaired physiology of digestion may be one of the major factors causing intoxication in insects.

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