# Evaluation of the toxic effect of insecticide chlorantraniliprole on the silkworm *Bombyx mori* (Lepidoptera: Bombycidae)

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## ABSTRACT

The silkworm *Bombyx mori* feeds exclusively on mulberry leaves and is highly sensitive to pesticides in general. Although mulberry plantations are free of agrochemicals, pesticide drift can occur. Chlorantraniliprole, a novel insecticide of the anthranilic diamides class, has been used to control pests in field crops. In this study, we investigated the biological effects of different concentrations of chlorantraniliprole on *B. mori* silkworm commercial Brazilian hybrids. To evaluate the toxicity of chlorantraniliprole, bio- assays were carried out and data on the lethal concentrations, symptomatology, morphology and variables of silk production were collected. Results indicated that *B. mori* is extremely sensitive to chlorantraniliprole, even in low concentrations. The highest silkworm mortality rates were observed in the two highest chlorantraniliprole concentrations, 0.2 and 0.1 ppm. Although lower chlorantraniliprole concentrations did not cause death of all the silkworm larvae, various symptoms of toxicity were observed: feeding cessation, regurgitation, late development and incomplete ecdysis. Such symptoms reflect the morphological changes we observed in the midgut epithelium, which affected nutrient uptake and metabolism, and even the production of cocoons. Exposed larvae also produced thin-shelled cocoons, which constitutes a serious economic problem because this type of cocoon is not useful for the silk industry. The results provided herein confirm the toxicity of chlorantraniliprole in silkworm larvae. Therefore, we strongly suggest that, competent authorities of the National Health Surveillance Agency, in pesticide management should take measures to reduce or eliminate the use of chlorantraniliprole in areas nearest to silkworm cultivation.

**Keywords:** Silkworm, Pesticide Drift; Mortality; Symptomatology; Chlorantraniliprole

## 1. INTRODUCTION

The silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae), an insect originally from China, was domesticated 5000 years ago for silk production [1]. Silkworms are no longer found in nature and are totally dependent on man to survive. *Bombyx mori* most likely evolved from *B. mandarina*, a wild silkworm species with similar characteristics and reproduction to *B. mori* [2]. Silkworms are herbivorous and feed exclusively on fresh mulberry leaves during the larval phase [3].

The practice of sericulture includes mulberry cultivation, egg production, larvae creation and maintenance until cocoon production and, finally, silk yard production by the industrial sector. Brazil's weather is favorable for

cultivating both mulberry and silkworms, which makes sericulture a good alternative for this country, especially for small farmers.

*Bombyx mori* is highly susceptible to insecticides, and the application of insecticides near sericulture projects is not advisable because the silkworms can be harmed by chemicals on the leaves, either through consumption of contaminated leaves or through other contact with the insecticides [4]. Hence, mulberry plantations intended for silkworms are free of pesticides. However, the complete elimination of pesticide drift is impossible and insecticides that are employed in other crops, especially those that are applied through aerial spraying, can damage sericulture activity.

Chlorantraniliprole is a novel insecticide belonging to a potent class of anthranilic diamides that is usually safe for mammals [5] and is commonly used in rice, coffee, sugar cane, apple and peach crops. Chlorantraniliprole eliminates Lepidoptera insects [6], as well as other pest orders such as Coleoptera, Diptera, Isoptera and Hemiptera [7]. This insecticide, once ingested, acts as a selective agonist for ryanodine receptors, a class of intracellular calcium channels. Ryanodine receptors play an important role in muscle function because the contraction of muscle cells requires exact liberation of calcium from intracellular deposits in the cytoplasm. Chlorantraniliprole causes unregulated $Ca^{2+}$ release from the sarcoplasmic reticulum of muscle cells [8], which impedes the insect's ability to regulate muscle function and leads to permanent muscle contraction. Poisoning symptoms include rapid cessation of feeding, lethargy, regurgitation, muscle paralysis and, ultimately, insect death [9-11].

The effects of chlorantraniliprole in different insects have been well-studied in many taxa: *Choristoneura rosaceana* [12], termites *Reticulitermes flavipes* [13], honey bees *Apis mellifera* and bumble bees *Bombus terrestris* [8] and *Leptinotarsa decemlineata* [14]. However, little information is described about the effects of chlorantraniliprole on *B. mori* [11]. Although this insecticide is not used in mulberry fields, it can cause loss of cocoon production in Brazilian silkworm farms near areas where chlorantraniliprole is used. Therefore, in this study, we investigated the effects of different concentrations of chlorantraniliprole on commercial Brazilian hybrids of the *B. mori* silkworm.

Recently, big crops such as sugarcane are using new kinds of insecticide to do the control of *Diatrea saccharalis* larvae in Paraná State, Brazil. The areas of mulberry plantation, which are close to the sugarcane crops, are affected by aerial spraying of the insecticide, which results in *B. mori* larvae death. Studies made by the agriculture secretary of Paraná State show a loss of cocoons equal to 8,626 kilograms. This State is the biggest producer of cocoons from Brazil, responsible for 92% of the total production in this country and due to this recent problem with cloranthraliniprole insecticide. This work was developed with the intention of describing the symptomatology of the intoxication in *B. mori* larvae that were exposed to this insecticide. The description of this symptom induced in laboratory can help the comprehension of the toxic effect caused by cloranthraliniprole in the larvae, which has a commercial interest for several countries. Those data can further help the Brazilian sericulture, as the results can assist either the farmer who works with sericulture or the company behind all the production.

## 2. MATERIAL AND METHODS

### 2.1. *Bombyx mori* Culture

The bioassays were carried out at the Fiação de Seda BRATAC laboratory at Bastos city, São Paulo, Brazil with the commercial hybrids of *B. mori* from this company. Silkworm larvae were reared under hygienic conpditions and in controlled environments. Rearing rooms were adjusted to $28°C \pm 0.5°C$ and $90\% \pm 5\%$ of relative humidity (RH) for the creation of 1st instar silkworm larvae, while 2nd instar larvae were reared at $27°C \pm 0.5°C$ and with $85\% \pm 5\%$ of RH. Larvae of the 3rd, 4th and 5th instars were reared at 25 to $26°C \pm 0.5°C$ and with RH of $70\% \pm 5\%$. Artificial white lighting was employed in the rearing rooms to submit silkworm larvae to fourteen-hour daily of photoperiod with 10 hours of dark. During the experiments, larval mortality and symptoms of exposure were recorded.

### 2.2. Lethal Concentration

Treatments consisted of three replicates, with 20 silkworm larvae each. The following chlorantraniliprole concentrations, in ppm (parts per million), were used: 0.2, 0.1, 0.05, 0.025, 0.0125, $6.25 \times 10^{-3}$, $3.13 \times 10^{-3}$ and $1.57 \times 10^{-3}$. Control groups consisted of silkworm larvae that were fed exclusively on mulberry leaves free of chlorantraniliprole.

Silkworm larvae were exposed once to mulberry leaves containing chlorantraniliprole at different days and different instars, depending on the treatment, as follows: 1st day of the 1st instar, 2nd day of the 1st instar, 1st day of the 2nd instar, 3rd day of the 2nd instar, 1st day of the 3rd instar, 3rd day of the 3rd instar, 1st day of the 4th instar, 3rd day of the 4th instar, 1st day of the 5th instar and 5th day of the 5th instar. Besides, bioassays also included 5th instar larvae that were exposed twice to chlorantraniliprole: on the 1st and 2nd days of the 5th instar and on the 5th and 6th days of the 5th instar.

For the lethal concentration bioassays, mulberry leaves were immersed in aqueous solutions containing dif-

ferent concentrations of chlorantraniliprole, as mentioned above. Leaves were dried at room temperature and then offered to *B. mori* larvae. Each replicate included 20 silkworm larvae that were fed once a day in petri-dishes with different amounts of mulberry leaves containing chlorantraniliprole, as follows: larvae exposed to the insecticide in the $1^{st}$ or $2^{nd}$ days of the $1^{st}$ instar were fed with 7 cm$^2$ of mulberry leaves, while larvae exposed in the $1^{st}$ or $3^{rd}$ days of the $2^{nd}$ instar were fed with 16 cm$^2$; larvae in the $1^{st}$ or $3^{rd}$ days of the $3^{rd}$ instar were fed with 10 g of mulberry leaves and those in the $1^{st}$ or $3^{rd}$ days of the $4^{th}$ instar and in the $1^{st}$ or $5^{th}$ days of the $5^{th}$ instar were fed with 30 g of mulberry leaves. Treatments involving silkworm larvae exposed twice to chloran- traniliprole were fed with 30 g of mulberry leaves when exposure occurred in the $1^{st}$ and $2^{nd}$ days of the $5^{th}$ instar and with 100 g of mulberry leaves when exposure occurred in the $5^{th}$ and $6^{th}$ days of the $5^{th}$ instar. Before and after chlorantraniliprole exposure, larvae were fed in abundance with mulberry leaves free of chlorantraniliprole.

Mulberry trees used in the bioassays have been grown in the experimental field of the company Fiação de Seda BRATAC, Bastos city in São Paulo state, Brazil. Mulberry leaves used for silkworm feeding were taken from 75-days mulberry trees. Silkworm larvae of the $1^{st}$, $2^{nd}$ and $3^{rd}$ instars were fed with young mulberry leaves taken from the top part of the principal branche, while old leaves were offered for $4^{th}$ and $5^{th}$ silkworm larvae.

### 2.3. Symptomatology

After chlorantraniliprole applications, symptoms related to insecticide exposure were registered immediately after the first day of exposure: feeding cessation, regurgitation, late development and irregular ecdysis. These symptoms were classified according to the intensity, where 0 represents the absence of symptoms and 5 represents the highest level of the symptom. In addition, the following productive variables were collected: mean number of normal cocoons (NC), mean weight of normal cocoon (NCW), mean number of thin-shelled cocoons (TSC), mean weight of thin-shelled cocoons (TSCW) and mean number of live pupae (LP). Although no measurements were taken to distinguish thin-shelled cocoons from normal cocoons, thin-shelled cocoons can be visually and unambiguously differentiated from normal cocoons since their shells are thinner and translucent.

### 2.4. Morphological Analysis

The effects of chlorantraniliprole in *B. mori* midgut tissue were evaluated. Larvae of $5^{th}$ instar were exposed to two different concentrations of this insecticide: 0.2 ppm and 0.1 ppm, in addition to the control group. On the $4^{th}$ day after exposure to chlorantraniliprole, larvae were anesthetized and dissected. Segments of the midgut wall were removed and fixed in DuBosq Brasil, dehydrated in ethanol and preserved in paraffin. Sequential cuts were made on an Olympus CUT4055 microtome at thicknesses ranging between 5 and 7 μm. Sections were stained with hematoxylin-eosin for general tissue analysis. Images were obtained with on Olympus BX 60 photomicroscope.

### 2.5. Statistical Analysis

The experimental delineation was completely randomized with three replicates per treatment (total 108 treatments). Twenty silkworm larvae were used per replicate. The data were compared with an analysis of variance, followed by the Scott-Knott means test (0.05%) using the statistical software SISVAR 5.3 [15].

## 3. RESULTS AND DISCUSSION

This insecticide is frequently applied by spray in sugarcane plantations next at silkworm producers in south of Brazil, causing damaging and lost of cocoon production. In the last years, Brazilian silkworm producers have been reporting great losses in cocoon production. Apparently, these losses are related to the period of time when insecticides are aerial sprayed in crops near silkworm farms. Recently, chlorantraniliprole has been employed in crops to eliminate Lepidoptera insects, especially sugarcane crops. In this study, we performed bioassays to verify if the symptoms caused by chlorantraniliprole in silkworm larvae are similar to those observed by producers in silkworm farms. To evaluate the toxicity and symptomatology of chlorantraniliprole in Brazilian hybrids of *B. mori*, we exposed groups of silkworm larvae to different concentrations of chlorantraniliprole.

### 3.1. Lethal Chlorantraniliprole Concentration

We analyzed the mortality of silkworm larvae after they were exposed to eight different concentrations of chlorantraniliprole (**Table 1**). The two highest chlorantraniliprole concentrations, 0.2 ppm and 0.1 ppm, resulted in 100% larval mortality in most silkworm larvae instars, though not when exposure occurred on the $1^{st}$ and $3^{rd}$ day of the $2^{nd}$ instar. Complete larval mortality was also observed in the groups exposed to 0.05 ppm in the $5^{th}$ day of the $5^{th}$ instar and in the $5^{th}$ and $6^{th}$ days of the $5^{th}$ instar. Silkworm larvae that were exposed to a concentration of 0.025 ppm on the $5^{th}$ and $6^{th}$ days of the $5^{th}$ instar also had a high mortality rate of 98.33%. Overall, exposures of silkworm larvae on the $5^{th}$ and $6^{th}$ days of the $5^{th}$ instar to the four highest chlorantraniliprole concentrations resulted in a 100% rate of larval mortality,

**Table 1.** *Bombyx mori* larvae mortality considering instar, day of exposure and concentration of the insecticide chlorantraniliprole.

| Instar | Day of exposure | Chlorantraniliprole concentration (ppm) | Mortality (%) |
|--------|-----------------|------------------------------------------|---------------|
| 1st instar | 1st day | 0.2 | 100.00 a |
| 1st instar | 1st day | 0.1 | 100.00 a |
| 1st instar | 1st day | 0.05 | 80.00 b |
| 1st instar | 1st day | 0.025 | 0.00 f |
| 1st instar | 1st day | 0.0125 | 0.00 f |
| 1st instar | 1st day | $6.25 \times 10^{-3}$ | 0.00 f |
| 1st instar | 1st day | $3.13 \times 10^{-3}$ | 0.00 f |
| 1st instar | 1st day | $1.57 \times 10^{-3}$ | 0.00 f |
| 1st instar | 1st day | Control group | 0.00 f |
| 1st instar | 2nd day | 0.2 | 100.00 a |
| 1st instar | 2nd day | 0.1 | 100.00 a |
| 1st instar | 2nd day | 0.05 | 96.66 a |
| 1st instar | 2nd day | 0.025 | 0.00 f |
| 1st instar | 2nd day | 0.0125 | 0.00 f |
| 1st instar | 2nd day | $6.25 \times 10^{-3}$ | 0.00 f |
| 1st instar | 2nd day | $3.13 \times 10^{-3}$ | 0.00 f |
| 1st instar | 2nd day | $1.57 \times 10^{-3}$ | 0.00 f |
| 1st instar | 2nd day | Control group | 0.00 f |
| 2nd instar | 1st day | 0.2 | 100.00 a |
| 2nd instar | 1st day | 0.1 | 85.00 b |
| 2nd instar | 1st day | 0.05 | 0.00 f |
| 2nd instar | 1st day | 0.025 | 0.00 f |
| 2nd instar | 1st day | 0.0125 | 0.00 f |
| 2nd instar | 1st day | $6.25 \times 10^{-3}$ | 0.00 f |
| 2nd instar | 1st day | $3.13 \times 10^{-3}$ | 0.00 f |
| 2nd instar | 1st day | $1.57 \times 10^{-3}$ | 0.00 f |
| 2nd instar | 1st day | Control group | 0.00 f |
| 2nd instar | 3rd day | 0.2 | 88.33 b |
| 2nd instar | 3rd day | 0.1 | 55.00 c |
| 2nd instar | 3rd day | 0.05 | 0.00 f |
| 2nd instar | 3rd day | 0.025 | 0.00 f |
| 2nd instar | 3rd day | 0.0125 | 0.00 f |
| 2nd instar | 3rd day | $6.25 \times 10^{-3}$ | 0.00 f |
| 2nd instar | 3rd day | $3.13 \times 10^{-3}$ | 0.00 f |
| 2nd instar | 3rd day | $1.57 \times 10^{-3}$ | 0.00 f |
| 2nd instar | 3rd day | Control group | 0.00 f |
| 3rd instar | 1st day | 0.2 | 100.00 a |
| 3rd instar | 1st day | 0.1 | 100.00 a |
| 3rd instar | 1st day | 0.05 | 5.00 f |
| 3rd instar | 1st day | 0.025 | 0.00 f |
| 3rd instar | 1st day | 0.0125 | 0.00 f |
| 3rd instar | 1st day | $6.25 \times 10^{-3}$ | 0.00 f |
| 3rd instar | 1st day | $3.13 \times 10^{-3}$ | 0.00 f |

**Continued**

| | | | |
|---|---|---|---|
| 3$^{rd}$ instar | 1$^{st}$ day | $1.57 \times 10^{-3}$ | 0.00 f |
| 3$^{rd}$ instar | 1$^{st}$ day | Control group | 0.00 f |
| 3$^{rd}$ instar | 3$^{rd}$ day | 0.2 | 100.00 a |
| 3$^{rd}$ instar | 3$^{rd}$ day | 0.1 | 98.33 a |
| 3$^{rd}$ instar | 3$^{rd}$ day | 0.05 | 33.33 d |
| 3$^{rd}$ instar | 3$^{rd}$ day | 0.025 | 13.33 e |
| 3$^{rd}$ instar | 3$^{rd}$ day | 0.0125 | 0.00 f |
| 3$^{rd}$ instar | 3$^{rd}$ day | $6.25 \times 10^{-3}$ | 0.00 f |
| 3$^{rd}$ instar | 3$^{rd}$ day | $3.13 \times 10^{-3}$ | 0.00 f |
| 3$^{rd}$ instar | 3$^{rd}$ day | $1.57 \times 10^{-3}$ | 0.00 f |
| 3$^{rd}$ instar | 3$^{rd}$ day | Control group | 0.00 f |
| 4$^{th}$ instar | 1st day | 0.2 | 100.00 a |
| 4$^{th}$ instar | 1$^{st}$ day | 0.1 | 100.00 a |
| 4$^{th}$ instar | 1$^{st}$ day | 0.05 | 3.33 f |
| 4$^{th}$ instar | 1$^{st}$ day | 0.025 | 1.66 f |
| 4$^{th}$ instar | 1$^{st}$ day | 0.0125 | 3.33 f |
| 4$^{th}$ instar | 1$^{st}$ day | $6.25 \times 10^{-3}$ | 6.66 f |
| 4$^{th}$ instar | 1$^{st}$ day | $3.13 \times 10^{-3}$ | 1.66 f |
| 4$^{th}$ instar | 1$^{st}$ day | $1.57 \times 10^{-3}$ | 3.33 f |
| 4$^{th}$ instar | 1$^{st}$ day | Control group | 0.00 f |
| 4$^{th}$ instar | 3$^{rd}$ day | 0.2 | 100.00 a |
| 4$^{th}$ instar | 3$^{rd}$ day | 0.1 | 100.00 a |
| 4$^{th}$ instar | 3$^{rd}$ day | 0.05 | 15.00 e |
| 4$^{th}$ instar | 3$^{rd}$ day | 0.025 | 5.00 f |
| 4$^{th}$ instar | 3$^{rd}$ day | 0.0125 | 0.00 f |
| 4$^{th}$ instar | 3$^{rd}$ day | $6.25 \times 10^{-3}$ | 5.00 f |
| 4$^{th}$ instar | 3$^{rd}$ day | $3.13 \times 10^{-3}$ | 1.66 f |
| 4$^{th}$ instar | 3$^{rd}$ day | $1.57 \times 10^{-3}$ | 3.33 f |
| 4$^{th}$ instar | 3$^{rd}$ day | Control group | 1.66 f |
| 5$^{th}$ instar | 1$^{st}$ day | 0.2 | 100.00 a |
| 5$^{th}$ instar | 1$^{st}$ day | 0.1 | 100.00 a |
| 5$^{th}$ instar | 1$^{st}$ day | 0.05 | 13.33 e |
| 5$^{th}$ instar | 1$^{st}$ day | 0.025 | 3.33 f |
| 5$^{th}$ instar | 1$^{st}$ day | 0.0125 | 3.33 f |
| 5$^{th}$ instar | 1$^{st}$ day | $6.25 \times 10^{-3}$ | 1.66 f |
| 5$^{th}$ instar | 1$^{st}$ day | $3.13 \times 10^{-3}$ | 3.33 f |
| 5$^{th}$ instar | 1$^{st}$ day | $1.57 \times 10^{-3}$ | 1.66 f |
| 5$^{th}$ instar | 1$^{st}$ day | Control group | 0.00 f |
| 5$^{th}$ instar | 5$^{th}$ day | 0.2 | 100.00 a |
| 5$^{th}$ instar | 5$^{th}$ day | 0.1 | 100.00 a |
| 5$^{th}$ instar | 5$^{th}$ day | 0.05 | 100.00 a |
| 5$^{th}$ instar | 5$^{th}$ day | 0.025 | 56.66 c |
| 5$^{th}$ instar | 5$^{th}$ day | 0.0125 | 1.66 f |
| 5$^{th}$ instar | 5$^{th}$ day | $6.25 \times 10^{-3}$ | 0.00 f |

**Continued**

| | | | |
|---|---|---|---|
| 5th instar | 5th day | $3.13 \times 10^{-3}$ | 1.66 f |
| 5th instar | 5th day | $1.57 \times 10^{-3}$ | 0.00 f |
| 5th instar | 5th day | Control group | 0.00 f |
| 5th instar | 1st - 2nd days | 0.2 | 100.00 a |
| 5th instar | 1st - 2nd days | 0.1 | 100.00 a |
| 5th instar | 1st - 2nd days | 0.05 | 56.66 c |
| 5th instar | 1st - 2nd days | 0.025 | 3.33 f |
| 5th instar | 1st - 2nd days | 0.0125 | 1.66 f |
| 5th instar | 1st - 2nd days | $6.25 \times 10^{-3}$ | 0.00 f |
| 5th instar | 1st - 2nd days | $3.13 \times 10^{-3}$ | 0.00 f |
| 5th instar | 1st - 2nd days | $1.57 \times 10^{-3}$ | 0.00 f |
| 5th instar | 1st - 2nd days | Control group | 0.00 f |
| 5th instar | 5th - 6th days | 0.2 | 100.00 a |
| 5th instar | 5th - 6th days | 0.1 | 100.00 a |
| 5th instar | 5th - 6th days | 0.05 | 100.00 a |
| 5th instar | 5th - 6th days | 0.025 | 98.33 a |
| 5th instar | 5th - 6th days | 0.0125 | 0.00 f |
| 5th instar | 5th - 6th days | $6.25 \times 10^{-3}$ | 5.00 f |
| 5th instar | 5th - 6th days | $3.13 \times 10^{-3}$ | 0.00 f |
| 5th instar | 5th - 6th days | $1.57 \times 10^{-3}$ | 3.33 f |
| 5th instar | 5th - 6th days | Control group | 0.00 f |
| Overall mean | | | 28.34 |
| VC (%) | | | 23.78 |

(a-f) in the columns are different by the Scott-Knott test ($P < 0.05$).

which indicates that larvae are more susceptible to the detrimental effects of this insecticide under such conditions.

Exposure to chlorantraniliprole at a concentration of 0.05 ppm resulted in variable rates of mortality, as seen in **Table 1**. The highest silkworm larvae mortality (96.66%) occurred when the insecticide was applied once on the 2nd day of the 1st instar, on the 5th day of the 5th instar or twice on the 5th and 6th days of the 5th instar. High mortality (80%) was also observed when exposure occurred on the 1st day of the 1st instar. On the other hand, the lowest rates of larval mortality were observed following exposure on the 1st or 3rd days of the 2nd instar, the 1st day of the 3rd instar or on the 1st day of the 4th instar.

Few larvae died when they were exposed to a concentration of 0.05 ppm of chlorantraniliprole in the early days of the instar. This finding can be explained by the fact that, once ecdysis occurs, silkworm larvae are lethargic and eat less than in the last days of each instar. When chlorantraniliprole exposure occurred in the last days of an instar, the larvae likely consumed more contaminated leaves and therefore suffered a higher mortality rate.

The other chlorantraniliprole concentrations (0.025, 0.0125, $6.25 \times 10^{-3}$, $3.13 \times 10^{-3}$ and $1.57 \times 10^{-3}$ ppm) resulted in lower or no silkworm larval mortality. An exception occurred when larvae were exposed to chlorantraniliprole at a concentration of 0.025 ppm on the 5th day of the 5th instar or on the 5th and 6th days of the 5th instar, which resulted in 56.66% and 98.33% larval mortality rates, respectively. These lower concentrations are not generally fatal to *B. mori* larvae, but they may cause damage to silkworms, in the end days of the last instar like discussed above.

High concentrations of chlorantraniliprole, such as 0.2 ppm and 0.1 ppm, caused 100% larval mortality in most of the larval instars, except when the insecticide was applied in the 1st day of the 2nd instar, in this case only for 0.1 ppm, or in the 3rd day of the 2nd instar (**Table 1**). Although lower concentrations of this insecticide cause little or no larval mortality, they still result in damage such as cessation of feeding and production of a thin-shelled cocoon, which is economically not viable. Hence, our results demonstrated that Brazilian hybrids of the *B. mori* silkworm are extremely sensitive to chloran- traniliprole. In experiments conducted in China was detected a high sensitivity to chlorantraniliprole in the *B. mori* silkworm and identified a threshold value of $LC_{50}$ at

0.02045 ppm of chlorantraniliprole working with $3^{rd}$ instar larvae [11].

We were not able to detect a median lethal concentration unique, $LC_{50}$, for all treatments with the chlorantraniliprole concentrations used in this study. The results of our bioassays have demonstrated that three treatments had LC50 was well defined, such as, $2^{nd}$ instar in $3^{rd}$ day at concentration of 0.1 ppm, $5^{th}$ instar in $5^{th}$ day at concentration 0.025 ppm and in the treatment with two applications of insecticide, in $5^{th}$ instar in the $1^{rst}$ - $2^{nd}$ days at concentrations of 0.05 ppm. However, high concentrations of this insecticide were responsible for 100% larval mortality. The lethal concentrations (LC) for all treatments are 0.2 ppm of chlorantraniliprole and 0.1 ppm and 0.5 ppm causes high mortality in all treatments (**Table 1**).

The value of LC50 presented by [11] is similar our results but with larvae in $5^{th}$ instar instead with larvae $3^{rd}$ instar, probably due to genetic differences of silkworms and climatic conditions.

The value of LC50 presented by [11] is similar our results but with larvae in $5^{th}$ instar instead with larvae $3^{rd}$ instar, probably due to genetic differences of silkworms and climatic conditions.

### 3.2. Symptomatology Analysis

The concentrations under 0.1 ppm of the insecticide do not cause mortality, but the symptoms were common in the sub-lethal chlorantraniliprole concentrations and result in lost productivity. Between the symptoms in sublethal concentrations are important the difficult of reach the cocoon stage. In most treatments, we observed near complete feeding cessation by silkworm larvae, as well as regurgitation. So, the symptoms included feeding cessation, regurgitation, late development and irregular ecdysis (incomplete larvae ecdysis; **Figures 1(A)** and **(B)**). Another common symptom at sublethal concentrations was the presence of rosary-shaped excrements (**Figure 1(C)**), which may have been due to the peristaltic movement of the intestine being compromised by the muscle myofibrils.

In addition to feeding cessation, regurgitation and rosary-shaped excrements, we observed the production of thin-shelled cocoons by silkworm larvae (**Figure 1(D)**). This kind of cocoon has very thin shell which is not useful for spinning. Hence, sericulture companies do not accept thin-shelled cocoons and silkworm producers have to assume for financial loss. The symptoms varied in relation the insecticide's concentration and day of exposure of life circle. For the farmers do not is interesting lost productivity either for lethal doses or not lethal, be cause the symptoms are very damaging for the production.
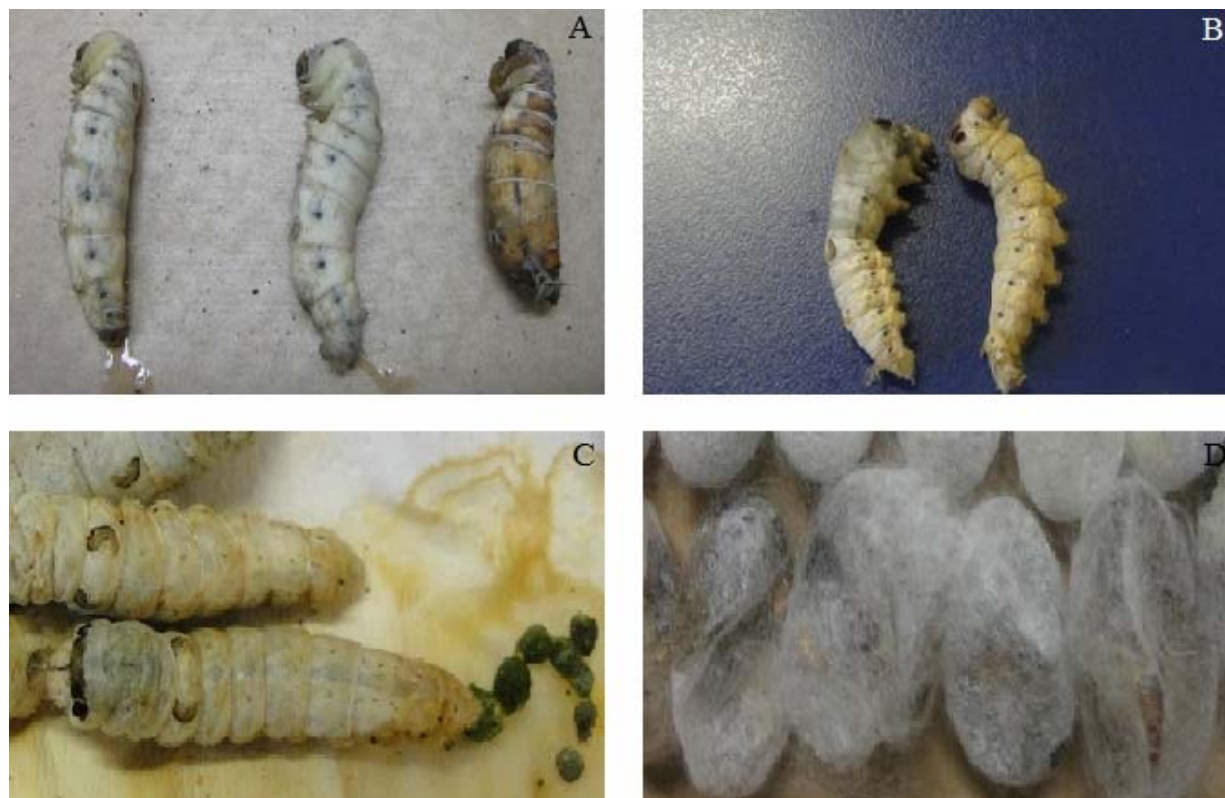


**Figure 1.** Symptomatology observed in *Bombyx mori* Brazilian silkworm hybrids exposed to chlorantraniliprole: incomplete ecdysis (A and B), rosary-shaped excrements (C), and thin-shelled cocoons (D).

We observed symptoms similar to those found by [11]. They also reported that symptoms emerged very quickly, in agreement with our observations. The observed symptoms are most likely related to the unregulated calcium release, which controls muscle myofibril activity [9], and alterations in feeding lead to changes in the conversion of ingested and digested food, which may eventually cause abnormalities in subsequent development [16].

We also evaluated productive variables of silkworm cocoons and pupae. Analyses were conducted only for treatments in which exposure to chlorantraniliprole occurred in 4th and 5th silkworm instars; treatments with concentrations equal to 0.2 ppm and 0.1 ppm were excluded from the analysis because they were lethal, causing 100% of larval mortality. **Table 2** illustrates the data obtained for the following variables: mean number of normal cocoons (NC), mean weight of normal cocoon (NCW), mean number of thin-shelled cocoons (TSC), mean weight of thin-shelled cocoons (TSCW) and mean number of live pupae (LP).

In general, there was little variation among treatments. There was no statistical variation among treatments in which chlorantraniliprole exposure occurred in the 4th larval.

When the larvae were exposed during later stages of the 5th instar, there was greater statistical variation, mainly in the NC, NCW and LP variables. After exposure on the 1st day of the 5th instar, there were no significant differences and only modest variation in the variable NCW. Low values of NCW were observed at chlorantraniliprole concentrations of 0.025 ppm and 0.0125 ppm, with values equal to 2.23 and 2.26 g, respectively.

When exposure to chlorantraniliprole occurred on the 5th day of the 5th instar, the response was variable in each variable except TSCW. A chlorantraniliprole concentration of 0.05 ppm resulted in the production of only thin-shelled cocoons by the silkworms, so the variables NC, NCW and LP could not be assessed. The NC, NCW, TSC and LP for the groups exposed to 0.025 ppm were significantly different from the other chlorantraniliprole concentrations, and their values were 6.66 cocoons, 2.13 g, 7.66 cocoons and 8.66 pupae, respectively.

Treatments with chlorantraniliprole also involved two exposures of this insecticide at different ages of the 5th instar. When chlorantraniliprole was applied twice in the 5th instar, on the 1st and 2nd days, there was no variation among NCW, TSC and, consequently, TSCW in the treatments. The NC and the LP, had the lowest values in the treatment with chlorantraniliprole at a concentration of 0.05 ppm, reaching values of 8.33 cocoons and 11.33 pupae, respectively.

Finally, our experiment also evaluated exposure to chlorantraniliprole twice on the 5th and 6th days of the 5th

instar. At a concentration of 0.05 ppm, there was total mortality, and no further data are available. NC, NCW and LP varied at the chlorantraniliprole concentration of 0.025 ppm and had the overall lowest values at this concentration: 0.33 cocoons, 0.70 g and 0.33 pupae, respectively. It is important to note that the TSCW was less than half of the NCW for most of the treatments. Moreover, the NC decreased significantly when high concentrations of chlorantraniliprole were applied on the 5th day of the 5th instar.

## 3.3. Morphological Alterations

The morphological features of the columnar epithelial cells in the midgut of *B. mori* larvae were altered by chlorantraniliprole exposure. The apical columnar cell surface exhibited protrusions in addition to the conspicuous microvilli related to apocrine vesicles. Some of these vesicles contained cell nuclei (**Figures 2(B)** and **(D)**; black arrows).

Cell fragments enclosed by a membrane with morphology similar to apoptotic bodies were observed along the midgut epithelium. The term apoptotic body is often used during the process of death by classical apoptosis to define the membrane-bound portion of the cell containing nuclear and cytoplasmic residue from fragmentation during apoptosis [17]. The presence of the nucleus in the structures observed in light microscopy was confirmed to be the result of the death of epithelial cells of the midgut. Columnar cells are most abundant in the midgut epithelium of insects and are responsible for processing food, secreting digestive enzymes and absorbing the final digestion products [18].

Their malfunction affects the entire metabolism, including silk production.

The regenerative cells presented in the midgut epithelium underwent hypertrophy and hyperplasia (**Figure 2(D)**), changing their appearance. The regenerative cells are responsible for cell renewal [18]. It is assumed that these cells are the precursors of others cells types, including columnar cells [18,19]. However, the regeneration of columnar cells did not occur due to changes and hypertrophy resulting from exposure.

We also observed the intestinal wall and its layers of longitudinal and circular muscles, which work in peristalsis. These muscles were disorganized, possibly due to the disarray of their myofibrils (**Figures 2(B)** and **(D)**; MM). **Figure 2(A)** shows normal muscle control for comparison. The midgut is responsible for the food digestion and absorption of necessary nutrients for the development and maintenance of normal metabolic function of the insect. Thus, the microscopic changes observed in response to chlorantraniliprole at concentrations of 0.2 ppm and 0.1 ppm harmed the development of their normal organic functions and was responsible for

**Table 2.** Productive evaluation[1] of silkworm *Bombyx mori* cocoons and pupae exposed to several concentrations of chloran- tranili- prole insecticide.

| Instar | DE | [] (ppm) | NC | NCW | TSC | TSCW | LP |
|---|---|---|---|---|---|---|---|
| 4th instar | 1st | 0.05 | 19.00 a | 2.46 a | 0.33 b | 0.97 a | 19.33 a |
| 4th instar | 1st | 0.025 | 19.00 a | 2.65 a | 0.33 b | 0.98 a | 19.66 a |
| 4th instar | 1st | 0.0125 | 19.33 a | 2.55 a | 0.00 b | 0.00 a | 19.33 a |
| 4th instar | 1st | $6.25 \times 10^{-3}$ | 19.66 a | 2.41 a | 0.33 b | 1.40 a | 18.66 a |
| 4th instar | 1st | $3.13 \times 10^{-3}$ | 19.00 a | 2.48 a | 0.33 b | 0.73 a | 19.66 a |
| 4th instar | 1st | $1.57 \times 10^{-3}$ | 19.33 a | 2.47 a | 0.66 b | 0.83 a | 19.33 a |
| 4th instar | 1st | Control group | 19.66 a | 2.47 a | 0.33 b | 1.00 a | 20.00 a |
| 4th instar | 3rd | 0.05 | 17.33 a | 2.37 a | 0.33 b | 0.80 a | 17.00 a |
| 4th instar | 3rd | 0.025 | 18.66 a | 2.37 a | 0.66 b | 1.71 a | 19.00 a |
| 4th instar | 3rd | 0.0125 | 19.33 a | 2.31 a | 0.0 0b | 0.00 a | 20.00 a |
| 4th instar | 3rd | $6.25 \times 10^{-3}$ | 17.66 a | 2.30 a | 0.33 b | 0.68 a | 19.00 a |
| 4th instar | 3rd | $3.13 \times 10^{-3}$ | 19.00 a | 2.29 a | 0.33 b | 0.82 a | 19.66 a |
| 4th instar | 3rd | $1.57 \times 10^{-3}$ | 18.66 a | 2.40 a | 0.0 0b | 0.00 a | 19.33 a |
| 4th instar | 3rd | Control group | 19.33 a | 2.46 a | 0.66 b | 1.57 a | 19.66 a |
| 5th instar | 1st | 0.05 | 16.66 a | 2.35 a | 0.00 b | 0.00 a | 17.33 a |
| 5th instar | 1st | 0.025 | 16.66 a | 2.23 b | 0.66 b | 1.74 a | 19.33 a |
| 5th instar | 1st | 0.0125 | 18.66 a | 2.26 b | 0.33 b | 0.99 a | 19.33 a |
| 5th instar | 1st | $6.25 \times 10^{-3}$ | 19.33 a | 2.29 a | 0.00 b | 0.00 a | 19.66 a |
| 5th instar | 1st | $3.13 \times 10^{-3}$ | 19.00 a | 2.30 a | 0.33 b | 0.79 a | 19.33 a |
| 5th instar | 1st | $1.57 \times 10^{-3}$ | 17.00 a | 2.29 a | 1.00 b | 1.53 a | 19.66 a |
| 5th instar | 1st | Control group | 19.33 a | 2.41 a | 0.66b | 1.46 a | 20.00 a |
| 5th instar | 5th | 0.05 | -* | -* | 0.66 b | 1.40 a | -* |
| 5th instar | 5th | 0.025 | 6.66 b | 2.13 b | 7.66 a | 2.17 a | 8.66 d |
| 5th instar | 5th | 0.0125 | 16.66 a | 2.11 b | 0.00 b | 0.00 a | 19.66 a |
| 5th instar | 5th | $6.25 \times 10^{-3}$ | 19.66 a | 2.16 b | 0.66 b | 1.03 a | 20.00 a |
| 5th instar | 5th | $3.13 \times 10^{-3}$ | 18.66 a | 2.27 a | 0.66 b | 0.64 a | 20.00 a |
| 5th instar | 5th | $1.57 \times 10^{-3}$ | 18.33 a | 2.16 b | 0.33 b | 0.64 a | 20.00 a |
| 5th instar | 5th | Control group | 18.33 a | 2.20 b | 0.00 b | 0.00 a | 20.00 a |
| 5th instar | 1st - 2nd | 0.05 | 8.33 b | 1.85 b | 0.33 b | 0.60 a | 11.33 c |
| 5th instar | 1st - 2nd | 0.025 | 17.33 a | 2.07 b | 0.66 b | 1.00 a | 16.00 b |
| 5th instar | 1st - 2nd | 0.0125 | 18.00 a | 2.17 b | 0.00 b | 0.00 a | 19.00 a |
| 5th instar | 1st - 2nd | $6.25 \times 10^{-3}$ | 18.00 a | 2.20 b | 0.00 b | 0.00 a | 20.00 a |
| 5th instar | 1st - 2nd | $3.13 \times 10^{-3}$ | 17.33 a | 2.08 b | 0.00 b | 0.00 a | 20.00 a |
| 5th instar | 1st - 2nd | $1.57 \times 10^{-3}$ | 18.66 a | 2.12 b | 0.00 b | 0.00 a | 20.00 a |
| 5th instar | 1st - 2nd | Control group | 16.33 a | 1.99 b | 0.00 b | 0.00 a | 20.00 a |
| 5th instar | 5th - 6th | 0.05 | -* | -* | -* | -* | -* |
| 5th instar | 5th - 6th | 0.025 | 0.33 c** | 0.70 c** | 0.00 b** | 0.00 a** | 0.33 e** |
| 5th instar | 5th - 6th | 0.0125 | 17.00 a | 2.03 b | 0.33 b | 0.60 a | 20.00 a |

**Continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5th instar | 5th - 6th | $6.25 \times 10^{-3}$ | 16.00 a | 2.27 b | 2.33 b | 1.53 a | 19.00 a |
| 5th instar | 5th - 6th | $3.13 \times 10^{-3}$ | 19.33 a | 2.02 b | 0.00 b | 0.00 a | 20.00 a |
| 5th instar | 5th - 6th | $1.57 \times 10^{-3}$ | 18.00 a | 2.05b | 0.00 b | 0.00 a | 19.33 a |
| 5th instar | 5th - 6th | Control group | 17.00 a | 1.98 b | 0.00 b | 0.00 a | 20.00 a |

[1]Productive evaluation: DE—Day of exposure to chlorantraniliprole, []—Chlorantraniliprole concentration (ppm), NC—mean number of normal cocoons, NCW—mean weight of normal cocoon, TSC—mean number of thin shell cocoons, TSCW—mean weight of thin shell cocoons weight, LP—mean number of alive pupae. Means followed by distinct letters (a-d) in the columns are different by the Scott-Knott test (P < 0.05); [*]Missing plots; [**]Value of one experimental plot.
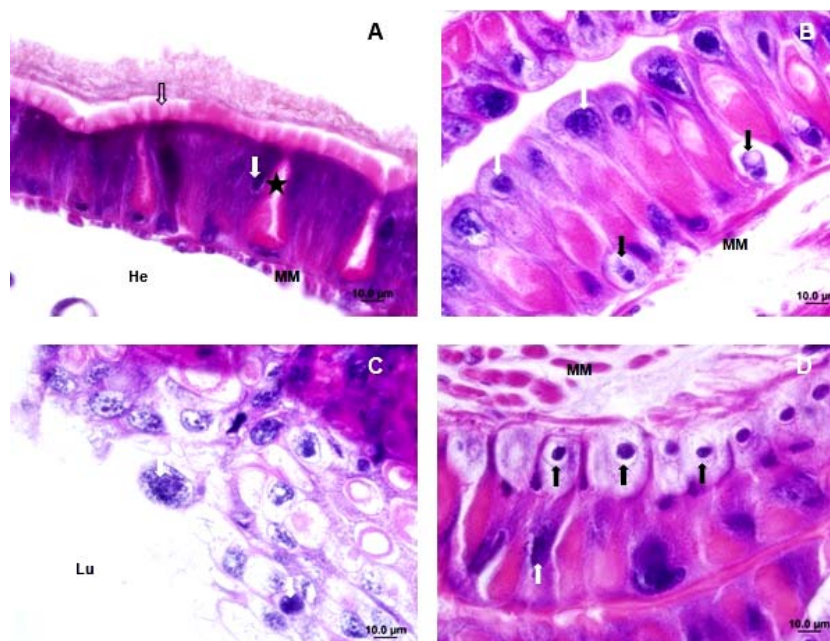


**Figure 2.** *Bombyx mori* 5th instar larvae midgut epithelial cells. (A) Morphology of the control group midgut epithelium showing columnar cell nuclei (white arrow), a goblet cell lumen (★), microvilli (hollow arrow), muscle layer (MM) and hemocoel (He). (B), (C) and (D) Morphologic changes to the midgut epithelium caused by chlorantraniliprole: (B) The nucleus of the columnar cells (white arrow) in the dilated cellular apex and an abundance of hypertrophic regenerative cells (black arrows) in the basal region of epithelium. (C) Part of the cytoplasm of columnar cells is released by budding, culminating in the expulsion of the cell nucleus to the intestinal lumen (Lu), as seen in detail in (D). Hematoxilin-eosin stain.

many of the symptoms such as irregular development, rosary-shaped excrements, and even the production of thin-shelled cocoons.

## 4. CONCLUSIONS

Aerial application of insecticides to agricultural fields may cause damage to silkworm production when the chemicals drift to nearby mulberry plantations that provide food for the silkworms. Several authors have reported disturbances to the silkworm life cycle and cocoon production due to different types of pesticides [16,20,21].

Although most of the studies reported insecticides that belong to classes which do not include chlorantraniliprole, which is an anthranilic diamide, the symptoms of intoxication are very similar to those we observed. Chlorantraniliprole is damaging to Brazilian silkworm hybrids, even in low concentrations, as revealed by our study.

Hence, actions should be taken to avoid this damage.

Previous report proposed that authorities in pesticide management should take measures to reduce the influence of chlorantraniliprole on silkworm rearing and forbid its use in sericulture areas [11]. Although chlorantraniliprole is not used in mulberry plantations, it can injure silkworms whenever this insecticide is applied incorrectly near mulberry fields and drifts into them.

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