2.13 Fipronil effect on the frequency of anomalous brood in honeybee reared *in vitro*

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Abstract

Larvae of honeybee workers were exposed to the insecticide fipronil during the feeding phase. To evaluate the effect of fipronil in the post-embryonic development of africanized *Apis mellifera*, bioassays of toxicity were done. The bioassays were performed by acute exposure applying 1µL of distilled water for control (I) and for experiments: 0.5 ng a.i./µL of fipronil; 5 ng a.i./µL of fipronil and 20 ng a.i./ µL of fipronil. Triplicates were performed for all treatments. The results showed that the rate of anomalous pupae in exposed honeybees was statistically significant in relationship to the control (p <0:03). The most frequent abnormalities were: high pigmentation on the proximal and distal larval body and body malformation, such as absence of head and limbs. Pink eye pupa and white eyed pupae presented malformations in their larval bodies, but with the eye developed. It is assumed that the fat body is related to the high rate of anomalies, since this tissue has proteins linked to the process of metamorphosis. Furthermore, the fat body may be participating in the regulation of juvenile hormone during the process of metamorphosis, and consequently in the release of ecdysteroid hormones that are involved in the change from larva to adult. The high rate of abnormalities in the pupal stage of individuals exposed to fipronil raises concerns about the impacts caused in the colonies of bees and population decline of pollinators.

Keywords: bees, larvae, pupae, metamorphosis, anomalies, fipronil.

1. Introduction

Fipronil (phenylpyrazoles - $C_{12}H_4Cl_2F_6N_4OS$) is an agrochemical widely used in Brazil for pest control, such as termites, beetles, caterpillars and for drilling in plantations of cotton, potato, corn, soy and sugar cane, by many ways of use. Fipronil is a neurotoxic molecule which acts directly on the central nervous system (CNS) of the insects, blocking the chloride channels acting on the gamma-aminobutyric acid receptor (GABA). Therefore, the insecticide is a serious CNS disruptor, causing abnormalities in normal nerve impulses in insects, such as hyperarousal, convulsions and paralysis, taking them to death. Fipronil is highly toxic to non-target insects, with LD_{50} in adult of africanized *Apis mellifera* L. (Hymenoptera: Apidae) of 1.06 ng a.i./ µL / bee[1-5].

The toxic effects of fipronil are dose-dependent and can shorten the lifespan of bees, killing and disrupting their physiological homeostasis[6-8]. The insecticide has sublethal effects on the viability, survival and colony population, and consequently the effects on the bee population are unpredictable and highly variable, resulting in a very difficult[9,10] impact evaluation and diagnosis on bees.

Adult bees and larvae can be contaminated by pollen and nectar collected from plantations where fipronil is applied[11]. Potential risks to bee larvae occur during the feeding phase, because the worker larvae are fed by nurse bees 143 times during the whole larval phase[12,13]. Additionally, during the larval feeding stage the nurse works while touching the larvae and the walls of the alveoli and may contaminate both the larva and the wax[14,15]. Based on the above, the relationship between fipronil insecticide with the frequency of anomalies in pupae of Africanized *A. mellifera* was analyzed.

2. Material and Methods

First instar larvae of africanized *A. melifera* workers were collected from brood combs obtained from an apiary located in rural area in Piedade, state of São Paulo, Brazil, and individually transferred to previously sterilized polyethylene well plates. The wells were inserted in cell culture microplates with 24 wells, containing larval food. Then the microplates were kept in incubator B.O.D (34-35 C°, 95.5 % of humidity) during the all larval development. The larvae were fed daily with a micropipette with 1 µL larval food from day 1-5 [14-17]. The bioassays were performed by acute exposure, applying 1µL of water and different concentrations of fipronil solutions on the larval tegument at the 4th day of incubation. Distilled water (I) was used for control, but for experiments: II) 0.5 ng a.i./ µL of fipronil; III) 5 ng a.i./ µL of fipronil and IV) 20 ng a.i./ µL of fipronil[3,18]. Triplicates with 24 larvae were performed for all treatments, totalizing 72 larvae per group. The post-embryonic development (larval and pupal stages) was monitored. Anomalous pupae were collected, classified and counted under a stereomicroscope Zeiss Stemi DV4. The statistical analyses were performed, using the variance test-ANOVA (F test) and the Student's t test ($p < 0.05$) with Assistant program, version 7.7 beta[19].

3. Results and Discussion

Different anomalies were observed between the control and the treatments of 20 and 5 ng a.i./µL/larvae, with an exception for treatment 0.5 ng a.i./µL/larvae that was also different from the control (Table 1). These results confirmed the negative impact on the larval development of the bee after exposure to fipronil. The results also showed that the impact on the larval development is dose-dependent (Table 1).

Treatment	mean values ng a.i./µL/larva of anomalies	F	P
Control	0.33333 **c**	4.7347*	0.0349
20	5.33333 **a**		
5	4.33333 **ab**		
0.5	0.66667 **bc**		

Table 1 Analysis of variance and mean values of anomalies in the pupal stage larvae treated with fipronil. Mean value of anomalies followed by the same letter are not statistically different, according to T test at P=5%. ANOVA (F test); *statistical differences ($p < 0.05$).

The anomalies were more frequent during the pupal development. Many anomalies of different types were observed for each pupal stage in treatments. The anomalous individuals within the domes were lying on the bottom of the alveoli, whereas normal larvae stood upright, such as in natural conditions (Figure 1A, B; Figure 2B, C; Figure 3A, B, C). White-eyed pupa were the more frequently observed pupa with a malformation of the head, thorax and abdomen, and absence of appendices (Figure 1A, B).

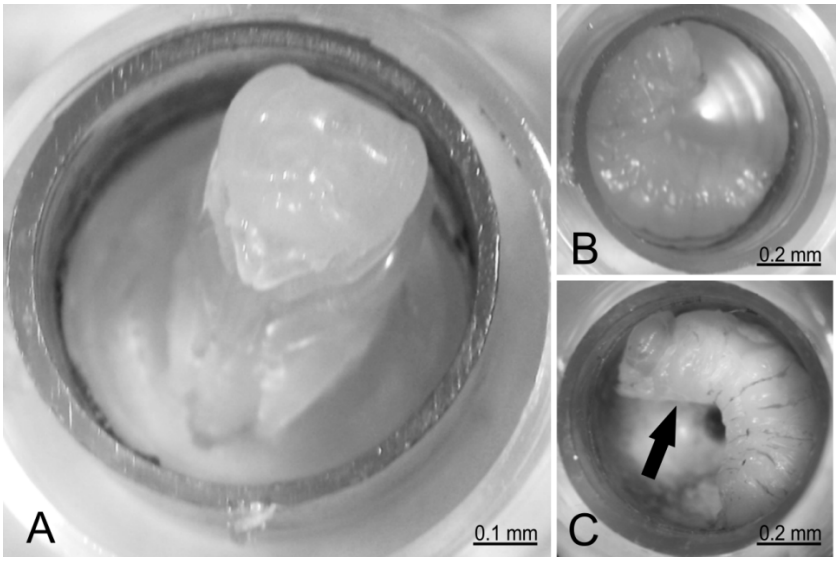

Figure 1 White eyed pupa. A. Control. B and C treatments anomalous pupa. Notice the absence of appendices (arrow).

Among the pink eyed pupae also anomalous individuals were present, with incomplete development of the head and thorax (Figure 2B.). Some individuals also had a larval body with developed eyes (Figure 2C.). Pupae with dark pink eyes presented more evidence of incomplete development of the head and thorax (Figure 3B.) and some larvae showed dark pigmentation in middle-distal portion of the body (Figure 3C). Additionally, some individuals presented a more frequent development of the eye but with poor development of the thorax and abdomen (Figure 3C).

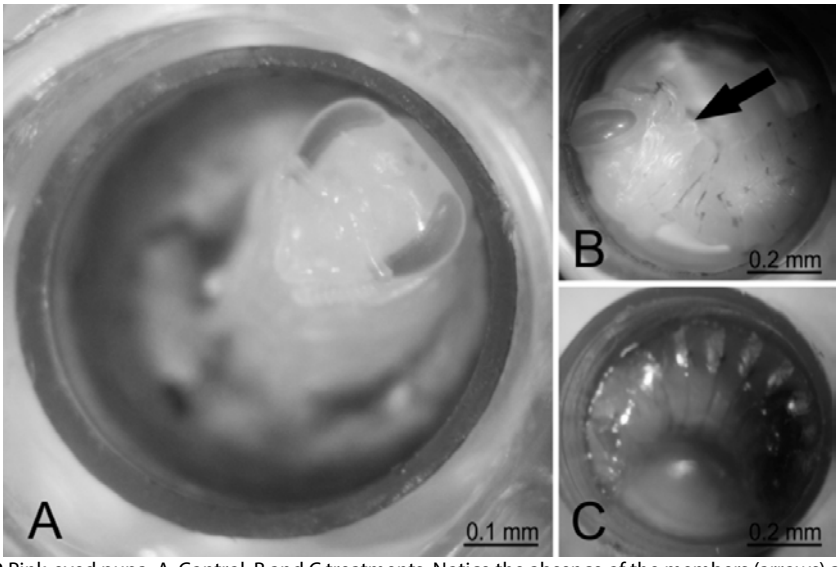

Figure 2 Pink-eyed pupa. A. Control. B and C treatments. Notice the absence of the members (arrows) and in C dark pigmentation of the middle-posterior body with eye and larval body (red arrow).

Anomalous undefined pupas were also observed (Figure 3A, B, C). The most frequent abnormality was the arrest of metamorphosis demonstrated by pupae with necrosis in head and thorax (dark coloration) and absence of appendices eversion. (Figure 3A, B, C).

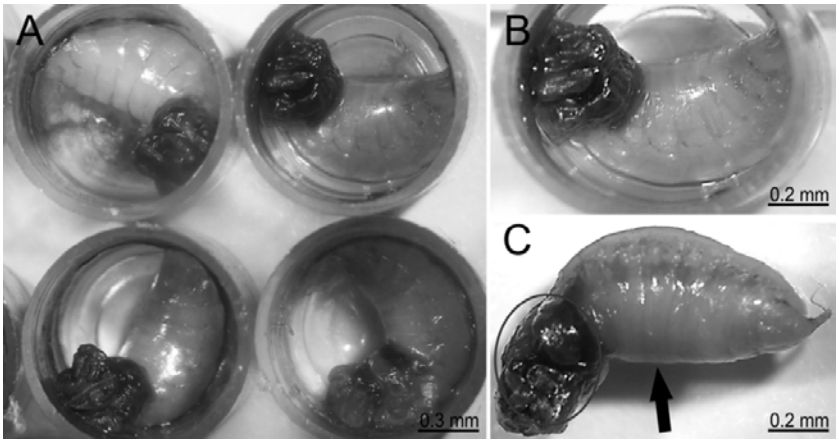

Figure 3 A and B. Anomalous pupa showing necrosis in head and thorax (dark coloration) and absence of appendices eversion. **C.** Anomalous pupa with the whole body presented anomalies. Malformation and dark pigmentation of the head and thorax, as anomalous development of the abdomen.

It is assumed that the fat body has a role related to the abnormalities in the individuals exposed to fipronil during the post-embryonic development, since this tissue acts in the intermediary metabolism of bees, synthesizing and storing proteins related to the transport of important hormones for metamorphosis, such as *hexamerins*[20,21].

The fat body fills the insect body cavities and it is the predominant tissue in larvae and pre-pupae. The fat body is directly in contact with the *hemocoel*. Assuming that the natural metabolites and even insecticides are present in the *hemocoel*, it is suggested that the action and the interaction of the insecticide molecules with the fat body is very quick [21-23]. According to these authors, in *A. mellifera* the fat body can represent up to 60% of the larval body weight[24]. The fat body is composed primarily of two cell types, trophocytes with functional differentiation, and oenocytes. The cells of this tissue have extensive plasticity, which is demonstrated by the multiple roles they play, and the fat body may be the target of morphogenetic hormones of insects[25,26].

The proteins produced during the larval stage and stored in the hemolymph are named *storage proteins*[27]. Some of these proteins belong to the class of hexamerins and are synthesised in large quantities by trophocytes and often also by oenocytes (cells responsible for lipid synthesis and hydrocarbons[26]) of larval fat body[28]. The hemolymph protein storage is a response to intense food intake, where in up to 90% of the total circulating proteins may be accumulated[29,30]. These proteins are used in the intermediary metabolism and the post-larval development, acting as a source of amino acids for the reconstruction of the adult tissues[22,31-34].

In this context, many authors demonstrated the role of certain hexamerins in the transport of metabolites or hormones, such as ecdysteroids (Ecd) and juvenile hormone (JH)[35-38]. According to these authors, protein from the hemolymph, which includes the hexamerins, form a complex with JH binding proteins, aiding their transport to target cells and tissues[39]. Indirectly, the hexamerins could be related to the regulation of the hemolymph titers of JH and consequently its role in regulation of the larval and pupal development[35-39]. The above may help understanding the fat body's relationship with the anomalies observed in the ontogenetic development and pupae of africanized *A. mellifera*.

According to these authors, there was a synthesis *de novo* of Ecd in the abdomen of *Aedes aegypti* (Diptera, Culicidae) grown in laboratory conditions, suggesting the presence of an abdominal source of these hormones in these insects[40,41].

The JH-III in bees is synthesized by a pair of symmetrical retrocerebral glands controlled by the central nervous system and located in the thorax, the *corpora allata*. The gland and also the prothoracic glands synthesise the Ecd hormone[44-53]. As shown in this study, most of the anomalies in treated pupae are found on the head and thorax, where the organs that synthesise JH and Ecd are situated[54].

Furthermore, JH-III plays a function in storage and control of protein granules in trophocytes, and is also considered to be responsible of production and control of the levels of JH binding protein in the hemolymph[42,43]. The maximum titer of JH-III synthesis is reached in worker larvae, and decreases in the early pupal stage[42,53,54]. The Ecd hormones are involved in the change from larva to adult (metamorphic process), so at the end of the 5th instar of worker larvae, Ecd titer starts to increase[42]. Additionally, there is evidence that oenocytes synthesise Ecd. This hormone regulates several metabolic processes during development and still is involved in the synthesis of lipids and hydrocarbons in *cuticulogenesis*[22].

The results also indicate that larvae exposed to fipronil are not completing the changes to the last larval instar, probably maintain a higher titer of JH in the abdominal region, but also do not perform the activation of pupal genes by Ecd hormone, consequently inhibiting or disrupting the expression of the genes for adulthood.

During the larval period, the presence of JH and ecdysone induce epidermal cells to produce the larval cuticle. When there is a reduction of circulating JH at the end of the larval period, the metamorphosis and pupation starts [53,54]. Therefore, intense new cuticle synthesis is required prior to *apolysis* of the larval cuticle that occurs in pre-pupae[54-56]. However, in this study it was observed that the necessary *apolysis* of the cuticle in the metamorphic process did not occur in anomalous pupae.

4. Conclusion

The larval exposure to fipronil proved extremely deleterious pupae of africanized *A. mellifera* reared under laboratory conditions. This is corroborated by research[15,57-63] that exposed concern and discussed the relationship of contamination of larvae by pesticides and their impact on bee colonies.

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