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Adult And Egg Mortality of Rhynchophorus Ferrugineus Oliver (Coleoptera: Curculionidae) Induced By Thiamethoxam And Clothianidin

Vincenzo Di Ilio [1,2], Nabawy Metwaly[3], Francesco Saccardo[3], Emilio Caprio[4]
[1]*(Dep. BCP, Rothamsted Research, Harpenden, United Kingdom)*
[2]*(Dep UTAGRI ECO, ENEA Casaccia Research Centre, Rome, Italy)*
[2]*(Dep. DAFNE, Università della Tuscia, Viterbo, Italy)*
[2]*(Dep. Agricolture, Università degli Studi di Napoli Federico II, Napoli, Italy)*
Corresponding Author: Vincenzo Di Ilio

Abstract: *The red palm weevil (RPW) is the major pest of palms in the Mediterranean region. One of the most interesting control solutions for this pest is endotherapy, comprising injections of biologically active substances directly into the stem of the palm. The objective of the present work was to study the ovicidal and adulticidal properties of two neonicotinoid insecticides (clothianidin and thiamethoxam) under laboratory conditions, to obtain evidence for application of endotherapy in the control of RPW infestations. Our results show that both commercial compounds display a dose-dependent action and exhibit different modes of action: clothianidin is more rapid in its action, but in general is less effective for control of the adult stages, while thiamethoxam is more effective, but its action requires longer to show efficacy. The eggs are much less sensitive to treatments, especially for clothianidin.*

Keywords: *Rhynchophorus ferrugineus, neonicotinoids, adults, eggs, clothianidin, thiamethoxam*

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I. Introduction

The red palm weevil (RPW), *Rhynchophorus ferrugineus* Oliver (Coleoptera, Curculionidae) is a large weevil which was inadvertently introduced into the Mediterranean region and that lives and grows only in association with palms. The insect is causing serious damage to the whole palm heritage of the Mediterranean region, and particularly in Italy, for biotic reasons related to its life cycle and its biology [1-3]. This insect is native to Southeast Asia and Melanesia [4] and, in 1906, was first described as a serious pest of coconut palm in India by Lefroy [5]. In 1920 Buxton [6] originally documented RPW on date palm. Over the last 20 years, the weevil spread from East to West [7], affecting many countries bordering the Mediterranean Sea including Israel, Jordan and Palestine [8], Egypt [9], Spain [10] and Italy [11]. Currently, RPW can be considered present in almost the entire Mediterranean area [12], where it is causing the loss of thousands of palm trees. The RPW larvae cause, in a short time, the death of the palm by feeding on internal tissues of the leaf rachis and subsequently affecting the apical meristem [13] and causing an irreversible damage that cause death of the palm [14]. *R. ferrugineus* has a gregarious behavior and males produce an aggregation pheromone that attracts other individuals to maximize reproductive efficiency [15]. The average lifespan of an RPW female is about 120 days. A mated female produces 100-200 eggs during its life, of which the fertility is estimated to be between 50 - 75%. The female lays eggs in small pits produced by the rostrum at the base of the leaves or young shoots, in wounds or scars on the plant, or in the stems of young palms [16]. First instar larvae emerge after 2-3 days of embryonic development and immediately feed on succulent tissues [17]. The larval development lasts 60-90 days [18], passing through four larval stages. At maturity, the larva constructs a pupal chamber with the fibers of the plant. The duration of the pupal stage is very variable and lasts, according to several authors, from 13 to 50 days [4] [16].

In the case of the infestation on *Phoenix canariensis* Chabaud, larvae develop mainly in the upper part of the palm. *P. canariensis* constitutes an important architectural element in Mediterranean cities where there are legal limitations on the use of chemical insecticides. In addition, RPW has been reported on other palms: *Cocos nucifera* L., *Livistona decipiens* Beccari, *Sabal umbraculifera* Martius, *Phoenix dactylifera* L., *Trachycarpus fortunei* (Hook) and *Washingtonia spp* [7] [12].

Currently, the most effective control measures are preventive, while the intervention on already attacked plants remains problematic. Several insecticides have been used to limit infestations of RPW, but the results are often unsatisfactory [19-21]. Regarding the biological control of RPW, some organisms have been tested in the laboratory, but no efficient antagonists have yet been identified [22,23]. In this context, one of the most interesting techniques is endotherapy [24-26]. This methodology involves the application of plant protection products through injections into the stem. Endotherapy has many advantages over traditional methods of pesticide application, such as low environmental impact and an insignificant impact on beneficial insects. However, some aspects remain to be investigated, such as quantitative and qualitative translocation inside the palm as well as phytotoxicity of the injected active principles.

Neonicotinoids are insecticides with systemic properties that, regardless of the manner of application, can translocate throughout all plant tissues making them toxic to insects [27,28]. Neonicotinoid applications virtually protect the plant from direct damage caused by herbivorous insects and indirectly from damage by plant viruses that are transmitted by insects. With the discovery of thiamethoxam [29] and its metabolite clothianidin [30] in 1999, neonicotinoids have become the most widely used insecticides [31-33].

The aim of the laboratory trials presented here was to verify the ranges of concentrations of thiamethoxam and clothianidin necessary to ensure effective control of RPW adults feeding on palm tissues. Preliminary results of fecundity tests carried out in our laboratories, but not reported here, showed that neither thiamethoxam nor clothianidin interfere directly with the production of eggs and sperm. However, since the detection of RPW larvae at the early stages of infestation remains difficult, we set up laboratory trials to verify egg toxicity of these two active principles to give new insights for the application of the endotherapic method as a preventive intervention.

II. Materials And Methods

The experimental work focused on analysis of the ovicidal and adulticidal effects caused by the two commercial insecticides thiamethoxam and clothianidin by defining the variable LC50.

2.1 Insects

The rearing of adults and larvae of RPW, as well as all the experiments, were carried out in the laboratories at the Centre ENEA Casaccia (Roma – Italy). Rearing and tests were carried out in climatic chambers at 27°C, 60% (relative humidity) and with a photoperiod of 16/8 h (light/dark). Adults of both sexes, obtained by field captures, were maintained together in plastic containers of various dimensions, closed with a grid lid to allow for ventilation of the indoor environment. A layer of coconut fibre was placed on the bottom of the container to absorb excess moisture. The coconut fibre also furnished a shelter for the beetles. Some apples were provided in the rearing cages for adult feeding and as a substrate for oviposition.

2.2 Chemicals

Two commercial products were chosen for these experiments. Actara 25 wg (Syngenta Crop Protection, Canada) is a systemic insecticide, active ingredient thiamethoxam, which acts by contact and ingestion and which contains a concentration of 25% of the active ingredient. The other compound is Dantop 50 wg (Sumitomo Chemical Italy), a systemic insecticide having clothianidin (50%) as an active ingredient.

2.3 Egg toxicity

The eggs used in the tests were randomly chosen from a pool of eggs obtained from apples exposed for 48 hours to mature females RPW in the rearing cages. A small square of filter paper (approx. 0.5 cm2) was impregnated with 70 µl of test solution and placed within each well of 24-well plates. A single egg was then placed over the moist filter paper in each well and was left to hatch. After 72 h after the initiation of treatment, the number of first instar larvae in the plates was recorded to calculate the hatching rate.

As well as the control, in which eggs were placed in the presence of distilled water only, four treatment groups were set up for the assays with thiamethoxam and four treatment groups were prepared to study the clothianidin effects. The commercial formulations were firstly dissolved in distilled water and then serially diluted to obtain the test solutions to be used in the experimental trials. The treatment groups treated with thiamethoxam were labeled as AC. The concentrations of thiamethoxam used in each AC treatment group were set as follows: AC1 (1250 µg/ml), AC2 (250 µg/ml), AC3 (50 µg/ml), AC4 (10 µg/ml). The treatment groups treated with clothianidin were labeled as DA. The concentrations of clothianidin used in each DA treatment group were set as follows: DA1 (1500 µg/ml), DA2 (300 µg/ml), DA3 (60 µg/ml), DA4 (12 µg/ml).

Totally, 108 eggs per GT were subjected to experimentation, distributed in 6 replicates of 18 eggs each. To ascertain the ovicidal properties of the products, we considered the hatching rate to be defined as the number of first instar larvae per number of eggs treated in each treatment group.

2.4 Adult toxicity

For the execution of the experimental study, adults were used which had been collected from pheromone traps placed in the field and then maintained in laboratory conditions as described before. Adult pairs, comprising a male and female RPW randomly selected from the rearing cages, were confined in Phytatray plastic boxes (Sigma- Aldrich), the lid of which was drilled with 6 holes of about 5 mm diameter to allow for ventilation of the internal environment.

The different solutions of the two insecticides in varying concentrations were incorporated in an artificial diet prepared in the laboratories of the unit UTAGRI at the Centre ENEA Casaccia (Rome, Italy), according to the method described by Tomic-Carruthers34). The diet comprises a mixture of tap water, agar, cellulose, pharmamedia isolated soy protein, sucrose, ascorbic acid, methyl paraben, sorbic acid, β-sitosterol, myo-inositol, folic acid, choline chloride, ferrous sulfate, corn oil and hydrochloric acid. The commercial formulations were initially dissolved in distilled water and then serially diluted to obtain the test solutions to be incorporated into the artificial diet in the proportion 1/4 (weight/weight). The control group was fed with a mixture prepared in the same way but containing only distilled water in the same proportion. 10 g of each of the obtained mixtures were placed in a 5 cm diameter Petri dish and offered to each confined pair. The treatment groups exposed to Actara were labeled as "TH". The concentrations of thiamethoxam administered in each AC treatment group were as follows: TH 1 (50 µg/ml), TH 2 (5 µg/ml), TH 3 (0.5 µg/ml), TH 4 (0.05 µg/ml). The treatment groups treated with Dantop were labeled as "CL". The concentrations of clothianidin used in each treatment group were as follows: CL 1 (60 µg/ml), CL 2 (6 µg/ml), CL 3 (0.6 µg/ml), CL 4 (0.06 µg/ml).

The mortality of individuals was recorded daily. Sixteen replicates, divided into four separate stages, were carried out for each treatment group. The duration of the experiment was set at 96 hours. Analysis of the mortality was carried out on data obtained after 72 and 96 h from the initiation of treatment.

2.5 Statistical analysis

The data were processed with the program SPSS for Windows (v. 15.0). LC50 was calculated using the Probit analysis. The values of LC50 related to the two active principles were obtained considering the concentration of thiamethoxam and clothianidin in their respective commercial products (Actara and Dantop).

III. Results

3.1 Egg toxicity

In the experimental conditions set, the death of the eggs is to be intended as unhatched eggs, and the hatching rate is defined as number of first instar larvae by number of eggs assayed. In the Control group (absence of treatment) the hatching rate resulted 0.73 (± 0.11), in line with what reported by other authors in natural conditions16). This indicates that the experimental conditions set are optimal. The trend lines in Fig. 1, A and B show that both Actara and Dantop induce mortality of the RPW eggs with a dose-dependent pattern. The analysis of variance (ANOVA) performed on the data of hatching eggs subjected to treatment with Actara shows that there are significant differences between the different treatment groups ($F = 3.174$, $p = 0.036$, d.f. = 24). However, the results presented in Fig. 1A indicate that the Actara induced an appreciable mortality of the eggs only in the treatment groups at higher concentrations (AC1 and AC2). Indeed, the LSD test (Least Significant Difference; $\alpha = 0.05$) confirms that the hatching rate in the Control group is significantly different from the treatment groups AC1 and AC2, while there are no significant differences between the control group and groups AC3 and AC4. The Probit analysis (95% confidence limit) showed that the estimated value of LC50 after 72 hours of the eggs undergoing treatment with thiamethoxam is 44 µg/ml (Upper Bound 111.65 µg/ml, Lower Bound 9.35 µg/ml). The product Dantop (Fig. 1B) did not show evident ovicidal properties in the experimental conditions set. In fact, the ANOVA analysis detected no significant differences between the control and the other treatment groups ($F = 1.526$, $p = 0.233$, d.f. = 24). These data are confirmed by the Probit analysis (95% confidence limit) that showed that the estimated value of the LC50 for clothianidin is 1084.72 µg/ml (Upper Bound 299915.12 µg/ml, Lower Bound 276.57 µg/ml).

3.2 Adult toxicity

Fig. 2 A and B show adult mortality in the different treatment groups, respectively, 72 and 96 hours after initiation of treatment. The mortality rate has been defined as the number of dead adults per number of adults in each treatment group. From the analysis of the figures, it is clear that, under the experimental conditions adopted, the level of activity of thiamethoxam and clothianidin is strongly dose-dependent. With respect to thiamethoxam, the analysis of variance performed on the data of mortality after 96 hours of treatment (Fig. 2B) shows that highly significant differences exist between the different treatment groups and the control group ($F = 8.523$, $P < 0.01$; DF = 19). In particular, the LSD test confirms that the control group is significantly different from all other treatment groups ($\alpha = 0.05$). The same test, however, shows no significant differences between TH2, TH3 and TH4. The analysis of mortality data for the formulation Dantop after 96 hours (Fig. 3) showed that highly significant differences exist between the different treatment groups ($F = 59.000$, $P < 0.01$, DF = 19). The LSD test ($\alpha = 0.05$) confirms that each group is significantly different from all the others. After 72 h from the beginning of treatments (Fig. 2), the one way Anova test shows that the differences between treatment groups are even more pronounced. With respect to thiamethoxam, a highly significant difference can be observed between the control and all the other treatment groups (F = 10.395, P< 0.01, DF = 19). Furthermore, the LSD test (α = 0.05) shows that there are no significant differences among the groups treated with the higher doses of thiamethoxam (TH 1, TH 2 and TH 3). The clothianidin after 72 h (Fig. 2) shows a marked dose dependence, since there are highly significant differences among all the treatment groups (F = 80.500, P< 0.01, DF = 19). The LSD test confirms that the mortality recorded for all treatment groups is statistically different from each other, except for the lowest treatment (CL 4), which does not induce a mortality significantly different from the control group (α = 0.05). The statistical analysis, performed on mortality data collected 72 and 96 h after the beginning of treatment, highlights the fact that the clothianidin formulation expresses a more marked dose-dependence with respect to the product thiamethoxam. This conclusion can be graphically seen in Fig. 2 and 3, observing the greater steepness of the trend line of clothianidin with respect to the thiamethoxam linear trend after both 72 and 96 h of treatment, evident also looking at the angular coefficients of the line equations indicated in Fig. 2 and 3. Fig. 4 and 5 show the daily trend of mortality induced respectively by thiamethoxam and clothianidin. Firstly, it can be observed that the pattern of mortality induced by thiamethoxam is more linear (Fig. 4) with respect to that elicited by Dantop, which presents a more pronounced sigmoid trend (Fig. 5). These trends are more evident if we consider the highest concentrations of both insecticides (TH 1, TH 2 and CL 1, CL 2). In Fig. 4 and 5, it can also be observed that, after 96 hours, mortality induced by clothianidin at the higher concentration (CL 1) appears to be higher than the one elicited by thiamethoxam (TH 1). This situation is opposite at the lower concentrations (TH 4 and CL 4), where thiamethoxam expresses a greater toxicity than clothianidin. The results of the Probit analysis are displayed in Table 1. They confirm the above observations. In fact, after 96 hours of treatment, the value of LC50 reported for thiamethoxam is 0.39 μg/ml, lower than that reported for clothianidin, where the LC50 is 1.44 μg/ml. Conversely, if one considers the value of LC50 after 72 hours, the condition is the opposite. The registered value of the LC50 after 72 hours for thiamethoxam is 1.35 μg/ml, while treatments with clothianidin give an LC50 value of 1.65 μg/ml. A possible interpretation of this phenomenon is that the clothianidin is more rapid in its action, but in general expresses a lower toxicity on the adult stages of RPW. Thiamethoxam, on the contrary, seems to be generally more effective, but its action appears slower than clothianidin. Finally, our data shows that there are no differences in mortality induced by treatment between males and females, so we can conclude that males and females are equally susceptible to treatments with both thiamethoxam and clothianidin.

IV. Discussion

Our laboratory trials indicate both clothianidin and thiamethoxam to be effective in control programs against the adults of RPW. Other preliminary experiments not reported here, showed that the two neonicotinoids do not affect the reproductive system of males and females. As expected, under the experimental conditions adopted, the effectiveness of both thiamethoxam and clothianidin is significantly dose-dependent, (Fig. 2 and 3): the higher iis thedose administered, the greater is the observed mortality among adults. Anyhow, some differences in the mode of action of the two insecticides can be observed. After 96 hours of treatment both thiamethoxam and clothianidin show the same mode of action as shown by the trend lines in Fig. 3, but in the same figure can be observed that clothianidin is more effective at the higher doses, while at the lower doses the most effective is thiamethoxam. In that sense, the differences between two active principles are more evident after 72 hours of treatment as revealed also by the difference in the values of the angular coefficients of the line equations relative to thiamethoxam and clothianidin (Fig. 2). Moreover, looking at the daily trends of the mortality displayed in Fig. 4 and 5, it was recorded that clothianidin induces a high mortality after only 48 hours at the higher doses, while the lower doses cannot induce an appreciable mortality of the RPW adults. As regards thiamethoxam, a high level of mortality is reached after 96 hours with the exception of the lower concentration. Taken together these findings suggest that clothianidin is more rapid in its action, but in general less effective for the control of the adult stages, whereas the thiamethoxam is more effective, but its action requires a longer time.Eggs, on the contrary, are much less sensitive to treatments than adults under laboratory conditions. This is also evident if considering the value of the angular coefficients of the trend lines displayed in Fig. 1 and 2. Data show that clothianidin express a weaker ovicidal activity than thiamethoxam that is in general more effective in the control of RPW eggs yet considering that an appreciable reduction of the hatching rate was recorded only at very high concentrations.

V. Conclusion

R. ferrugineus has been recently introduced in the Mediterranean region and lives only in association with palms. This insect, native to Southeast Asia, is affecting many countries bordering the Mediterranean sea and is causing severe damage to the whole palm heritage of the Mediterranean region since the infestations lead to a rapid decay of the palms. Larvae feed on internal tissues of the leaf rachis and affecting the apical meristem cause the irreversible damage that precede the death of the palm. After a variable period, related to the environmental conditions, larvae pupate into a cocoon made with the fibers of the plant before emerging as adults. In the Mediterranean region RPW shows a clear preference for the species *P. canariensis*, that constitutes an important architectural element in Mediterranean cities where there are legal limitations on the use of chemical insecticides. For this reason, the most effective control measures are preventive, while the intervention on already attacked plants remains problematic due to the difficulty to detect the infestation at an early stage. Several insecticides have been used to limit infestations of RPW, essentially spraying the crown of the palm, but the results are often unsatisfactory. As regards the biological control, a variety of putative agents have been tested in the laboratory, but no efficient antagonists have yet been identified. In this context, one of the most interesting solutions is represented by the endotherapy. This methodology involves the application of plant protection products through direct injections into the stem and, although some aspects remain to be investigated, it has many advantages over traditional methods of pesticide application, such as low environmental impact and negligible effects on beneficial insects. Neonicotinoids are good candidates for the endotherapic treatments as they feature systemic properties that, regardless of the manner of application, can translocate throughout all plant tissues making them toxic to insects. Neonicotinoid applications virtually protect the plant from direct damage caused by herbivorous insects and from the indirect damage caused by plant pathogens transmitted by insects. In this context the purpose of our work is to identify the ranges of concentrations of thiamethoxam and clothianidin necessary to target RPW adults feeding on palm tissues.

Our experiments demonstrate that the two nicotinoids are both suitable for the control of RPW and that clothianidin and thiamethoxam act following different modes of action and the effectiveness of both thiamethoxam and clothianidin is significantly dose-dependent both on adults and on eggs. On adults our findings demonstrate that clothianidin acts rapidly compared with the thiamethoxam, but this latter is more effective over a longer period. Both the compounds show a very weak action on eggs even if we recorded a lower hatching rate only using thiamethoxam at the highest concentrations.

In this context our results indicate that both the compounds are theoretically suitable for the control of RPW adults however further experiments are needed to verify the quantity of active principles and the quality of the residues that reach the apical bud of the plants for a correct application of the endotherapic control method. In addition, it is of paramount importance to get evidence of the effects of both thiamethoxam and clothianidin on the larval stages of RPW. Experiments are now in progress to clarify these issues.

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Figures and Tables

To ensure a high-quality product, diagrams and lettering MUST be either computer-drafted or drawn using India ink. Figure captions appear below the figure, are flush left, and are in lower case letters. When referring to a figure in the body of the text, the abbreviation "Fig." is used. Figures should be numbered in the order they appear in the text.Table captions appear centered above the table in upper and lower case letters. When referring to a table in the text, no abbreviation is used and "Table" is capitalized. (10)

Table 1 LC50 values, expressed in micrograms per milliliter, referred to adults and relative to the two active ingredients after 72 and 96 hours after the initiation of treatment.

	Active Ingredient	95% Confidence Limits		
		LC50	Lower bound	Upper bound
96 h	Thiamethoxam	**0.39**	0.05	1.34
	Clothianidin	**0.96**	0.39	2.30
72 h	Thiamethoxam	**1.35**	0.30	5.41
	Clothianidin	**1.10**	0.46	2.62

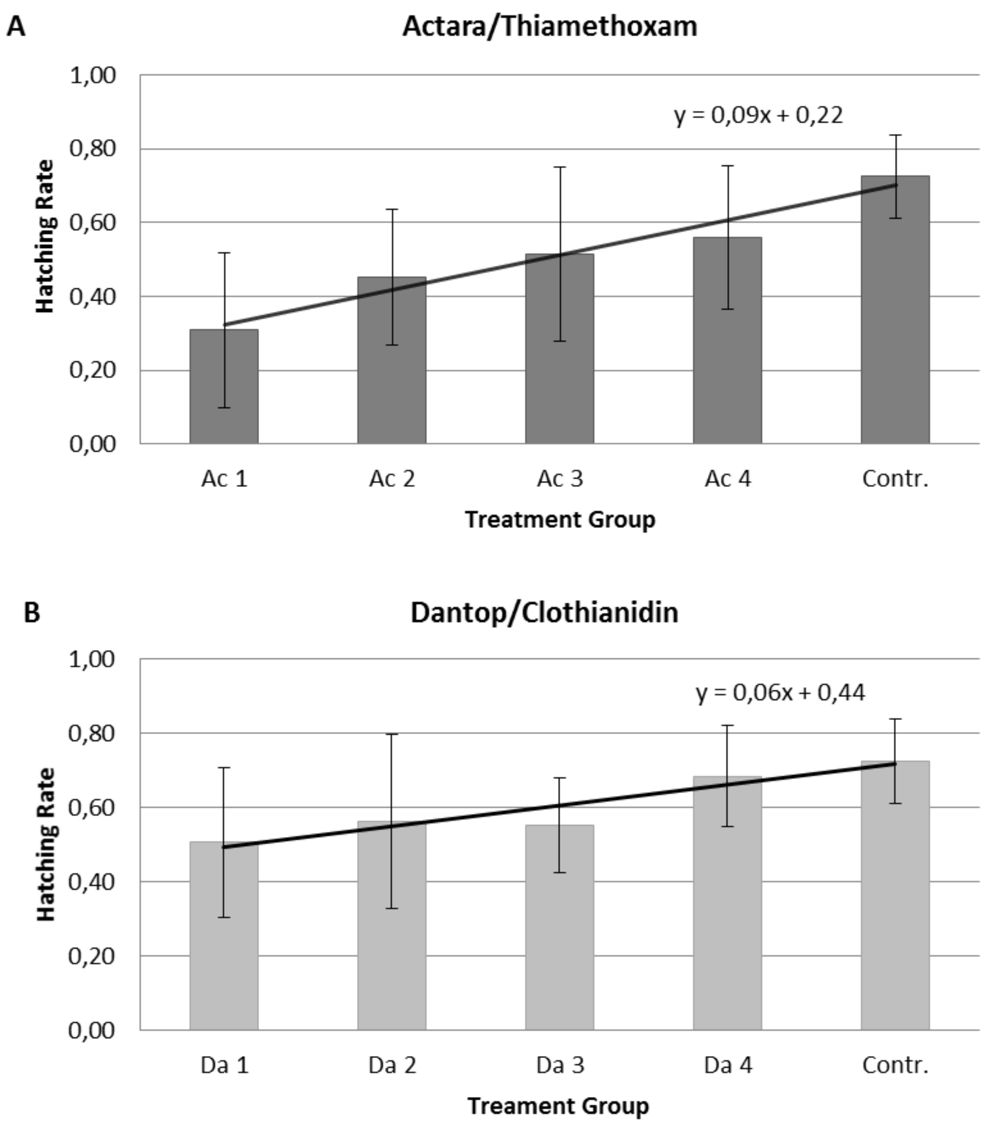

Figure 1. Eclosion rate in the different treatment groups of the eggs induced by application with Tiamethoxam (A) and Clothianidin (B). Data are expressed as number of eggs hatched by number of eggs treated, after 72 hrs from the beginning treatment. Standard Deviation is shown as error bar. Trend lines and their equations are also displayed.

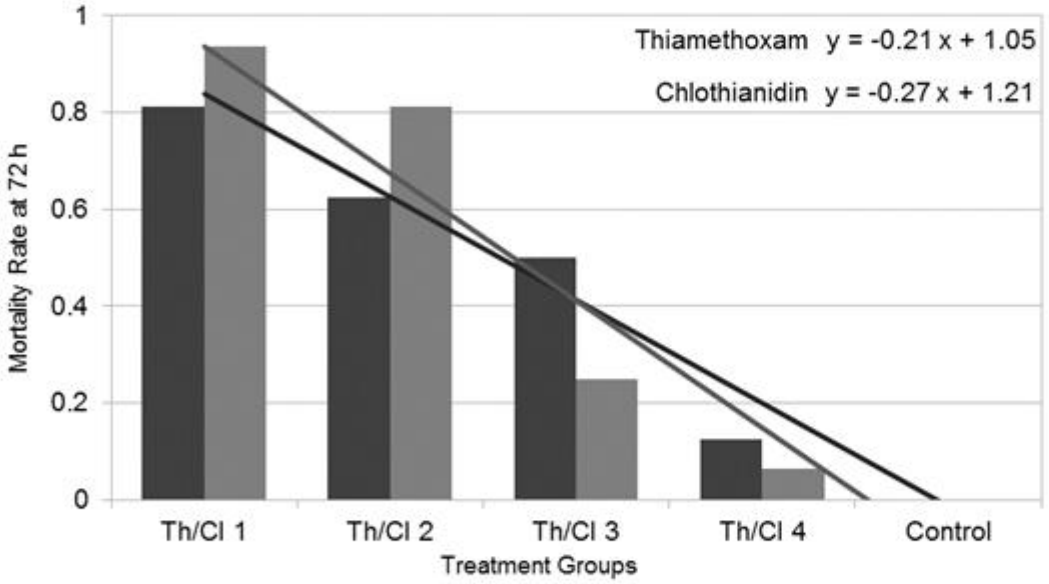

Figure 2. Mortality rate of adults after 72 hours after the beginning of treatment with thiamethoxam (dark grey bars) and clothianidin (light grey bars). The mortality rate is defined as the number of adults dead by the number of adults in each treatment group. Trend line referred to thiamethoxam is in dark grey, while that referred to clothianidin is displayed in light grey. Equations of the trend lines referred to Thiamethoxam and Clothianidin are shown in the graphs.

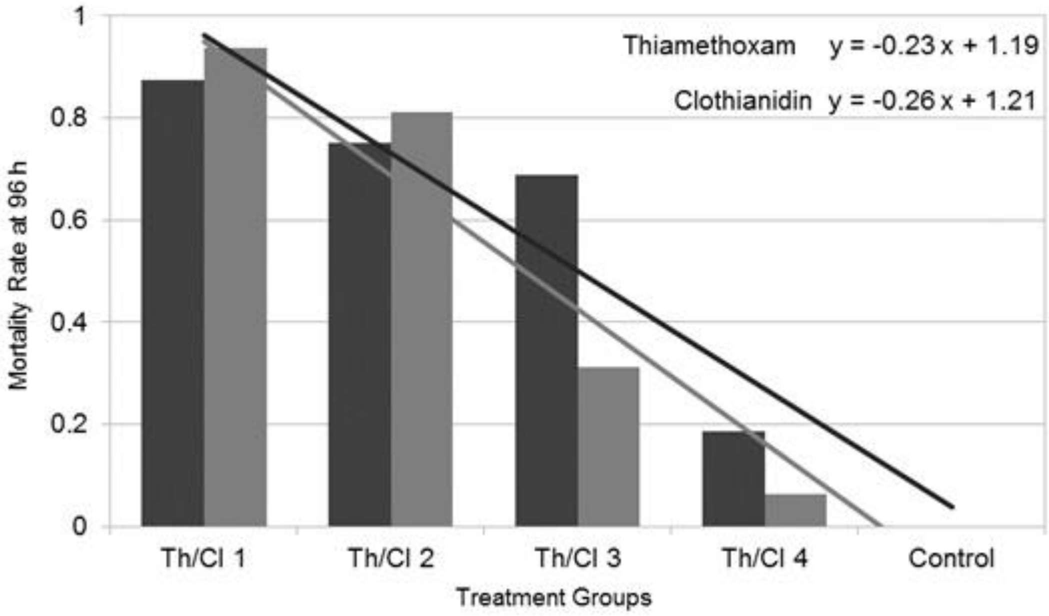

Figure 3. Mortality rate of adults after 72 hours after the beginning of treatment with thiamethoxam (dark grey bars) and clothianidin (light grey bars). The mortality rate is defined as the number of adults dead by the number of adults in each treatment group. Trend line referred to thiamethoxam is in dark grey, while that referred to clothianidin is displayed in light grey. Equations of the trend lines referred to Thiamethoxam and Clothianidin are shown in the graphs.

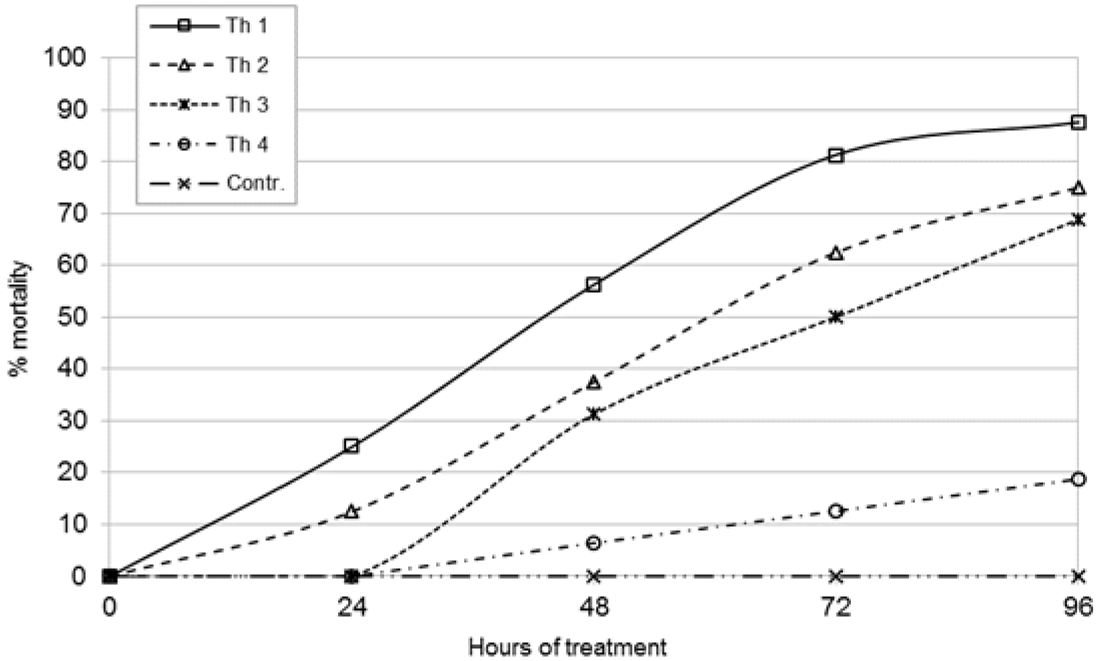

Figure 4. Mortality trends in the different treatment groups induced by Thiamethoxam. Data are expressed as the percentage of dead individuals.

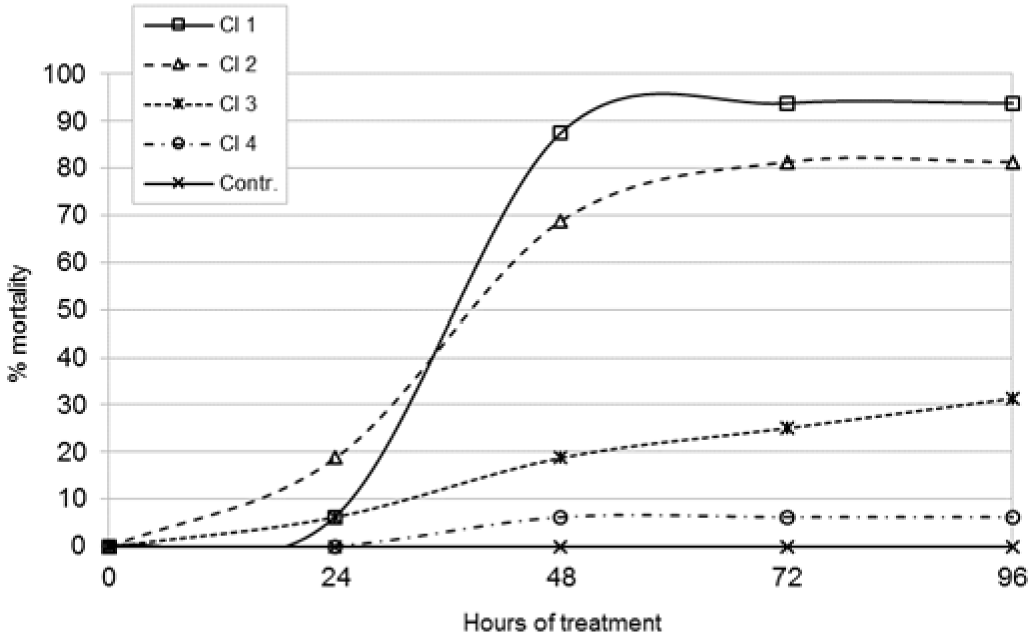

Figure 5. Daily mortality trends in the different treatment groups induced by Clothianidin. Data are expressed as the percentage of dead individuals.

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