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Effects of Topical Application of Hexaflumuron on Adult Sugar Beet Weevil, *Aubeonymus mariaefranciscae*, on Embryonic Development: Pharmacokinetics in Adults and Embryos

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The pattern of penetration, distribution, and excretion of labeled hexaflumuron on young and old adults of a new sugar beet pest, *Aubeonymus mariaefranciscae* Roudier (Coleoptera: Curculionidae), and its transovarial effects were investigated. Small differences between young and old adults in penetration and excretion were observed, though at the end the amount of hexaflumuron inside the body reached similar levels. Penetration of [^{14}C] hexaflumuron in females was a little higher than in males, particularly during the first 3 days after application, but excretion was strongly lower in males, resulting in higher retention of insecticide in their bodies than in females. Retention of hexaflumuron in the body was high compared to other insect species. Young adult weevils retain higher amounts of hexaflumuron in the reproductive system than the older ones. Moreover, the low percentage of radiolabeled hexaflumuron recovered in the eggs impaired the embryo development, leading to inhibition of egg hatch. Treatment with hexaflumuron on *A. mariaefranciscae* adults resulted in obvious symptoms of disorganization in the integument ultrastructure of embryos. The procuticle was particularly affected as a result of low residual amounts of hexaflumuron. Both endo- and exocuticle were totally unstructured and the typical lamellated organization was absent, resulting in an amorphous structure appearance. This effect seems to be related to irregular deposition of chitin–protein layers, leading to mechanical weakness and death of the embryo later on.

INTRODUCTION

The weevil *Aubeonymus mariaefranciscae* Roudier is a new sugar beet pest first recorded in 1979 in Montemayor (Cordoba) and Ecija (Sevilla) in southern Spain (1) and described as a new species in 1981 (2). Adults produce characteristic notches on the leaves, as well as cavities in the petioles and in the roots. The larvae feed on the sugar beet roots, which they tunnel extensively.

Direct damage by autumn populations is especially devastating, particularly by injuring sugar beet seedlings. However, adults and larvae can also produce severe damage during the spring by feeding on the storage roots. At present, it is estimated that about 3000 ha of sugar beet are infested by this curculionid, and there is evidence that it is spreading to other areas (3). All these factors make this insect a major pest of sugar beet in southern Spain.

The control of *A. mariaefranciscae* currently relies heavily on foliar applications of the organophosphate parathion-methyl and soil applications of the carbamate aldicarb. Due to the risk of these broad-spectrum insecticides to the environment, it would be useful to use more selective insecticides, such as benzoylphenyl ureas (BPUs).

The effects of benzoylphenyl ureas on the insect molting physiology have been reviewed in detail by Retnakaran *et al.* (4). Effects on reproduction in different insect orders by BPUs have also been reported, primarily as a reduction on egg hatching (5–9). In most cases, the embryo was fully developed in the egg but the larva failed to hatch (10). Accordingly, Marco and

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Castanera (11) reported on the potential of an acyl-urea, hexaflumuron, for the control of *A. mariaefranciscae* populations. They found a drastic reduction of egg hatching when adults were fed with treated leaves. More recent studies by the same authors confirmed that application of hexaflumuron on *A. mariaefranciscae* adults resulted in a significant period of inhibition of egg hatching; the effect was dependent on the method of application of the insecticide (oral–topical), the physiological reproductive stage (old–young), and the mating regime (12). They have reported that the effects of topical treatments were dominant to that of ingestion of hexaflumuron by adults. However, the differences in inhibitory activity on egg hatching observed when treating adults of different age (young vs. old) and when applying hexaflumuron to males and/or females could not be explained.

As such, in continuation of our study, here we report on the pattern of penetration through the cuticle and excretion through the feces of ^{14}C-labeled hexaflumuron, as well as its distribution and retention in different parts of the body of young and old females and males of *A. mariaefranciscae*. These data should provide better insights on the importance of the pharmacokinetic processes, leading to a differential susceptibility of adults of both sexes of different age. Hence, we have assessed the accumulation of labeled hexaflumuron in the eggs deposited by treated adults.

In addition, we aimed to elucidate the inhibitory mode of action of hexaflumuron toward egg hatching. We have compared sections of the integument ultrastructure of embryos originating from treated adults with those of the untreated controls by means of an electron microscope.

MATERIALS AND METHODS

Chemicals. Hexaflumuron, 1-[3,5-dichloro-4-(1,1,-2,2-tetrafluoroethoxy)-phenyl]-3- (2,6-difluorobenzoyl) urea, uniformly labeled with ^{14}C in the aniline ring with a specific activity of 22.2 mCi/mmol, was provided by Dow Elanco (U.K.) to study its penetration, distribution, and excretion in adults.

Consult 10EC (100 g hexaflumuron/L), provided by DowElanco Espana, was used to study transovarial effects.

Insect. Adults of *A. mariaefranciscae* were collected from a sugar beet field in Ecija (Sevilla, Spain). The insects were reared on sugar beet plants, cv. Eva, in a growth chamber (Conviron S10H, Controlled Environments, Winnipeg, Canada) at 26 ± 1°C, 80 ± 10% RH, and 16:8 (L:D) h photoperiod. Collection and manipulation of eggs was performed with a stereomicroscope. The eggs were removed from the leaf petioles by a fine camel's-hair brush, transferred onto moistened filter paper inside plastic containers (1.5 by 3.5 by 5.5 cm), and incubated at identical conditions as above. Neonate larvae were reared on an artificial diet as described in Marco *et al.* (13) to obtain adults of a known age.

Adults topically treated were considered old when females were at the end of the oviposition period (adults collected in the field) and young when females started to lay eggs (adults reared on the artificial diet). The mean duration of the oviposition period was 40.6 ± 7.8 days under the conditions mentioned above. Adults were sexed by observing the presence (males) or absence (females) of a depression between the third and fourth abdominal tergites, whereas the diet reared weevils were sexed at the pupal stage by observing the presence (females) or absence (males) of two cuticular nodes in the last abdominal tergite (14).

Topical application with radiolabeled [^{14}C]hexaflumuron. Adult males and females of *A. mariaefranciscae* were individually treated by applying 0.5 μl of an acetone solution containing [^{14}C]hexaflumuron on the pronotum with a Hamilton microapplicator. The average amount of radioactivity was 46601 ± 3979 cpm for old adults and 36716 ± 1056 cpm for young adults. Each treated adult was confined onto a plastic petri dish containing two Whatman filter papers No. 1 on the bottom, and they were provided with fresh leaves of sugar beet *ad libitum*.

For each assay, four males and four females as well as their feces plus the filter paper were collected at different time intervals (1, 2, 3, 7,

and 14 days) after the topical treatment. The adults were kept at normal rearing conditions. For young adults, we also collected samples at 21 days after treatment with ^{14}C-label. They were killed by freezing and kept at −20°C until analyzed.

Penetration and excretion of [^{14}C]hexaflumuron. To assess the amount of labeled compound that had penetrated in the body and the rate of penetration, each sample containing two adults was washed twice in 1 ml of acetone for 10 min. A preliminary assay demonstrated that the efficiency of recovery of these two washes reached 92% of the amount of radioactivity applied. The amount of acetone from the washes was concentrated to dryness in a scintillation vial and 10 ml of Luma Safe Plus (Lumac, Belgium) was added. The radioactivity was determined by liquid scintillation counting (Kontron, LKB, Belgium).

In order to measure the retention and excretion of [^{14}C]hexaflumuron excreted at each interval, the feces and the filter paper together with leaf remnants of each sample (originating from two adults) were combusted in a Biological Material Oxidizer Model 306 (BMO, Harvey Instruments Co., USA). The combusted gases (+[^{14}C]dioxide) were collected with 8 ml of "Carbosorb I" (Lumac, Belgium) and 9 ml of "Carboluma" (Lumac, Belgium). The amount of radioactivity was expressed as a percentage of the total radioactivity recovered from each sample. Data are means (±SD) of two replicates of two adults each. A logarithmic fitting with curve-fitting option of Excel Microsoft was performed; the quality of fitting is evaluated on the curve correlation coefficient R^2.

Determination of [^{14}C]hexaflumuron radiolabel in the cuticle, gut, and reproductive system and eggs laid. The distribution of radioactivity in the body was determined by dissecting the adults in an embryo glass with wax to separate the integument (including cuticle + muscles + peritoneal fat body + head), the food canal (including the content + visceral fat body + malpighian tubules), and the reproductive system. Petri dishes containing females as well as the leaves inside were examined to separate the eggs laid. The dissected tissues were washed in Grace's physiological solution (Sigma Co., Bornem, Belgium) and then combusted separately as mentioned above. Data are presented as means (±SD) of two replicates of two adults each. The amount of radioactivity is expressed as a percentage of the total radioactivity recovered from each sample.

Effect of hexaflumuron on the ultrastructure of the embryo integument via adults. Four replicates of 10 adults each (5 females and 5 males) were topically treated with Consult 10EC dissolved in an analytical grade acetone to make a solution of 62.5 mg a.i./ml. A microliter dispenser (Burkard 900-x) was used to apply 0.5 μl/weevil of this solution on the pronotum of the thorax, equating to ca. 0.03 μg a.i./adult (average adult weight ≈ 6 mg). Control weevils were treated with 0.5 μl of acetone. Each replicate and control was placed in a plastic pot arena (7 cm diameter by 7 cm high) filled with moistened sand where a detached sugar beet leaf was inserted and confined within another plastic container (6 cm diameter by 15.5 cm high) at 26 ± 1 C, 80 ± 10% RH, and a 16:8 (L:D) photoperiod.

On a daily basis, eggs deposited on sugar beet leaves were removed and transferred into plastic containers (1.5 by 3.5 by 5.5 cm) with moistened filter paper. The movement of the embryo could be observed within the eggshell. Further, when the embryo was completely formed, it failed to hatch. Then the eggs were frozen up until the moment they were prepared for the electron microscope.

Embryos were released by pressing the eggshell gently with a fine camel's-hair brush and cut into sections. We used conventional processing for electron microscopy as follows: embryos were embedded in 2% agar and fixed in Karnovsky liquid fixative (5% glutaraldehyde and 4% formaldehyde) in 0.025 M cacodylate buffer for 5 h. After three washes of 30 min each in the same buffer, they were postfixed in 1% osmium tetroxide and washed again. Samples were dehydrated first in an ethanol series (30–100%) and then in 100% ethanol: propylene

oxide (1:1) for 5 min. Dehydrated samples were embedded in vials with propylene oxide:Epon (1:1, v:v) for 45 min at room temperature and in Epon for 45 min. Semithin sections (1 μm) were observed under phase contrast without any staining for general structure observation. Ultrathin cross sections of embryos from treated adults and controls were mounted on Formvar-coated copper grids, stained with uranyl acetate for 30 min and lead citrate for 2 min, and examined in a transmission electron microscope (Philips EM 420, Holland).

RESULTS AND DISCUSSION

Penetration and Excretion of [^{14}C]Hexaflumuron

After topical application, the penetration curve of [^{14}C]hexaflumuron followed a similar profile in both males and females of young and old age (Figs. 1A and 1B). The radiolabeled insecticide was absorbed more rapidly the first 3 days in young adults of both sexes; then the slope decreased, indicating that the penetration rate was lowering reaching a maximum concentration. After 2 weeks, 80 and 73% of the applied amount of [^{14}C]hexaflumuron had penetrated in young females and males, respectively, and 75 and 70% in old females and males. Fitting of the data to a logarithmic curve was good for both young (females: $R^2 = 0.99$; males: $R^2 = 0.98$) and old (females: $R^2 = 0.89$; males: $R^2 = 0.96$) adults. Comparison of the rates of penetration (dy/dx) of hexaflumuron in young and old adults demonstrated that the insecticide was absorbed somewhat more rapidly in old adults (Figs. 1A and 1B).

Further, we found a coincidence of patterns for the different physiological ages, and a single model of absorption of hexaflumuron was estimated. Pooling of the data of young and old adults resulted in a logarithmic fitting that was good; $R^2 = 0.99$ for both males and females (Fig. 1C). This finding suggests that the penetration pattern of hexaflumuron is independent from the age of adults. Likewise, our data indicated that absorption through the cuticle was slow, which has been reported to be a general phenomenon for BPUs (4). Many studies in different laboratories have demonstrated that the oral toxicity of this group of BPUs is higher than by contact or topical treatment, particularly for larval stages (4, 15), or that there are only small differences (8, 16). Only a few species, among them *A. mariaefranciscae* adults (unpublished data), have been reported to be more susceptible to BPUs by contact application compared to ingestion of treated leaves (17, 18). So, when we apply the current model curve (Fig. 1C), 29 and 14% of the [^{14}C]hexaflumuron applied had penetrated in females and males, respectively, after 1 day (Figs. 1A and 1B). However, these low percentages resulted in a strong inhibition of egg hatching. These low values agree with the results of Smagghe *et al.* (19), who reported that only 15% of diflubenzuron had penetrated in larvae of *Spodoptera exigua* after 24 h of topical treatment and 31% in *Spodoptera littoralis*, while the compound was highly toxic to about the same extent in both species.

In females, 50% of the radioactivity applied had penetrated after 3.2 days, while in males this percentage was only reached after 5.2 days (Fig. 1C). This means that penetration is occurring 1.6 times faster in females than in males. In all different intervals, the amount of hexaflumuron in the body was higher in females than in males. Two weeks after treatment, 77% of the [^{14}C]hexaflumuron applied was attained in females and 72% in males.

Typically, the profile of the excretion curves of [^{14}C]hexaflumuron in young and old females and males were similar (Figs. 2A and 2B). Both young and old males had excreted about the same amount of hexaflumuron after 2 weeks: 41 and 45%, respectively. Conversely, the percentage of product excreted differed depending on the age of the females. In young females, the half-life value (50% excreted) was 4.8 days, whereas this was 8.9 days in old females. After 2 weeks of topical treatment, 66 and 55% of radiolabel applied was eliminated in young and old weevils, respectively. These values were calculated with the estimated curve for which the goodness of fit was 0.9 for young and 0.8 for old females.

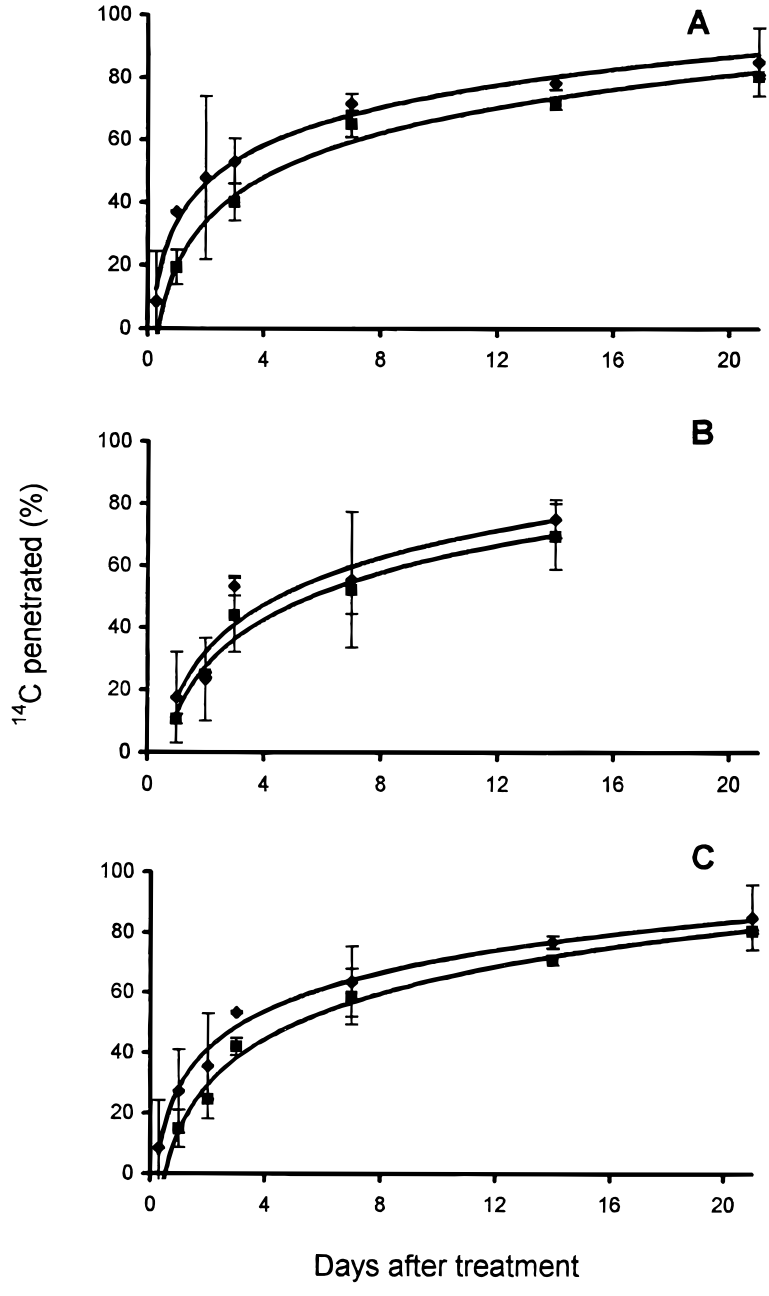

FIG. 1. Percentage (means ± SD) of the applied [^{14}C]hexaflumuron absorbed through the weevil's cuticle at different time intervals in young and old females (♦) and males (■) of *A. mariaefranciscae*. The curves represent the fitting to a logarithmic function. (A) For young females ($Y = 17.61\text{Ln}X + 33.83$; $R^2 = 0.99$) and males ($Y = 20.33\text{Ln}X + 19.80$; $R^2 = 0.98$); (B) for old females ($Y = 21.92\text{Ln}X + 17.02$; $R^2 = 0.89$) and males ($Y = 21.84\text{Ln}X + 12.27$; $R^2 = 0.96$); (C) for the total of females ($Y = 18.26\text{Ln}X + 28.56$; $R^2 = 0.99$) and males ($Y = 21.84\text{Ln}X + 14.19$; $R^2 = 0.99$).

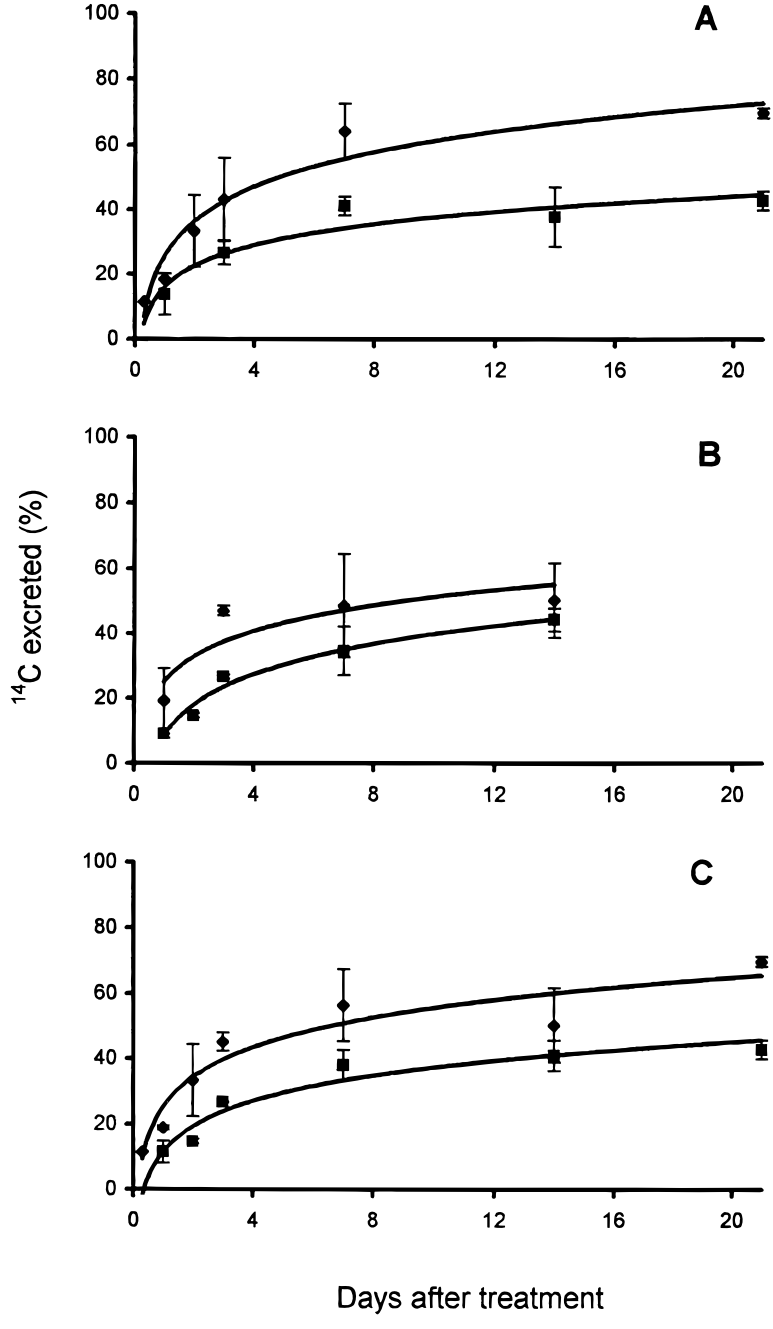

FIG. 2. Percentage (means ± SD) of the applied [^{14}C]hexaflumuron excreted through the feces at different time intervals in young and old females (◆) and males (■) of *A. mariaefranciscae*. The curves represent the fitting to a logarithmic function. (A) For young females ($Y = 15.47\text{Ln}X + 25.67$; $R^2 = 0.94$) and males ($Y = 9.39\text{Ln}X + 15.98$; $R^2 = 0{,}89$); (B) for old females ($Y = 11.41\text{Ln}X + 25{,}04$; $R^2 = 0.77$) and males ($Y = 13.68\text{Ln}X + 8.41$; $R^2 = 0.97$); (C) for the total of females ($Y = 13.21\text{Ln}X + 25.23$; $R^2 = 0.91$) and males ($Y = 11.23\text{Ln}X + 11.45$; $R^2 = 0.94$).

A single model of excretion rate was made with data of young and old adults (Fig. 2C). The fitting to a logarithmic curve was good for females ($R^2 = 0.91$) and males ($R^2 = 0.94$). According to this model, 2 weeks after the topical application females had excreted 1.5 times more of the radioactivity applied than males (60 and 41%, respectively). This pattern can help to explain the strong inhibitory effect of hexaflumuron in *A. mariaefranciscae*. In contrast, Still and Leopold (20) observed no differences between male and female adults of the boll weevil, *Anthonomus grandis*, in their ability to eliminate [^{14}C]diflubenzuron.

In the current assays, it should be remarked that the rate of excretion is low compared to other insects studied so far. Granett *et al.* (21) showed that diflubenzuron was very rapidly excreted in two lepidopteran species, *Choristoneura occidentalis* and *Orgyia pseudotsugata*, being eliminated 90 and 86%, respectively, after 24 h of ingestion. Approximately 20 h after topical application, 50% of the absorbed compound had been excreted. Still and Leopold (20) reported percentages of 29 and 8% of radioactivity applied in *A. grandis* at 6 and 13 days after dipping in diflubenzuron, respectively. On the other hand, our data agree with those of Auda *et al.* (22). They found that hexaflumuron was more toxic to last instar larvae of *Leptinotarsa decemlineata* compared to *S. littoralis* and *S. exigua* and concluded that it was probably due to its greater retention in the larval body. Only 57% of the ingested [^{14}C]hexaflumuron was recovered in the feces of the beetle after 24 h, while more than 90% was recovered at the same period for both lepidopterans. Likewise, excretion of another new BPU, flufenoxuron, was found to be slow in larvae of *S. littoralis* after oral and topical application (16). Taken together, retention of hexaflumuron in the body of *A. mariaefranciscae* adults is high compared to some other pests, including different species in which retention has been considered to be high.

A summary of the data shows that there are small differences between young and old adults in penetration and excretion, but at the end the amount, in absolute values, of hexaflumuron inside the body reaches somewhat the same levels. Penetration of [^{14}C]hexaflumuron in females is a little higher than in males, but excretion is strongly lower in males, resulting in higher retention of insecticide in their bodies than in females. Further, in young females high amounts of hexaflumuron were absorbed during the first 3 days after application. So, our current results confirm the high retention of hexaflumuron in the body of the insects, which can make this compound interesting for field treatments in the long term.

Accumulation of [^{14}C]Hexaflumuron in the Integument, Gut, Reproductive System, and Eggs Deposited

The level of radioactivity in the body tissues was maintained for at least 2 weeks after topical application. Here, the pronotum residue has acted as a reservoir. It was clear that high amounts of the recovered radioactivity in old adults, both females and males, were present in the integument (Figs. 3C and 3D). After 1 day, 82% was retained in females and 76% in males. Further on, this amount remained almost constant in females, whereas it decreased slightly in males. After 2 weeks the female and male integument yielded, respectively, 74 and 60% radioactivity applied. In contrast, the percentages recovered in the integument of young adults were conspicuously lower (Figs. 3A and 3B). In young females, the amount decreased slightly between 1–2 days (64%) and 2 weeks after the treatment, reaching 40%. The latter percentage was only half of that recovered in old females at that time. For young males, the values of radioactivity recovered ranged between 73 and 57%.

We demonstrated that the levels of [^{14}C]hexaflumuron recovered from the alimentary canal were low and irrespective of sex and physiological age. The values ranged between 11 and 20% from the beginning until the end of the experiments. These results suggest that the passing of hexaflumuron through the food canal is in balance with the rate of accumulation/transport in the body, leading to elimination out of the

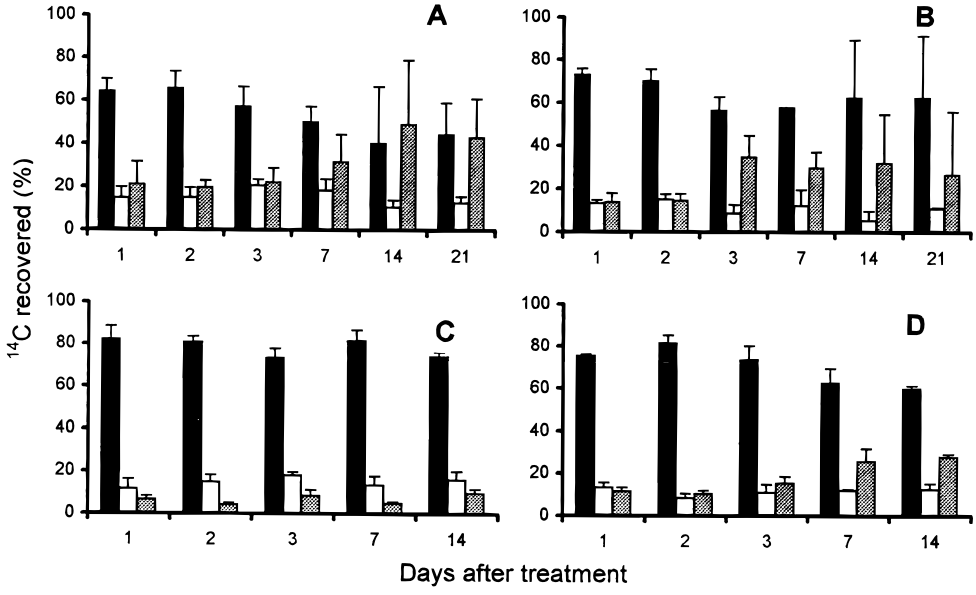

FIG. 3. Percentage (means ± SD) of the applied [^{14}C]hexaflumuron recovered in the integument (■), gut (□), and reproductive system (⊡) at different time intervals. (A) Young females (data include eggs laid); (B) young males; (C) old females; (D) old males of *A. mariaefranciscae*.

body via feces or reabsorption from the gut in the hemolymph. Van Laecke *et al.* (23) found that diflubenzuron and chlorfluazuron were transported very quickly to the hemolymph through the gut of last instar larvae of *S. exigua* when larvae were reared on treated artificial diet. For BPUs, a differential rate of transportation from the gut to the biochemical site of action has been reported to be essential for their toxicity (24).

With respect to the accumulation of hexaflumuron in the reproductive system, we found a striking differential pattern that depended on the age and sex of *A. mariaefranciscae* adults (Fig. 3). Radioactivity was much more absorbed in the reproductive system of young females than in that of old ones. At 1 day after treatment, three times more radiolabel was recovered in the reproductive tissues (ovaries and eggs) of young (21%) compared to old (7%) specimens. Moreover, these differences increased with time. After 2 weeks, nearly 50% of the radioactivity recovered in young females was present in the ovaries plus eggs laid, whereas at this time only 9.7% of the recovered radioactivity was in the reproductive system of the old females. Likewise, differential levels of radioactivity were found in the gonads of young males compared to old individuals, although they were not as obvious as in females. After 2 days of treatment, the percentages recovered were similar. But 1 day later the amount in the gonads of young adults had increased to 35%, which is about two times more than in the old males (15%). Further, the gonads of both groups yielded similar levels after treatment until the end of the assay.

It seems that young adult weevils retain higher amounts of hexaflumuron in the reproductive system than the older ones. The radioactivity recovered in young males could be enough to affect spermatogenesis in some way and a part of the product could be transferred to the female during the mating process, leading to nonsuccessful fertilization. For females, it is reasonable to conclude that the different amounts of hexaflumuron in the reproductive system of young vs. old weevils might be related to the physiological age of the ovaries. It is well known that the ovaries of young females are very active for accumulating vitellogenins from the hemolymph

in order to form the eggs (24). Therefore, hexaflumuron molecules could have been taken up in the ovaries through the process of adsorptive endocytosis.

Our results show that a very small part of the applied amount of [^{14}C]hexaflumuron had incorporated into the eggs from young and old females during the first 3 days, but that the amount increased with time (Fig. 4). Between 2 days and 2 weeks after treatment the amount of hexaflumuron recovered in the eggs deposited ranged from 12.0 $\pm$ 4.8 to 46.9 $\pm$ 3.9 cpm per egg, representing a percentage of the amount applied between 0.03 and 0.13%. The latter percentage is equal to about 2. 3 pmol of hexaflumuron that had accumulated in one egg. Although this amount was low, it was enough to cause the death of the embryo. Previous studies with *A. mariaefranciscae* have shown that topical application of hexaflumuron on adults at the beginning of the ovipositing period resulted in a period of complete inhibition of egg hatching for over 6 weeks (12). This effect can be explained with the accumulation of hexaflumuron currently observed in eggs deposited.

Moreover, the low percentage of radiolabeled hexaflumuron recovered in the eggs seems to be responsible for the embryocidal effects that we have observed. In a study by Ivie and Wright (5), it was shown that house flies, *Musca domestica*, and stable flies, *Stomoxys calcitrans*, treated with diflubenzuron secreted low levels of the insecticide to the eggs (1% or less). Similar to our conclusions, this low amount was enough to cause the ovicidal toxicity in these species.

Topical treatments with diflubenzuron have been found to be effective against adults of *A. grandis*, where the compound was transported to the ovaries and eggs, leading to egg mortality (26). Likewise, field trials with diflubenzuron have been demonstrated to be effective for this species by ovicidal activity (27, 28). Egg-sterilizing effects were also observed in the beetle *Carpophilus hemipterus* when adults were exposed to artificial diets treated with different BPUs (29).

The effect of age of the treated adult on egg viability could not be observed in this study because of the small number of eggs laid by old females. However, it is expected to be one of the effects of diflubenzuron on eggs through the female (30, 31). Additionally, Holst (32) had observed that ovicidal effects of diflubenzuron in the diet of adults of *Epilachna varivestis* decreased more quickly in the older adults than in the younger ones. Otherwise, topical treatment with diflubenzuron on *Riptortus clavatus* decreased egg hatching irrespective of adult age (33).

In summary, we believe that the high retention in the body and the low doses required to produce inhibition of egg hatch confirm the usefulness of hexaflumuron in the management of this curculionid pest.

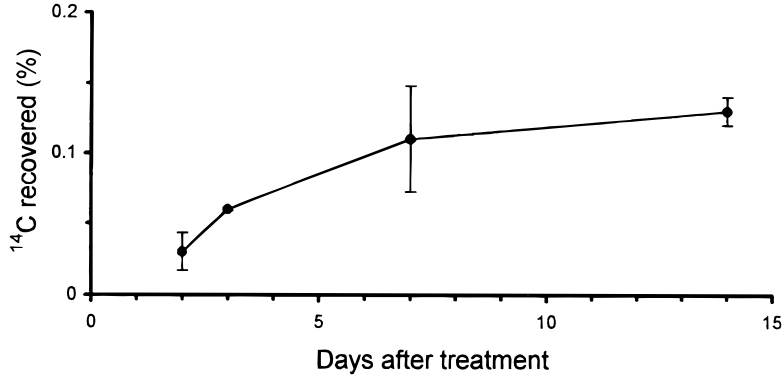

FIG. 4. Percentage (means $\pm$ SD) of the applied [^{14}C]hexaflumuron recovered in the eggs laid by *A. mariaefranciscae* at different time intervals.

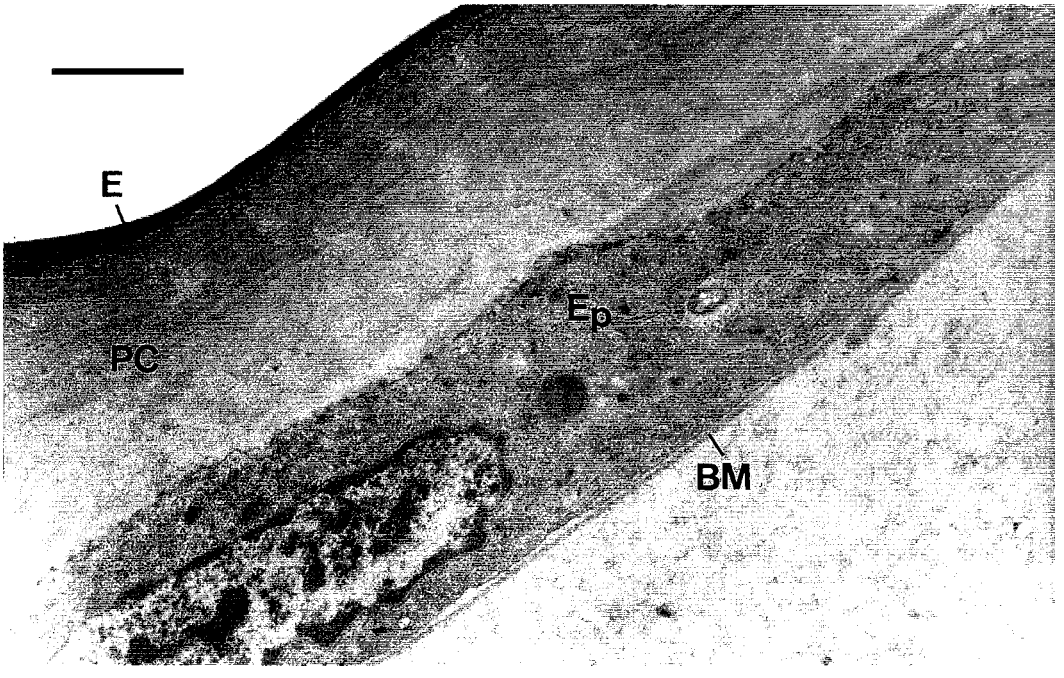

FIG. 5. General appearance of the fine structure of the integument of a control embryo of *A. mariaefranciscae*. Procuticle (PC) is clearly lamellated. Epicuticle (E) and procuticle have uniform thickness and electron density. Epidermis (Ep) is 1 cell thick, separated from hemocoel by a basal membrane (BM). Bar, 1 μm.

Effects of Hexaflumuron on the Ultrastructure of the Embryo Integument

Under the electron microscope, the integument of control embryos of *A. mariaefranciscae* consists of a thin electron-dense epicuticle, followed by a thick procuticle and the epidermis cells that are separated from the hemocoel by a thin basal membrane. The procuticle was clearly lamellated, which is in accord with the general organization of chitin-containing cuticles (34). The epidermis showed a 1-cell-thick organization. In addition, regular microvilli with typical plasma membrane plaques at their tip were observed (Figs. 5 and 6), indicating secretion of lamellated insect cuticle (35). In the epicuticle, three layers could be distinguished in accord with Binnington (36): inner epicuticle, outer epicuticle, and superficial layer. Typically, the epi- and procuticle showed an homogeneous electron density, and the three layers, i.e. epicuticle, procuticle, and epidermis, were parallel and uniform in thickness and structure (Figs. 5 and 6).

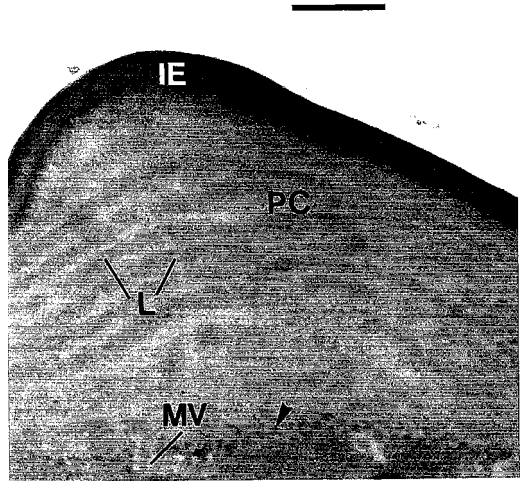

FIG. 6. Epicuticle and procuticle of a control embryo. Procuticle (PC) is conspicously lamellated (L). Typical microvilli (MV) at the apical border of the epidermal cell show regular appearance and dense plaques at the tips (arrowhead). Inner epicuticle (IE) has regular thickness. Bar, 0.5 μm.

Treatment with hexaflumuron resulted in obvious symptoms of disorganization in the integument ultrastructure. The procuticle showed empty spaces and electron-dense bodies (Figs. 7 and 8), giving the cuticle an heterogeneous appearance. Both endo- and exocuticle were totally unstructured and the typical lamellated organization was absent, resulting in an amorphous structure. These ultrastructural effects agree with those of Grosscurt (31) in embryos of *L. decemlineata* just prior to egg hatch when the gravid females had previously ingested leaves treated with diflubenzuron. It is a well-known phenomenon that BPUs interfere in cuticular deposition, resulting in a conspicuous lack of the endocuticular lamellated structure. This has been reported in other studies with different insects (37, 38). The lamellar structure reflects a regular deposition of chitin–protein layers. Lack of this could result because of an alteration in deposition and may lead to mechanical weakness and death of the embryo later on. Likewise, we observed in the procuticle of treated embryos some electron-dense opaque patches. Hassan and Charnley (39) described that this effect might represent short-chain chitin accumulations. On the other hand, we observed that microvilli displayed small and irregular plasma membrane plaques (Fig. 8). A similar effect was reported in larvae of the spruce budworm, *Choristoneura fumiferana*, treated with a sublethal dose of chlorfluazuron (40). Taken together, the current findings may strengthen the hypothesis that the disrupted procuticle in treatment is mainly formed by proteins, because hexaflumuron had inhibited synthesis of chitin or hindered its correct deposition in the procuticle. Hence, the thickness of procuticle in control embryos ranged between 1.5 and 1.9 μm, whereas treatment with hexaflumuron provoked only a slight reduction. This phenomenon of an unchanged thickness in treated and control insects may suggest that protein synthesis or its incorporation into the cuticle is not affected (31,

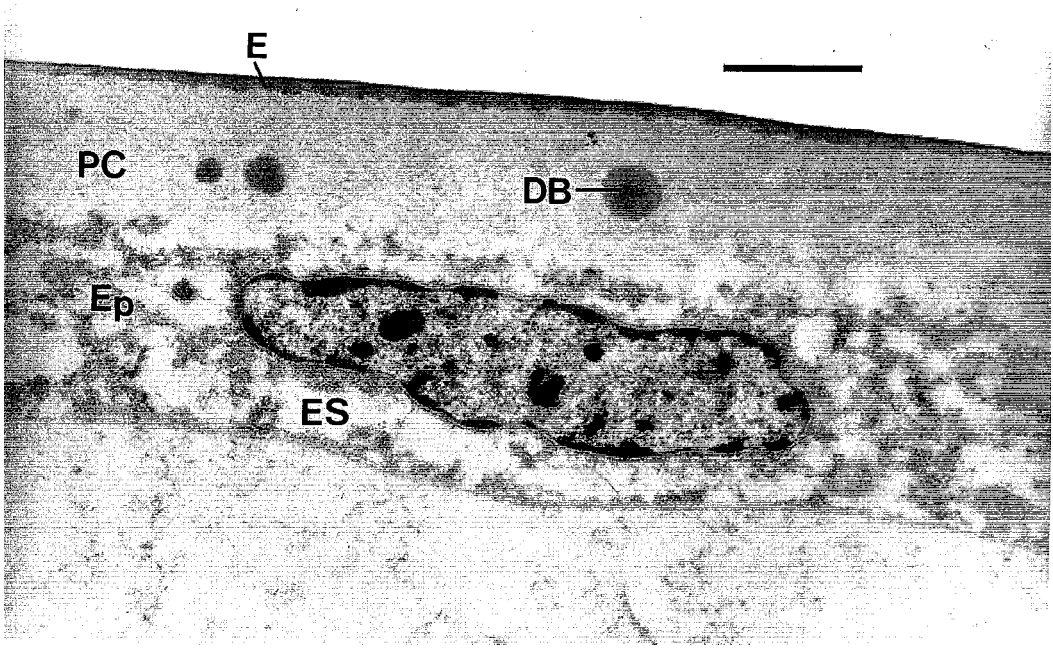

FIG. 7. General appearance of the fine structure of the integument of an embryo from *A. mariaefranciscae* adults topically treated with hexaflumuron showing abnormalities in cuticular layers. Procuticle (PC) displays electron-dense bodies (DB) and lack of lamellate disposition. Epidermis (Ep) shows large areas of empty spaces (ES) and irregular epicuticle (E). Bar, 1 μm.

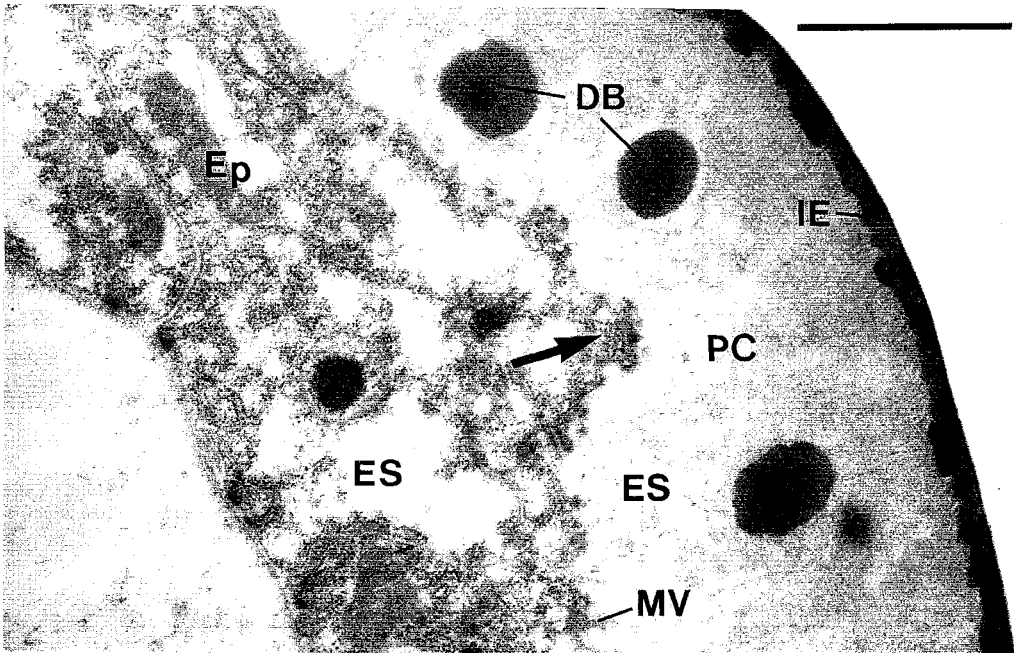

FIG. 8. Detail of the integument of an embryo from treated adults. Note cytoplasmic extrusions from epidermis (Ep) to the procuticle (PC)(arrow). The profile of the microvilli (MV) is irregular and the density of the plaques at the tips is reduced. The inner epicuticle (IE) displays irregular thickness and electron density. DB, dense-electron bodies; ES, empty spaces. Bar, 1 μm.

39), while the production of chitin was inhibited. Other studies of Binnington *et al.* (41), Degheele (42), and Soltani *et al.* (43) demonstrated a drastic reduction in cuticular thickness and explained this effect due to a reduced chitin and protein content. Likewise, the current studies revealed abnormalities in the epicuticle due to hexaflumuron (Fig. 7), resulting in a lack of uniform thickness of the thicker lighter layer. The latter corresponds to the inner thick proteinous epicuticle. Similar observations were reported by Degheele (42) in *Mamestra brassicae* larval cuticle of larvae treated with diflubenzuron and in embryos of *L. decemlineata* contaminated via the female (31). The latter effects suggest that treatment with BPUs may additionally interfere with cuticular protein deposition. In addition, this study revealed abnormalities in the epidermis of sections from treated embryos. As in the rest of the integument, the thickness is not uniform. Apical microvilli of the epidermal cells are irregular, the cytoplasm displays many vacuolated spaces, and clear symptoms of cellular degeneration were seen. Likewise, noticeable extrusions of the cytoplasm to the procuticle were visible (Fig. 8). Similar effects have previously been observed in larvae of *Manduca sexta* after feeding with diflubenzuron (39).

Grosscurt (31) indicated that the ovicidal effect of BPUs applied on adults can be due to the inhibition of chitin synthesis in the embryo that produces disturbances in the structure of the integument and a general weakening of the cuticle. In addition, the muscular fibers cannot anchor firmly to the cuticle and the embryo is not strong enough to break the chorion. Finally, the embryo dies into the egg shell, which is consistent with our data that chitin formation is impaired. A study on the control of the white pine weevil, *Pissodes strobi*, with diflubenzuron (44), and a recent study of Wilson and Cryan (45) with another BPU, lufenuron, support this

point of view. On the contrary, our data do not suggest effects on the vitellogenesis process as reported by Soltani and Soltani-Mazouni (46) after topical treatment of females of *Cydia pomonella* with diflubenzuron. In conclusion, our current results on pharmacokinetics and electron microscope support the notion that aberrations of the embryo integument are the result of low residual amounts of hexaflumuron, provoking embryo mortality, when hexaflumuron was applied on *A. mariaefranciscae* adults.

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