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Source: Journal of Orthoptera Research, 24(2) : 77-81

Published By: Orthopterists' Society

URL: https://doi.org/10.1665/034.024.0206

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The effects of diflubenzuron on the cuticle and on hemolymphatic ecdysteroids of fifth instar nymphs of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae)

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Abstract

The effect of diflubenzuron (DFB), a benzoylphenyl urea insecticide, on the cuticle in newly molted fifth-instar nymphs of *Schistocerca gregaria* was examined. While there was an increase in dry weight of the cuticle in untreated nymphs, DFB treated nymphs had a reduced cuticle dry weight, with significant reductions in chitin levels. In a second series of experiments, DFB significantly reduced ($p < 0.05$) ecdysteroid titers in the hemolymph during nymphal development, leading to disruption of molting.

Key words

EIA, insecticide, insect growth regulators, molting, physiology, endocrinology, benzoylphenylurea

Introduction

Locusts, like all arthropods, have the characteristic of being able to synthesize a cuticle and grow by molts. The cycle of renewal of the cuticle is controlled by three types of hormones: brain hormone, juvenile hormone, and ecdysteroids, which, in addition, are involved in regulating other mechanisms of development (Nation 2002).

Ecdysteroids are steroid hormones that control various aspects of the development of insects (Bellés 1998, Lafont *et al*. 2005, McBrayer *et al*. 2008) and are therefore a potential target for insecticides (Dinan 1989). Disruption of production or lack of ecdysteroids is a potential mechanism of insecticide action. Considerable knowledge has been accumulated over the past thirty years on the physiology and endocrinology of insects, which has helped develop procedures to select insecticides that interfere with the metabolism of specific insect development or endocrine mechanisms (Dhadialla *et al*. 2005). These compounds are referred to as Insect Growth Regulators (IGRs) (Retnakaran *et al*. 1985, Dhadialla *et al*. 1998, Kostyukovsky & Trostanetsky 2006). Growth regulators are synthetic molecules that act on the physiology of insects by disrupting their development.

Although several families of chitin synthesis inhibitors are known, only the benzoylphenylureas (BPUs) are marketed as a pesticide (Spindler-Barth & Spindler 2001). Several studies have described the biochemical and biological effects of BPUs (Retnakaran *et al*. 1985, Reynolds 1987, Ishaaya 1990, Cohen 2001, Abo-Elghar *et al.* 2004) but past studies have not completely elucidated their mechanism of action. Hypotheses have been proposed, including that BPUs interfere with the formation of the cuticle due to an increase in chitinase activity (Ishaaya & Casida 1974, Merzendorfer & Zimoch 2003) or that of the chitin by activation of the proteolytic activity of chitin synthase (Leighton *et al.* 1981). The BPU diflubenzuron (DFB) is known to interfere with the molting process by disrupting cuticle secretion in immature stages of insects (Tunaz & Uygun 2004, Dhadialla *et al.* 2005, Mommaerts *et al.* 2006). From a biochemical perspective, it was long thought that DFB blocking of the synthesis of chitin is probably acting on the biosynthesis of chitin synthase. However, this hypothesis has not been confirmed *in vitro* (Cohen 2001). To date, it is not known whether inhibition of chitin synthase is the primary biochemical site of action, as DFB does not inhibit enzyme activity *in vitro* (see *e.g.*, Cohen 2001). Another mechanism of action could be that it interferes in the ecdysteroid regulation of chitin synthesis (Merzendorfer 2006).

The hypothesis of DFB interfering with hormones was originally formulated by Yu & Terriere (1975, 1977) followed by Soltani *et al.* (1984, 1989), Fournet *et al.* (1995) and Tail *et al.* (2008, 2010). Therefore, in this paper we tested the hypothesis that DFB may affect the level of hemolymph ecdysteroids in last-instar nymphs of *S. gregaria*.

Materials and methods

Rearing and synchronization of locusts.—Fourth-stage nymphs of *S. gregaria* were placed in separate cages, and as they molted to the fifth instar, they were collected and distributed into smaller cages (20 × 30 × 15 cm) so that nymphs of the same age could be used for the various comparative tests. Rearing was at a temperature of 30°C, at 80% relative humidity, and a photoperiod of 12:12 L:D. Locusts were fed daily with *Triticum durum* wheat, *Brassica oleracea* cabbage, *Pennisetum dichotomum* grass and a protein supplement containing wheat bran. The same conditions of temperature and humidity were selected to perform our experiments.

DFB treatment.—DFB (DIMILIN® 25 WP, Chemtura) was applied by ingestion. Three batches of 10 newly molted (0-4 h after molting) fifth-stage gregarious nymphs were fed 30 mg/mL of DFB in water for 24 hours. After this period, untreated food was then presented to the treated locusts. Ten individuals of the same age, fed with an untreated substrate, constituted the control group.

Sampling.—After cutting off one or two legs, 10 to 15 µl of hemolymph were collected from each specimen using a calibrated capillary tube. Each hemolymph sample was added to 300 µL of methanol and stored at -20°C for subsequent assay of ecdysteroids.

The abdomens of control or treated nymphs were sampled 0-4 h after molting and every 24 h thereafter during the first four days of development. The integument was isolated under a binocular microscope and discarded from adhering muscle mass and adipose tissue using forceps. The cuticle material was carefully freed from

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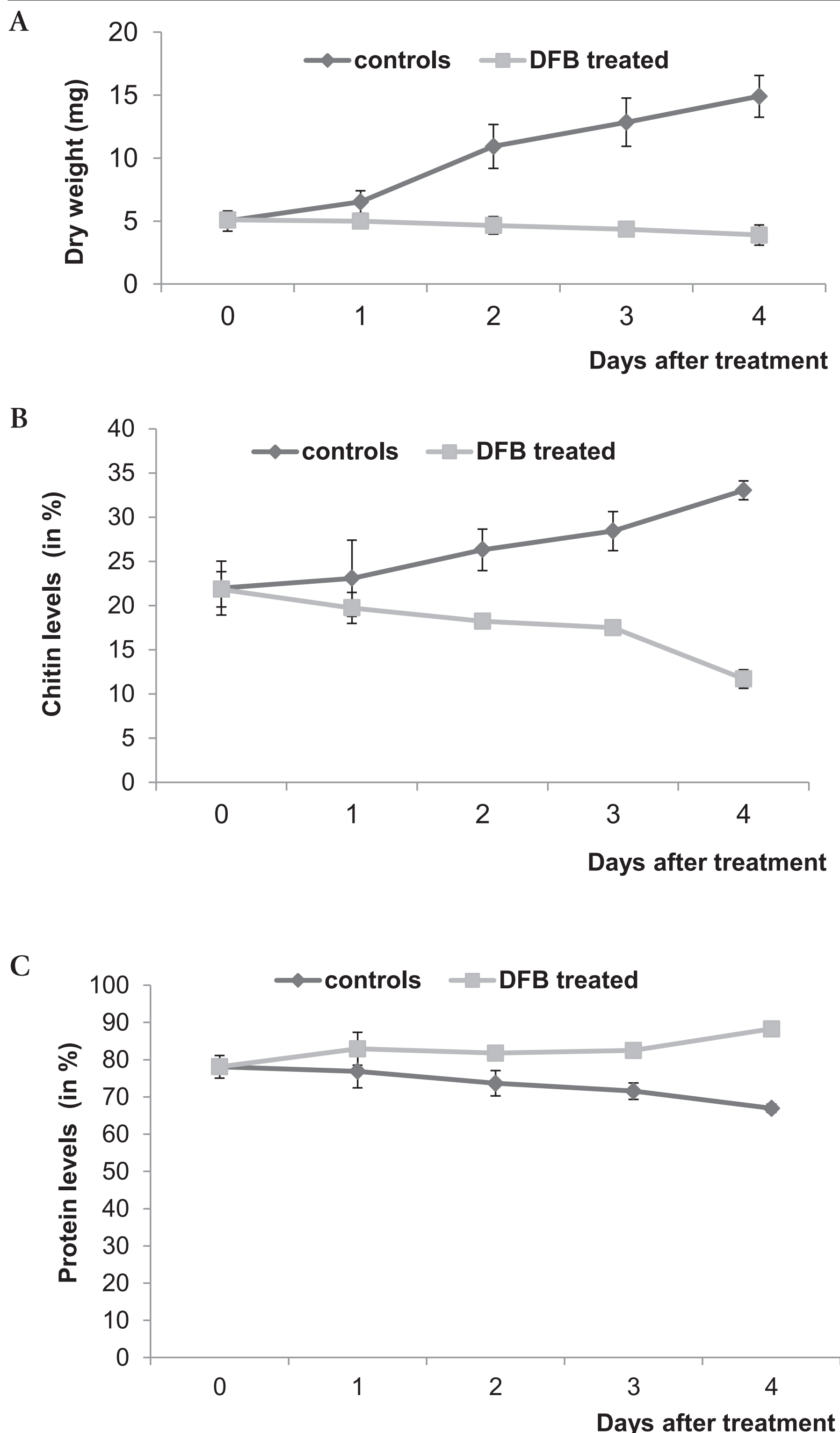

Fig. 1. Changes in mean dry weight (mg) (A), chitin levels (in %) (B), and protein levels (in %) (C), of the cuticle of control and DFB-treated 5th instar nymphs of *S. gregaria* (mean ± SD, n = 30).

the underlying tissue and washed with ether-chloroform 1:1 (v:v) at room temperature for 24 h to remove all fat traces. After rinsing with alcohol (96%), the cuticle was dried in an oven at 60°C until obtaining a dry weight (P1).

Determinations of total protein and chitin of abdominal sternal cuticle of nymphs.—The separation of protein components and chitin was performed by extended basic hydrolysis as indicated by Bordereau & Anderson in Abbaci & Hamza (2004). Each sample of dry weight (P1) was introduced into a sealed tube and heated at 110°C for 2-3 h in the presence of 2.5 N sodium hydroxide. The solid chitinous residue was washed successively with distilled water, 1 N hydrochloric acid, absolute ethanol, and ether. It was then dried to a dry weight (P2) and weighed. The difference (P1-P2) is the weight of the protein in the sample and corresponds to the total protein content of the cuticle.

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Extraction and purification of ecdysteroids.—Each hemolymph sample preserved in methanol was sonicated for 5 min and then centrifuged (5000 rpm, 10 min). The supernatant was removed. The operation was repeated: both supernatants were combined and evaporated using nitrogen.

Enzyme immunoassay (EIA) of ecdysteroids.—Ecdysteroid measurements were performed in individual samples. Each dry extract was dissolved in 500 µL EIA buffer. Ecdysteroids were quantified by EIA adapted from the method described by Porcheron *et al.* (1989). The enzymatic tracer was 2-succinyl-20-hydroxyecdysone coupled to peroxidase; o-phenylenediamine was used as peroxidase substrate (Marco *et al.* 2001). Ecdysteroid titres were calculated using a calibration curve established with 20-hydroxyecdysone (20E). Results are given as pmol 20E equivalents per mL hemolymph. In the assay, 20E and ecdysone are nearly equally recognized by the polyclonal AS 4919 antibodies used (Porcheron *et al.* 1989).

Statistics.—Single factor analysis of variance (ANOVA) was used to analyze the test results in the control and treated series using SPSS software, version 13.0. Comparisons of means were performed at a significance level of 95% using the SNK test (Student-Newman-Keuls). Data were presented as mean (± standard deviation) based on a methodology that is specified in the results.

Results

Effects of DFB on the cuticle of mature nymphs.—In control *S. gregaria* nymphs, there was an increase in dry weight and in chitin levels and a decrease in protein synthesis of the abdominal cuticle of fifth-instar nymphs during the four days after treatment (Fig. 1).

In treated specimens, DFB prevented the increase in the dry weight of the cuticle (Fig. 1A) ($F = 16.07$; df= 1; $P < 0.01$) and in chitin levels (Fig. 1B) ($F = 26.62$; df=2,353; $P< 0.001$) and, by contrast, increased the proportion of cuticular proteins (Fig. 1C) ($F = 37.96$; df=2,487; $P < 0.001$).

Effects of DFB on changes in rates of ecdysteroids in the hemolymph of nymphs.—In controls, low levels of ecdysteriods (75 to 155 pmol /mL hemolymph) during the first three days were followed by a sharp increase (559 pmol /mL hemolymph) about day 4, and further increases for several days thereafter. During day 9, there was a spectacular increase in hormone concentration, reaching a peak of 2596 pmol followed by a rapid decline to 43 pmol the day before fledging (Fig. 2).

However, in treated nymphs, hormone levels were significantly lower than those of controls, and there was an extension in the duration of the stage. Concentrations were low during the first five days (23-67 pmol), and increased only gradually over the next five days (88 to 210 pmols) to reach a maximum of 460 pmol and then declined before fledging (Fig. 2). DFB significantly ($F=12.79$; df=1; $p<0.01$) reduced ecdysteroid titers in hemolymph of fifth instar nymphs of *S. gregaria*.

Discussion

After molting, the weight of the insect cuticle corresponds to that of exocuticle (Nøhr & Andersen 1993). In controls, the dry weight of the abdominal sternal cuticle of last-instar nymphs of *S. gregaria* increased gradually during the first four days of molting. This increase in weight that occurred was a result of deposition of new endocuticular layers. The weight of the cuticle decreased towards the end of the stage due to a gradual degradation of proteins and chitin to prepare for the next molt (Nøhr & Andersen 1993).

After treatment with DFB, there was a decrease in the dry weight of the tested cuticle. This can be explained by the fact that DFB prevents the deposition of new endocuticular layers in treated specimens. Similar results have been reported in other insect species following treatment with DFB in *Cydia pomonella* (Soltani & Soltani-Mazouni 1992) and in *Spodoptera littoralis* and *S. exigua* (Smagghe *et al.* 1997). Our experiments on *S. gregaria* also showed that the proportion of protein and chitin altered as development during the fifth instar

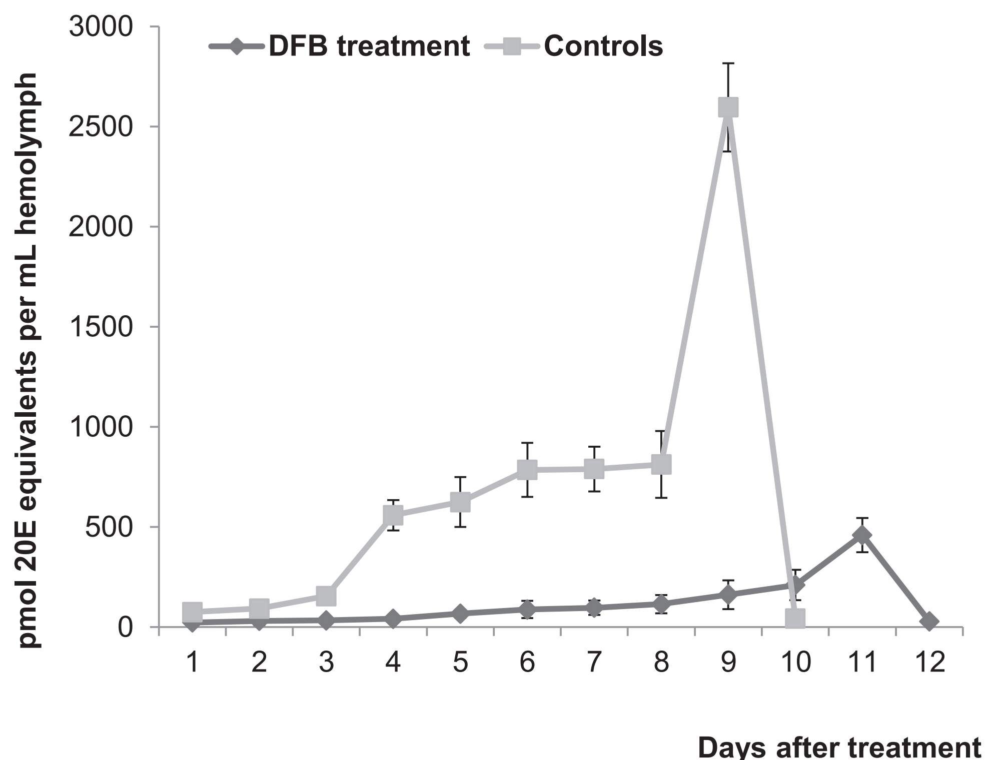

Fig. 2. Changes in the concentration of hemolymph ecdysteroids in control and DFB-treated 5th instar nymphs of *S. gregaria* (mean ± SD, n = 30) (pmol of 20E equivalents per mL hemolymph).

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progressed. Immediately after apolysis, the newly formed cuticle consisted essentially of protein. Thereafter, the protein secretion decreased with the amount of protein declining to 70%, at the same time as chitin content increased to 30%. Similarly, the study of Cassier *et al*. (1980) on *Locusta migratoria* showed that the first cuticular secretions were essentially proteins; pre-ecdysial layers were richer in protein than post-ecdysial layers; reflecting a slowdown in protein synthesis.

DFB significantly reduced the chitin content of the abdominal sternal cuticle in the last two nymphal stages of *S. gregaria*. Our results confirm and extend previous studies. Zhang & Zhu (2006) noted that DFB significantly reduced the content of chitin in the larvae of *Anopheles quadrimaculatus* depending on the dose. Similarly, Salokhe *et al.* (2006) reported a significant reduction in chitin and an increase in total protein of larvae of *Tribolium castaneum* contaminated by sublethal doses of flufenoxuron. Thus, sublethal concentrations of flufenoxuron alter expression of developmentally regulated proteins and chitin formation in a stage-specific manner, thereby resulting in developmental abnormalities in *T. castaneum*. Vincent & Clarke (1985) noted that DFB significantly reduced the content of chitin from the intersegmental membrane in adults of *L. migratoria*. Mehrotra (1987) also showed a significant reduction in the content of chitin in *S. gregaria* wings injected with a dose of 5 µg of DFB. In addition, sublethal exposure of *Culex quinqefasciatus* mosquitoes affected life table parameters including survival, fecundity and blood feeding rate (Suman *et al.* 2010).

Our tests on the last-instar nymphs of *S. gregaria* indicated, however, that DFB application increased cuticular protein during the first four days of post-molt compared to controls. On the other hand, other studies on the cuticular proteins showed that they were not affected by DFB or its analogues (Retnakaran *et al.* 1985). The nymphs of *T. molitor* treated with DFB presented an additional protein band (Soltani *et al.* 1984).

Sclerotization is a process related to proteins. It has been shown that the filling of the proteins in the cuticular sclerotized areas is slightly affected by DFB (Hillerton & Vincent 1979). The hypothesis is that proteins are not stabilized by tanning; the incorporation of new protein is inhibited. More specifically, Clarke *et al.* (1977), by studying the effects of DFB on the production of peritrophic membrane in *L. migratoria*, first observed an increase in the fibrous appearance of this membrane in treated individuals and then a reduction in weight. They explain this reduced production of peritrophic membrane of treated insects by an inability of the cuticular structure to stabilize proteins. It appears, in conclusion, that the effects of DFB on the cuticle of insects cannot be reduced to a direct action of the insecticide on chitin synthesis.

The integument is the target organ of morphogenetic hormones and their actions are qualitative and quantitative. Thus, in the desert locust secretory activity of epidermal cells is stimulated *in vitro* and *in vivo* and maintained by the presence of ecdysteroids (Cassier *et al.* 1980). The prothoracic glands secrete during nymphal stage fluctuating amounts of ecdysone (Gilbert *et al.* 2002, Lafont *et al.* 2005, Vandersmissen *et al.* 2007).

The concentration of hemolymph ecdysteroids in untreated fifth instar nymphs of *S. gregaria* remained low (75 pmol/ml hemolymph) during the first half of the instar. Then the concentration rose rapidly to a peak (2596 pmol/mL hemolymph) when the insects had accomplished 90% of the stage. Finally, just before fledging ecdysteroids levels fell sharply (43 pmol/mL hemolymph). Similarly, Cassier *et al.* (1980) reported that circulating hormonal amounts were low during the first third of the last stage of *L. migratoria*. They rose to a maximum about 48 h before molting. These significant fluctuations in ecdysteriods can be correlated to cuticulogenesis. The highest levels of ecdysteroids are reached a few days before molting at the time of digestion of the old cuticle and the establishment of a new one. The latter's pre-ecdysial layers are particularly rich in protein.

Molting takes place when levels of ecdysteroids are low. At the end of this stage, an accelerated synthesis of cuticular proteins is associated with a decrease in the rate of ecdysteroids and in the proteosynthetic activity of fats as well (Cassier *et al*. 1980). However, in treated nymphs, DFB inhibited the production of hormones and reduced dramatically the rates of ecdysteroids in the hemolymph throughout the last nymphal stage. This hormonal imbalance caused by DFB explains the different morphological and biochemical abnormalities observed in our study. Tearing of the old cuticle or molting, observed in DFB treated nymphs, indicates, according to Retnakaran *et al.* (1997), that the molting hormone is secreted. The low hormone levels affect the chitin synthesis and thus disturb the normal secretion and cuticular exuviation in treated specimens. On the other hand, the accumulation of proteins in the nymphal cuticle treated with DFB is probably due to a decrease in protein utilization by tissues, including the cuticle, due to interference with DFB secretion of the post-exuvial cuticle which is controlled originally by ecdysteroids.

Conclusion

These preliminary results seem to confirm our hypotheses that DBF affects levels of ecdysteroids in desert locust nymphs. Our present results show that DFB reduces cuticle dry weight, with significant reductions in chitin levels of *S. gregaria*, due to interference with DFB secretion of the post-exuvial cuticle which is controlled originally by ecdysteroids.

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