Diflubenzuron effects on reproduction and hemolymph ecdysteroid levels in female locusts Schistocerca gregaria (Forskål, 1775) (Orthoptera, Acrididae)

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Diflubenzuron effects on reproduction and hemolymph ecdysteroid levels in female locusts *Schistocerca gregaria* (Forskål, 1775) (Orthoptera, Acrididae)

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Abstract

The insecticide diflubenzuron (DFB), a benzoylphenyl urea, decreases fecundity in insects by a still unknown mechanism. The objective of this study was firstly to see the effects of DFB on female reproduction, after its ingestion by *Schistocerca gregaria* females five days following imaginal molt. DFB significantly delayed the timing of the first and of the second laying when compared to control females. DFB-treated females laid eggs only twice, while control females had, as typical, up to five ovipositions. DFB action on ovarian development and hemolymphatic ecdysteroid levels were also evaluated in females. Ecdysteroid titres in control females fluctuated according to the ovarian cycle, reaching a peak of around 170 pmol/mL hemolymph during the vitellogenic phase. In DFB-treated females, hemolymph ecdysteroid titres remained very low and oocyte growth was reduced. The treatment caused early oocyte resorption and any eggs deposited never hatched.

Key words

Schistocerca gregaria, ecdysteroids, hemolymph, EIA, diflubenzuron, reproduction, vitellogenesis

Introduction

Efforts have been made during the last 30 years to synthesize new insecticides which interfere more specifically with the hormonal regulation of molting and development in insects (see *e.g.*, Dhadialla *et al.* 2005).

Ecdysteroids are steroid hormones which regulate many aspects of insect reproduction and development (see *e.g.*, Gäde *et al.* 1997, Bellés 1998, Lafont *et al.* 2005, Raikhel *et al.* 2005). These hormones are present in all adult female insects, follicle cells in the ovary being the main site of their production, as first demonstrated for locusts (Goltzené *et al.* 1978). In many species these maternal ecdysteroids are stored by vitellogenic oocytes as inactive conjugates, which later on, release free active hormones used to control early embryonic development (Gäde *et al.* 1997, Lafont *et al.* 2005). In addition, in part these ovarian ecdysteroids are secreted into insect hemolymph, and have been assigned different functions in accordance with different insect species and their reproductive patterns. Their role in controlling vitellogenesis is well documented in mosquitoes and flies (Bellés 1998, Raikhel *et al.* 2005).

Ecdysteroid production or actions are potential targets for insecticides (Dinan 1989). The considerable knowledge obtained during the past years in insect physiology and endocrinology allows the development of new methods for the selection of insecticides which could interfere with specific metabolic or endocrine pathways in insects. The efficiency of such strategies was demonstrated using various insect growth regulators which disrupt endocrine regulation of the molting process such as juvenile hormone analogues or chitin synthesis inhibitors (Dhadialla *et al.* 2005).

The insecticide Diflubenzuron (DFB), the first commercialised benzoylphenyl urea derivative (BPU), has been proven to act as a chitin synthesis inhibitor. It alters cuticle composition and causes abortive moulting and death in immature stages (see reviews Tunaz & Uygun 2004, Dhadialla *et al.* 2005, Mommaerts *et al.* 2006). 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). DFB is used to control a wide range of phytophagous insects, including certain major pests such as migratory locusts (Matthews 2005, Holt & Cooper 2006). It has been shown to be very toxic against *Schistocerca gregaria* nymphs (Coppen & Jepson 1996). BPUs have been shown to be more effective by ingestion than by contact or topical application, because their penetration through cuticle is relatively low (Smagghe *et al.* 1997, Medina *et al.* 2002, Dhadialla *et al.* 2005). These compounds are mainly larvicides and, in addition, some BPUs, including DFB, also possess a strong ovicidal activity, possibly due to inhibition of embryonic cuticle formation (Grosscurt 1978, Medina *et al.* 2002).

In comparison to the large literature dealing with its effects as a chitin synthesis inhibitor, only a few studies have been published about the effects of DFB on insect reproduction. It affects reproduction in adult females of *Tenebrio molitor* (Soltani 1987), by decreasing fecundity and the duration of the oviposition period and in *Oxya japonica* (Lim & Lee 1982) and *Cydia pomonella* (Soltani & Soltani-Mazouni 1992) by disturbing growth and development of oocytes. Negative effects on fecundity have been also reported by using other BPU derivatives (*e.g.*, Perveen & Miyata 2000, Kellouche & Soltani 2006). The most frequently observed effect is a decrease in egg production and a reduction of egg hatching. This has been reported in insect species from several different orders : Hemiptera (Kim *et al.* 1992), Orthoptera (Lim & Lee 1982), Coleoptera (Soltani 1987, Mani *et al.* 1997, Marco *et al.* 1998), Diptera (Smith & Wall 1998), Neuroptera (Rumpf *et al.* 1998, Medina *et al.* 2002).

To date, nothing has been reported on the action of DFB on reproduction in locust females and on the evolution of ecdysteroid titres. Therefore, our work aimed to 1) ascertain the effects of DFB on reproduction in females of *S. gregaria* and 2) measure, using an enzyme immunoassay, its effects on hemolymph ecdysteroid levels, in order to assess correlations with reproductive disruption.

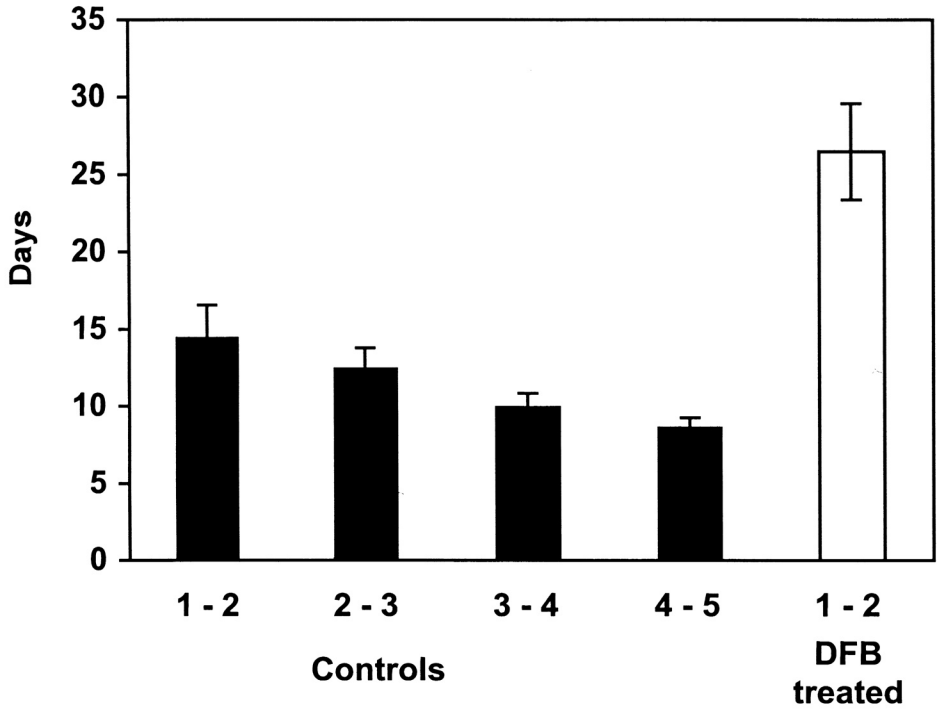

Fig. 1. Time interval (in days, mean ± $s_{\bar{x}}$, n = 8) between two successive egg-layings. On the abscissa, the numbers correspond to the egg-layings: 1-2, between first and second; 2-3, between second and third; 3-4, between third and fourth; 4-5, between fourth and fifth.

Materials and methods

Insect rearing and synchronization.— Fifth instar larvae were obtained by laboratory mass rearing. Immediately after the imaginal molt, adult insects were transferred to cages (20 × 30 × 15 cm) for experiments. In all cases, the temperature was maintained between 29 °C and 32 °C, with relative humidity close to 80 % and a photoperiod of 12 h L: 12 h D.

DFB treatment.— DFB (Dimilin® 25 WP, Chemtura) was applied by feeding. Insects were given cabbage leaves and wheat bathed in a Dimilin solution (30 mg/mL distilled water, as recommended by the INPV unit for locust control, Algeria). Treatments began five days after the imaginal molt and lasted for three days. This time schedule was chosen because it corresponded to the period of ovarian growth, from 5 to 8 days after imaginal molt.

Effects on reproduction.— Each treated female was immediately paired with an untreated male in an individual cage containing food. The moment of first oviposition, the interval between two successive egg-layings and the number of eggs deposited by each female were recorded. Insects of the same age, fed under similar conditions but omitting DFB, were used as controls. Eggs were incubated at 30 °C.

Determination of ovarian development.— To evaluate the effect of DFB on oocyte growth in *S. gregaria*, the oocyte lengths from the experimental and control groups were measured. For each female dissected, the lengths of 10 terminal oocytes in each ovary were measured using an ocular micrometer under a binocular microscope. Thus, 20 terminal oocytes per female were used to calculate the mean oocyte length. The mean length in the experimental group was compared to the mean length in the control group. The number of resorption bodies was also counted.

Tissue collection and ecdysteroid extraction.— Hemolymph samples (15 µL) were collected from individual females using a calibrated capillary tube after puncturing the membrane between thorax and metathoracic leg. Each individual sample was mixed with 300 µl methanol and kept at -20 °C. For extraction, each sample was centrifuged (5000 rpm for 10 min), and the supernatant collected. The pellet was resuspended in 300 µL methanol and centrifuged again. The two supernatants were pooled and evaporated to dryness under 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 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.— Results are presented as the mean ± $s_{\bar{x}}$ of measurements established on individual samples. The numbers of animals tested per series are given with the results. Comparison of mean values was made by Student's *t* test at the 5 % significance level.

Results

Rhythm of egg-laying.— DFB-treated females laid eggs only twice, whereas control females could have up to five ovipositions. This difference is not due to a lethal effect of DFB on females, as their life span was not significantly different from that of control females

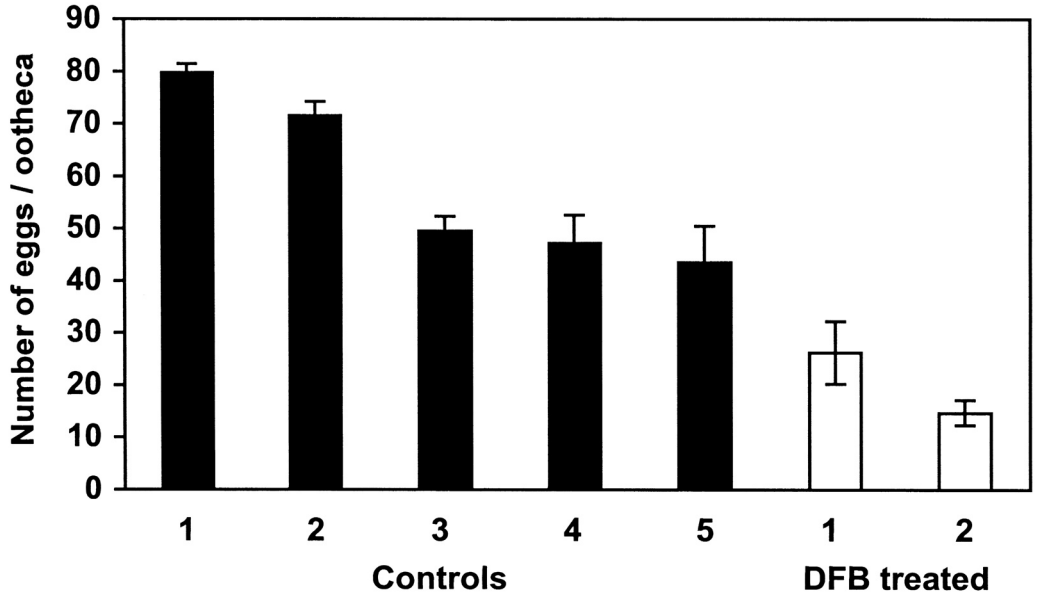

Fig. 2. Changes in the number of eggs per pod (mean ± $s_{\bar{x}}$, n = 8) in DFB-treated (open bars) and control females (closed bars). On the abscissa, the numbers correspond to egg-layings: 1 to 5 for controls, 1 and 2 for experimental females.

(respectively 47.3 and 50.0 d). The first egg laying occurred at about 12.7 (± 0.7) d after the imaginal molt in control females (n = 9), and at 16.2 (± 1.2) d after treatment by DFB (n = 9). Thereafter, the time interval between successive egg-layings for the same female was also modified by DFB treatment: the second egg-laying occurred 26.5 ± 3.1 d after the first one, instead of 14.5 ± 2.1 in control animals (p = 0.001, Fig. 1). In control females, the time interval between successive egg-layings decreased with each subsequent egg-laying (Fig.1).

Egg number per ootheca.— The number of eggs per ootheca decreased by almost threefold in ootheca from DFB-treated females, 26 ± 6 eggs per female, compared to controls, 80 ± 2 eggs per female (significant p = 0.0001). Twenty controls and eight treated oothecae were assessed, and we observed that the number of eggs per ootheca decreased according to the rank of egg-laying (Fig. 2). Eggs obtained from DFB-treated females, did not hatch, even after 45 d. Emergence from eggs laid by control females occurred in 74 % of total eggs, and after 14 to 16 d of incubation.

Action of DFB on oocyte resorption.— During the first gonotrophic cycle, the resorption of the terminal oocytes occurred progressively in treated females as shown in Fig. 3. The proportion of atresic oocytes was greater in treated females, with a maximum value reached 8 days after treatment (83.9 ± 3.8 % of total ovarioles) compared to controls (10 ± 3 %). Treatment with DFB induced a significant ($p<0.05$) resorption of terminal oocytes, which is consistent with the reduced egg-pod size.

Effects of DFB on oocyte growth.— During the first gonotrophic cycle, the length of basal oocytes increased from less than 2.1 ± 0.02 mm (previtellogenesis) up to 5.4 ± 0.03 mm on day 11 (end of vitellogenesis) (Fig. 4A). DFB treatment resulted in a significant ($p<0.05$) decrease of the basal oocyte final size (3.2 ± 0.1 mm) on day 12. From day 13, ovarioles only contained previtellogenic oocytes in both cases, but the size of basal oocytes was smaller in DFB-treated females.

Action of DFB on hemolymph ecdysteroid levels.— During the first gonotrophic cyle, the ecdysteroid levels were evaluated from individual hemolymph samples collected from 9 to 16 d after the imaginal molt in the same groups of females used to analyse the effects of DFB on oocyte growth. In control females, ecdysteroid titres increased progressively from a basal level on days 9 and 10 after the imaginal molt (Fig. 4B), corresponding to previtellogenesis (Fig. 4A), up to a maximum reached on day 11 (169.8 ± 15.7 pmol/mL hemolymph), at the moment when the size of the terminal oocytes was maximal. In DFB-treated females, ecdysteroid titres were quite steady and low, with the highest value on day 12 (23.2 ± 4.3 pmol/mL). The lowest ecdysteroid levels correlated with the decrease of terminal oocyte size, from day 13 (*i.e.*, 5 d after treatment) and thereafter.

Discussion

In *S. gregaria*, as in most insect species, vitellogenesis takes place after the imaginal molt, and vitellus is almost exclusively formed from the food ingested during adult life. Ovaries of *S. gregaria* are of panoistic type; *i.e.*, the feeder trophocytes are absent and the follicular base is the only trophic tissue (Launois-Luong 1978). Ovulation results in the discharge of the fully developed terminal oocytes into the paired oviducts where, in *S. gregaria*, they are thought to remain for up to 72 h prior to oviposition (Popov *et al*. 1990).

Although the life span of females was not significantly affected by DFB treatments, we showed that those treatments had deleterious effects on female reproduction at several levels. Treated locust females laid eggs with a different rhythm in comparison with control ones: only twice, instead of five times in controls, and these two egg-layings were delayed for about 3 and 12 d relative to controls (Fig. 1). Fecundity was rather low, as the number of eggs per ootheca was three times lower than in control animals. In treated locusts, most of the basal oocytes did not complete their growth, and their early degeneration resulted in a great number of resorption bodies (Fig. 3). Similar reductions of fecundity in insect females have been observed after BPU treatments, either by ingestion (Lim & Lee 1982, Soltani 1987) or by topical application (Soltani & Soltani-Mazouni 1992, Perveen & Miyata 2000, Hami *et al*. 2004, Kellouche & Soltani 2006).

Moreover, none of the eggs laid by DFB-treated locust females

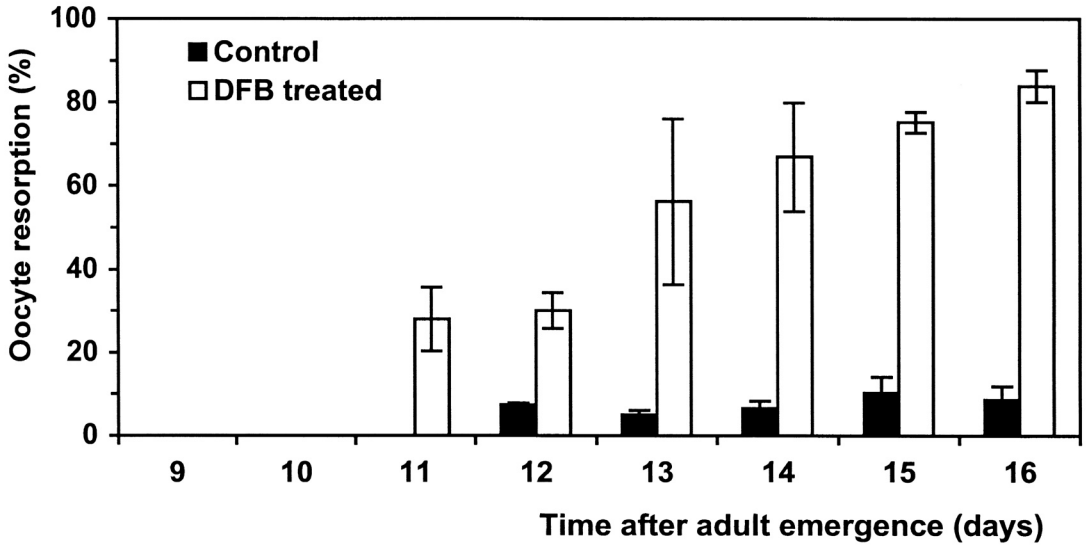

Fig. 3. Proportion of resorption bodies (in %) relative to the total number of ovarioles in adults treated (or not) by DFB during the first gonotrophic cycle (mean ± $s_{\bar{x}}$, n = 10). Day 9 corresponds to one day after the end of DFB treatment.

ever hatched. Ovicidal activity of BPUs has been studied in many species, pest insects in particular. Egg hatching was reduced or suppressed either by a direct effect or after exposure of adults to the compound (Grosscurt 1978, Medina *et al*. 2002, Hami *et al*. 2004, Kostyukovsky & Trostanetsky 2006). The loss of egg viability induced by DFB treatment could result from interference with the process of oogenesis and vitellogenesis, leading to few metabolites in eggs as reported in *Cydia pomonella* (Soltani & Soltani-Mazouni 1992). DFB applied to lacewing adults very strongly inhibited egg hatch: fully developed embryos died whithin the egg shell (Medina *et al*. 2002). Inhibition of chitin deposition could explain this ovicidal effect (Grosscurt 1978, Medina *et al*. 2002).

Oocyte growth was monitored during the first gonotrophic cycle, and appeared to be reduced in DFB-treated females: the maximal size reached was lower than in controls, and the size of previtellogenic oocytes during the second gonotrophic cycle was only half that of control ones (Fig. 4A). So, DFB treatment significantly affected the growth and development of oocytes, as was also observed after topical application of DFB to young pupae of *Cydia pomonella* (Soltani & Soltani 1988, Soltani & Soltani-Mazouni 1992) and likewise topical application of other BPUs in *Spodoptera litura* (Perveen & Miyata 2000) and *T. molitor* (Hami *et al*. 2004).

This reduced fertility could be a consequence of a disturbed vitellogenesis, as suggested in *T. molitor* (Soltani-Mazouni & Soltani 1994). In this species, ingested DFB reduced fertility and, in addition, interfered with the ovarian synthesis of DNA during oocyte maturation, without significant effect on the fine structure of follicular cells and basal oocytes (Soltani-Mazouni 1994). A significant decrease in ovarian protein content was reported after DFB treatment in *C. pomonella* (Soltani & Soltani-Mazouni 1992) and after chlorfluazuron application in S. *litura* (Perveen & Miyata 2000). As DFB and other growth regulators, accumulate in the reproductive system of females and males of *T. molitor* (Chebira *et al.* 2006), this could explain their strong inhibiting effects on reproduction.

We think that the observed effects on oogenesis and vitellogenesis in treated *S. gregaria* females were really due to ingested DFB. Even if, in adult insects, DFB treatments disturbed the production of the peritrophic membrane in the gut (Clarke *et al*. 1977), they did not suppress it and were not considered as equivalent to starvation. It is noticeable that in starved insects, oocyte resorption is considerably increased and ovarian lipid, carbohydrate and protein contents are significantly reduced (Clarke *et al*. 1977). In contrast, in BPU-treated females, only the protein level of ovaries was reduced (Perveen & Miyata 2000). In fat body, the major site of vitellogenin production, DFB had no significant effect on protein content and synthesis during the sexual maturation of *T. molitor* females, in contrast with starved animals (Soltani-Mazouni & Soltani 1995).

In parallel to the oocyte growth profile, we measured ecdysteroid levels in hemolymph. In control animals, ecdysteroids were present during the entire first cycle of reproduction (Fig. 4B). Levels peaked (at about 0.17 µM) on the 11th day after the imaginal molt, at the end of vitellogenesis when oocyte size was maximal (Fig. 4A), then declined in coincidence with ovulation. These results are in agreement with those previously obtained in the same species (Tawfik *et al*. 1997) concerning both the timing during the first ovarian cycle and the maximal values of hemolymph ecdysteroids (about 0.5 µM of 20E equivalents).

Ecdysteroids are produced in ovaries by the follicle cells surrounding terminal oocytes: this is well documented in locusts. The highest concentrations are reached when egg chambers attain their maximal size, in *Locusta migratoria* (Lagueux *et al*. 1977, Goltzené *et al*. 1978, Tawfik *et al*. 2002) and *S. gregaria* (Tawfik *et al*. 1999). At this time, ovarian ecdysteroids represent more than 95% of all body ecdysteroids. In *L. migratoria*, free ecdysteroids are involved during the late stages of oocyte maturation, in the control of meiotic reinitiation (Lanot *et al.* 1987). Most of these ecdysteroids are stored in the eggs, mainly as maternal conjugates, to be used during early embryonic life.

What could then be the role of these circulating ecdysteroids in adult females? Whether they are secreted (as opposed to leaking) from ovaries has been questioned (Lagueux *et al*. 1977), and this problem is still open. It could be that they are involved in cyclicity of vitellogenesis, as demonstrated in the case of cockroaches (Bellés 1998). In *L. migratoria* and *S. gregaria*, vitellogenesis and oocyte growth are mainly controlled by juvenile hormone and neurohormones such as ovary-maturing parsins (OMP), characterized as gonadotropins (Girardie & Girardie 1996, Girardie *et al.* 1998). However, the induction of precocious vitellogenesis in *L. migratoria* by daily injections of 20E in adult females would suggest that ecdysteroids could also have a gonadotrophic action in locusts (Girardie & Girardie 1996). Moreover, in *S. gregaria*, OMP injection induced a precocious rise of circulating ecdysteroids (Girardie *et al.*

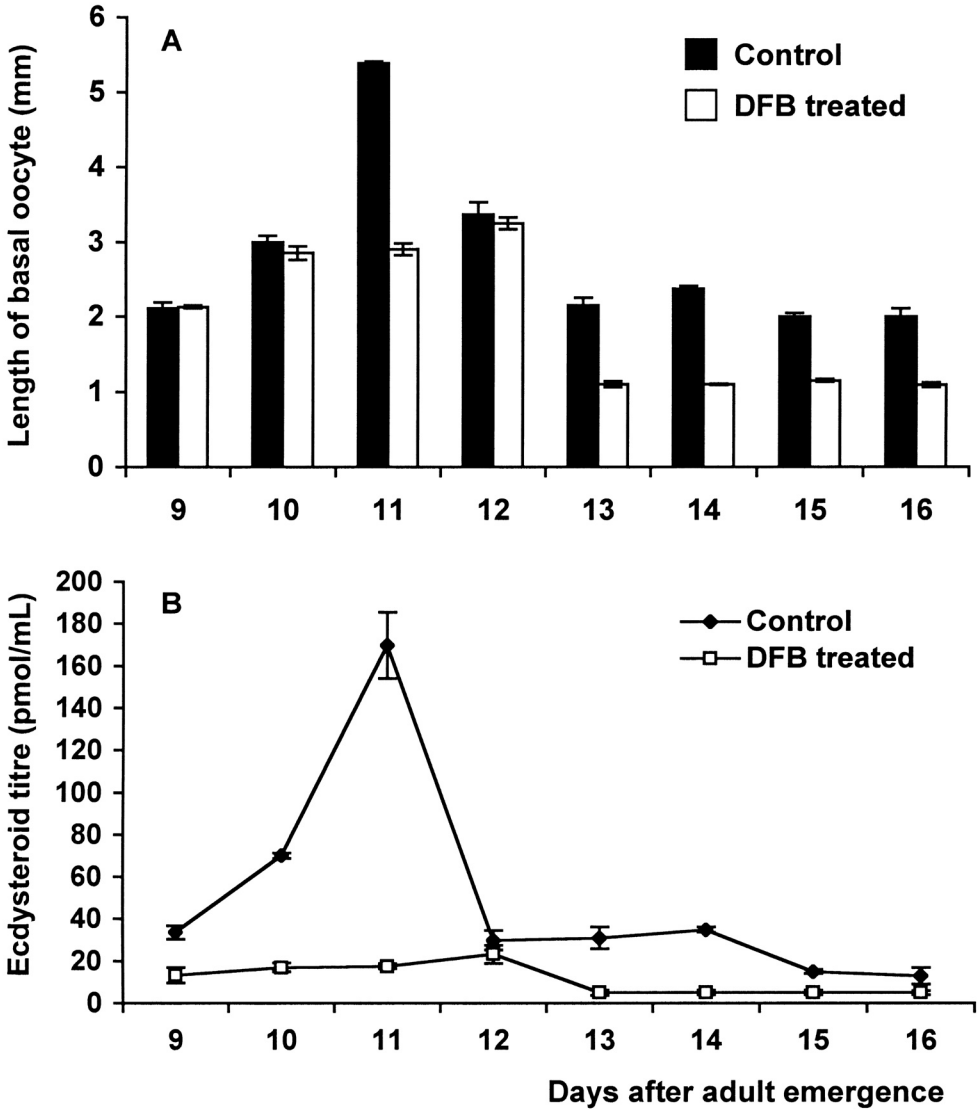

Fig. 4. A. Growth of oocytes (length of basal oocytes in mm). B. Fluctuations of hemolymph ecdysteroids (pmol of 20E equivalents per mL) in control and DFB-treated females during the first gonotrophic cycle (mean ± $s_{\bar{x}}$, n = 10). Day 9 corresponds to one day after the end of DFB treatment.

1998). It has been suggested that OMP stimulates oocyte growth by inducing ovarian ecdysone production, but this is not supported by direct evidence (Girardie & Girardie 1996, De Loof *et al.* 2001).

Following DFB treatment in *S. gregaria*, ecdysteroid titres in hemolymph were reduced: they remained low throughout the first ovarian cycle (Fig. 4B). In parallel, the size of basal oocytes was reduced (Fig. 4A). In *C. pomonella*, a topical application of DFB to young pupae disrupted oocyte development and prevented the appearance of the second ovarian ecdysteroid peak in adult females (Soltani & Soltani 1988).

We suggest that the reduced hemolymph levels in *S. gregaria* could reflect a reduced ovarian synthesis, itself connected with the reduced size of follicle chambers. It has been reported that DFB could strongly reduce ecdysteroid secretion *in vitro* from ovaries of adult females of *Gryllus bimaculatus* (Lorenz *et al*. 1995), but the precise cellular mode of action remains unknown. As a consequence of reduced ecdysteroid accumulation, eggs would contain reduced levels of maternal ecdysteroid conjugates, insufficient to sustain embryonic development. This is consistent with earlier data obtained by feeding *L. migratoria* females with modified sterols which could not be transformed into ecdysteroids (Costet *et al*. 1987).

In newly laid eggs of *S. gregaria* females, we measured ecdysteroid content before and after enzymatic hydrolysis, to compare free and conjugated ecdysteroids in control eggs and after DFB treatment (unpub. results). DFB significantly ($p < 0.02$) reduced the levels of both free and conjugated ecdysteroids, about three times, compared to the control (respectively 7.6 ± 3.1 *vs* 26.2 ± 12.6 pmol per egg before hydrolysis, and 160.9 ± 62.2 *vs* 419.2 ± 128.6 pmol per egg after hydrolysis; n = 5). These preliminary results seem to confirm our hypotheses. Measurements of ecdysteroid levels in ovaries and throughout embryonic development after DFB treatment are in progress. Our present results show that DFB reduces fecundity and fertility of *S. gregaria*, via a still unknown mode of action on ovaries, which is, however, independent of its inhibiting effect on chitin formation.

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