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Full Length Research Paper

Effects of sublethal doses of chlorfluazuron on the ovarian biochemical constituents of *Spodoptera litura*

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This paper describes the effects of sublethal doses (LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}) of chlorfluazuron on the amounts of ovarian constituents during various stages of development in common cutworm, *Spodoptera litura* (F.). Chlorfluazuron was applied topically to newly ecdysed fifth instars under laboratory conditions. In newly emerged adults, the amount of ovarian protein in LD_{10} and LD_{30} treated larvae was significantly reduced, but carbohydrate and lipid were not affected compared with their respective controls. The order of relative proportion of the amount of ovarian DNA in three developmental stages was: newly emerged adults>newly pupae>newly ecdysed larvae. Moreover, the order of the amount of RNA was: newly ecdysed larvae>newly emerged adults> newly pupae. The amount of utricular DNA was greater (14.2 µgmg^{-1} of tissue) after first mating than the second one in the spermatheca. However, the amount of ovarian DNA was reduced more than ovarian RNA and it decreased rapidly, whereas amount of DNA decreased steadily. On the 5th day of pupae, ecdysteroid titer was 3.5 ± 0.8 pgmg^{-1}. Initially, it was increased slowly then gradually, and furthermore, sharply during the 5th day of pupae to before adult emergence. It was peaked on the 1st day after adult emergence (52.0 ± 1.5 pgmg^{-1}) and decreased thereafter. Therefore, it is concluded that sublethal doses of chlorfluazuron reduced the amounts of ovarian constituents during ovarian development and oogenesis in *S. litura*. These reductions increased with an increase in dose from LD_{10} to LD_{30}. The effects of chlorfluazuron on the amounts of ovarian constituents are presumed to be responsible for the reduction in fecundity caused by sublethal exposure to chlorfluazuron.

Key words: Chlorfluazuron, ecdysteroid, nucleic acids, oogenesis, *Spodoptera litura,* sublethal doses.

INTRODUCTION

In many insects, oviposition requires the development of the ovary, egg maturation, mating and in some cases feeding of the female. Ovarian development, which includes oocytes growth and vitellogenesis, is known to be under hormonal control (Engelmann, 1979). Ovarian growth and regulation of oogenesis have been widely studied (Anderson, 1974). Post ovarian development of insects are initiated and controlled by hormones from both the neurosecretory cells of the brain and the prothoracic glands (Muller, 1963).The amounts of carbohydrates, protein, and nucleic acids depend on the morpho-functional state of organisms (Dudash, 1979). As a rule, the DNA directs the synthesis of RNA and protein. It relays a specific underlying code that relates given triplets of RNA nucleotides into specific amino acids. The code had been thought to be the same in all organisms, but exceptions have been seen particularly in mitochondria. On the other hand, the DNA also directs the synthesis of DNA itself (Muller, 1963). The marked changes in the content of protein, RNA and DNA were noted during growth and development (Dudash, 1979). Free active ecdysteroid hormones are released at a specific time during oogenesis to trigger oocytes formation in insects (Bownes et al., 1988).

Reproductive inhibition induced by benzoylphenyl ureas (BPUs) has been reported most widely when applied on adult insects (Holst, 1974; Fytizas, 1976; Wright and Spates, 1976). It has been reported that treatment of adult insects with diflubenzuron (DFB), a benzoyl phenyl urea, disrupts the secretion of adult cuticle in numerous insect species (Hunter and Vincent, 1974; Ker, 1977; Clark et al., 1977), the production of peritrophic membrane in the migratory locust, *Locusta migratoria* (L.) (Orthoptera: Acrididae) (Hunter and

Vincent, 1974) and the mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (Soltani, 1984; Soltani et al., 1987). In addition, when it was applied to adult females, this caused a decrease in fecundity in the Mexican bean beetle, *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae) and *L. decemlineata* (Holst, 1974) and adversely affected eggs viability in the stable fly, *Stomoxys calcitrans* (L.) and the house fly, *Musca domestica* (L.) (Diptera: Muscidae) (Wright and Spates, 1976).

Philip and Loughton (1979) reported that haemolymph treated with DFB and cyclohexamide inhibited RNA and protein synthesis in the fourth and fifth-instar larvae of the *L. migratoria*. Shakoori and Saleem (1989) reported the effects of sublethal treatments of malathion and malathion-permethrin on sixth-instar larvae of the red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae). Permethrin (200 ppm) and malathion (20 ppm) mixtures increased the activities of choline esterase (70.8%) and raised the concentrations of cholesterol (21%), DNA (24%) and RNA (8%). Miltin et al. (1985) reported that when the boll weevil, *Anthonomus grandis* (Boheman) (Coleoptera: Curculionidae) was treated with DFB, the biosynthesis of DNA was inhibited in females, but RNA was not, neither was protein synthesis affected. Diminished sexual function may, therefore, result in part from inhibition of DNA by DFB.

Chlorfluazuron is an effective treatment against major lepidopteran and coleopteran pests because it disrupts chitin deposition during ecdysis (Retnakaran et al., 1989), resulting in the development of malformed-larvae (Omatsu et al., 1991). It is also effective against immature insects with relatively slow but strong action (Hashizume, 1988). Topical application of lethal-doses of chlorfluazuron to newly-ecdysed fifth-instar larvae of the common cutworm, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae), causes significant mortality in subsequent life stages. Furthermore, application of sublethal doses to larvae (LD_{10} or LD_{30}) significantly reduced fecundity and fertility of subsequent adults, and hatchability of their eggs (Perveen, 2000a). The objectives of this research were to determine the effects of sublethal doses of chlorfluazuron (LD_{10}or LD_{30}) on the amounts of ovarianprotein, lipid, carbohydrates, DNA, and RNA, and ecdysteroid titres in different developmental stages of *S. litura*, a major crop pest around the world (Skibbe et al., 1995).

MATERIALS AND METHODS

Insect rearing

Insects were reared in the laboratory under controlled conditions (temperature: 25 ± 1°C; photoperiod: 16L: 8D h; R.H: 50 to 60%). Larvae were fed on the artificial diet Insecta LF® (Nihon Nohsan Kohgyo Inc., Yokohama, Japan) and adults on a 10% sucrose solution. Eggs laid on Rido® cooking paper (Lion, Tokyo, Japan) were collected daily, and kept in 90 ml plastic cups (4 cm diameter × 4 cm high) for hatching.

Application of chlorfluazuron

Chlorfluazuron (Atabron®; analytical grade; purity: 99.9%) was obtained from Ishihara Sangyo Kaisha (Osaka, Japan) and stored at 4°C. Using a micro-applicator and micro-syringe, sublethal doses of chlorfluazuron diluted in 2.0 µl of acetone were applied topically to the dorsum of newly ecdysed fifth instars of *S. litura*. LD_{10} (1.00 ng $larva^{-1}$) or LD_{30} (3.75 ng $larva^{-1}$) were calculated based on the results of toxicity data of larval tests at adult emergence. Treated and control (untreated or solvent treated) batches of larvae were kept in paper towel-padded 860 ml plastic cups (13 cm in diameter × 9.5 cm high; n = 150 for each batch). Other procedures followed Perveen (2006).

Analysis of ovarian protein, carbohydrate and lipid

Paired ovaries were collected from newly emerged adults in the control and treated batches, and homogenized collectively in a homogenizing tube containing aqueous trichloroacetic acid (TCA: 1.0 ml; 100 g/L) and then centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was used for carbohydrate determination and the precipitate was washed with ether and chloroform (1.0 ml; 1:1 by volume). After further centrifugation at 5000 rpm for 10 min at 4°C, the chloroform supernatant was used for lipid determination. The amount of protein was determined in an aliquot using the Coomassie blue method.

Analysis of ovarian nucleic acids

Nucleic acids (DNA and RNA) were extracted following Schmidt and Thannhauser (1945) with some modification following Munro (1966), and their level were measured by the diphenylamine method of Burton (1956) and orcinol reaction of Schneider (Schneider, 1957), respectively. Paired ovaries were collected from relevant stages of the control and treated batches; each ca. 14 to 28 mg were crushed in 2.0 ml 70% ethanol, and then centrifuged at 1180 *g* for 15 min. Other procedures followed Perveen et al. (2010) and the amount of oovarian nucleic acids, DNA and RNA were calculated by the following formula: µg DNA / mg of pair ovaries = total µg from curve × dilution / 1× weight of ovaries × 0.1 × 1000; µg RNA / mg of pair ovaries = total µg from curve × dilution/2 × weight of ovaries × 0.1 × 1000, respectively.

Estimation of utricular nucleic acids

The utriculus is that part of spermatheca in which the sperm are stored after mating. Therefore, the utriculi were collected after first and second mating of the control and treated batches and weighed and 10% homogenate was prepared in glass-distilled water. The rest of the procedure was the same as described in estimation of ovarian nucleic acids.

Analysis of egg ecdysteroid titer

Paired ovaries were collected from newly emerged adults in the control and treated batches, were crushed in 300 µl 70% methanol, and then centrifuged at 10 625 *g* for 10 min. The third supernatant was dried under nitrogen gas (N_2) and 1.0 ml 5% methanol was added. This solution was passed through a C_{18} sep-pak® (Millipore) for fractionation. Procedures for testing of these samples followed Perveen et al. (2010). Anti-ecdysteroid antiserum was obtained from L.I. Gilbert and W.E. Bollenbacher (University of North Carolina, Chapel Hill, USA). [^{3}H] ecdysone (1.85 TBq mmol $pgmg^{-1}$) was obtained from Du Pont (North Carolina, Kannapolis, USA)

Table1. Effects of sublethal dosesof chlorfluazuron on utricular DNA of the spermatheca of adult females after first and second matings after topical application to newly ecdysed fifth instars of *Spodoptera litura*.

Treatment[a]	n	After first mating[b] (mean ± SD) µg mg of tissues^{-1}	After second mating[b] (mean ± SD) µg mg^{-1} of tissue
Control	5	2.04 ± 0.06[a]	1.75 ± 0.08[a]
LD_{10}	5	1.25 ± 0.09[b]	1.19 ± 0.07[b]
LD_{30}	5	0.85 ± 0.08[c]	0.75 ± 0.08[c]

[a]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n, number of females used; [b]Data were analyzed using one-way ANOVA at $P<0.05$ .Means within columns followed by different letters are significantly different by Scheffe`s F-test at 5%; $F_{4,20} = 123.1$; $P<0.05$.

and 20-hydroxyecdysone from Rohto Pharmaceutical (NewYork, USA) and samples were dried. Subsequent procedures for each constituent followed Perveen (2010a) and Perveen et al. (2010); ovarian protein, carbohydrate, lipids, DNA and RNA were measured in µg mg of pair ovaries^{-1} and ecdysteroid titres in pg mg of pair ovaries^{-1} with three replications each.

Statistical analysis

Data were analyzed by using one-way analysis of variance (ANOVA) (Abacus Concepts Super ANOVA, 1989) at $P<0.05$ and Scheffe's *F*-test (Scheffe, 1953) at 5%.

RESULTS AND DISCUSSION

S. litura has paired ovaries which branch into four polytrophic. Meroistico ovarioles are located on the ventral side of body cavity, making several loops of ovarioles with all basal oocytes developing simultaneously. Each ovariole is differentiated into three portions according to the developmental stages of oocytes: the yellowish green pedicle, where fully matured ova are stored; the reddish orange vitellarium, which contains developing oocyte and trophocyte follicles, which undergo accumulation of yolk proteins, and chorio-genesis; and the whitish, germarium, which contains oogonia, from which germ cells proliferate and follicles are formed (Riakhel and Dhadialla, 1992; Etman and Hooper, 1979).

During mating, sperm packed in spermatophore were transferred by male into the bursa copulatrix of female. Sperm are stored here for few hours then poured through spermathecal duct into spermatheca, the depository organ for sperm, which consists of two parts, utriculus and legna. Generally, sperm were stored in utriculus, however, no sperm were observed in legna. The DNA content of utriculus was greater after first mating (2.04 ± 0.06 µg mg of tissue^{-1}) than the second one (1.75 ± 0.08 µg mg of tissue^{-1}). However, during the second mating, a greater number of sperm were transferred by male (Perveen*,* 2008). Perhaps more sperm were destroyed during traveling from the bursa copulatrix to utriculus of spermatheca. However, in the study, significant reduction of DNA content (38.7 and 32% after first and second mating, respectively) by the LD_{10} and more significant reduction (58 and 57% after first and second mating, respectively) by LD_{30} were observed compared with the controls. The reason was that DNA and RNA contents are directly proportional to number of sperm present in testes. The number of sperm inseminated during first and second mating significantly reduced in LD_{10}-treated males of *S. litura* and more significantly reduced in LD_{30}-treated males (Perveen*,* 2008). Therefore, DNA content after first and second mating significantly reduced in LD_{10}-treated females of *S. litura* and more significantly reduced in LD_{30}-treated females (Table 1).

Chlorfluazuron is known to be a chitin synthesis inhibitor but its effects on insect reproduction have not been widely studied. The effects of chlorfluazuron on amounts of ovarian constituents during ovarian development and oogenesis are presented here for the first time. Previous work (Perveen, 2000a) demonstrated that sublethal doses of chlorfluazuron (LD_{10} and LD_{30}) applied topically to fifth instar larvae of *S. litura* reduced the fecundity and fertility of the subsequent adult females and the hatchability of their eggs. Other effects of similar sublethal doses were; decrease in the mass of ovaries, number of mature ova, and size of basal oocytes (Perveen and Miyata, 2000) and reduction in the amounts of ovarian constituents including protein, carbohydrates, lipid, nucleic acids and ecdysteroid titres (at the present). However, effects of same doses in males were decreased volume and mass of testes; and the size and number of spermatocytes (Perveen, 2000b); and reduction in the amounts of testicular constituents including protein, carbohydrates, lipid, nucleic acids and ecdysteroid titers (Perveen, submitted) and also delayed initiation of mating (Perveen*,* 2008). Moreover, the same doses also reduced activity of oviposition stimulation factors (Perveen, 2009). Subsequently, Perveen (2006) demonstrated that eggs laid by treated females were up to 66% smaller and more likely to be infertile, than those laid by untreated females. Sublethal doses of chlorfluazuron have an effect on different stages of embryogenesis (Perveen, 2010a) and reduces amount of egg constituents including protein, carbohydrates, lipid, nucleic acids and ecdysteroid titres (Perveen, 2010b).

In the control, the amount of ovarian protein of newly

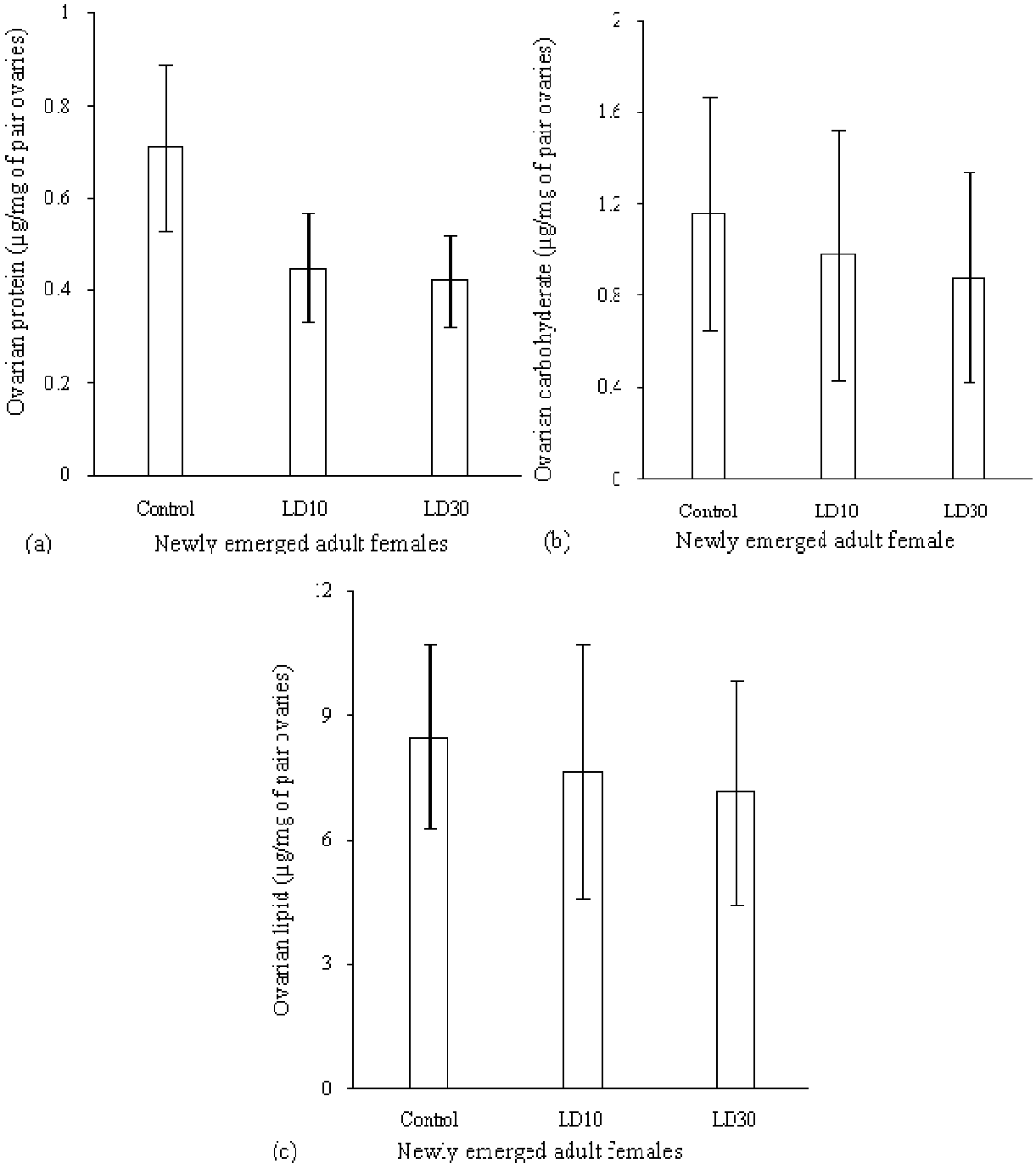

Figure 1. Effects of sublethal doses of chlorfluazuron (LD_{10}, 1.00 µg larva^{-1} or LD_{30}, 3.75 µg larva^{-1}) on ovarian protein (a), carbohydrate (b) and lipids (c) of newly emerged female adults of *S. litura*; data were analyzed using one-way ANOVA (Abacus Concepts Super ANOVA, 1989) at $P<0.05$. Only means of treatments (LD_{10} or LD_{30}) for protein were significantly different when compared with controls according to Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars indicate±SD; for protein, $F_2 = 21.2$, $P<0.001$, between LD_{10} and LD_{30} treatments $P = 0.6385$; for carbohydrate: $F_2 = 2.4$, $P <0.0963$; for lipids: $F_2 = 2.9$; $P <0.0611$; n = 15 to 21 for each point (Perveen and Miyata, 2000).

emerged female adults was 0.71 ± 0.18 µg mg of pair ovaries^{-1}. However, it reduced by 36 and 41%, respectively, in LD_{10} and LD_{30}-treated newly-emerged female adults (Figure 1a). In the control, the amount of ovarian carbohydrates of newly emerged female adults was 1.16 ± 0.51 µg mg of pair ovaries^{-1}. However, it was reduced by 15.5 and 24%, respectively, in LD_{10} and LD_{30}-treated newly-emerged female adults (Figure 1b). In the

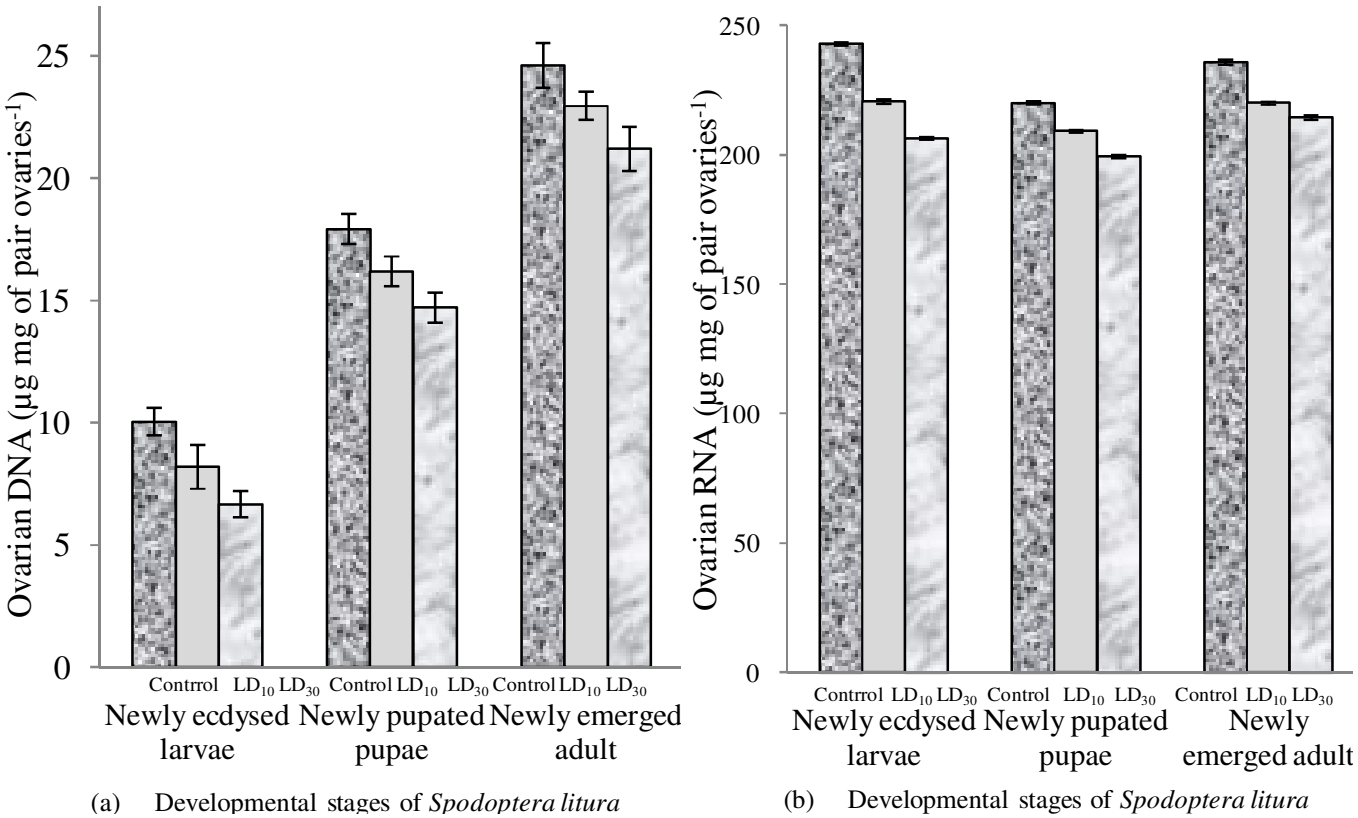

Figure 2. Effects of sublethal doses of chlorfluazuron ($LD_{10,}$ 1.00 µglarva^{-1}or LD_{30}, 3.75 µglarva^{-1}) on ovarian DNA (a) and RNA (b) over three developmental stages of *S. litura*. Data were analyzed using one-way ANOVA (Abacus Concepts Super ANOVA, 1989) at $P<0.05$; all means were significantly different according to Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars indicate ±SD; for DNA: $F_{8,188}$= 541.17; for RNA:$F_{8,180}$ = 3983.2; 1P<0.05; n = 21 for each point.

control, the amount of ovarian lipid of newly emerged female adults was 8.49 ± 2.23 µg mg of pair ovaries^{-1}. However, it reduced by 10 and 16%, respectively, in LD_{10} and LD_{30}-treated newly-emerged female adults (Figure 1c). Difference for ovarian carbohydrates and lipid amounts were not significant when compared with the controls according to Scheffe's *F*-test at 5% (Figure 1). In the control too, the amount of ovarian DNA of newly ecdysed sixth instars was 10.03 ± 0.56 µg mg of pair ovaries^{-1} and it increased by 44.0% in pupae and 59.2% in adult females compared with larvae. Sublethal dose of chlorfluazuron LD_{10} significantly ($P<0.05$) reduced the amount of DNA (larvae: 18.3%; pupae: 9.7%; adults: 6.7%) and LD_{30} more significantly ($P<0.05$) reduced it (larvae: 33.6%; pupae: 17.9%; adults: 13.9%) compared with their respective controls for each developmental stage (newly ecdysed sixth instars; newly ecdysed pupae; newly emerged female adults; n = 21; Figure 2a).

Moreover, in the control, the amount of ovarian RNA of newly ecdysed sixth instars was 242.90 ± 11.69 µg mg of pair ovaries^{-1}. It decreased by 9.4% in pupae as well as in adults and only increased 2.9% in female adults compared with larvae. Sublethal dose of chlorfluazuron LD_{10} significantly ($P<0.05$) reduced amount of RNA (larvae: 9.2%; pupae: 5.0%; adults: 6.7%) and LD_{30} more significantly ($P<0.05$) reduced it (larvae: 15.1%; pupae: 9.4%; adults: 9.0%) compared with their respective controls for each developmental stage as in the case of DNA (n=21; Figure 2b). In addition, after first mating in the control, utricular DNA in spermatheca of females was 2.04 ± 0.06 µg mg of tissues^{-1}. It was reduced by 38.7 and 58.3% in LD_{10}-and LD_{30}-treated larvae, respectively. In the control after second mating, utricular of DNA in spermatheca was 1.75 ± 0.08 µg mg of tissues^{-1}. It was reduced by 32.0 and 57.1% in LD_{10}-and LD_{30}-treated larvae, respectively (n = 5; Table 1).

Ecdysteroid titres in ovaries were observed after each consecutive 24 h on seven day-old female pupae to four day-old adult females. Preliminary data from the controls indicate that the ovarian ecdysteroid titers measured *in vivo*, changed during vitellogenesis in *S. litura* in a characteristic way: the titres were low during pre-vitellogenesis (on the 4th day: 3.5 ± 0.8 pg mg^{-1}; on the 3rd day: 4.5 ± 0.5 pg mg^{-1}, after adult emergence), increased during vitellogenesis (on the 2nd day: 18.0 ± 3.16 pg mg^{-1}; on the 1st day: 27.0 ± 3.3 pg mg^{-1}; on the 0 day after adult emergence: 35.0 ± 1.6 pg mg $^{-1}$, after adult emergence), peaked at choriogenesis (on the 1st day

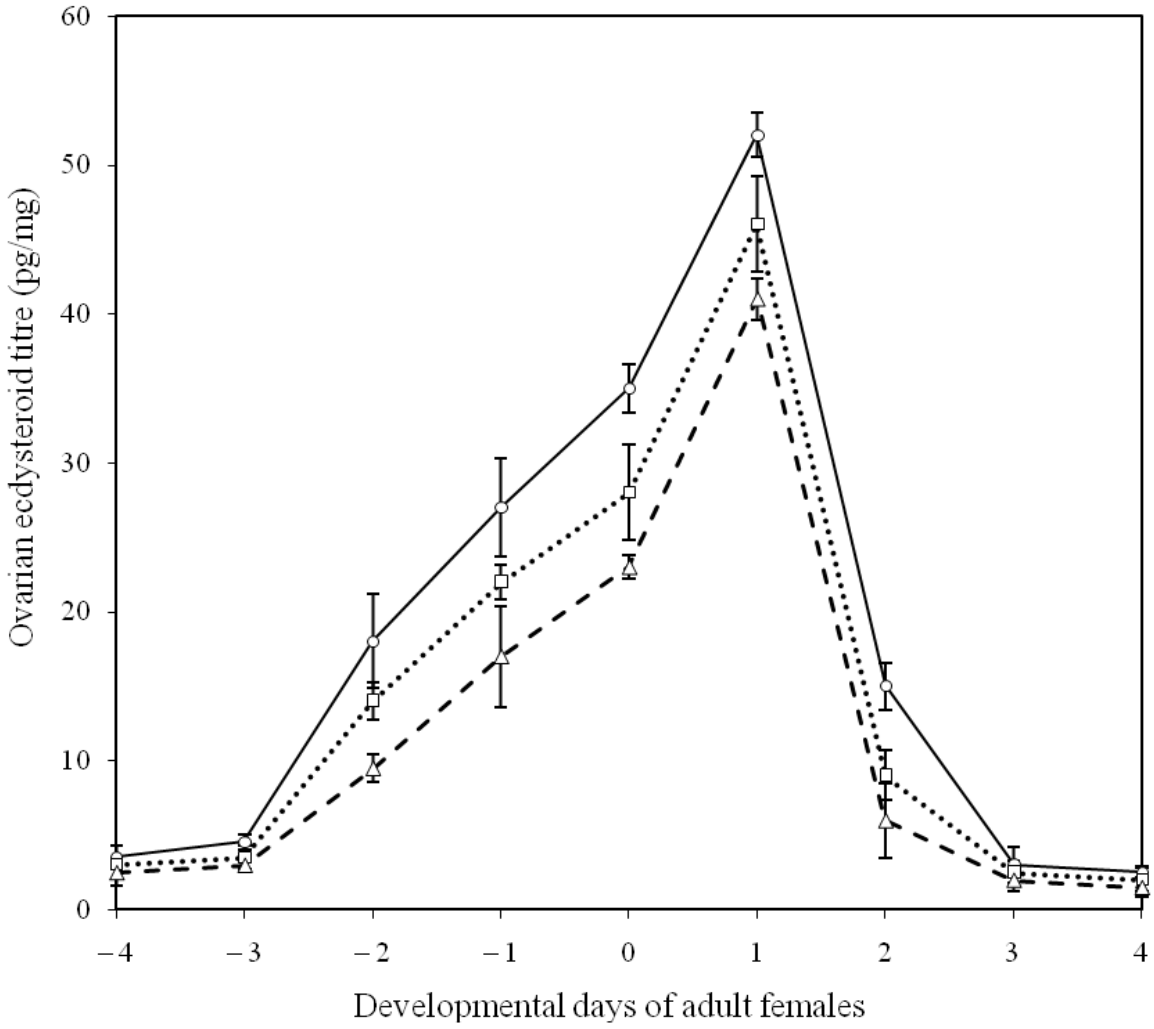

Figure 3. Effect of sublethal dosesof chlorfluazuron (LD_{10}, 1.00 µg larva^{-1} or LD_{30}, 3.75 µg larva^{-1}) on the ovarian ecdysteroid titre of *S. litura* from seven day-old female pupae to four day-old adult females. Controls, O; LD_{10}, □; LD_{30}, Δ; data were analyzed by one-way ANOVA (Abacus Concepts Super ANOVA, 1989) at $P<0.05$; all means were significantly different according to Scheffe's *F*-test (Scheffe, 1953)at 5%; vertical bars indicate ±SD; n = 5 for each point.

after adult emergence: 52.0 ± 1.5 pg mg^{-1}) and decreased when the insects started to deposit eggs (on the 2nd day: 15.0 ± 1.58 pg mg^{-1}) and decreased thereafter (Figure 3). Chlorfluazuron at the two tested sublethal doses significantly affected ecdysteroid titres accumulated *in vivo* by ovaries. It significantly ($P<0.05$) decreased the titres in the LD_{10}-treated females and more significantly ($P<0.05$) decreased it in the LD_{30}-treated females. However, the pattern was the same as in the control throughout the time course in treated batches with both doses (Figure 3).

Chlorfluazuron has three main effects on ovarian development and oogenesis in *S. litura*. The first was described by Perveen and Miyata (2000); topical application of sublethal doses decreased ovarian mass and length of different parts of ovarioles. The second is that the same doses reduced number of mature ova and size of basal oocytes during oogenesis (Perveen and Miyata, 2000). The third presented in this paper, is that sublethal doses reduced the amounts of ovarian constituents during oogenesis; ovarian protein, lipid and carbohydrate, DNA, RNA amounts and ecdysteroid titres. The reduction in the amounts of ovarian constituents is responsible for the changes in ovarian development and oogenesis. Finally, the reduced number of mature ova and size of oocytes following chlorfluazuron treatment (Perveen and Miyata, 2000) may be responsible for the reduction in the amounts of egg constituents. These effects increase with an increase in dose from LD_{10} to LD_{30}.

These results therefore demonstrate that chlorfluazuron affects the amounts of ovarian constituents during

ovarian development and oogenesis of *S. litura*. Substantial work has been done on the mode of action of chlorfluazuron, providing insight into the mechanisms by which it affects ovarian development and oogenesis. The precise biochemical mechanisms by which chlorfluazuron affect reproduction and ovarian development could reveal the nature of the effects of sublethal doses. Future research should explore the biochemical nature of the action of sublethal doses of chlorfluazuron in *S. litura*.

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