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Full Length Research Paper

Effects of sublethal doses of chlorfluazuron on the testicular biochemical constituents of *Spodoptera litura* (Lepidoptera: Noctuidae)

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The effects of sublethal doses (LD_{10}: 1.00 ng/larva; LD_{30}: 3.75 ng/larva) of chlorfluazuron on the amounts of testicular biochemical constituents of the common cutworm, *Spodoptera litura* (F.) was described. Chlorfluazuron was applied topically to newly ecdysed fifth-instar larvae under laboratory conditions. Sublethal doses of chlorfluazuron significantly reduced the amount of protein with no effect on carbohydrate and lipid. The relative proportion of the amounts of control testicular DNA was: 7.52±0.9 µg/mg at the day before adult emergence> 4.54±0.44 µg/mg at newly emerged adults> 3.52±0.49 µg/mg at 1st day after adult emergence. Sublethal doses of chlorfluazuron significantly ($p < 0.05$) reduced the amounts of testicular, seminal vesicular and aedeagular DNA compared with the controls. Similar reduction was observed in RNA as found in DNA. Three peaks in ecdysteroid titres were observed during 80 to 208 h. Compared with the controls, all peaks were reduced in LD_{10} or LD_{30} treated testis as follows: 11.5 or 21%; 12 or 22%; 12.5 or 23%, respectively. Sublethal doses of chlorfluazuron reduced the ecdysteroid titres with no effect on its pattern. Sublethal doses of chlorfluazuron reduced the amounts of biochemical testicular constituents during the development of testes in *S. litura*.

Key words: Chlorfluazuron, ecdysteroid titre, nucleic acid, protein, *Spodoptera litura*, sublethal doses, testicular biochemical, seminal vesicle, aedeagus.

INTRODUCTION

The common cutworm, *Spodoptera litura* (F.) larva has bright yellow colour testes which are distinctly paired, reniform and situated between the 5th and 6th abdominal segments. Each of the lateral testicular lobes is made up of four follicles. The testicular lobes are enclosed within two thick, double-layered peritoneal sheaths. This forms a common envelope to all follicles of testis (Etman and Hooper, 1979). The seminal vesicles are paired and elongated organs. They are connected with vas efferent (paired) at one hand and at other hand, with vas deferens (paired). The vas deferens is directly connected with testis (single in adult) and pours semen into seminal vesicles. The seminal vesicles are not merely a depository or storage organs for spermatozoa, but a place for their maturation (Kasuga et al., 1985; Mann, 1984). The accessory reproductive glands of *S. litura* are paired tubular structures. Their secretions pour through ductus ejaculatorious duplex into seminal vesicles contribute to the seminal fluid and formation of the spermatophore (Sridevi et al., 1989b). During copula formation, aedeagus enters into female corpus bursa and endophallus enters ductus bursa and complete transfer of spermatophore to female bursa copulatrix takes place (Etman and Hooper, 1979).

In Lepidoptera, sperm development initiates in larvae (Munson, 1906; Machida, 1929). Secondary spermatocytes are present in early last instars persist through middle to last stadium. The sperm bundles form as a result of maturation of spermatids in adult stages (Sridevi et al., 1989). Two distinct types of spermatozoa are found in Lepidoptera, eupyrene and apyrene. Eupryene spermatozoa are nucleated and can fertilize the eggs, whereas apyrene completely lack the nuclear structure (Doncaster, 1911; Goldschmidt, 1916). Like eupyrene sperm, the apyrene sperms are produced in large number (Friedlander and Gitay, 1972). Holt and North

(1970) suggest that in *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) apyrene sperm might aid the transport of eupyrene sperm in the female reproductive tract.

Studies *in vitro* and *in vivo* indicate that high titres of Juvenile hormone inhibit spermatogenesis and sperm mitosis and meiosis require sufficient nucleic acids and ecdysteroid titres (Dumser, 1980a). Post testicular development of insects has been initiated and controlled by hormones from neurosecretory cells of the brain and prothoracic glands (Garbini and Imberski, 1977). The marked changes in the content of protein, RNA and DNA are noted in testicular development and spermiogenesis during growth of insects. Dudash (1979) reported that the amounts of carbohydrates, protein and nucleic acids depend on the morpho-functional state of organisms. Nucleic acids synthesize in the zygote play an inductive role in initiation of testicular development (Perveen, 2000b). Free active ecdysteroid hormones are released at a specific time in spermatogenesis to trigger sperm maturation in insects (Sridevi et al., 1989). The potential use of acylureas as integrated pest control agents has received considerable attention over the last three decades (Perveen, 2000). One class of these compounds is employed as insect growth regulators. Application of diflubenzuron to adult females caused a decrease in fecundity in *Epilachna varivestis* Mulsant Coleoptera: Coccinellidae) and *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Holst, 1974; Fytizas, 1976) and adversely affected egg viability in *Stomoxys calcitrans* (L.) and *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-instars of the *L. migratoria*. Shakoori and Saleem (1989) reported the effects of sublethal treatments of malathion and malathion-permethrin on sixth-instars of *Tribolium castaneum* Herbst (Coleoptera: Tenibrionidae). Permethrin (200 ppm) and malathion (20 ppm) mixtures increased the activities of choline estrase (70.8%) and raised the concentrations of cholesterol (21%), DNA (24%) and RNA (8%). Miltin et al. (1977) reported when *Anthonomus grandis* (Coleoptera: Curculionidae) was treated with DFB, the biosynthesis of DNA was inhibited in females, but RNA was not and no protein synthesis was 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 its action being relatively slow but strong (Hashizume, 1988). Topical application of lethal doses of chlorfluazuron to newly ecdysed fifth-instar larvae of the tobacco cutworm, *S. litura* (F.) (Lepidoptera: Noctuidae), cause significant mortality in subsequent life stages. Furthermore, application of sublethal doses to larvae (LD_{10}: 1.00 ng/larva; LD_{30}: 3.75 ng/larva) significantly reduced fecundity and fertility of subsequent adults and hatchability of their eggs (Perveen, 2000).

The objectives of this study were to determine, the effects of sublethal doses of chlorfluazuron (LD_{10} or LD_{30}) on the amounts of testicular protein, 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: 16L8D; 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 microapplicator and micro-syringe, sublethal doses of chlorfluazuron (LD_{10}: 1.00 ng/larva; LD_{30}: 3.75 ng/larva) diluted in 2.0 µl of acetone were applied topically to the dorsum of newly-ecdysed fifth instars of *S. litura*. Larvae were kept for an appropriate time lapse that solvent of doses evaporated. LD_{10} and LD_{30} values were calculated basis of toxicity data from larval tests at adult emergence. Treated and control (untreated) 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 were followed according to Perveen (2006).

Analysis of testicular protein, carbohydrate and lipid

Testes were dissected from newly emerged adults of control and treated batches and homogenized collectively in a homogenizing tube containing aqueous trichloroacetic acid (TCA: 1.0 ml; 100 gl^{-1}) 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 in an aliquot was determined using the Coomassie blue method (Perveen, 2000b).

Analysis of testicular nucleic acids

Nucleic acids (DNA and RNA) were extracted following Schmidt and Thannhauser (1945) with some modification following Munro (1966). Their levels were measured by the diphenylamine method of Burton (1956) and the orcinol reaction of Schneider (1957), respectively. Testes from relevant stages (single from larval and pupal; and double from adult during different developmental days) with uniform length and weight for each control and treated batches

were selected and crushed in 2.0 ml 70% ethanol, then centrifuged at 1180 *g* for 15 min. Other procedures followed Perveen et al. (2010) and the amounts of testicular DNA and RNA were calculated by the following formulas: µg DNA /mg of testis(s) = total µg from curve × dilution/1× weight of testis(s)× 0.1×1000; µg RNA /mg of testis(s) = total µg from curve × dilution/2× weight of testis(s)× 0.1×1000, respectively.

Analysis of testicular ecdysteroid titres

Testes of the same weight, size and age group from control or treated batches of relevant stages (as described in analysis of testicular nucleic acids) were dissected and crushed in 300 µl 70% methanol, 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, Kannapolis, United States of America). [^{3}H]-ecdysone (1.85 TBq/mmol) was obtained from Du Pont (North Carolina, Kannapolis, United States of America)) and 20-hydroxyecdysone from Rohto Pharmaceutical (New York, United States of America) 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/testis tissues or µg mg/seminal vesicle tissues or µg mg/aedeagus tissues and ecdysteroid titres in pg mg/testis (following represent as µg/mg or pg/mg) with three replications each.

Statistical analysis

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

RESULTS

Effects on amounts of testicular protein, carbohydrate and lipid

Sublethal doses of chlorfluazuron significantly ($p < 0.001$) reduced the amount of protein by the LD_{10} and more significantly ($p < 0.0001$) reduced by the LD_{30} in newly emerged adult males compared with the controls, measured in µg/mg. In LD_{10}- and LD_{30}-treated newly emerged adult males, the amounts of protein estimated were 0.69±0.04 and 0.46±0.08 µg/mg, respectively, compared with control which was 0.95±0.03 µg/mg (Figure 1a). The amounts of carbohydrate and lipid of testis were not significantly ($p < 0.05$) reduced in the same stage of males by these doses (Figure 1b and c) (Perveen, 2000b).

Effects of chlorfluazuron on amount of testicular DNA

In control, the amount of testicular DNA increased since newly ecdysed sixth-(last)-instars till pre-pupation and remained relatively constant until 9th day after pupation. Sublethal doses, LD_{10} significantly ($p < 0.05$) reduced testicular DNA since newly ecdysed sixth instars through prepupation to 9th day pupae compared with control, measured in µg/mg of testis (F-value: 893.56; df: 09; n=5) (Figure 2a). In the same way, LD_{30} significantly ($p < 0.05$) reduced testicular DNA, during the same developmental stages compared with LD_{10} (Figure 2a).

In LD_{10}- and LD_{30}-treated 2nd day of sixth-instars, amounts of DNA were 3.24±0.21 and 2.88±0.38 µg/mg, respectively, compared with control which was 3.52±0.26 µg/mg. In LD_{10}- and LD_{30}-treated 4th day of sixth-instars, amounts of DNA were 5.04±0.21 and 3.90±0.27 µg/mg, respectively, compared with control (6.42±0.31 µg/mg) (Figure 2a).

In LD_{10}- and LD_{30}-treated pre-pupae, amounts of DNA were 6.02±0.4 and 4.54±0.31 µg/mg, respectively, compared with control which was 7.50±0.35 µg/mg. In LD_{10}- and LD_{30}-treated 2nd day after pupation, amounts of DNA were 5.90±0.4 and 4.30±0.56 µg/mg, respectively, compared with control which was 7.30±0.16 µg/mg. In LD_{10}- and LD_{30}-treated 5th day after pupation, amounts of DNA were 5.76±0.29 and 4.22±0.53 µg/mg, respectively, compared with control which was 7.12±0.59 µg/mg. In LD_{10}- and LD_{30}-treated 9th day after pupation, amounts of DNA were 5.54±0.39 and 4.2±0.71 µg/mg , respectively, compared with control which was 7.04±0.34 µg/mg of testis (Figure 2a).

In the control, the order of relative proportion of amount of DNA in testis of adults was: 7.52±0.9 µg/mg at day before adult emergence> 4.54±0.44 µg/mg at newly emerged adults> 3.52±0.49 µg/mg at 1st day after adult emergence. However, in LD_{10}-treated insects, on the day before adult emergence, amount of DNA was decreased (by 13.3%) compared with the controls. In the same stage in LD_{30}-treated insects, it was lowered (by 15.6%) compared with those LD_{10}-treated ones. The same trend was observed in newly emerged and 1st day after adult emergence of treated insects with both doses (F-value: 4305.81; df: 09; n=5) (Figure 2b).

Effects of chlorfluazuron on amount of testicular RNA

In the controls, order of relative proportion of the amount of testicular RNA according to three developmental stages was: newly emerged adults> newly ecdysed larvae> newly ecdysed pupae. Sublethal doses, LD_{10} ($p < 0.05$; compared with controls) and LD_{30} ($p < 0.05$; compared with LD_{10}) significantly reduced amount of testicular RNA during each developmental stage (F-value: 7024.83; df: 09; n=10–19). In LD_{10}- and LD_{30}-treated newly ecdysed sixth-instar larvae, amounts of RNA were 7.30±0.72 and 5.40±0.70 µg/mg , respectively, compared with control which was 8.65±0.54 µg/mg. In LD_{10}- and LD_{30}-treated newly ecdysed male pupae,

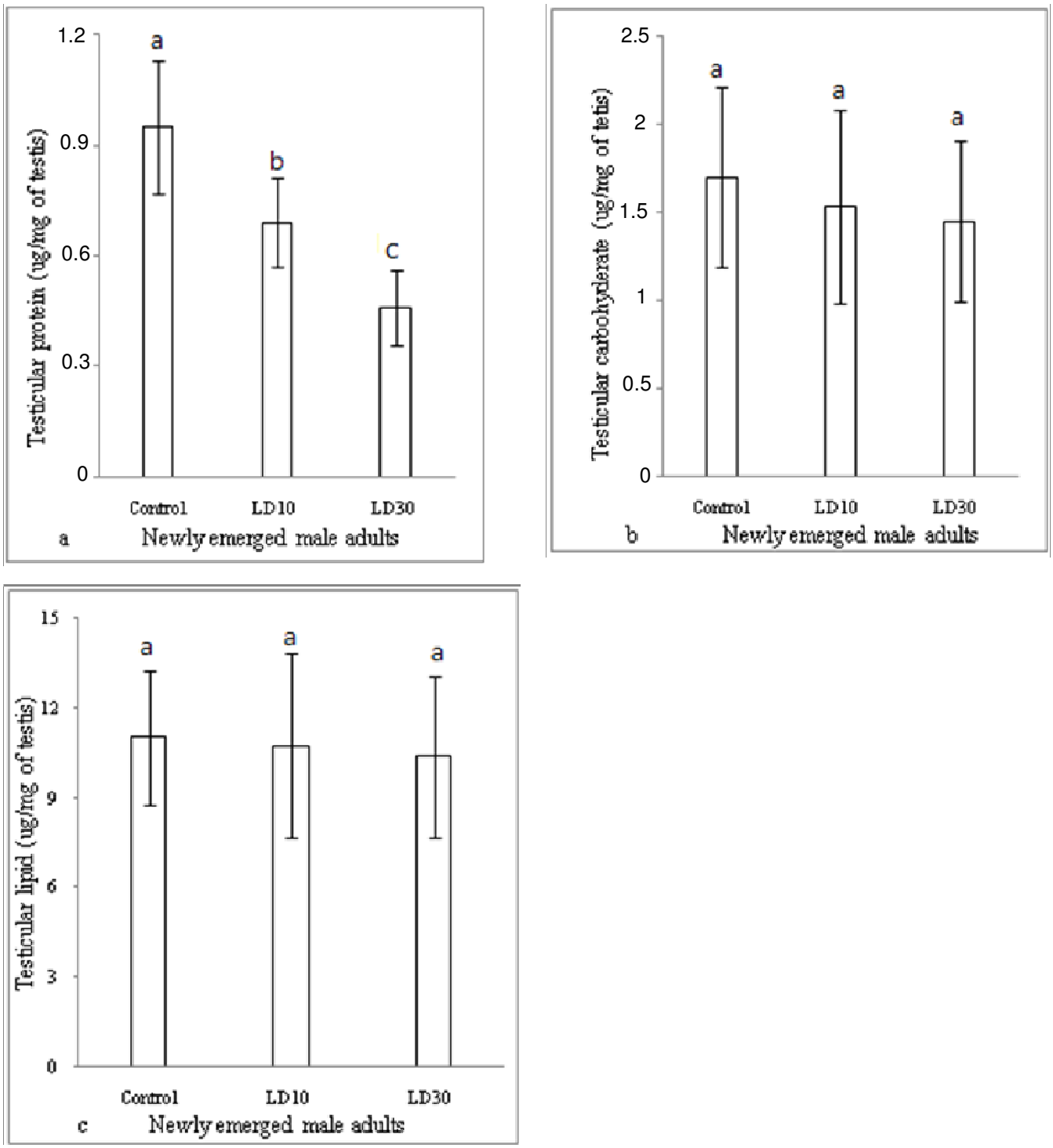

Figure 1. Effects of sublethal doses of chlorfluazuron (LD_{10}: 1.00 µg larva^{-1} or LD_{30}: 3.75 µg larva^{-1}) on amounts of testicular protein (a), carbohyderate (b) and lipids (c) at newly emerged male adults of *S. litura*; mean values were analyzed using one-way ANOVA (Abacus Concepts, 1989) at $p < 0.05$; bars contain different letters show significant differences by Scheffe's *F*-test at 5% (Scheffe, 1953); vertical bars: ±SD; n= 11 to 15 for each point (Source: Perveen, 2000b).

amounts of RNA were 6.84±0.60 and 5.34±0.75 µg µg/mg , respectively, compared with control which was 8.01±0.49 µg/mg. In LD_{10}- and LD_{30}-treated newly emerged male adults, amounts of RNA were 8.01 ± 0.51 and 6.74±0.50 µg/mg, respectively, compared with the control which was 9.33±0.76 µg/mg (Figure 3).

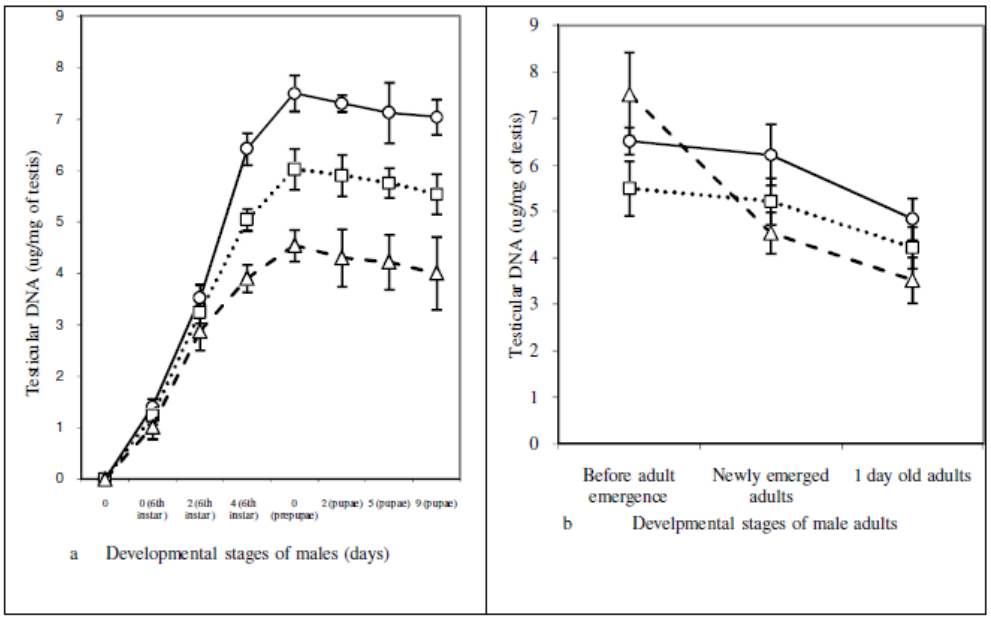

Figure 2. Effect of sublethal doses of chlorfluazuron (LD_{10}: 1.00 µg $larva^{-1}$ or LD_{30}: 3.75 µg $larva^{-1}$) on testicular DNA during developmental of *Spodoptera litura*: from newly ecdysed sixth-instars to 0 to 9 days old pupae (a); before adults emergence to 1 day old adults (b); controls: O; LD_{10}: □; LD_{30}: Δ; mean values analyzed by RM-ANOVA (Abacus Concepts, 1989) at $p < 0.05$ and Scheffe`s *F*-test (Scheffe, 1953) at 5%; F-value: 893.56, df: 09 (for a); F-value: 4305.81, df: 09 (for b); vertical bars: ±SD; n= 5 for each point; paired larval testes and fused single pupal testis were considered as testes pair equivalent.

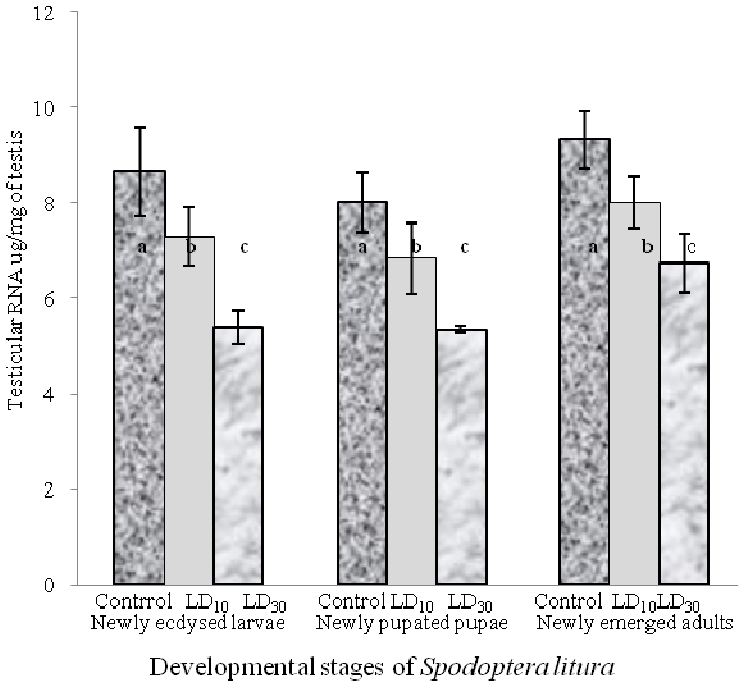

Figure 3. Effects of sublethal doses of chlorfluazuron (LD_{10}: 1.00 µg $larva^{-1}$ or LD_{30}: 3.75 µg $larva^{-1}$) on testicular RNA over three developmental stages of adults of *S. litura*; mean values were analyzed using one-way ANOVA (Abacus Concepts, 1989) at $p < 0.05$; bars contain different letters show significant differences by Scheffe's *F*-test at 5% (Scheffe, 1953); vertical bars indicate ±SD; F-value: 7024.83; df: 09; n= 10 to 19 for each point.

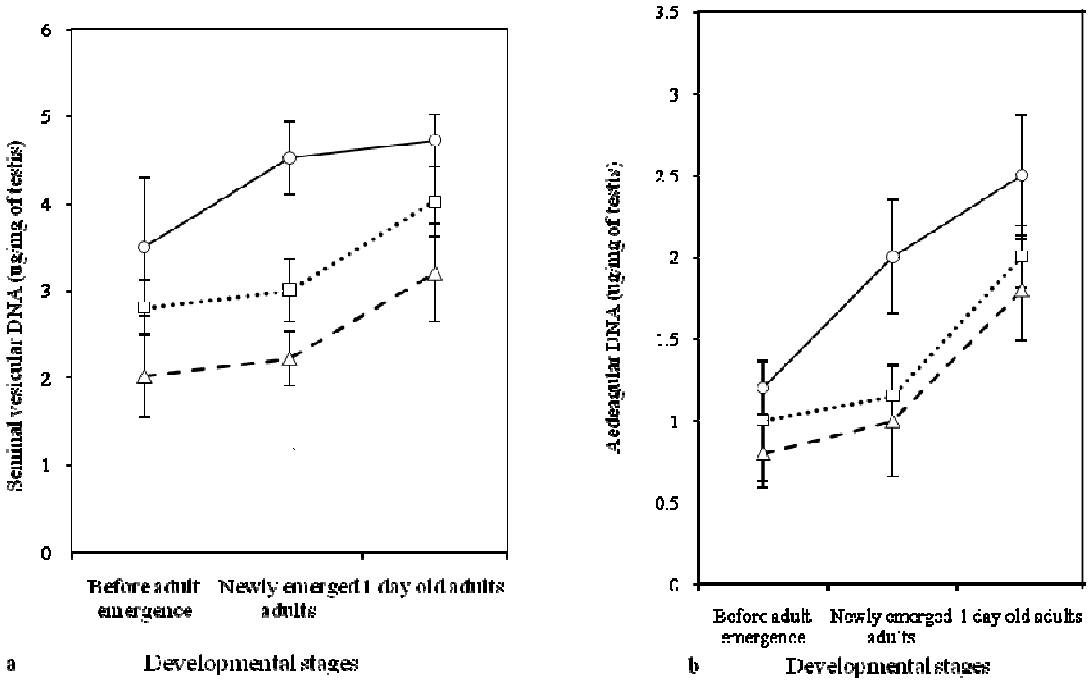

Figure 4. Effects of sublethal doses of chlorfluazuron (LD_{10}: 1.00 µg $larva^{-1}$ or LD_{30}: 3.75 µg $larva^{-1}$) on seminal vesicular DNA (a) and aedeagular DNA (b) over three developmental stages of *S. litura*; controls: O; LD_{10}: □; LD_{30}: Δ; mean values analyzed by one-way ANOVA (Abacus Concepts, 1989) at $p < 0.05$ and Scheffe`s *F*-test (Scheffe, 1953) at 5%; vertical bars: ±SD; F-value: 10.73, df: 09 (for a); F-value: 10.81, df: 09 (for b); n= 5 for each point.

Effects of chlorfluazuron on amount of seminal vesicular DNA

In controls, order of relative proportion of the amount of seminal vesicular DNA according to developmental days of adults was: 1st day after adult emergence> newly emerged adults> day before adult emergence. However, in LD_{10}- and LD_{30}-treated day before emergence adults, amounts of seminal vesicular DNA were 2.80±0.32 and 2.02±0.48 µg/mg, respectively, compared with the control which was 3.50±0.79 µg/mg of seminal vesicles. In LD_{10}- and LD_{30}-treated newly emerged adults, amounts of seminal vesicular DNA were 3.0±0.36 and 2.22±0.32 µg/mg, respectively, compared with the control which was 4.52±0.42 µg/mg of seminal vesicles. In LD_{10}- and LD_{30}-treated 1st day after adult emergence, amounts of seminal vesicular DNA were 4.02±0.4 and 3.2±0.56 µg/mg , respectively, compared with the control which was 4.72±0.30 µg/mg (F-value: 10.73; df: 09; n=5) (Figure 4a).

Effects of chlorfluazuron on amount of aedeagular DNA

In the controls, order of relative proportion of amount of aedeagular DNA according to developmental days of adults was: 1st day after adult emergence> newly emerged adult> day before adult emergence. In LD_{10}- and LD_{30}-treated day before adult emergence, amounts of aedeagular DNA were 1.0±0.37 and 0.8±0.21 µg/mg, respectively, compared with control which was 1.20±0.16 µg/mg of aedeagus. In LD_{10}- and LD_{30}-treated newly emerged adults, amounts of aedeagular DNA were 1.15±0.18 and 1.0±0.34 µg/mg of aedeagus, respectively, compared with control which was 2.00±0.35 µg/mg. InLD_{10}- and LD_{30}-treated 1st day after adult emergence, amounts of aedeagular DNA were 2.0±0.19 and 1.8±0.31 µg/mg, respectively, compared with control which was 2.50±0.37 µg/mg (F-value: 10.73; df: 09) (n=5; Figure 4b).

Effects on testicular ecdysteroid titre

In the controls, very low ecdysteroid titre was present in newly-ecdysed sixth-instar larvae. It was not significantly increased until 16 h of 2 days old sixth-instar larvae. However, after 16 h of 2 days old sixth-instar larvae till the prepupal stage (80 h), it significantly ($p < 0.05$) increased. Between 80 to 208 h (5 days old pupae), three peaks of ecdysteroid titre were observed. The first peak was during 80 to 88 h; the second one was at 88 to 136 h and final was at 136 to 208 h old. After that the titre

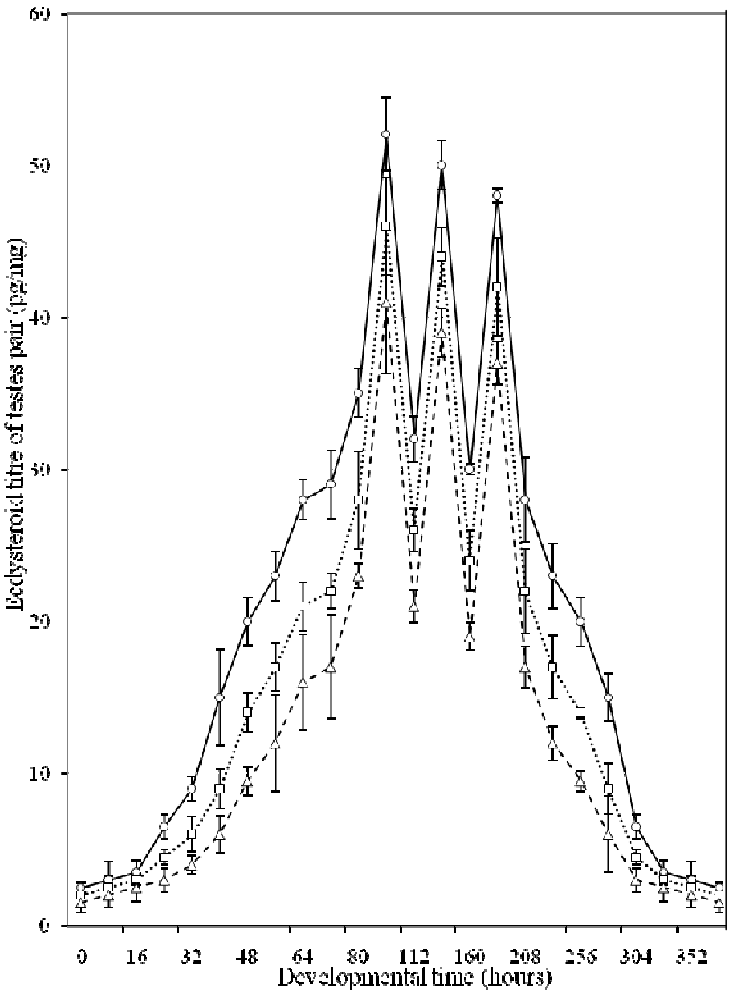

Figure 5. Effects of sublethal doses of chlorfluazuron (LD_{10}: 1.00 µg $larva^{-1}$ or LD_{30}: 3.75 µg $larva^{-1}$) on testicular ecdysteroid titres each consecutive 8 h of *S. litura* from 2 days old sixth-instar larvae (0 h) to pre-pupae (88 h) and after each consecutive 24 h from pupae (112 h) to 2 days old adults (376 h); controls: O; LD_{10}: □; LD_{30}: Δ; mean values analyzed by one-way ANOVA (Abacus Concepts, 1989) at $p < 0.05$ and Scheffe`s *F*-test (Scheffe, 1953) at 5%; vertical bars: ±SD; F-value: 10.73; df: 09; n= 5 for each point.

gradually decreased till 376 h (2 days old adults). Sublethal doses of chlorfluazuron affected the ecdysteroid titre of testes during development of sixth-instar, pupae and adults. It was significantly ($p < 0.05$) decreased by the LD_{10} during all developmental stages of *S. litura* compared with the control. It was also significantly ($p < 0.05$) decreased by the LD_{30} during the same developmental stages when compared with LD_{10}. However, in LD_{10}- and LD_{30}-treated males the pattern of ecdysteroid titre secretion was the same as in controls (Figure 5).

DISCUSSION

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 testicular biochemical constituents 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 instars 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 decreases in the weight of ovaries, number of mature ova and size of basal oöcytes (Perveen and Miyata, 2000), decreased in the amount of ovarian biochemical constituents including protein, carbohydrates, lipid, nucleic acids and ecdysteroid titires (Perveen, submitted), delayed initiation of mating (Perveen, 2008) and reduced activity of

oviposition stimulation factors (Perveen, 2009). Moreover, LD_{10} and LD_{30} treatment of males significantly ($p < 0.01$) reduced (65.8 and 88.6%) the number of inseminated eupyrene sperm (Perveen, 2008). 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 effects on different stages of embryogenesis (Perveen et al., 2010, 2010a) and reduced amount of egg biochemical constituents including protein, carbohydrates, lipid, nucleic acids and ecdysteroid titres (Perveen, 2010b). Future research should be explored the biochemical nature of sublethal doses of chlorfluazuron in *S. litura*.

Chlorfluazuron has three main effects on testicular development in *S. litura.* The first, was described by Perveen (2000b), topical application of sublethal doses of chlorfluazuron was decreased by the weight and size of testes in LD_{10}- or LD_{30}-treated males. The second is that the same doses reduced the number and size of cysts, eupyrene and apyrene sperm bundles during spermatogenesis (Perveen, 2000b). The third aspect is that the same doses reduced the amount of testicular biochemical constituents during testicular development and spermatogenesis, that is, protein, lipid, carbohydrate (Perveen, 2000b), DNA and RNA amount and ecdysteroid titre (Figures 1 to 5). The second and third aspects act as factors which were responsible for the first aspect. Following chlorfluazuron treatments may be responsible for the reduction in the amounts of testicular constituents. These effects increased with an increase in dose from LD_{10} to LD_{30}. The testicular phenotype caused by the inhibition of chitin synthetase in testes will be explored in future.

In this study, in newly emerged adult males, sublethal doses of chlorfluazuron significantly ($p < 0.01$) reduced the amount of protein by the LD_{10} and more significantly ($p < 0.001$) by the LD_{30} compared with controls, however, amounts of carbohydrate and lipid of testis were not significantly reduced, it means protein is more sensitive to sublethal doses of chlorfluazuron than carbohydrate and lipid. In this study, in the control, the order of relative proportion of amount of DNA in testis of adults was: before adult emergence> newly emerged adults> after adult emergence, it may be due to amount of DNA utilized for emergence of adult. Moreover, very low ecdysteroid titre was present in the control of newly-ecdysed sixth-instar larvae, when the testes were very small. After 16 h of 2 days old sixth-instar larvae till the prepupal stage (80 h), it significantly ($p < 0.05$) increased. In controls, three peaks of ecdysteroid titre were observed. The first peak appeared during 80 to 88 h, when the testes increased in size, simultaneously, an increase in spermatogenesis. The second peak was appeared at 88 to 136 h when sixth instar larvae ecdysed into pupae. Final peak appeared at 136 to 208 h when larval-paired testes started to fuse to form a single testis in adults. At present, beside the effects of sublethal doses of chlorfluazuron on amount of testicular DNA, effects on amount DNA of sperm have also been compared in two conditions, that is, during storing in seminal vesicles and during transferring (running) in aedeagus. In the LD_{10}- and LD_{30}-treated *S. litura* during three developmental stages, that is, day before emergence newly emerged and 1st day after emergence of adults, th amounts of seminal vesicular and aedeagular DNA were decreased almost with the same patterns (Figure 4a, b).

In this study, comparatively higher reduction of amount of DNA than RNA by sublethal doses of chlorfluazuron may be correlated with the fact that chlorfluazuron is a growth retardant. Tabassum (1994) reported that DFB reduced 33, 21 and 32% RNA and 44, 50 and 43% amounts of DNA after 24, 72 and 144 h, respectively, using a glass film method. Naqvi et al. (1992) reported amount of nucleic acid reduction in *M. domestica* when treated with solfac (cyfluthrin 5%). The reduction of amount of testicular DNA and RNA by chlorfluazuron in this study was higher than reported as described. The reduction of amount of testicular nucleic acids, consequently caused reduction in the amount of testicular protein in *S. litura* as reported by Perveen (2000b).

These results demonstrate that chlorfluazuron affects the amounts of testicular biochemical constituents during testicular development of *S. litura*. Substantial work has been done on the mode of action of chlorfluazuron providing insight into the mechanisms by which it affects testicular development and spermatogenesis. The precise biochemical mechanisms by which chlorfluazuron affect reproduction and testicular development could reveal the nature of the effects of sublethal doses. Future research should explore the mode of action of chlorofluazuron according to its chemical structure.

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