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Biochemical Analyses of Action of Chlorfluazuron as Reproductive Inhibitor in *Spodoptera litura*

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1. Introduction

Chlorfluazuron is highly effective against insect pests because it disrupts chitin synthesis during the moulting process (Retnakaran et al., 1989) and causes malformed larvae in *Spodoptera litura* (Omatsu et al., 1991). Chlorfluazuron had been used as chitin synthesis as well as reproductive inhibitor (Perveen, 2006). Toxicity and effects of chlorfluazuron on reproduction and viability of *S. litura* had been examined. The LD_{50} was found to be 12.0 ng larva^{-1} when evaluated up to pupation and 9.9 ng larva^{-1} up to adult emergence. Lethal dosages of chlorfluazuron when applied to newly-ecdysed fifth instars had a devastating effect on the *S. litura* population by killing them during larval, pupal, and adult stages. Reduction in the body weight was also observed in the larvae and pupae when treated with a sublethal dose (LD_{30}: 3.75 ng larva^{-1}) and in the adults when treated with sublethal doses (LD_{10}: 1.00 ng larva^{-1}; LD_{30}: 3.75 ng larva^{-1}) as newly-ecdysed fifth-instar larvae of *S. litura,* although the number of matings per female and life span of adult females and males remained unaffected by the same treatments. In insect pest management, the purpose of research is to maintain the pest population below a level of economic loss. Topical application of sublethal doses of chlorfluazuron (LD_{10} or LD_{30}) on newly-ecdysed fifth-instars did not kill the whole population of *S. litura* but reduced it, by affecting its reproduction. When sublethal doses were applied only to females or only to males, or both sexes, the results from these observations suggest that the fecundity was reduced to a similar degree when only females or only males or both sexes were treated with LD_{10} or LD_{30} doses as newly-ecdysed fifth-instars. However, the fertility and hatchability were affected more when only males were treated with LD^{10} and much more when treated with LD_{30} (Perveen, 2000a). However, there were no significant differences observed between larval and pupal treatment in the reduction of these biological parameters (Perveen, 2009a). Effects of chlorfluazuron on ovarian development and oögenesis (Perveen and Miyata, 2000), testicular development and spermatogenesis (Perveen, 2000b), insemination and number of inseminated sperm (Perveen, 2008) and haemolymph-borne oviposition-stimulating factors (Perveen, 2009b) in *S. litura* had been reported. The effect of sublethal doses of chlorfluazuron on embryogenesis of *S. litura* has also been reported during the

eight embryonic developmental stages (0-84 hours after oviposition) (Perveen, 2009c). Chlorfluazuron has proved significantly affected to the biological and reproductive parameters of *S. litura*. The biochemical analyses of effects of sublethal doses of chlorfluazuron and their efficacy on reproductive system of *S. litura* have been undertaken during the present research work. That research work will help in the development of a new group of pesticides that may be cheaper and less hazardous to the environment and non-target organisms which is the main desired outcome of the present work.

2. Experimental procedures

2.1 Insect rearing

Experiments were conducted with *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) taken from a stock that was established from eggs obtained from Aburahi Laboratory of Shionogi Pharmaceutical (Koga-Shiga-Pref., Japan). The larvae of *S. litura* were reared in the laboratory under controlled conditions on the artificial diet Insecta LF® (Nihon Nohsan-kohgyo, Kanagawa, Japan). The rearing temperature was maintained at 25±1 °C, with a L16:D8 hour photoperiod and 50-60% r.h. To facilitate observations, the dark period was set from 06:00 to 14:00 hours. Adults were fed on a 10% sucrose solution soaked in cotton. The eggs, which were laid on Rido® cooking paper (Lion, Tokyo, Japan), were collected every 3rd day and kept in 90 ml plastic cups (4 cm in diameter4×4 cm high) for hatching under the same environmental conditions (Perveen, 2000a).

2.2 Chlorfluazuron and its application

Chlorfluazuron (Atabron®) in powder form at 99.9% purity was obtained from Ishihara Sangyo Kaisha and was stored at 4 °C until use. It was diluted with acetone (analytical grade; Wako Pure Chemical Industries, Tokyo, Japan) for the test concentrations (Perveen, 2000a).

Using a micro-applicator (Kiya, Tokyo, Japan), sublethal doses (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) of chlorfluazuron diluted in 2.0 µl of acetone were applied topically using a micro-syringe (Ito, Fuji-City, Japan) to the dorsum of the third or fourth thoracic segments of newly-ecdysed fifth-instars of *S. litura* of similar weight (approximately 131 mg) and size (1.6 cm long). The LD_{10} or LD_{30} values were calculated based on the results of the toxicity data of larval tests at adult emergence (Perveen, 2000a). A batch of larvae treated with 2.0 µl of acetone was kept as a control to determine any effects of the solvent. Treated, untreated, and control 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) and provided with food on alternate days. These batches were kept under the same environmental conditions as those used for the rearing stock culture. Treated, untreated, and control larvae were examined daily and mortality was recorded until adult emergence. The mortality was observed in the control larvae were the same as in the untreated ones. Therefore, their data were not used for further analysis. Pupae were sexed immediately following pupation. The larvae, pupae and adults taken from treated and untreated batches were use separately for analysis of effects of chlorfluazuron on ovarian, testicular and egg constituents. When the male and female adults were emerged, they were used for the experiments in the sections described below (Perveen, 2000a).

2.3 Quantitative determination of ovarian constituents

2.3.1 Estimation of ovarian protein, carbohydrate and lipid

Paired ovaries were collected from newly emerged adults in the untreated and treated (LD_{10} or LD_{30}) batches (n=15–21) and analysed individually as follows:

Protein was extracted according to Le Bras and Echaubard (1977). The ovaries were homogenised in aqueous trichloroacetic acid (TCA: 1.0 ml; 100 g l^{-1}) and centrifuged at 1844 g for 10 min at 4 °C. The supernatant (1) was used for carbohydrate determination while the precipitate was washed with ether and chloroform (1.0 ml; 1: 1 by volume) to remove lipids. After centrifugation as before, the chloroform supernatant (2) was used for lipid determination and the second precipitate, thus obtained, was suspended in distilled water (1.0 ml) and the amount of protein was determined in an aliquot using the Coomassie blue method (Bradford, 1976) at 595 nm with bovine serum albumin (Sigma Chemical Corp., Tokyo, Japan) as a standard.

Carbohydrate content was determined by the anthrone method (Duchateau and Florkin, 1959; Mokrasch, 1954) as used previously for haemolymph carbohydrate determination (Soltani, 1990). After ovarian extraction by TCA and centrifugation as before, an aliquot was taken from the supernatant (1) for carbohydrate quantification at 625 nm using trehalose (Merck Chemical Corp., Tokyo, Japan) as a standard.

Lipids were measured according to Goldsworthy et al. (1972). After lipid extraction an aliquot (0.1 ml) from supernatant (2) was mixed with concentrated sulphuric acid (1.0 ml) and heated for 10 min at 100°C. After cooling, an aliquot was taken and mixed with a solution of 13 mM vanillin in 11.8 M phosphoric acid (Wako Pure Chemical Industries Ltd., Tokyo, Japan). Absorbance was measured at 545 nm in a microplate reader with a computer, using a standard lipid solution of cholesterol (Wako Pure Chemical Industries Ltd., Tokyo, Japan) containing 1.0 g 100 ml^{-1} as a standard.

Data on ovarian constituents were expressed as µg pair of ovaries^{-1} and µg mg of ovaries^{-1} (Perveen and Miyata, 2000).

2.3.2 Estimation of ovarian nucleic acids

Estimation of nucleic acids (RNA and DNA) in the ovaries was determined separately at three different stages of development of *S. litura,* which were as follows: (1) newly ecdysed last (sixth)-instars; (2) newly-pupated pupae and (3) newly-emerged female adults. Paired ovaries were collected from the relevant stages of the untreated and treated (LD_{10} or LD_{30}) batches (n=10) and weighed 81.1 mg and 10% homogenate was prepared in distilled water. DNA and RNA were extracted according to the procedure of Schmidt and Thannhauser (1945) with some modified according Munro (1966). DNA and RNA were measured by the diphenylamine method of Burton (1956) and orcinol reaction of Schneider (1957), respectively (Perveen and Miyata, 2000).

2.3.2.1 Extraction of DNA and RNA

Ovaries from 4–8 insects ca. 14–28 mg were used for each batch (untreated and treated). Ovaries were crushed in 2.0 ml 70% ethanol and centrifuged at 1180 g for 15 min. The supernatant was discarded and 2.0 ml of 70% ethanol was added in the precipitate and centrifuged at same speed. This process of ethanol washing was repeated three times. The supernatant was discarded and 2.0 ml methanol and ether (3:1 ratio) was added and boiled for 3 min, the solution was cooled and centrifuged for 15 min at 1180 g. The supernatant was discarded and the precipitate was treated twice with the ethanol and ether mixture (1:1). The

supernatant was discarded and the (1:1) was desiccated in a vacuum under the pressure of KOH (pellets) for 2 hours and then 2.0 ml of ice-cold water were added to the precipitate and Allowed to dissolve and was kept in refrigerator for 1 hour. Two ml 20% ice-cold PCA was added. The mixture was incubated at 4 °C in the refrigerator for 18–24 hours. The product contained RNA and precipitate contained DNA. Two ml of 10% PCA was added, mixed, shaken well and heated at 75 °C for 45 min. The supernatant represented the DNA extracted (Perveen, accepted).

2.3.2.2 Estimation of ovarian DNA and RNA

A DNA extract (0.4 ml) was taken for the test and 0.4 ml 10% PCA for the blank. Distilled water 0.6 ml and then 2.0 ml diphenylamine reagent were added to each tube. They were mixed and boiled for 15 min. Blue colour indicated the presence of DNA. Absorbance was read 660 nm against a blank. The amount of DNA of the ovaries was calculated by the following formula (Perveen, accepted):

$$\mu\text{g DNA mg ovary}^{-1} = \frac{\text{total } \mu\text{g from curve} \times \text{dilution}}{1 \times \textit{weight of ovaries} \times 0.1 \times 1000}$$

An RNA extract of 0.2 ml was taken for each test in a separate test and 1.8 ml distilled water added to it and 2.0 ml was added to the blank. Then 2.0 ml of orcinol reagent was added to the tests and the blank. These were mixed well and boiled for about half an hour. When green colour appeared, the absorbance was read at 660 nm against the blanks. The amount of RNA of ovaries was calculated by the following formula (Perveen, accepted):

$$\mu g \text{ RNA mg ovary}^{-1} = \frac{\text{total } \mu\text{g from curve} \times \text{dilution}}{2 \times \textit{weight of ovaries} \times 0.1 \times 1000}$$

2.3.2.3 Estimation of utricular DNA of spermatheca

The *utriculus* is that part of spermatheca in female in which the sperm are stored after mating. Therefore, estimation of the nucleic acids were determined in the *utriculi* which were collected after first and second mating of the untreated and treated (LD_{10} or LD_{30}) batches (n=5) and weighed and a 10% homogenate was prepared in distilled water. The rest of the procedure was the same as described above in section 1.2.2 (Perveen, accepted).

2.3.3 Estimation of ovarian ecdysteroid titre

Analysis of the ecdysteroid titre in ovaries was determined in 7 day-old female pupae to 4 day-old female adults after each consecutive 24 hours. Ovaries of the untreated and treated batches (n=5–14) were used. Ovaries were dissected in 0.9% NaCl solution and incubated in Ringer's solution (Barbosa, 1974) for 8 hours. Five pairs of ovaries with 300 µl 70% methanol were homogenized at 10625 g for 10 min. The supernatant obtained was added to the previous one and 500 µl of hexane was added and centrifuged at 1844 g for 5 min. The 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 (Rees and Issac, 1985). Then 1.0 ml 70% methanol was added and this solution was dried by N_2 gas (Perveen, accepted).

2.3.3.1 RIA methods

These samples were tested by the method described by Horn et al. (1976) and Gilbert et al. (1980). However, the ecdysteroid titre, antiecdysteroid antiserum was obtained from Prof.

Dr. LI Gilbert and Prof. Dr. WE Bollenbacher (University of North Carolina). The [^{3}H] ecdysone (1.85 TBq mmol^{-1}) was obtained from Du Pont Company Ltd., North Carolina, USA and 20-hydroxyecdysone from Rohto Pharmaceutical Company, New York, USA (Perveen, accepted).

Samples were dried and added to 50 µl distilled water. The standard groups (2–3 replicates), 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 and 0.2 ng 20-hydroxyecdysone 50 µl^{-1} were made. The [^{3}H] ecdysone solution (9.8 ml distilled water and 200 µl ^{3}H] was made and added to 50 µl of [^{3}Hecdysone 0.01 µci sample^{-1}) was vortexed. Then, 50 µl antiserum (appropriate dilution of the antiserum) was added and vortexed. It incubated at 5 °C for overnight. Then 150 µl saturated solution of ammonium sulphate was added. It was again incubated on ice for 0.5–1 hour and centrifuged at 1180 g for 5 min. The supernatant was removed by aspiration and 300 µl 50% sat. ammonium sulphate was added and vortexed. Again the tubes were centrifuged at 1180 g for 5 min. The supernatant was removed by aspiration and 20 µl distilled water was added and vortexed. The 300 µl of scintillator was added and vortexed. Tubes were then radio-assayed (Perveen, accepted).

2.4 Quantitative determination of testicular constituents

2.4.1 Estimation of testicular protein, carbohydrate and lipid

Testes were dissected from newly emerged adults in the untreated and treated (LD_{10} or LD_{30}) batches (n=11–15), and analysed individually by the same procedure as described above in section 1.2.1. Data were expressed as µg of testicular protein, carbohydrate and lipids in mg of testis^{-1} and µg testis^{-1} (Perveen and Miyata, 2000).

2.4.2 Estimation of testicular nucleic acids

Analysis of nucleic acids (RNA and DNA) in testes was determined separately at the different stages of development of *S. litura,* i.e., larvae, pupae and adults. The testes were dissected from the relevant stage of untreated and treated (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) batches (n=5) in Ringer' solution. Testes from 4–8 moths were pooled, about 14–28 mg weighed then a 10% homogenate was prepared in distilled water and the further procedure used was the same as described above in section 1.2.2 (Perveen, accepted).

2.4.3 Estimation of seminal vesicular DNA

Seminal vesicles (paired) are wide tube like structure arising from the testis. During mating, mature sperm transferred from the seminal vesicles through vas efferent and packed into spermatophore. Here, sperm are stored for some time. For estimation of DNA in seminal vesicles, they were collected from individuals, before adult emergence, newly emerged adults and 1 day-old adults of untreated and treated (LD_{10} or LD_{30}) batches (n=5) and weighed. A 10% homogenate was prepared in distilled water. The further procedure was the same as described above in section 1.2.2 (Perveen, accepted).

2.4.4 Estimation of aedeagular DNA

The *Aedeagus* is a 1.0 cm tube, present in the area of collum (part of spermatophore) formation of the cuticular secondary segment of the ductus ejaculatorius simplex. For the estimation of DNA, it was collected from individuals, before adult emergence, newly adult emergence and 1 day-old adults of the untreated and treated (LD_{10}: 1.00 ng larva^{-1} or LD_{30}: 3.75 ng larva^{-1}) batches (n=5) and weighed. A 10% homogenate was prepared in distilled

water. The further procedure was the same as described above in section 1.2.2 (Perveen, accepted).

2.4.5 Estimation of testicular ecdysteroid titre

Analysis of the ecdysteroid titre in testes was determined in newly-ecdysed sixth-instars to pre-pupae (72 hours) after each consecutive 8 hours. Testes of the untreated and treated batches (n=5) were used. Testes were dissected in 0.9% NaCl solution and incubated in Ringer's solution for 8 hours. Five pairs of testes with 300 µl 70% methanol were homogenized at 10625 g for 10 min. Then the same procedure was used as described above in section 1.2.3 (Perveen, accepted).

2.5 Data analysis

Data were analyzed by using analysis of variance (ANOVA) (Concepts, 1989; Minitab, 1997; Walpol and Myers, 1998) at $P<0.0001$ and Scheffe's F-test (multiple range) (Scheffe, 1953) at 5%.

3. Results

3.1 Effects on ovarian constituents

3.1.1 Effects on ovarian protein

Quantitative determination of the protein content in each pair of ovaries of newly emerged female adults showed that sublethal doses of chlorfluazuron significantly reduced the amount of protein in the ovaries (calculated in µg protein mg ovary^{-1} or µg protein pair of ovaries^{-1}: $P<0.0001$) compared with the controls, but there was no significant difference ($P=0.6385$) between the LD_{10} and LD_{30} treatments (Table 3.1). The protein content in the control newly emerged female adults was 0.71±0.18 µg mg ovary^{-1} or 53.9±18.5 µg pair ovaries^{-1}. However, in the LD_{10}-treated, newly emerged female adults, it was 0.45±0.12 µg mg ovary^{-1} or 28.8±13.1 µg pair ovaries^{-1}. In the LD_{30}-treated, newly emerged female adults, it was 0.42±0.10 µg mg ovary^{-1} or 24.5±10.6 µg pair of ovaries^{-1} (Table 3.1) (Perveen and Miyata, 2000).

Treatments[1]	n	Ovarian protein content[2]	
		(M±SD) µg pair of ovaries^{-1}	(M±SD) µg mg of ovaries^{-1}
Control	15	53.9±18.5^{a}	0.71±0.18^{a}
LD_{10}	21	28.8±13.1^{b}	0.45±0.12^{b}
LD_{30}	17	24.5±10.6^{b}	0.42±0.10^{b}

[1]Source: Perveen and Miyata, 2000; LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.1. Effect of sublethal doses of chlorfluazuron on the ovarian protein contents in newly-emerged adult female after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen and Miyata, 2000).

3.1.2 Effects on ovarian carbohydrate

The carbohydrate content of ovaries was reduced, but the reduction was not significant ($P<0.0963$ and $P<0.0611$, respectively, when calculated for µg mg ovary^{-1} or µg pair of ovaries^{-1}) for the LD_{10} or LD_{30} treatments compared with controls (Table 3.2). In control, newly emerged female adults, the amount of carbohydrate estimated was 1.16±0.51 µg mg ovary^{-1} or 86.2±36.9 µg pair of ovaries^{-1}. However, in the LD_{10}-treated newly-emerged female adults, it was reduced by 15.5%, when considered in mg ovary or 24.5%, when considered per pair of ovaries. In the LD_{30}-treated newly-emerged female adults, it was reduced by 24%, when considered in mg ovary or 38.1%, when considered per pair of ovaries (Table 3.2) (Perveen and Miyata, 2000).

Treatments[1]	n	Ovarian carbohydrate content[2]	
		(M±SD) µg pair of ovaries^{-1}	(M±SD) µg mg of ovaries^{-1}
Control	15	86.2±36.9[a]	1.16±0.51[a]
LD_{10}	21	65.1±47.3[a]	0.98±0.55[a]
LD_{30}	17	53.4±39.5[a]	0.88±0.46[a]

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.2. Effect of sublethal doses of chlorfluazuron on the ovarian carbohydrate contents in newly-emerged adult female after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen and Miyata, 2000)

3.1.3 Effects on ovarian lipid

The lipid content of ovaries was reduced, but the reduction was not significant ($P<0.0963$ and $P<0.0611$, respectively, when calculated for µg mg ovary^{-1} and µg pair ovaries^{-1}) by the LD_{10} or LD_{30} treatments compared with the controls (Table 3.3). In control, newly-emerged female adults, the amount of lipid estimated was 8.49±2.23 µg mg^{-1} or 643.6±199.1 µg pair ovaries^{-1}. However, in the LD_{10}-treated newly-emerged female adults, it was reduced by 10%, when considered in mg of ovaries or 22.6%, when considered per pair of ovaries. In the LD_{30}-treated newly-emerged female adults, it was reduced by 16%, when considered in mg of ovaries or 32.2%, when considered per pair of ovaries (Table 3.3) (Perveen and Miyata, 2000).

Treatments[1]	n	Ovarian lipid content[2]	
		(M±SD) µg pair of ovaries^{-1}	(M±SD) µg mg of ovaries^{-1}
Control	15	643.6±199.1[a]	8.49±2.23[a]
LD_{10}	21	498.4±274.2[a]	7.64±3.08[a]
LD_{30}	17	436.6±245.2[a]	7.14±2.71[a]

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.3. Effect of sublethal doses of chlorfluazuron on the ovarian lipid contents in newly-emerged adult female after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen and Miyata, 2000)

3.1.4 Effects on ovarian DNA

Quantitative determination of the DNA of the ovaries of newly-ecdysed sixth-instar larvae, pupae and adults showed that the concentration of DNA was greater in larvae, and then it decreased again in pupae and then increased in female adults. Sublethal doses of chlorfluazuron significantly ($P<0.001$) reduced the amount of DNA in the LD_{10}-treated and more significantly ($P<0.0001$) reduced in the LD_{30}-treated females compared with the controls, measured either in µg mg of ovary^{-1} or µg pair of ovaries^{-1} at each developmental stage (newly-ecdysed last-(sixth)-instar larvae; newly ecdysed pupae; newly-emerged female adults) (Table 3.4) (Perveen, accepted).

In the controls newly-ecdysed last-(sixth)-instar larvae, the amount of DNA estimated was 4.74±0.94 µg mg^{-1} ovary or 10.03±0.56 µg pair ovaries^{-1}. However, in the LD_{10}-treated larvae, it was reduced to 2.67±0.62 µg mg ovary^{-1} or 8.19±0.90 µg pair ovaries^{-1}. In the LD_{30}-treated newly-emerged female adults, it was reduced to 1.27±0.43 µg mg ovary^{-1} or 6.66±0.53 µg pair ovaries^{-1} (Table 3.4) (Perveen, accepted).

In the control newly-pupated female pupae, the amount of DNA estimated was 3.00±0.62 µg mg ovary^{-1} or 17.91±0.61 µg pair ovaries^{-1}. In the LD_{10}-treated newly-pupated female pupae, it was 1.51±0.75 µg mg^{-1} ovary or 16.18±0.61 µg pair ovaries^{-1}. In the LD_{30}-treated newly emerged female adults, it was 0.50±0.07 µg mg ovaries^{-1} or 24.61±0.92 µg pair ovaries^{-1} (Table 3.4) (Perveen, accepted).

In the control newly emerged female adults, the amount of DNA estimated was 5.98±0.61 µg mg ovary^{-1} or 24.61±0.92 µg pair ovaries^{-1}. In the LD_{10}-treated newly-pupated female pupae, it was 4.81±0.54 µg mg ovary^{-1} or 22.95±0.57 µg pair ovaries^{-1}. In the LD_{30}-treated newly-emerged female adults, it was 3.50±0.62 µg mg ovary^{-1} or 21.19±0.91 µg pair ovaries^{-1} (Table 3.4) (Perveen, accepted).

Treatment stages	Treat-ments[1]	n	Ovarian DNA contents[2]	
			(M±SD) µg mg^{-1}	(M±SD) µg pair ovaries^{-1}
Newly ecdysed larvae	Control	10	4.74±0.94^{a}	10.03±0.56^{a}
	LD_{10}	10	2.67±0.62^{b}	8.19±0.90^{b}
	LD_{30}	10	1.27±0.43^{c}	6.66±0.53^{c}
Newly pupated pupae	Control	10	3.00±0.62^{a}	17.91±0.61^{a}
	LD_{10}	10	1.51±0.75^{b}	16.18±0.61^{b}
	LD_{30}	10	0.50±0.07^{c}	14.7±0.62^{c}
Newly emerged adults	Control	10	5.98±0.61^{a}	24.61±0.92^{a}
	LD_{10}	10	4.81±0.54^{b}	22.95±0.57^{b}
	LD_{30}	10	3.50±0.62^{c}	21.19±0.91^{c}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.4. Effects of sublethal doses of chlorfluazuron on the ovarian DNA contents of different developmental stages after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, accepted).

3.1.5 Effects on utricular DNA in spermatheca

Quantitative estimation of the DNA of the utriculus of the spermatheca after first and second matings showed that it was greater after the first mating than the second mating

(Table 3.6). Sublethal doses of chlorfluazuron significantly (P<0. 001) lowered the amount of DNA in the utriculus of the spermatheca in the LD_{10}- and more significantly (P<0.0001) reduced in the LD_{30}-treated females compared with the controls, as measured in µg mg tissue^{-1} (Table 3.5) (Perveen, accepted).

In the control, after the first mating, the amount of DNA estimated in the utriculus of the spermatheca was 2.04±0.06 µg mg^{-1}. In the LD_{10}- treated larvae, it was reduced by 38.7%. In the LD_{30}-treated larvae, it was reduced by 58.3% (Table 3.5) (Perveen, accepted).

In the control after the second mating, the amount of DNA estimated in the utriculus of the spermatheca was 1.75±0.08 µg mg^{-1}. In the LD_{10}- treated larvae, it was reduced by 32.0%. In the LD_{30}-treated larvae, it was reduced by 57.1% (Table 3.5) (Perveen, accepted).

Treatments[1]	n	After first mating[b] (M±SD) µg mg^{-1}	After second mating[2] (M±SD) µg mg^{-1}
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]

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.5. Effects of sublethal doses of chlorfluazuron after the 1st and 2nd matings on the DNA contents of the *utriculus* of the spermatheca of adult females after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, accepted).

3.1.6 Effects on ovarian RNA

Quantitative determination of the RNA of the ovaries of newly-ecdysed sixth instar larvae, pupae and female adults showed that the concentration of RNA was the greatest in larvae then it decreased in pupae and then increased again in adult females as for DNA estimation in female ovaries (Table 3.6). Sublethal doses of chlorfluazuron significantly (P<0.001) lowered the amount of RNA in the LD_{10}-and more significantly (P<0.0001) lowered the RNA in the LD_{30}-treated females compared with the controls, measured in µg mg ovary^{-1} or µg pair ovaries^{-1} in each developmental stage in (newly-ecdysed sixth-instar larvae; newly-pupated pupae; newly-emerged male adults (Table 3.6) (Perveen, accepted).

In the control newly-ecdysed sixth-instar larvae, the amount of RNA estimated was 51.91±0.50 µg mg ovary^{-1} or 242.90±11.69 µg pair ovaries^{-1}. In the LD_{10}-treated larvae, it was 47.59±1.09 µg mg ovary^{-1} or 220.64±6.76 µg pair ovaries^{-1}. In the LD_{30}-treated newly-emerged female adults, it was 43.29±0.82 µg mg ovary^{-1} or 206.33±5.33 µg pair ovaries^{-1} (Table 3.5). In the control newly-pupated female pupae, the amount of RNA estimated was 25.70±0.47 µg mg ovary^{-1} or 220.07±6.15 µg pair ovaries^{-1}. In the LD_{10}-treated newly-pupated female pupae, it was 24.25±0.84 µg mg ovary^{-1} or 209.13±6.19 µg pair ovaries^{-1}. In the LD_{30}-treated newly emerged female adults, it was 22.19±0.75 µg mg ovary^{-1} or 199.45±4.09 µg pair ovaries^{-1} (Table 3.6) (Perveen, accepted).

In the control newly-emerged female adults, the amount of RNA estimated was 50.12±0.63 µg mg ovary^{-1} or 235.79±9.06 µg pair ovaries^{-1}. In the LD_{10}-treated newly-emerged female adults, it was 48.75±0.62 µg mg ovary^{-1} or 219.94±4.45 µg pair ovaries^{-1}. In the LD_{30}-treated newly-emerged female adults, it was 47.54±0.62 µg mg ovary^{-1} or 241.51±3.61 µg pair ovaries^{-1} (Table 3.6) (Perveen, accepted).

Treatment stages[1]	Treat-ments[1]	n	Ovarian RNA contents[2]	
			(M±SD) µg mg^{-1}	(M±SD) µg pair ovaries^{-1}
Newly ecdysed larvae	Control	10	51.91±0.50^{a}	242.90±11.69^{a}
	LD_{10}	10	47.59±1.09^{b}	220.64±6.76^{b}
	LD_{30}	10	43.29±0.82^{c}	206.33±5.33^{c}
Newly pupated pupae	Control	10	25.70±0.47^{a}	220.07±6.15^{a}
	LD_{10}	10	24.25±0.84^{b}	209.13±6.19^{b}
	LD_{30}	10	22.19±0.75^{c}	199.45±4.09^{c}
Newly emerged adults	Control	10	50.12±0.63^{a}	235.79±9.06^{a}
	LD_{10}	10	48.75±0.62^{b}	219.94±4.45^{b}
	LD_{30}	10	47.54±0.62^{c}	214.51±3.61^{c}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.6. Effects of sublethal doses of chlorfluazuron on the ovarian RNA contents of different developmental stages after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Perveen, accepted).

3.1.7 Effects on ovarian ecdysteroid titre

Ecdysteroid titre in ovaries was determined after each consecutive 24 hours on the 7 day-old female pupae through 4 day-old adult females. Preliminary data from controls indicated that the ovarian amounts of ecdysteroids, measured *in vivo*, changed during vitellogenesis in *S. litura* in a characteristic way: the amounts 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 $^{-1}$mg, after adult emergence), peaked at choriogenesis (on the 1st day 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 (Fig. 3.1).

Chlorfluazuron at the two tested sublethal doses significantly affected the amounts of ecdysteroids accumulated *in vivo* by ovaries. It significantly (P<0.001) decreased the amount in the LD_{10}-treated females and more significantly (P<0.0001) decreased it in the LD_{30}-treated females. However, the pattern of ecdysteroid production was the same in all three groups of insects (Fig. 3.1) (Perveen, accepted).

In the LD_{10}-treated females, during pre-vitellogenesis, the amount were (on the –4th day: 3.0±0.32 pg mg^{-1}; on the –3rd day: 3.5±0.8 pg mg^{-1}, after adult emergence), increased during vitellogenesis (on the –2nd day: 14.0±1.29 pg mg^{-1}; on the –1st day: 22.0±1.15 pg mg^{-1}; on the 0 day: 28.0±3.2 pg mg^{-1}, after adult emergence), peaked at the time of choriogenesis (on the 1st day after adult emergence: 46.0±3.2 pg mg^{-1}) and decreased when the insects started to deposit eggs (on the 2nd day after adult emergence: 9.0±1.66 pg mg^{-1}) and more significantly (P<0.0001) decreased thereafter (Fig. 3.1) (Perveen, accepted).

In the LD_{30}-treated females, during pre-vitellogenesis the amount was (on the –4th day: 2.5±0.92 pg mg^{-1}; on the –3rd day: 3.0±0.32 pg mg^{-1}, after adult emergence), increased during vitellogenesis (on the –2nd day: 9.5±0.93 pg mg^{-1}; on the –1st day: 17.0±3.39 pg mg^{-1}; on the 0

day: 23.0±0.8 pg mg^{-1}, after adult emergence), peaked on the time of choriogenesis (on the 1st day after adult emergence: 41.0±1.4 pg mg^{-1}) and decreased when the insects started to deposit eggs (on the 2nd day after adult emergence: 6.0±2.5 pg mg^{-1}) and more significantly (P<0.0001) decreased thereafter (Fig. 3.1) (Perveen, accepted).

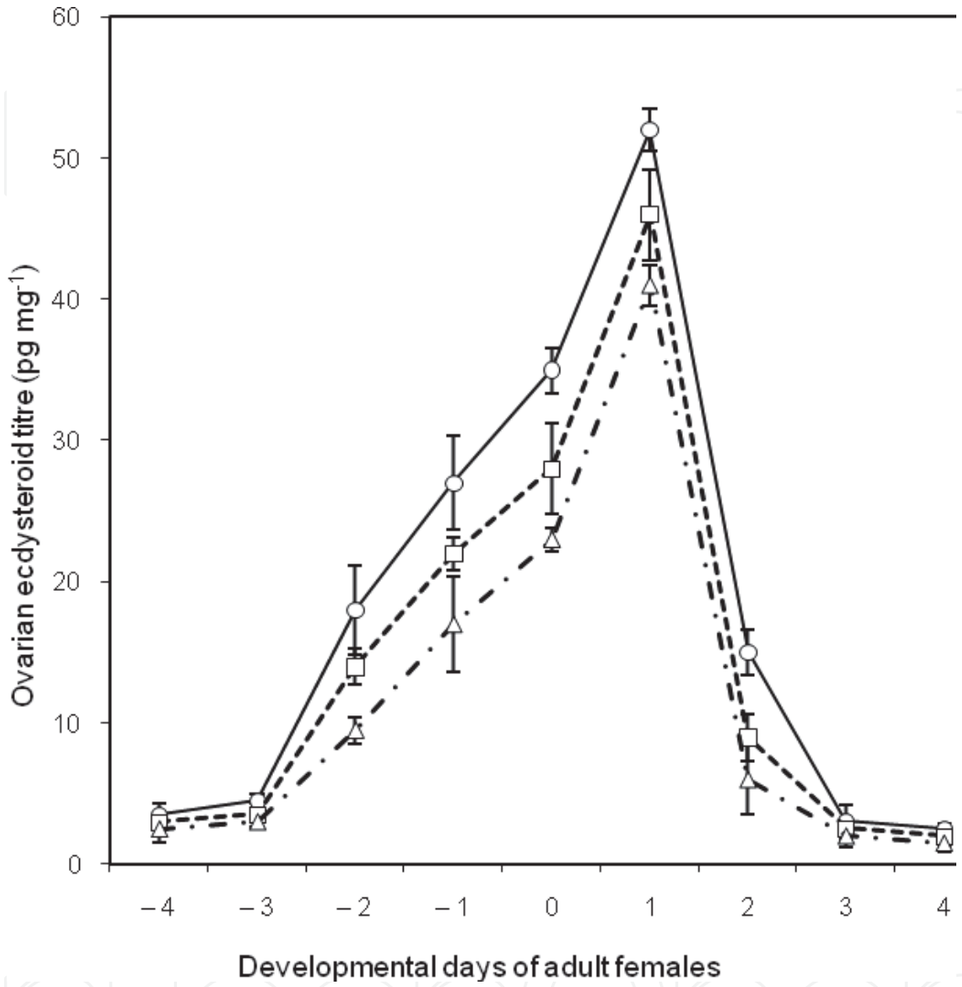

Fig. 3.1. Effect of sublethal doses of chlorfluazuron on the daily ovarian ecdysteroid titre of *Spodoptera litura* from 7 day-old female pupae to 4 day-old adult females; controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at P<0.0001 and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; n=5 for each point; (Source: Perveen, accepted).

3.2 Effects on testicular constituents

3.2.1 Effects on testicular protein

Quantitative determination of the constituents of the testis of newly-emerged adult males showed that sublethal doses of chlorfluazuron significantly (P<0.001) reduced the amount of protein by the LD_{10}-treated and more significantly (P<0.0001) reduced by the LD_{30}-treated males compared with the controls measured in µg mg testis^{-1} or µg testis^{-1} (Table 3.7) (Perveen, 2000b).

In the control newly-emerged adult males, the amount of protein estimated was 0.95±0.03 µg mg testis^{-1} or 3.25±0.44 µg testis^{-1}. In the LD_{10}-treated newly-emerged adult males, it was 0.69±0.04 µg mg testis^{-1} or 1.87±0.29 µg testis^{-1}. In the LD_{30}-treated newly-emerged adult males, it was 0.46±0.08 µg mg^{-1} or 0.92±0.21 µg testis^{-1} (Table 3.7) (Perveen, 2000b).

Treatments[1]	n	Testicular Protein contents[2]	
		(M±SD) µg mg^{-1}	(M±SD) µg testis $^{-1}$
Control	15	0.95±0.03^{a}	3.25±0.44^{a}
LD_{10}	13	0.69±0.04^{b}	1.87±0.29^{b}
LD_{30}	11	0.46±0.08^{c}	0.92±0.21^{c}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.7. Effects of sublethal doses of chlorfluazuron on the testicular protein contents of newly-emerged adults after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, 2000b).

3.2.2 Effects on testicular carbohydrate

Quantitative determination of the constituents of the testis of newly-emerged adult males showed that sublethal doses of chlorfluazuron significantly (P<0.0001) reduced the carbohyderate content of the testis when considered in µg testis^{-1}, but the reduction was not significant (P<0.0001) when considered as µg mg testis^{-1} compared with the control (Table 3.8) (Perveen, 2000b).

In the control newly-emerged male adults, the amount of carbohydrate estimated was 1.70±0.25 µg mg testis^{-1} or 5.43 ±1.39 µg testis^{-1}. In LD_{10}-treated newly-emerged male adults, it was reduced by 10 % when considered per mg testis or by 26% when considered per testis. In LD_{30}-treated newly-emerged male adults, it was reduced by 14.7% when considered per mg testis or by 41.8% when considered per testis (Table 3.8) (Perveen, 2000b).

Treatments[1]	n	Testicular carbohydrate content[2]	
		(M±SD) µg mg^{-1}	(M±SD) µg testis^{-1}
Control	15	1.70±0.25^{a}	5.43±1.39^{a}
LD_{10}	13	1.53±0.19^{a}	4.02±0.53^{b}
LD_{30}	11	1.45±0.29^{a}	3.16±0.67^{b}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.8. Effects of sublethal doses of chlorfluazuron on the testicular charbohydrate contents of newly-emerged adults after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, 2000b).

3.2.3 Effects on testicular lipid

Quantitative determination of the constituents of the testis of newly-emerged adult males showed that sublethal doses of chlorfluazuron significantly (P<0.0001) reduced the lipid

content of the testis when considered in µg testis^{-1}, but reduction was not significant (P<0.0001) when considered as µg mg testis^{-1} compared with the control (Table 3.9). In the control newly-emerged adult males, the amount of lipid estimated was 11.01±0.63 µg mg testis^{-1} and 37.19±6.62 µg testis^{-1}. In the LD_{10}-treated newly-emerged adult males, it was reduced by 2.6%, when considered per mg testis and 25% when considered per testis. In the LD_{30}-treated newly-emerged adult males, it was reduced by 5.9%, when considered per mg testis, and 72.2% when considered per testis (Table 3.9) (Perveen, 2000b).

Treatments[1]	n	Testicular Lipid contents[2]	
		(M±SD) µg mg^{-1}	(M±SD) µg testis^{-1}
Control	15	11.01±0.63^{a}	37.19±6.62^{a}
LD_{10}	13	10.72±1.08^{a}	27.77±2.84^{b}
LD_{30}	11	10.36±0.62^{a}	20.97±2.15^{b}

[1]Source: Perveen, 2000b; LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different (P<0.0001).

Table 3.9. Effects of sublethal doses of chlorfluazuron on the testicular lipid contents of newly-emerged adults after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, 2000b).

3.2.4 Effects on testicular DNA

Quantitative determination of the DNA of the testes of newly-ecdysed last-(sixth)-instar larvae through the 9th day after pupation to the 1st day after adult emergence showed that the concentration of DNA slowly and gradually increased until pre-pupation and remained constant until the 9th day after pupation (Fig. 3.2).

Sublethal doses of chlorfluazuron significantly reduced (P<0.001) the amount of DNA in LD_{10}-treated and more significantly (P<0.0001) reduced in LD_{30}-treated males compared with the control in the developmental stages described above measured in µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control newly-ecdysed last-(sixth)-instar larvae, the amount of DNA was 1.4±0.16 µg mg testis^{-1}. In the LD_{10}-treated larvae, it was 1.23±0.16 µg mg testis^{-1}. In the LD_{30}-treated larvae, it was 1.02±0.24 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control for the 2nd day sixth-instar larvae, the amount of DNA was 3.52±0.26 µg mg testis^{-1}. In the LD_{10}-treated larvae, it was 3.24±0.21 µg mg testis^{-1}. In the LD_{30}-treated larvae, it was 2.88±0.38 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control for the 4th day-sixth instar larvae, the amount of DNA was 6.42±0.31 µg mg testis^{-1}. In the LD_{10}-treated similar larvae, it was 5.04±0.21 µg mg^{-1}. In the LD_{30}-treated similar larvae, it was 3.90±0.27 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control pre-pupae, the amount of DNA was 7.50±0.35 µg mg testis^{-1}. In the LD_{10}-treated similar larvae, it was 6.02±0.4 µg mg testis^{-1}. In the LD_{30}-treated similar larvae, it was 4.54±0.31 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

In the control on the 2nd day after pupation, the amount of DNA was 7.30±0.16 µg mg testis^{-1}. In the LD_{10}-treated similar pupae, it was 5.90±0.4 µg mg testis^{-1}. In the LD_{30}-treated similar pupae, it was 4.30±0.56 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

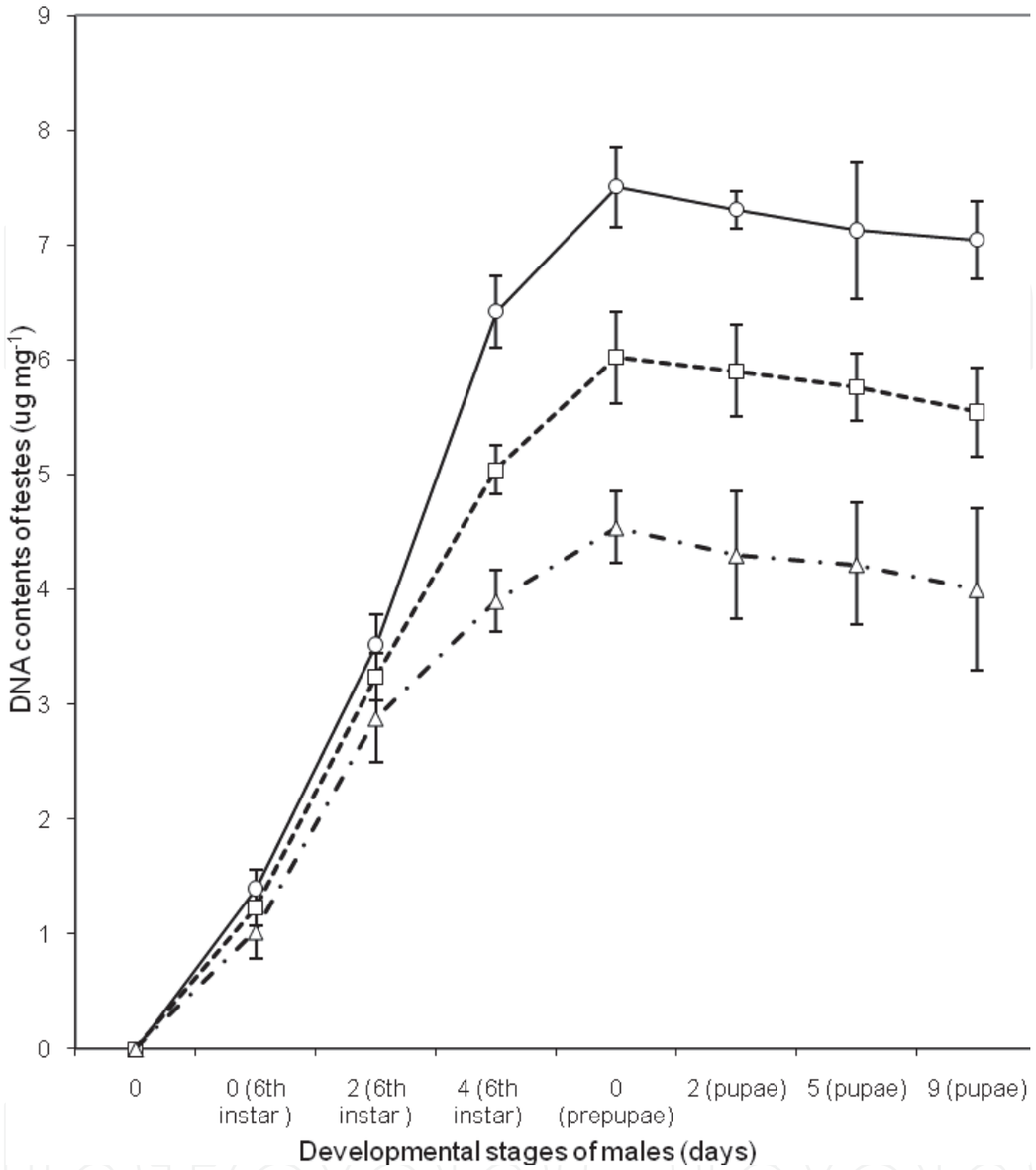

Fig. 3.2. Effect of sublethal doses of chlorfluazuron on the DNA contents of testes from newly-ecdysed sixth-instars to 9 day-old pupae of *Spodoptera litura*; controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at $P<0.0001$ and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; L: larval, P: pupal and A: adult developmental days; n=5 for each point; paired larval testes and fused single pupal testis were considered as testes pair equivalent; (Source: Perveen, accepted).

In the control on the 5th day after pupation, the amount of DNA was 7.12±0.59 µg mg testis^{-1}. In the LD_{10}-treated similar pupae, it was 5.76±0.29 µg mg testis^{-1}. In the LD_{30}-treated similar pupae, it was 4.22±0.53 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).
In the control on the 9th day after pupation, the amount of DNA was 7.04±0.34 µg mg testis^{-1}. In the LD_{10}-treated similar pupae, it was 5.54±0.39 µg mg testis^{-1}. In the LD_{30}-treated similar pupae, it was 43.2±0.71 µg mg testis^{-1} (Fig. 3.2) (Perveen, accepted).

When the DNA content was measured on the day before adult emergence, it slightly increased (7.52±0.9 µg mg^{-1}). Then, in newly-emerged adults, it sharply decreased (4.54 ± 0.44 µg mg^{-1}) and on the 1st day after adult emergence, it slowly decreased (3.52±0.49 µg mg^{-1}). In newly-emerged adults, the DNA content was 27.3% lowered compared with the LD_{10}-treated ones and 21% compared with the LD_{30}-treated ones. On the 1st day after adult emergence, it was decreased by 27.3% as compared to LD_{10}-treated ones and by 16. 6% compared with the LD_{30}-treated ones (Fig. 3.3) (Perveen, accepted).

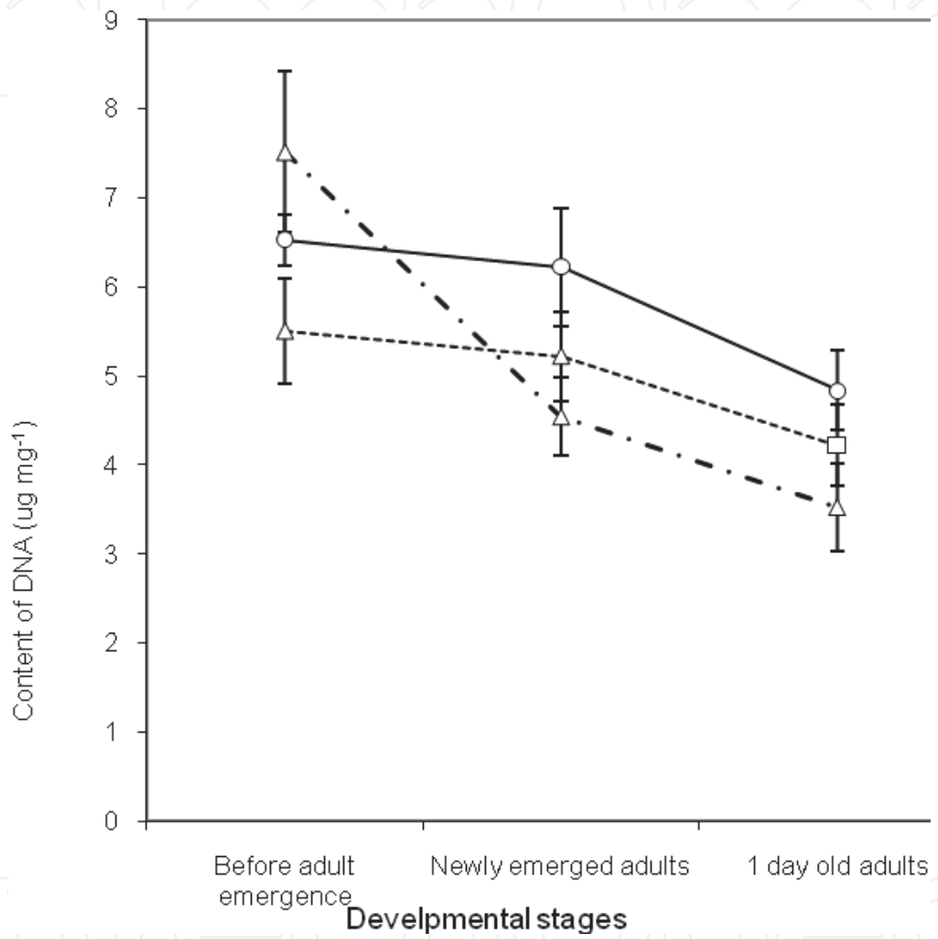

Fig. 3.3. Effects of sublethal doses of chlorfluazuron on the DNA contents of the testis over three developmental stages of *Spodoptera litura*; controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at P<0.0001 and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%.; vertical bars: SD; n=5 for each point; (Source: Perveen, accepted).

However, in trend insects the tendency was quite different. In LD_{10}-treated specimen on the day before adult emergence, the DNA content was significantly decreased (by 13.3%) compared with the controls. In the same stage in the LD_{30}-treated specimen, it was significantly lowered (by 15.6%) those LD_{10}-treated ones. The same trend was observed in newly-emerged adults and on the 1st day after adult emergence of treated insects with both doses, i.e., in newly-emerged adults, it was decreased (6.2±2 6.6 µg mg^{-1}) from the day before adult emergence, but it was the greatest than the controls and greater than LD_{30}-

treated ones in which it was (5.22±0.5 µg mg^{-1}), which is greater than the controls. On the 1st day after adult emergence, it was decreased (4.84±0.45 µg mg^{-1}) than in newly-emerged adults and LD_{10}-treated 1 day-old adults, but it was greater than the controls (Fig. 3.3) (Perveen, accepted).

3.2.5 Effects on seminal vesicular DNA

When the DNA content was measured in the seminal vesicles on the day before adult emergence, it was 3.50±0.79 µg mg^{-1}. In newly-emerged adults, it increased to (4.52±0.42 µg mg^{-1}) and on the 1st day after adult emergence, it slightly increased to (4.72±0.30 µg mg^{-1}). However, in treated insects, the tendency was quite different. In the LD_{10}-treated specimen on the day before adult emergence, the DNA content was significantly decreased (by 20%) compared with the controls. For LD_{30}-treated ones, it was significantly decreased (by 27.9%) than the LD_{10}-treated ones. The same trend was observed in newly-emerged adults and 1 day-old adults treated with both sublethal doses compared with the controls. However, in newly-emerged LD_{10}-treated adults, it was almost constant on the day before adult emergence. On the 1st day after adult emergence LD_{10}-treated males, it had increased level compared with the newly-emerged adults of the similar treatment. Similar tendency was observed in LD_{30}-treated ones during the same adult developmental days as in LD_{10}-treatment (Fig. 3.4) (Perveen, accepted).

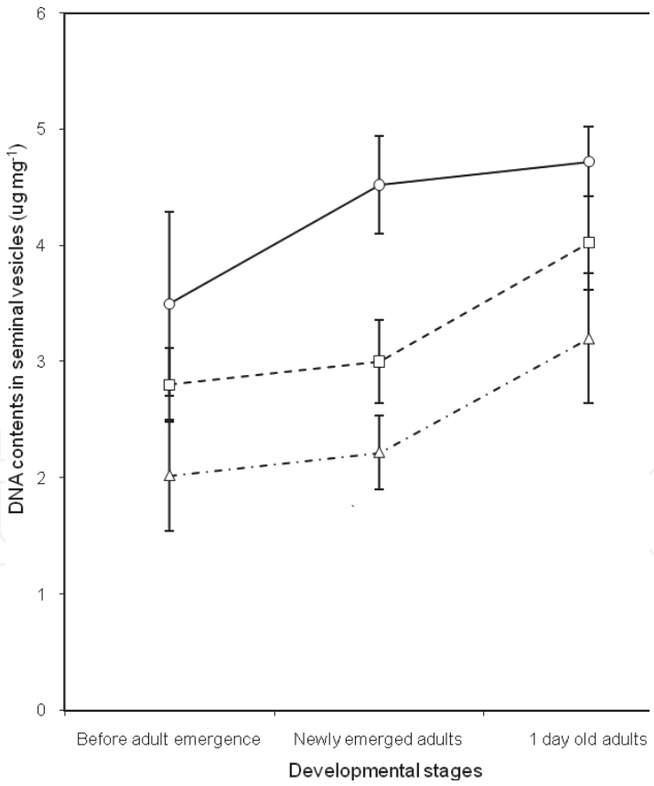

Fig. 3.4 Effects of sublethal doses of chlorfluazuron on the DNA contents of seminal vesicle over three developmental stages of *Spodoptera litura*; for controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at P<0.0001 and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; n=5 for each point; (Source: Perveen, accepted).

3.2.6 Effects on aedeagular DNA

When the DNA content was measured in aedeagus to the 1 cm tube (area of collum formation of the cuticular secondary segment of the ductus ejaculatorious simplex) on the day before adult emergence, it was 1.20±0.16 µg mg^{-1} and then in newly emerged adults, it increased to 2.00±0.35 µg mg^{-1} and on the 1st day after adult emergence, it slowly increased to 2.50±0.37 µg mg^{-1}. However, in treated insects the tendency was a little changed. In the LD_{10}-treated insects on the day before adult emergence, the DNA content was significantly ($P<0.0001$) decreased (by 16.6%) compared with the controls. In the same stage in the LD_{30}-treated ones, it significantly decreased ($P<0.0001$; by 20%) compared with the LD_{10}-treated ones. The same trend was observed in newly emerged adults and on the 1st day after adult emergence of treated insects with both sublethal doses when compared with the controls. However, in both LD_{10}- and LD_{30}-treated newly emerged adults, the level slightly increased from before adult emergence but it sharply increased on the 1st day after adult emergence compared with the newly emerged adults (Fig. 3.5) (Perveen, accepted).

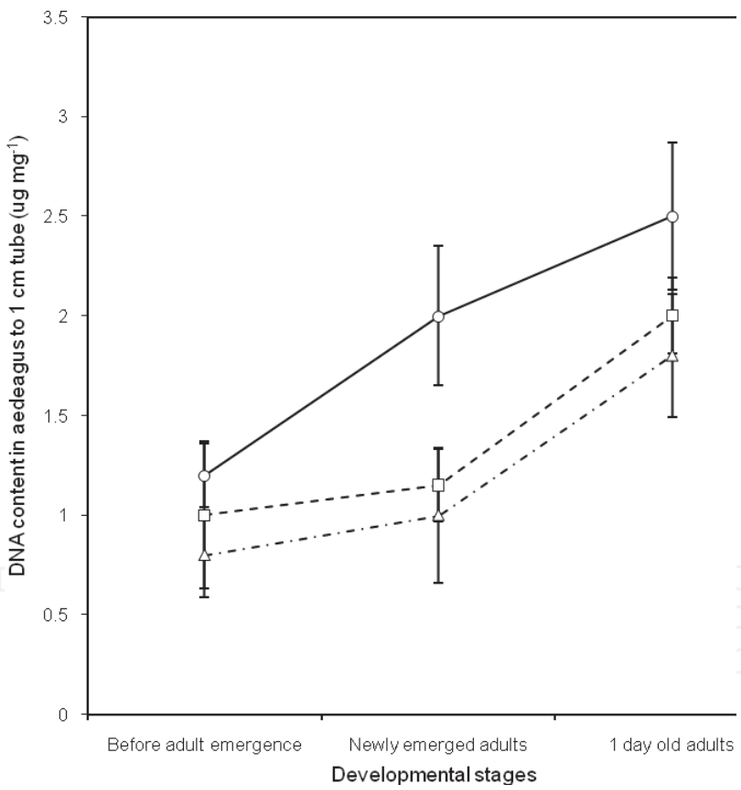

Fig. 3.5. Effects of sublethal doses of chlorfluazuron on the DNA contents of aedeagus to 1 cm tube over three developmental stages of *Spodoptera litura*; controls: O; LD_{10}: □; LD_{30}: Δ; data were analyzed by one-way ANOVA (Concepts, 1989) at $P<0.0001$ and followed by Scheffe's *F*-test (Scheffe, 1953) at 5%; vertical bars: SD; n=5 for each point; (Source: Perveen, accepted).

3.2.7 Effects on testicular RNA

Quantitative determination of the RNA content of the testis of newly-ecdysed sixth-instar larvae, pupae and male adults showed that the concentration of RNA was the greatest in larvae, decreased in pupae and again increased in adult males, the same as for DNA (Table 3.10) (Perveen, accepted).

Sublethal doses of chlorfluazuron significantly reduced ($P<0.001$) the amount of RNA in LD_{10}- and more significantly ($P<0.0001$) reduced in LD_{30}-treated males compared with the controls, measured in µg mg testis^{-1} or µg testis^{-1} in each developmental stage (newly-ecdysed sixth-instar larvae; newly-pupated pupae; newly-emerged male adults) (Table 3.10). In the control newly-ecdysed sixth-instars, the amount of RNA was 8.65±0.54 µg mg^{-1} and 16.32±0.82 µg testis^{-1}. In LD_{10}-treated larvae, it was reduced by 15.6% when considered per mg testis, and by 9.9%, when measured per testis. In LD_{30}-treated larvae, it was reduced by 37.6%, when considered per mg testis, and by 20.9%, when measured per testis (Table 3.10) (Perveen, accepted).

In the control newly-pupated male pupae, the amount of RNA was 8.01±0.49 µg mg testis^{-1} or 40.94±0.61 µg testis^{-1}. In the LD_{10}-treated newly-pupated male pupae, it was reduced by 14.5% when considered per mg testis, and by 4.5%, when measured per testis. In the LD_{30}-treated newly-pupated male pupae, it was reduced by 33.3%, when considered per mg of testis and by 5.4%, when measured per testis (Table 3.10) (Perveen, accepted).

In the controls newly emerged male adults, the amount of RNA was 9.33±0.76 µg mg^{-1} or 51.93±0.53 µg testis^{-1}. In the LD_{10}-treated newly-emerged male adults, it was reduced by 14.2%, when considered per mg testis and by 3.1%, when measured per testis. In the LD_{30}-treated newly-emerged male adults, it was reduced by 27.8% when considered per mg testis and by 7.7% when measured per testis (Table 3.10) (Perveen, accepted).

Treated stages	Treatments[1]	n	RNA contents[2] (M±SD) µg mg^{-1}	(M±SD) µg testis^{-1}
Larvae	Control	10	8.65±0.54^{a}	16.32±0.82^{a}
	LD_{10}	10	7.30±0.72^{b}	14.7±0.62^{b}
	LD_{30}	10	5.40±0.70^{c}	12.91±0.55^{c}
Pupae	Control	19	8.01±0.49^{a}	40.94±0.61^{a}
	LD_{10}	19	6.84±0.60^{b}	39.08±0.73^{b}
	LD_{30}	19	5.34±0.75^{c}	38.71±0.79^{c}
Adults	Control	19	9.33±0.76^{b}	51.93±0.53^{b}
	LD_{10}	19	8.01±0.51^{b}	50.30±0.91^{b}
	LD_{30}	19	6.74±0.50^{c}	47.94±1.14^{c}

[1]LD_{10}, 1.00 ng larva^{-1}; LD_{30}, 3.75 ng larva^{-1}; n: number of females used.
[2]Data were analyzed by one-way ANOVA (Concepts, 1989) and Scheffe`s F-test (Scheffe, 1953) at 5%. Means within a column followed by different letters are significantly different ($P<0.0001$).

Table 3.10. Effect of sublethal doses of chlorfluazuron on the RNA contents of the testes in different developmental stages after topical application to newly-ecdysed fifth-instars of *Spodoptera litura* (Source: Perveen, accepted).

3.2.8 Effects on testicular ecdysteroid titre

Measurment of the effects of sublethal doses of chlorfluazuron on the ecdysteroid titre of testis were conducted every 8 hours, from the 2 day-old sixth-instar larvae (0 hour) through to prepupae (88 hours) and after each 24 hours, from pupae (112 hours) to 2 day-old adults (376 hours). Sublethal doses were applied by same method as described for newly-ecdysed fifth-instar larvae of *S. litur* (as in Materials and methods). Ecdysteroid titre was measured in pg paired larval testes^{-1}; the fused single pupal testis was considered as testes pair equivalent (Perveen, accepted).

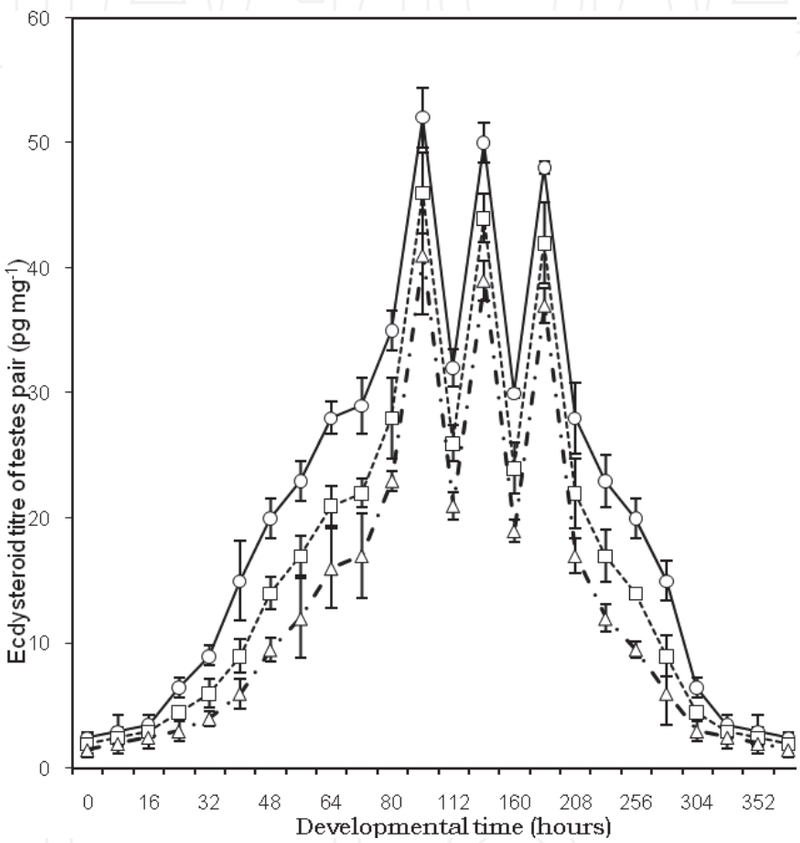

Fig. 3.6. Effects of sublethal doses of chlorfluazuron on the testicular ecdysteroid titre each consecutive 8 hours of *Spodoptera litura* from 2 day-old sixth-instars (0 hour) to pre-pupae (88 hours) and after each consecutive 24 hours from pupae (112 hours) to 2 day-old adults (376 hours); controls: O; LD_{10}: □; LD_{30}: Δ; data analyzed by one-way ANOVA at $P<0.0001$; after 16 hours all data are significantly different by Scheffe`s F-test at 5%.; vertical bars: SD; n=5 for each point; paired larval testes and fused single pupal testis were considered as testes pair equivalent (Source: Perveen, accepted).

Very low ecdysteroid titre was present in control newly-ecdysed sixth-instar larvae, when the testes were very small. It did not significantly increase until 16 hours of 2 day-old sixth-instar larvae. However, after 16 hours of 2 day-old sixth-instar larvae till the prepupal stage (80 hours), it significantly ($P<0.0001$) increased, as the testis increased in size and, simultaneously, spermatogenesis also increased and also when larval paired testes started to fuse to form a single testis (Fig. 3.6). Between 80 hours to 208 hours (5 day-old pupae), three

peaks of ecdysteroid titre were observed. The first peak was during 80–88 hours; the second one was at 88–136 hours; the third was at 136–208 hours old. At this time (third) spermatogenesis was at its peak and, simultaneously, sperm bundles were being transferred to the seminal vesicles. After that, the titre gradually decreased till 376 hours (2 day-old adults), (Fig. 3.6) (Perveen, accepted).

Sublethal doses of chlorfluazuron affected the ecdysteroid titre of testes during this all development time of the sixth-instar (last) larvae, pupae and adults. It was significantly ($P<0.001$) decreased by the LD_{10} and more significantly ($P<0.0001$) decreased by the LD_{30} during development of all stages of *S. litura*. However, in the LD_{10}- and LD_{30}-treated males the pattern of ecdysteroid titre production was the same as in the controls (Fig. 3.6) (Perveen, accepted).

4. Discussion

Effects of sublethal doses (LD_{10}: 1.00 ng larva^{-1}; LD_{30} : 3.75 ng larva^{-1}) of chlorfluazuron on biochemical were analysed to observe the causes of effects of chlorfluazuron on oÖgenesis and ovarian development (Perveen and Miyata, 2000; Perveen, 2009a); spermatogenesis and testicular development (Perveen, 2000b, 2009a); insemination (Perveen, 2008, 2009a), oviposition stimulating factors (Perveen, 2009b); and embryogenesis (Perveen, 2009c); and also ultimate effects on the fecundity, fertility and hatchability.

In the present research, a significant decrease in ovarian protein content in chlorfluazuron-treated females was observed (Table 3.1). Diflubenzuron also caused a decrease in ovarian protein content in *Cy. pomonella* (Soltani and Mazouni, 1992). Decrease in the ovarian protein content suggests interference by BPUs with vitellogenesis. In the current results, the decrease in ovarian protein content in chlorfluazuron-treated females was presumed to have several causes, such as the lack of protein in the ovarioles or interference of chlorfluazuron with the mechanism controlling yolk deposition. It has been reported that diflubenzuron could affect the protein content from other organs, such as the epidermis, in *T. molitor* (Soltani, 1984), ovaries in *C. pomonella* (Soltani et al., 1989a and b), and the concentration of haemolymph constituents in *T. molitor* (Soltani, 1990). In the control, newly emerged adult females, the amount of protein was 0.71±0.18 µg mg ovaries^{-1} and 53.9±18.5 µg pair ovaries^{-1}. However, in the LD_{10}-treated newly-emerged adult females, it was reduced up to 36.6% when calculated per mg ovaries and 46.6% per pair ovaries. In the LD_{30}-treated newly emerged adult females, it was reduced 40.8% when calculated per mg ovaries and 54.6% per pair ovaries (Table 3.1).

Maturation of insect testes also depends, among other factors, upon the materials that are taken up from the surrounding haemolymph and by materials synthesized by the testes *in situ*. These materials include protein, lipid and carbohydrate, all of which are required for development of the genital tract (Kunkel and Nordin, 1985; Kanost et al., 1990). In the present work, the decrease in testis protein content in chlorfluazuron-treated males may have several causes, such as lack of protein in the haemolymph of the males, and/or interference with the mechanism controlling spermatogenesis. In the control, newly emerged adult males, the amount of protein was 0.95±0.03 µg mg testis^{-1} and 3.25±0.44 µg testis^{-1}. In the LD_{10}-treated newly-emerged adult males, it was reduced up to 27.4% when calculated per mg of testis and 42.5% per testis. In the LD_{30}-treated newly-emerged adult males, it was reduced up to 51.6% when calculated per mg testis and 71.7% per testis (Table 3.7).

Barnby and Klocke (1987) found increased digestibility and reduced weight in azadirechtine-treated larvae of tobacco bud worm as a result of longer stay of protein in the gut and large accumulation of proteolytic enzymes. The reduction in larval weight and protein content was also observed in *H. armigera* larvae when fed on azadirechtine-treated diet (Javaid, 1989). The protein and peptide content were reduced in house fly larvae when fed on NFB- and nimocinol- (neem compounds) treated diets (Nizam, 1993). The present findings agree with those previous results. In the present work, sublethal doses of chlorfluazuron inhibited protein content in both ovaries and testes in newly emerged adults. IGR and other pesticides have been reported as protein inhibitors in various insect species, e.g. Chang et al. (1974), Philips and Loughton (1979), Ahmad and Naqvi (1985), Naqvi et al. (1986), Rizvi et al. (1986), Akhtar (1989), Javaid (1989), Naqvi et al. (1989), Masood (1990) Yasmeen et al. (1991) and Azmi (1993). Pesticides inhibit protein due to their poisonous effects. For examples, DDT, malathion, pyrethroids and IGRs reduced the protein content in susceptible strains of house flies, mosquitoes, stored grain pest and moths. In this work, reduction in protein content was observed in *S. litura* when sublethal doses were applied against newly-ecdysed fifth-instars. These results agree with the findings of previous workers. If differences are found, that may be due to differences in insect species or in methods or in IGR used.

Free amino acid composition of protein in the haemolymph of rice stem borer was affected by IGRs in the fat body (Chang et al., 1974). Ahmad and Naqvi (1985) reported on the toxic effect of dimilin against mosquitoes and flow rate of protein metabolites was affected. That may be due to changes at the molecular level. Grosscurt and Anderson (1980), Rizvi et al. (1986) and Hasan et al. (1987) reported changes in insect protein pattern after IGR treatment. Naqvi et al. (1986) reported similar results when cockroaches were treated with dimilin and penfluron. Naqvi et al. (1989) reported inhibition in the protein content of *Bl. germanica* and *M. domestica* larvae when treated with dimilin. Nizam (1993) reported 26% inhibition in protein content in *M. domestica* larvae when treated with dimilin. Similarly, in the present study, the protein content was reduced by the LD_{10} or LD_{30} doses in ovaries and testes. The percentage inhibition was greater than to Nizam (1993) results. This may be due to the different IGR used, application method or insect variations.

Activation instead of inhibition of some protein content was found in resistant strains of some insects (Naqvi et al., 1986; Akhtar, 1989). Nizam (1993) reported activation in malathion-treated larvae that were five-fold resistant to malathion, whereas IGRs and neem compounds inhibited protein content. However, in the present study, there was no activation but rather reduction in protein content in ovaries and testes. There is no report available that indicates activation of protein content in insects as result of application of chlorfluazuron. Diflubenzuron applied by dipping (10 mg ml^{-1}) at pupal ecdysis disturbed the development and also the changed the haemolymph titre of different carbohydrate metabolites. The pattern of haemolymphatic protein was also slightly affected after treatment (Soltani, 1990). In this study, the testis constituent of newly emerged male adults showed that sublethal doses of chlorfluazuron significantly reduced the amount of protein in the LD_{10}-treated and more significantly reduced in the LD_{30}-treated males compared with the controls, measured either in µg mg $testis^{-1}$ or µg $testis^{-1}$. However, the carbohydrate content of testes was not significantly lowered by these doses when measured in µg mg $testis^{-1}$ but compared with the controls were significantly lowered when measured in µg $testis^{-1}$. The lipid content of testes was not significantly lowered by these doses when measured in µg mg $testis^{-1}$. However, compared with the controls, they were significantly

lowered when measured in µg testis^{-1}. Moreover, these doses were reduced only protein contents but did not have effects on the charbohydrate and lipids contents in ovaries.

The proteins, lipids and carbohydrates present in the zygote of insects are a nutritional store for subsequent embryogenesis (Bownes et al., 1988). The morphological and temporal embryonic stages of *S. litura* affected by sublethal doses of chlorfluazuron applied topically to newly-ecdysed fifth-instars were allowed to description of effects of sublethal doses of chlorfluazuron exerted through eight embryonic stages during 0-84 hours of development of eggs after oviposition. In the current results, in chlorfluazuron-treated eggs, the decrease in protein content was presumed to have several causes, such as the lack of protein in the ovarioles development or interference of chlorfluazuron with the mechanism controlling yolk utilization. The protein-filled vesicles formed most of the volume of yolk in the oöcyte during oögenesis. Lipids were stored in insect eggs in the protein yolk bodies as well as in the triacylglycerol droplets (Telfer et al., 2009). Autoradiography of follicles labelled *in situ* with ^{3}H-glucose indicated that synthesis and deposition of glycogen were most rapid in the cortical cytoplasm of the oöcyte, with the assembled particles later moving into interstices between the lipid and protein yolk bodies deeper in the oöcyte (Mundall and Law, 1979). Presently, in chlorfluazuron-treated eggs, decreased in the protein, carbohydrate and lipid contents might be expected result of interference of chlorfluazuron in the similar mechanisms. The termination of vitellogenesis, a key step in late follicular development, normally occurred in the Cecropia moth, *Hyalophora cecropia* Linnaeus follicles when they had reached a length of 2 mm. But incubation in membrane-permeable analogues of cyclic adenosine monophosphate (cAMP) could induce the response in any vitellogenic follicle, regardless of its size (Wang and Telfer, 1996). The response was due to closure of the intercellular spaces (Wang and Telfer, 1997). Synthesis of the sulfated glycosaminoglycans deposited in the intercellular channels of the follicular epithelium was inhibited, water uptake causes the follicle cells to swell and close the emptied channels and tight junctions form between neighboring follicle cells. These were exactly the changes in nucleic acids exhibited by follicle cells during *in situ* termination of vitellogenesis (Wang and Telfer, 2000).

In the present work, the effect of chlorfluazuron on the DNA and RNA contents was estimated according to the method described by Burton (1956) and Munro (1966). The general growth of the insects is controlled by moulting hormone (MH) and juvenile hormone (JH) whose metabolism and degradation are checked by enzymes. If degradation continues then further action by that particular hormone will stop and ultimately growth of the insect is retarded. Later, it was suggested by Socha and Sehnal (1973), MH activated the synthesis of RNA and JH simultaneously induced the duplication of DNA. It means that growth hormones affect nucleic acid production and their quantity. A number of researchers (e.g. Attri and Ravi, 1980 a and b; Naqvi et al., 1989; Naqvi et al., 1993) worked on the aspect that IGR could act as a toxicant. They revealed a moderate to high level of inhibition of both nucleic acids. Philips and Loughton (1979) reported inhibition in RNA and protein synthesis. Sixty percent RNA inhibition was found after dimilin treatment. In the present work, both DNA and RNA were inhibited by the chlorfluazuron during ovaries, testes and egg development. However, inhibition was greater for DNA than RNA. Total RNA decreases rapidly whereas DNA content decreases steadily. Therefore, DNA was more sensitive to chlorfluazuron because it has longer half-life than dimilin. Chinzei and Tojo (1972) and Premkumar et al. (1991) described variations in DNA and RNA contents while studying them in *B. mori* and water scorpion, respectively. These reports are supporting by

the present findings. The percentage DNA and RNA was different after sublethal doses even when they were determined on same day of the same stage of *S. litura*. Sublethal doses of chlorfluazuron showed variation in their effects on DNA and RNA contents of ovaries and testes.

During mating sperm, were transferred from males by spermatophore into the bursa copulatrix of females. Sperm are stored here for a few hours and then, they are transferred into spermatheca of females, the depository organ for sperm storing, which consists of two parts, utriculus and legna. Mostly sperm are stored in utriculus, no sperm were observed in legna. The DNA content was estimated in utriculus after the 1st and 2nd matings. The DNA content in controls was 2.04±0.06 µg mg^{-1} and 1.75±0.08 µg mg^{-1} tissue after the 1st and 2nd matings, respectively, i.e., it was greater after first mating. However, after the second mating a greater number of sperm was transferred by the spermatophore. Perhaps more of the sperm were destroyed during travel from the *bursa copulatrix* to the *utriculus* of the spermatheca. However, significant reduction (1.25±0.09 µg mg^{-1} and 1.19±0.07 µg mg^{-1} after the 1st and 2nd mating, respectively) by the LD_{10} treatment and more significant reduction (0.85±0.08 µg mg^{-1} and 0.75±0.08 µg mg^{-1} after the 1st and 2nd mating, respectively) by the LD_{30} treatment were observed in DNA content compared with the controls. The reason was that DNA content is directly proportional to the number of sperm present in the testis. The number of sperm transferred to female during 1st and 2nd mating, in LD_{10}-treated *S. litura* significantly reduced and in LD_{30}-treated insects more significantly reduced (Perveen, 2008). Therefore, DNA content was also affected by sublethal doses (Table 3.5).

The DNA content in males significantly increased from newly-ecdysed sixth-instars until pre-pupae. Then, it remained constant from pre-pupae to before adult emergence (Fig. 3.2). After that in the control newly-emerged adults, it sharply and significantly decreased (Fig. 3.3). In I day-old adults, it decreased significantly but not very sharply. However, in LD_{10}-treated moth, the DNA content was significantly lowered and was even more significantly lowered in LD_{30}-treated moth compared with the controls (Figs. 3.2 and 3.3). However, the pattern was same from newly-ecdysed sixth-instars to before adult-emergence in both treatments. After that time variations were observed in both treatments compared with the controls, i.e. on the day of adult-emergence, the DNA content was significantly higher in treated insects compared with the controls. However, it was significantly lowered compared with the same treatment before adult-emergence. Again in 1 day-old adult, it was higher in treated insects compared with the controls; however, it significantly lowered compared with the same treatment (Figs. 3.2 and 3.3). These variations could be explained by the fact that the control newly-ecdysed sixth-instars to pre-pupae spermatogenesis was occurring and sperm were growing and developing, which required a greater quantity of DNA. Therefore, the DNA content significantly increased during these days. Then, from pre-pupae to before adult-emergence, the maturation of sperm took place, therefore, the DNA content did not significantly change but remained constant. After that, in control newly-emerged adults, sperm were transferred from the testis through the vas deferens to the seminal vesicles; therefore, the DNA content sharply and significantly decreased. This transfer of sperm continued till 1day-old adults. At that stage, it decreased significantly but not very sharply.

In LD_{10}-treated batches, the DNA content was significantly lowered and even more lowered still in LD_{30}-treated batches compared with the control during all larval development because of effect of chlorfluazuron. The pattern of decrease of DNA content was same from newly-ecdysed six-instars to before adult emergence in both treatments (Fig. 3.2) but different from controls. This was the time for spermatogenesis, growth and development of

sperm. After that, conditions were quite different in both treatments, i.e., on the day of adult emergence; the treatments significantly reduced the transfer of sperm from the testis through the vas deferens to the seminal vesicles. Therefore, the DNA content was significantly higher in the testis of treated insects compared with the control. However, it was significantly lowered compared with the same treatment before adult emergence (Fig. 3.3). Again, on the 1 day-old adults, the process of sperm transfer continued, therefore, the DNA content was increased in treated insects compared with the control. However, it was significantly lowered compared with the same treatment (Figs. 3.2 and 3.3).

In the seminal vesicles from before adult emergence to the 1 day-old adults of controls, the DNA content was continuously and significantly increased because sperm were transferred from the testis to the seminal vesicles through the vas deferens. Therefore, the DNA content sharply and significantly decreased in the testis. However, in the batches treated with both sublethal doses, it increased but the difference was not significant with treatments during adult development. In contrast, in the same batches of the testis, it significantly decreased. However, significant differences were observed with the LD_{10} and more significant differences with the LD_{30} compared with the controls over adult development. The reason was same, i.e. sperm were being transferred from the testis to the seminal vesicles but this was affected by the sublethal doses (Fig. 3.4).

When the DNA content of the control from *aedeagus* to the 1 cm tube was observed before adult emergence on the 1 day-old adults, it sharply and significantly increased because sperm were transferred from the seminal vesicles to this area. However, in batches treated with both sublethal doses, it was increased but the increase was not significant on the day of adult emergence because of treatments. But, it was significantly higher on the 1 day-old adults. Significant reduction with the LD_{10} treatment and even greater reduction with LD_{30} treatment were observed in the DNA content compared with the controls during adult development. The reason was that sperm were being transferred from the seminal vesicles to the aedeagus and to the 1 cm tube were affected by sublethal doses (Fig. 3.5).

The important point in the present results data was that both doses have a wide range of variations (Figs. 3.3-3.5) in their effect on DNA and RNA content. There were not only significant differences in DNA and RNA contents in the ovaries and testes when the treatments by both sublethal doses were compared with controls but significant differences were also observed between the sublethal doses (LD_{10} or LD_{30}) during different days of development of insect. It could be concluded that, primarily, chlorfluazuron has properties like other pesticides and, secondarily, it is a toxicant. That is why it is registered as one of the IGRs.

There are some previous reports on IGRs, effect on DNA and RNA. Philips and Loughton (1979) reported that actinomycine, dimilin (an IGR) and cyclohexamide inhibited RNA and protein synthesis in fourth instar nymphs of *L. migratoria*. About 60% inhibition of RNA was obtained by these compounds as compared with inhibition of RNA in the present work of about 3–8% by LD_{10} and 5–16% by LD_{30} doses in ovaries (Table 3.4) and 14–16% by LD_{10} and 27–38% by LD_{30} doses of chlorfluazuron in testes during different days of developmental stages which were observed (Figs. 3.2 and 3.3).

However, in the present work 19–50% inhibition of DNA was obtained as by LD_{10} and 41–83% by LD_{30} dose in ovaries (Fig. 3. 4) and 12–22% by LD_{10} and 27–43 %by LD_{30} dose in testis during different days of developmental stages which were observed (Figs. 3. 2 and 3.3). Saleem and Shakoori (1987) reported that the DNA content of *T. castaneum* remained unaltered when treated with sublethal doses (1.0 and 2.0 ppm) of permethrin. However, the

RNA content decreased 16% and 28% at these concentrations. Shakoori et al. (1988) reported that at higher doses of fenpropathrin, DNA and RNA contents were decreased up to 20% to 21%, respectively, in sixth-instar larvae of *T. castaneum*. Shakoori and Saleem (1989) reported that malathion was ineffective on the DNA and RNA content, but permethrin and malathion increased 28% and 27% the DNA and RNA activity after 120 hours of treatment. DNA increased up to 23% and 27% and RNA increased up to 14% and 18% by the pyrethroids, respectively. Tabassum (1994) agreed with earlier findings, where DNA and RNA were slightly inhibited by dimilin in *Tribolium* spp. In that case, dimilin inhibited by 33%, 21% and 32% the RNA content and by 44%, 50% and 43% the DNA content after 24, 72 and 144 hours, respectively, using a glass film method. Naqvi et al. (1992) reported nucleic acid inhibition in *M. domestica* when treated with NC and solfac.

Shakoori et al. (1985) reported that a sublethal dose (200 ppm) as well as a lethal dose (400 ppm) of a pyrethroid, fenpropathrin, against sixth-instar larvae of *T. castaenium* did not change the DNA and RNA content much under laboratory conditions. In another report of Saleem and Shakoori (1985) observed that the IGR, diflubenzuron, did not cause significant changes in DNA and RNA content in *Tribolium* spp. However, in the present research, the effects of sublethal doses (LD_{10} and LD_{30}) of chlorfluazuron on the inhibition of DNA and RNA content were observed and the inhibition of DNA and RNA were greater under for LD_{30} than LD_{10}. Thus, comparatively higher inhibition of DNA and RNA may be correlated with the fact that chlorfluazuron is a growth retardant.

In the present work, the highest inhibition level was observed after LD_{30} treatment by chlorfluazuron compared with the controls and the LD_{10} treatment which supports biodegradation of DNA concept (Stokes and Redfern, 1982; Jacobson et al., 1984). Different levels of inhibition may be due to different doses on female and male treatments (Table 3.4, 3.6 and 3.10; Figs. 3.2 and 3.3). DNA and RNA play an important part in living organisms and inhibition of nucleic acid consequently results in inhibition of proteins synthesis.

The DNA contents in testes was reduced significantly with LD_{10}-treatment and more significantly reduced with LD_{30}-treatment compared with the controls and the pattern of DNA contents was also the same, during larval development, however, the pattern of DNA was different during development of pupae and adults. The DNA contents were reduced in control as compared with LD_{10}- and LD_{30}-treatments. The RNA content in testes was reduced significantly with LD_{10}-treatment and more significantly reduced with LD_{30}-treatment compared with the controls, throughout larvae, pupae and adults. Nucleic acids synthesized in the zygote play an inductive role in blastulation and gastulation during early embryogenesis (Czihak and Horstadius, 1970). Recent results of significant decrease in nucleic acids in chlorfluazuron-treated eggs might be interfere in the same mechanism may clarify in future. Like many other insects, lepidopteron provision their eggs with high concentrations of ecdysteroids that were made available to developing embryos and prehatching larvae (Hoffman et al., 1980). The inhibition of DNA and RNA by chlorfluazuron in the present studies was higher than reported by Saleem and Shakoori (1985) and Shakoori et al. (1988). The higher inhibition may be because chlorfluazuron is an IGR. The growth disruption and abnormal growth caused by chlorfluazuron may be due to inhibition of nucleic acids and consequently, inhibition of protein synthesis. However, to confirm this needed to do more research work in this connection.

4.1 Effects on ecdysteroid titres of ovaries and testes

Ecdysone not only controls the ecdysis but also plays an important role in development and maturation of the reproductive system. It is not only produced by the prothoracic glands but

is also produced by the ovaries and testes themselves as well as their sheaths (Loeb et al., 1984; Loeb et al., 1986a and b; Gelman et al., 1988). It has been reported that diflubenzuron could affect ecdysteroid secretion from other organs such as: the epidermis in *T. molitor* (Soltani, 1984), ovaries in *C. pomonella* (Soltani et al., 1989a and b), and the concentration of haemolymph constituents in *T. molitor* (Soltani, 1990). In the mosquito, *A. aegypti*, the ovary begins to produce ecdysone after a blood meal. Ecdysone, after being converted to 20-hydroxyecdysone, stimulates the synthesis and secretion of vitellogenin by the fat-body. The vitellogenin is taken up by the growing oöcytes and becomes part of the yolk (Hagedorn et al., 1979). In the present research, in *S. litura*, according to current evidences would tend to suggest that ecdysteroid does not play the same role as described by Hagedorn and co-workers. Ecdysteroid titre was observed in 7 day-old pupae to 4 day-old adult female ovaries.

Soltani and Mazouni (1997) reported ecdysone levels that when ovaries from 4 day-old females of mealworm, i.e. at the end of vitellogenesis were cultured in media supplemented with diflubezuron, preliminary data indicated that ovarian amounts of ecdysteroids, as measured *in vivo*, changed during vitellogenesis of the *T. molitor* in a characteristic way. The amounts were low during pre-vitellogenesis, increased during vitellogenesis, peaked at the time of choriogenesis, and decreased when the insects started to deposit eggs and thereafter. Diflubezuron at the two tested doses (5 and 10 μg ml^{-1} medium) significantly decreased the amounts of ecdysteroid produced *in vitro* by ovaries. In contrast, the neurohormone stimulated the *in vivo* production of ecdysteroids by the ovaries. The neurohormone stimulated the *in vivo* production of ecdysteroid by the ovaries. In the present research, the ecdysteroid titre in the ovaries was determined after each consecutive 24 hours on the 7 day-old female pupae to 4 day-old adult females. Preliminary data from the controls indicated that the ovarian ecdysteroid, as measured *in vivo*, changed during vitellogenesis of *S. litura*, in the same characteristic way as reported in the meal worm. Chlorfluazuron at the two tested sublethal doses significantly affected the amounts of ecdysteroid produced *in vivo* by the ovaries. It significantly lowered in the LD_{10}-treated females and even more significantly lowered in the LD_{30}-treated females. However, the pattern of the levels of ecdysteroid titre secretion was the same as in the controls (Fig 3.1).

Spermiogenesis of the spermatocysts in the diapausing pupal testis of *M. brassica* was investigated *in vitro* with reference to ecdysteroids released from the testis. The quantity of the ecdysteroids and activity of spermiogenesis induction were measured in a medium conditioned with the culture of testes for 1 to 6 days (Shimizu et al., 1985). In the present research, ecdysteroid titre was observed in testes of *S. litura*. In the early development of last-(sixth)-instar larvae (from 0 to 16 hours), when the testes were very small, the ecdysteroid titre of the testes was very low and remained unchanged. As the testes increased in size and, simultaneously, spermatogenesis increased, the titre significantly increased until 80 hours. After 80 hours, when the larval paired testes fused to form a single testis, the titre was significantly reduced until the pre-pupal stage. Eighty hours after ecdysed of sixth-instars to 208 hours (5 day-old pupae), three peaks of ecdysteroid titre were observed. It is suggested that this times was the peak times of spermatogenesis. At 208 hours, the different developmental stages of sperm were present (Fig. 3.6).

The function of testis ecdysteroid is still open to speculation. However, ovarian and testicular ecdysteroids were important for the development and maturation of the reproductive tracts and play an important role in oögenesis and spermatogenesis. The

ecdysteroid, 20-hydroxyecdysone, is believed to be necessary for maximal spermatocyte mitosis and resumption of sperm development after diapause as well as testes fusion and genital tract maturation in lepidopterans (Nowock, 1972, 1973). Ecdysteroid titre in the present research suggest it, might perform the same function as described above. The testes of *H. virescens* and *L. dispar* were examined *in vitro* for the ability to produce ecdysteroids. Production was detected in the testes removed from larvae at mid- and late-periods in last-instar larvae, in the testes from pupae after the 3rd day of pulpal development and in testis taken from young male adults (Loeb et al., 1984). In the present research, the testes of *S. litura* were examined *in vivo* for the ability to produce ecdysteroid. Production was detected in the testes removed from sixth-instar larvae from 0 hour to 88 hours (pre-pupae) after each consecutive 8 hours and in testis from pupae (112 hours) to 2 day-old adults (376 hours) after each consecutive 24 hours. It was observed that very low ecdysteroid titres were present in the control newly-ecdysed sixth-instars. The titre was not significantly increase until 16 hours of 2 day-old sixth-instars. However, after 16 hours of 2 day-old sixth-instars till pre-pupae (80 hours), it was significantly increased. After 80 hours to 208 hours (5 day-old pupae), it was also significantly increased with three peaks of ecdysteroid titre observed in the testis. After that it slowly decreased till 376 hours (2 day-old adults) (Fig. 3.6).

It was the testis sheaths rather than the content that was physiologically active for the production of ecdysteroid (Gelman et al., 1988). Gonadal ecdysteroid can stimulate the production of growth factor from the sheath which in turn, promotes the growth, development and maturation of the genital tract (Loeb et al., 1996). Tissues from the irregular testis sheath and its extensions that form the follicle wall were responsible for the production of ecdysteroids (Loeb, 1986). In the present study there was might be the same source of secretion of ecdysteroid and they performed the same functions as described by Gelman et al. (1988) and Loeb et al. (1986, 1996). Soltani et al. (1989a and b) reported that dipping newly-ecdysed *T. molitor* pupae in an acetone solution of diflubenzuron prevented most of them (73%) from completing development. Such blocked insects did not secret the adult cuticle and remained apolysed. Their ecdysteroid level analysed by radioimmunoassy was not increase. However, injection of 20-hydroxyecdysone (2–10 μg) several days after diflubenzuron application allowed the secretion of a new cuticle but with an abnormal architecture but with a high content in N-acetyl-amino sugar as revealed by fluorescent wheat germ agglutinin (Soltani et al., 1984a and b). The effects of diflubenzuron were observed on the larval-larval and larval-pupal ecdysis cycles of *T. molitor*, after treatment at ecdysis. In both cases, the first part of the cycle, from ecdysis to apolysis was apparently not affected, but the pharate periods were lengthened; treated insects were generally unable to perform ecdysis and died. The ecdysteroid titres in the haemolymph of treated insects were measured with a radio-immunoassay and compared with the controls. During larval-larval cycles, the single ecdysteroid increase was not affected by the diflubenzuron treatment. In the present research, the effect of sublethal doses of chlorfluazuron on ecdysteroid in the ovaries and testes were determined. The titres were significantly lowered in LD_{10} and even more significantly lowered in the LD_{30} treated insects compared with the controls, from the 7 day-old female pupae to the 4 day-old female adults after each consecutive 24 hours in the ovaries and during the 2 day-old sixth-instar larvae (0 hour) to pre-pupae (88 hours) after each consecutive 8 hours and from pupae (112 hours) to 2 day-old adults (376 hours) after each consecutive 24 hours in the testes during life-span of *S. litura* (Figs. 3.1 and 3.6).

During larval-pupal development, a significant difference was observed; whereas two ecdysteroid peaks occurred in the controls; but the first peak was not modified (Soltani et

al., 1989a and b). During the present work, between 80 hours to 208 hours (5 day-old pupae), three peaks of ecdysteroid titre were observed in testes. The first peak was between 80 – 88 hours; the second between 88–136 hours and between third or last was between 136–208 hours old. After that, the titre slowly decreased (Figure 3.6). However, the ecdysteroid titre in testes was reduced significantly with LD_{10}-treatment and more significantly reduced with LD_{30}-treatment compared with the controls. Therefore, these reductions were responsible for reduction in different parameters of spermatogenesis and testicular development reported by Perveen (2000b).

Ecdysteroids control vitellogenesis and egg maturation in pharate adult females of the Indian meal moth, *Plodia interpunctella* (Hubner) (Shirk et al., 1990). They include ecdysone and 20-hydroxyecdysone, as well as hydrophilic conjugates of these and other ecdysteroids in both the silk moth, *Bombyx mori* Linnaeus (Ohnishi and Chami, 1977) and H. *cecropia* (Rubenstein et al., 1986). Assays of individual follicles in *Hyalophora* ovarioles showed that the ecdysteroids accumulate during both vitellogenesis and post-vitellogenic water uptake. In greater wax moth, *Galleria mellonella* Linnaeus the total accumulation was equivalent to 74 µg of ecdysone gram of $eggs^{-1}$ (Hsiao and Hsiao, 1979). That 20-hydroxyecdysone directly or indirectly triggers aspects in ovarian development became apparent during the metamorphic moults (Ohnishi et al., 1977). In the present research, the ecdysteroid titre was continuously increased in untreated eggs throughout the embryogenesis till hatching with the great variations during 0–84 hours of development (stages 1–8). When segmentation and blastulation were started during 0–4 hours (stages 1–2), a very low peak of ecdysteroid was observed. After that three peaks of ecdysteroid were observed which were correlated with events during embryogenesis. Initially, in the 32 hour old egg (at the late stage 6), when two head positions, the frontal and lateral positions were marked, the first lowest peak of ecdysteroid was appeared. Secondarily, in the 64 hour old egg (at the late stage 7), when organogenesis was established and the embryo was appeared larva-like, the middle peak of ecdysteroid was appeared. Finally, in the 84 hour old egg, when the embryo resembled the first instar; head darkness, appendages of the head segments, tracheal tubules and limbs were visible and the larva was ready to hatch at the late stage 8, the last highest peak of ecdysteroid was appeared.

It has been reported that DFB could affects ecdysteroid secretion from other organs, such as the epidermis, in the yellow mealworm, *Tenebrio molitor* Linnaeus (Soltani 1984), ovaries in *C. pomonella* (Soltani et al., 1989a and b), and the concentrations of haemolymph constituent in *T. molitor* (Soltani, 1990). In chlorfluazuron-treated eggs, the LD_{10} significantly ($P<0.05$) and LD_{30} more significantly ($P<0.05$) reduced the ecdysteroid titres during development of eggs (Fig. 3.10). However, the patterns of the ecdysteroid titres were the same as in untreated ones (Fig. 3.10). Moreover, the first and second peaks appeared 4 hours later in LD_{10}-treated and 8 hours later in LD_{30}-treated eggs compared with untreated ones (Fig. 3.10). Therefore, sublethal doses of chlorflazuron were extended their effects on embryogenesis of *S. litura* through reduction ecdysteroid titre in egg' contents during the eight embryonic developmental stages of progenies in F_1 generation.

5. Summary

In *Spodoptera litura*, sublethal doses of chlorfluazuron (LD_{10}: 1.00 ng $larva^{-1}$ or LD_{30}: 3.75 ng $larva^{-1}$) topically applied at newly-ecdysed fifth-instars of *S. litura*, reduction in parameters of oogenesis (Perveen and Miyata, 2000), spermatogenesis (Perveen, 2000b), insemination

(Perveen, 2008) and embryogenesis (Perveen, 2009c) were ultimate effects of reduction of biochemical of the ovaries and testes which were analysed here with reference to effects of sublethal doses of chlorfluazuron. The same doses of chlorfluazuron significantly reduced the protein, DNA, RNA contents and ecdysteroid titre with no effects on the carbohydrate and lipid contents in ovaries during developmental days of females, which resulted in reduction of ovarian weight, length of ovarioles, thickness of follicular epithelium, number of mature ova, cell density germarium and size of basal oöcytes. The ovarian ecdysteroid titre, measured *in vivo*, changed during vitellogenesis in a characteristic way: the titre was low during pre-vitellogenesis, increased during vitellogenesis and peaked at choriogenesis. Sublethal doses also significantly reduced the protein, carbohydrate, lipid, DNA, RNA contents and ecdysteroid titre during developmental days of males, by effecting size and weight of testis, thickness of testis sheath, size and number of eupyrene and apyrene sperm bundles. During testis development, three peaks of ecdysteroid titre were observed. The second peak was appeared at 88–136 hours when larval paired testes started to fuse to form a single testis. Sublethal doses also effects insemination by reducing spermataphore weight, number of inseminated sperm. Sublethal doses also significantly reduced the protein, carbohydrate, lipid, DNA, RNA contents and ecdysteroid titre during embryogenesis. Three peaks of ecdysteroid titre were observed which were correlated with events during embryogenesis. The last highest peak was appeared at 84 hours when larva was ready to hatch at the late stage 8. The embryo resembled the first instar; head darkness, appendages of the head segments, tracheal tubules and limbs were visible. However, patterns of reduction in protein, carbohydrate, lipid, DNA, RNA contents and ecdysteroid titre were the same in all tests. Finally, collective effects of all parameters have been resulted reduction in the fecundity, fertility and hatchability of *S.litura.*

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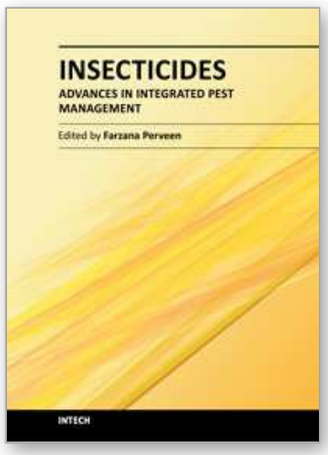

Insecticides - Advances in Integrated Pest Management

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This book contains 30 Chapters divided into 5 Sections. Section A covers integrated pest management, alternative insect control strategies, ecological impact of insecticides as well as pesticides and drugs of forensic interest. Section B is dedicated to chemical control and health risks, applications for insecticides, metabolism of pesticides by human cytochrome p450, etc. Section C provides biochemical analyses of action of chlorfluazuron, pest control effects on seed yield, chemical ecology, quality control, development of ideal insecticide, insecticide resistance, etc. Section D reviews current analytical methods, electroanalysis of insecticides, insecticide activity and secondary metabolites. Section E provides data contributing to better understanding of biological control through Bacillus sphaericus and B. thuringiensis, entomopathogenic nematodes insecticides, vector-borne disease, etc. The subject matter in this book should attract the reader's concern to support rational decisions regarding the use of pesticides.

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