Influence of insect growth regulator, BAY SIR 8514, on chitin synthesis and degradation of *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract: The present study has been conducted to investigate the effect of insect growth regulator BAY SIR 8514 on chitin and chitinase enzymes of 2nd in star of *Spodoptera littoralis* larvae, the effect of sublethal doses, LC_{25}, LC_{50} and LC_{90} shows a significant effect on antifeedant index, on the other hand larval treatment with the tested IGR doses revealed, certain histo-pathological symptoms such as (1) diorganisation, disintegration and vacuolization of oocytes and sperms. Insect treated with LC_{25} protein was found to have the highest number of bands (13 bands), while those treated with LC_{50} was found to have the lowest number (6 bands). The variability analysis of normal and treated insects showed some variations. Insects treated with LC_{25} showed 4 monomerphic and 11 unique bands, those treated with LC_{50} showed 4 monomerphic and 12 unique bands, those treated with LC_{90} showed 5 monomerphic and 5 unique.

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Key words: Insect growth, IGRs**,** BAY SIR 8514, pest control, chitin synthesis, lepidoptera.

1- Introduction

Chitin is the most common natural amino polysaccharide mainly synthesized in body wall of insect. Chitin is a linear polymer of B-(1-4) – linked N- acetyl- glucosamides. Chitin synthesis in bluiltup for growth and development. Cuticular exoskeleton in regularly shed and replaced by new one (**Merzendorfer and Zimoch, 2003**). Chitin is also found in internal structures of insects such as occurrence in trachae, it also a constituent part of peritrophic membrane that line the inner surface of mid gut of many insects (**Lehane, 1997; Barbehenn and Stannard, 2004**). Moreover, chitin synthesiz ing enzymes may prove promising as targets for new insecticides. Insect molting and metamorphosis are regulated by ecdysterone, a steroid hormone secreted cyclically, into the hemolymph by the prothoracic gland (**Sehnal, 1989**). Ecdysterone acts primarily on gene transcription by activating a nuclear receptor heterodimer consisting of the ecdysteroid receptor (Yao**, *et al.,* 1993**). Control of some pests depends on some techniques that based on natural compound which inhibit their growth and development.

An increase or an inhibition of glycosidase activity can induce beneficial effects because of chitinase belong to glycosidase. Glycosidases activate can be inhibited by using insect growth regulators.

Coordination of chitin syntheses and its degradation requires strict control of enzymes during development (**Merzendorfer and Zinoch, 2003**). The search for new compounds with satisfactory properties concerning their effects on the target pests as well as an environment. It is necessary to find new sources for pesticide production. Accordingly, attention has been given recently to synergistic affect between IGRS and Chitinase enzyme metabolism such as BAY SIR 8514 Moreover, due to unique properties. Chitin- itself attracting more and more interest in pest control management. In these paper, the effects of insect growth regulator will be discussed and we will focus on the antifeedant effect, degradation of chitin in body wall, ovary and testis as well as to investigate the effect of sublethal doses (LC_{25}, LC_{50}, LC_{90}) on the vira – like chitinase gene. We will use BAY SIR 85 14 (1-4 trifluromethoxyphyl)-3- (2- chlorobenzoylurea) as insect growth regulator. Genes and CDNAs encoding insect chitinases have been identified from several lepido- pterouss insects such as *Manduca sexta* **(Kramer *et al.,* 1993**) *Bombyx mori* and *Hyhpantria cunea* (**Rim *et al.,* 1998**) *Spodoptera lilloralis* (**Shinoda *et al.,* 2001**) *Choristoneura fumiferana* (**Zheng *et al.,* 2002**) and *Spodoptera frugiperda* (**Bolognese *et al.,* 2009)** *Spodoptera littoralis* **(Tyne and Possee 2005)**.

2- Material and methods

1- Insect rearing technique:

The colony of *Spodoptera littoralis* was supplied from Hadalsham field region Jeddah – Saudi Arabia. larval stages were reared on castor bean leaves. The culture and experiments were conducted at 27 $\pm$2 ºC and 55 $\pm$ 5% RH.

2- Toxicological studies

A Leaf dipping technique was carried out to evaluate insecticidal activity of IGR, BAY SIR 8514, (-4- (trifluoromethoxyphenyl) -3-2-

chlorobenzoylurea) against 2nd instar larvae of *S. littoralis*. Five concentrations of the tested IGR were used. One hundred of starved larvae distributed in four replicates (25 larvae in each replicate) were used for each concentration. And treated according to **Baker *et al.* (2012)**. Laval mortalitywas recorded. and corrected by Abbott's formula (1925) and analyzed by probit analysis (**Finney, 1971**) to obtain LC_{25}, Lc_{50} and LC_{90}. The data were expressed as means + standard errors. The statistical significance of differences between individual were determined by using one way ANOVA test. Levels of significance of each experiment was stated to be significant at ($P \leq 0.05$), high significant at ($P \leq 0.001$). The deterrent index was calculated according to **Lundergren (1975**). Larvae released. On treated Leal press (7.5 x 7.5 cm).

3- Histopathological Technique:

The full grown adults of the cotton leaf worm *S. littoralis* resulted from 2nd instar larvae treated with the tested IGR at the dose of LC 25, LC $_{50}$ and LC $_{90}$ were applied to examine the integument, ovary and testis. Samples of treated and untreated adults were taken for histological preparation in each treatment. The integument and both ovary and testis were removed and fixed in alcoholic Boun's solution for 24 hrs them processed in the usual manner for paraffin embedding. Serial sections of 5 microns were made by the rotary microtome. The sections were spread on clean glass microscopal slides by the usage of the hot plate for a period of 15 min. and then stained with haematoxylin for nucteus and oesin for cytoplasm and call wall. The virgin adults resulted from 2nd larval instar of *S. littoralis*. Check group and treated ones were dissected in ringer's saline solution under a dissecting Nikon microscope to remove gonads of adults. Testes and ovaries were fixed in alcoholic Bouin's fluid for 24 hrs. Washing and dehydration were carried out by an ascending series of ethyl alcohol infiltration with wax takes place by transferring to xylene – paraffin mixture, for 30 min, and then to three successive paraffin baths at 58 – 60 ºC., then embedded in pure paraffin. Sectioning takes place by using Rotary microtome at thickness of 5 microns paraffin ribbons were affixed on clean glass microscopal slides and flattened by the usage of hot plate for a period of 15 min. then staining takes place by using double stain. i.e., haematoxylin for nucleus and eosin for cytoplasm and cell wall sections were put in heamatoxylin for 5 min, and rinsed in distilled water destained in 70% acid alcohol until light pink colour of specimen was appeared and then neutralized in tap water, stained with eosin (15 sec) and washed in 2 changes of 95% ethyl alcohol then in absolute alcohol. Clearing in 2 changes of xylene, and then mounted with Canada balsam, covered with clean glass covers and left the slides to dry in an electric oven at 37 ºC. However, as for ovaries they differ in certain steps in which. After fixation and dehydration they transferred from 95% ethyl alcohol to methyl benzoate for clearing until it settled in the bottles bottom, washed with benzene for 5 min and then transferred to benzene and wax mixture, then running to three bathes of paraffin wax and then complete the routine technique that previously described for testis preparation.

4- RAPD analysis:

RAPD Analysis – PCR reactions were run with 10 – mer RAPD primers OPA-01 through OPA-10, obtained from Oberon Technologies (Alameda, CA). Each 25 µl reaction was carried out with a single primer in the following mixture: 12.5 µl 2x go Taq form promega (Madison, WI), 1.0 µl primer (10 pmol / µl), 1.0 µl genomic DNA (20 ng /µl), and 10.5 µl dH_2O. the samples were amplified in a thermocycler with the following program: I cycle at 94ºC for 5 minutes, followed by 50 cycles at 94ºC for 30 seconds, 35 ºC for I minute, and 72ºC for 2 minutes, followed by I cycle at 72 ºC for 5 minutes, and a hold at 4ºC. PCR products were fractionated on a 1% agarose gel.

Sequencing procedure – Bands of interest were cut out of the gel and purified using the QIA quick gel extraction Kit (QIAGEN, USA). The DNA obtained was then legated into the pGEM- Transformation of cells followed the manufacturer's protocol (promega, Madison, WI). The transformation products were plated onto AMP / X- gal / IPTG LB agar plates and incubated at 37 ºC overnight. The plasmid with the DNA insert, were removed from each plate and incubated overnight at 37ºC. The plasmid was purified using the QIAprep Miniprep spin column (QIAGEN, USA). The isolated DNA was then sequenced by GENEWIZ (South Plainfield, NJ)

3- Results

Results recorded in table (1) showed that BAY SIR 8514 had a significant effect on the larval mortality, the corresponding concentration LC $_{25}$, LC $_{50}$ and LC $_{90}$ were 0.9, 1.2 and 1.8 ppm, respectively for 2nd instar larvae of *Spodoptera littoralis* treated with BAY SIR 8514 as insect growth regulator(IGR).

Table (1): Toxicological impact of BAY SIR 8514 against 2nd instar larvae of *Spodoptera littoralis.*

Larval instar	Toxicity in ppm			Slope Function
	LC 25	LC 50	LC 90	
2nd instar	0.9	1.2	1.8	2.0

Results in table (2) revealed that the tested IGR induce antifeedan't activity on 2nd instar larvae of *S. littoralis* treated with LC 25, LC 50 and LC $_{90}$ all tested doses cause antifeedant response with positive direction an dose increase.

Table (2): Antfeedant index in matching of BAY SI R 8514 against 2nd larval instar of *Spodoptera littoralis*.

Larval instar	Antifeedant index			Slope function
	LC_{25}	LC_{50}	LC_{90}	
2nd instar	2.1	3.6	9.8	3

Histopathological studies were carried out to discuss the effect of LC 25, LC50 and IC 90 of the tested IGR, on the different insect tissues. It was indicate sever histopathological changes, where observed in the structure of larval integument when treatment was carried out by IGR. These changes include elongation and disorganization of epidermal cells which completely separated from cuticle (Fig 1, 2, 3, 4). However, treatment of 2nd instar larvae with LC 90 induced the greatest histopathological damage by decreasing in the cuticular thickness in some areas and detachment from degenerated epidermal cells. Also, the histo- pathological damage was recorded in both ovaries and testis tissues.

All tested doses LC_{25}, LC_{50} and LC_{90} of BAY STR 8514 when used as chitinase inhibitor cause sever damage in both tissues of the ovaries and testis. Our results clearly indicated that all the tested doses were affected in inducing destruction in the follicular epithelium and vitelline membrane, shrinkage in the ova as well as degeneration in the nurse calls Fig. (5, 6, 7, 8). On the other hand the histopathological examination indicated the degeneration of the epithelial sheath and in distinction in the testicular follicle Fig. (9, 10, 11, 12), at all tested doses.

It is quite clear that the treatment of 2nd larval instar with LC90 created abnormalities in the testis of resulting males as shown in Fig 12, such as vacuolization's, destruction in the epithelial sheath indistinguishing the testicular follicle and degeneration of spermatogonia in some areas as well as dameged sperimatids were detected.

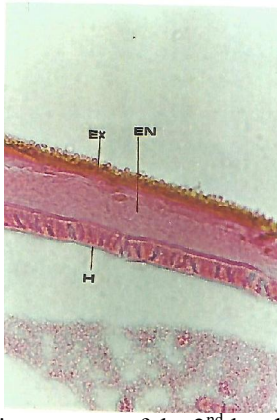


Fig 1: section of the integument of the 2nd laval instar of *S. littoralis*

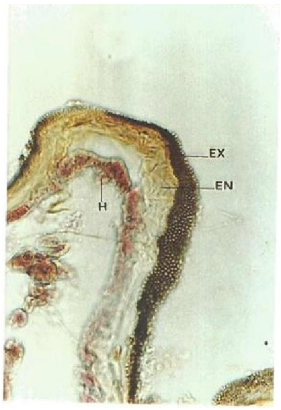


Fig 2: section of the integument of the 2nd larval instar of *S. Littoralis* Treated with LC25 of BAY SIR 8514.

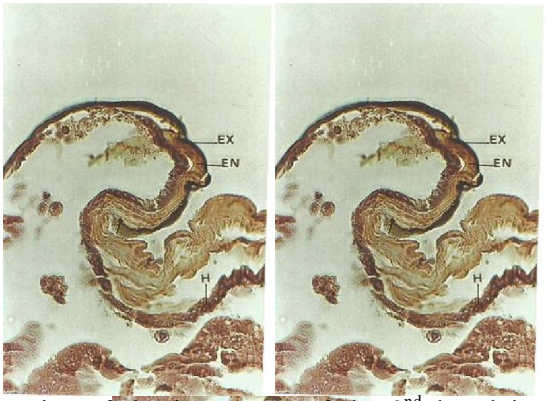


Fig 3: section of the integument of the 2nd larval instar of *S. littoraisl* treated with LC50 of BAY SIR 8514

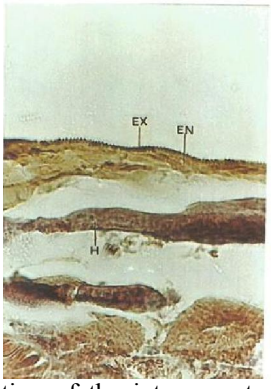


Fig 4 section of the integument of the 2nd larval instar of S. littoraisl treated with LC90 of BAY SIR 8514.

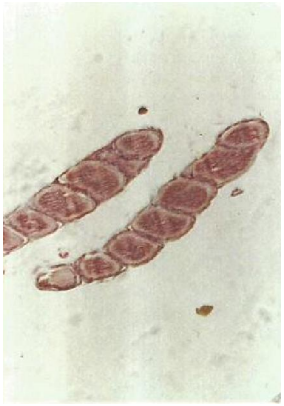


Fig 5: L.S. of the normal ovary of newly emerged female of *S. littoralis*

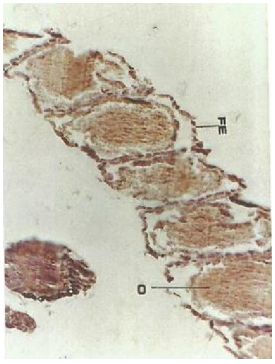


Fig 7: L.S of the ovary of newly emerged female of S. littoral in treated as 2nd larval instar with LC50 of BAY SIR 8514 showing highly degeneration of nurse cells and follicular epithelium.

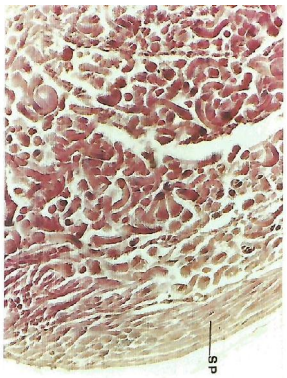


Fig 9: L.S. of the ovary of newly emerged female of S. littoralis treated with LC25 of BAY SIR 8514 showing highly degeneration of nurse cells and follicular epithelium.

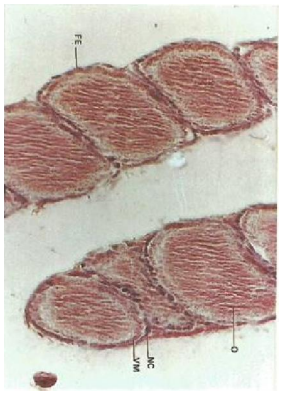


Fig 6: L.S. of the ovary of newly emerged female of *S. littoralis* treated with LC25 of BAY SIR 8514 showing highly degeneration of nurse cells and follicular epithelium.

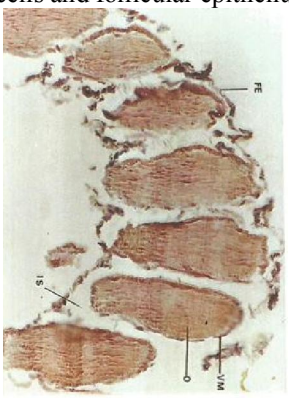


Fig8: L.S of the ovary of newly emerged female of S. littoral in treated as 2nd larval instar with LC50 of BAY SIR 8514 showing highly degeneration of nurse cells and follicular epithelium

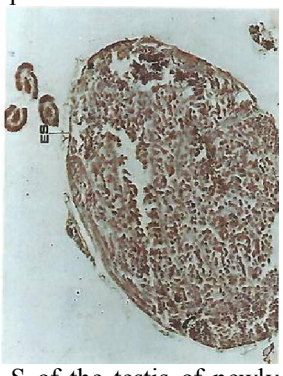


Fig 10: L.S of the testis of newly emerged male of *S.littoralis* treate as 2nd larval instar with LC50 of BAY SIR 8514 showing highly degeneration

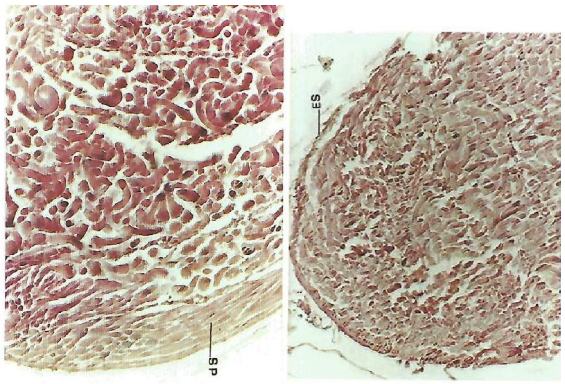


Fig 11: L.S of newly emerged male of *S. littoralis* treate as 2nd larval instar with LC50 of BAY SIR 8514

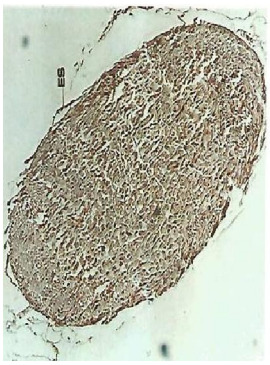
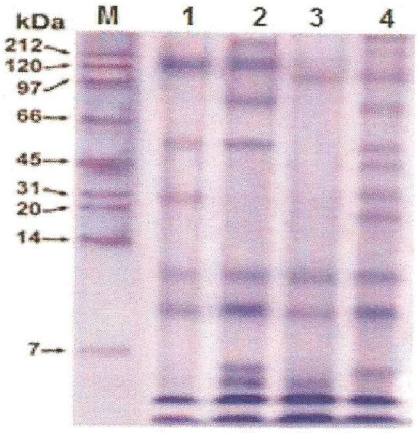


Fig 12: L.S of newly emerged male of S. littoralis treated as 2nd larval instar with LC90 of BAY SIR 8514

RAPD – analysis

Haemolymph chitinase showed a great difference in bonds with MW of 174.02, 147.61, 113.32, 7234, 47.50, 38.24, 29.53 and 21.41 KDa and R_m of 0.021, 0.058, 0.11,8 0.221, 0.316, 0.425 and 0.498.



Changes in the haemolympht chitinase enzyme banding patterns of *S. littoralis* second instar larvae treated with BAY SIR 8514 with sub lethal doses.

M: marker protein.

Lane 1: protein bands of insect treated with LC25.

Lane 2: protein bands of insect treated with LC50

Lane 3: protein bands of insect treated with LC90

Lane 4: protein bands of insect treated with water

Regarding the effect of LC_{25} of IGR on haemolymph protein profile of *S. littoralis* adults (2-4 days old), results were presented in the plate. The haemolymph protein was separated into 15 bands with MW ranged from 180.53 to 2.55 kDa and R_m from 0.012 to 0.987, the variability analysis was four monomorphic bands with 11.70, 7.74, 3.00 and 2.55 KDa and with 0.624, 0.733, 0.945 and 0.987 R_m three polymorphic bands with MW of 106.78, 67.68 and 28.77 kDA with R_m of 0.132, 0.236 and 0.431 while eight bands were unique with 180.53, 100.55, 48.38, 43.46, 22.76, 17.38, 9.33 and 5.29 kDa with 0.012, 0.146, 0.312, 0.337, 0.484, 0.545, 0.687 and 0.816 Rm the number of bands of treated (12 bands) exceeded that of control (7 bands) by five bands. The control was characterized by bands with MW of 106.78, 67.68 and 28,77 kDa and Rm of 0.132, 0.236 and 0.431. treated was characterized by bands with MW of 180.53, 100.55, 48.38, 43.46, 22.76, 9.33 and 5.29 kDa and Rm of 0.012, 0.146, 0.312, 0.337,0.484, 0.545, 0.687 and 0.816.

4- Discussion

Chitin in one of the most important biopolymers in nature, it junctions on scaffold material supporting the cuticles of epidermis, tracheae and some genetal ducts. Insect growth and development depends on the chitin synthesis and degradation. For this reason insect produce chitin and clzitinolytic enzymes in different tissues.

Chemical compounds that interfere with chitin metabolism, such as insect growth, regulator BAY STR 8514 have been of special interest for the control of lepidopteron pests, we will discusse the chitin and chitins changes an a result of administrating the tested IGR. Chitins on belong to glycosidase, Glycoside –ase activities can be inhibited. Efforts have been made to find chitinase inhibitors (**Solver man and Roosevelt, 1996**). BAY SIR 8514 may have a common mode of action and block a postcatalyitc step of chitin synthesis (**Nauem and smagghe, 2006**, **Asahora *et al.*, 2008**, **van lceuwne *et al.*, 2012**). Siso. Several intercalafina deyeo seem to distrupt the formation of micro fibrils

We have recently revisited the different effects induced by difiubenzuron, which have been previously found in several different insect species, in a single model beetle, the red flour beetle, Tribolium castaneum (**Merzendorfer *et al.*, 2012**) after oral application of difiubenzuron, we observed abortive molting. Defects in egg- hatching and reduced chitin amounts in the larval cuticle, the pm, and eggs. Electron microscopic

examination of the larval cu- ticle from 1. castancum revealed major structural changes and a loss of lamellate structure of the procuticle in sum mary benzoylureas Cause a variety of symptoms that at fact different tissues and cells (epidermis, tracheal sys- tem midgut epithelium, and acolytes) and possibly yield synergistic effects that eventually cause iethatiy during moltmg or egg hatching.

In addition to the above factors the maturation of an insect egg depends on the materials that are taken up from the surrounding haemolemph and materials synthesized by the ovary. These materials include protein, lipids and carbohydrates all of which required for embryonic structure (kanost ***et al.,*** 1990) subsequently the reduction in total number of eggs per female could be due to interference of the IGR with oogenesis. They induce decrease in the concentration of yolk proteins, carbohydrates, lipids and inhibition in both DNA and RNA synthesis in the ovaries of females treated as larval instars, moreover they caused vacuolation of murse cells and oocytes of the ovaries (Shaurub ***et al.,*** 1998).

Reduction in the percentage of egg- hatch obtained in the present study may be due to defects in te differentiation of oocytes and sperms (Meola & Mayer, 1980 and Horowitz ***et al.,*** 1992) Emam and degheel (1993) found that treating 4th instar larvae of S littordis with sublethal doses of chlorfluazuron and difiubenzuron induced decrease in adult emergence from 80% in control to 14 and 23% respectively, also viability of deposited eggs and progeny formation was reduced for explanation, Sallam (1999) indicated that ovicidal activity of flufenoxuron in eggs deposited by S. Littoralis that emerged from treated larvae could be due to disturbance in cuticle formation of the embryo, developed embryos were not able to perforate the surrounding vitelline membrane, it could be due to a weakened chitinous mouth parts that was insufficiently rigid to affect hatching Whiting, 2000 Butter ***et al.,*** 2003, salokhe ***et al.,*** 2008). RAPDs can be used to differentiate between protein bands – sauencing of fxagments of DNA produced by PC R analysis. Habibi ***et al.*** (2001) found that the saliva of Emposea jabae fed on broad bean four extra bands became visible compored with treatment simple diot. Huerta ***et al.*** (2007) reported that change in diet coulol have produced the difference aloserved. Causative analysis of silkworm proteins by Al- Akkad ***et al.,*** 2003) showed obvious variations in the number and position of bands wheel fed on different ducts. Our results demonstrated appearance and disappearance of protein bands in haemoly mphprofile after IGR administration – this may be att.: buted to their involve ment in the immune reactions, overall.

Treatment with IGR affect the synthesis and degradation – of chitin and chitinse enzymes.

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List of Abbreviations

B : Basement membrane.
CM : Circular muscle layer.
EN : Endocuticle.
EP : Epithelial Cells.
EX : Exocuticle.
F : Fat Cells.
H : Hypodermis.
L : Lunem.
L.M : Longitudinal muscle layer.
N : Nucleus.
P : Peritrophic membrane.
RE : Regenerative cells.
S : Sloughed Cells.
V : Vacuole.
FS : Epithelial sheath.
FF : Follicular epithelium.
IS : Interstitial space
NC : Nurse Cells
O : Oocyte.
SC : Spermatocyte.
SG : Spermatogonia.
SP : Sperms.
ST : Spermatids.
T : Testicular Follicles.
V : Vacuole.
VM : Vitelline membrane.