Title of the thesis: Studies on the effect of a new Insect Growth Regulator on the biology and biochemical parameters of *Tribolium castaneum*.

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Studies On The Effect Of A New Insect Growth Regulator On The Biology And Biochemical Parameters Of *Tribolium castaneum*.

A THESIS SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

BY

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JANUARY-2005

CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Studies on the effect of a new Insect Growth Regulator on the biology and biochemical parameters of *Tribolium castaneum*."** submitted by **MS. Shaila G. Salokhe** was carried out by the candidate under our supervision/ guidance at the Department of Biotechnology, University of Pune and Department of Entomology, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis

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ACKNOWLEDGEMENTS

I express my sincere gratitude to my guide, Dr. J. K. Pal and co-guide, Dr. S. N. Mukherjee for their expert guidance and encouragement throughout the course of the present work, which submitted in the form of dissertation. I acknowledge both for providing all the necessary facilities for my work.

I am thankful to Dr. Anita Kar (Reader, School of Health Science, University of Pune) for permitting me to use gel documentation system for protein analysis and photography unit from her laboratory.

I am grateful to The Director, NCL and Mr. Tungikar V. B. (Scientist Incharge, Entomology section, NCL), for permitting me to utilize laboratory facilities as guest worker and providing an opportunity to consult some of the great minds from NCL. My sincere thanks are due to Dr. Islam Khan (Scientist, Biochemical Division, NCL) for his valuable suggestions during my experimental work on chitin analysis and for laboratory and other facilities. The research would have merely remained concept without Dr. Sushama Gaikwad's help in biochemical analysis and also a very warm and friendly feeling to be comfortable to work with. I am also thankful to Dr. Chakraborty, Dr. Khaire, Dr. Amutha and Manoj from NCL for their technical assistance during my work at NCL.

My sincere thanks are due to Dr. Pushpa Pawar (Scientist, Entomology Section, NCL) a core person to introduce me to my research guide, Dr. Mukherjee and her help at each step to make my pursuit to reach an ultimate end. I am also thankful to Dr. A. Sen (Scientist, Entomology Section, NCL)

for his valuable suggestions throughout my work at NCL. I acknowledge the homely and friendly atmosphere created by Dr. Tare, Dr. Raina, Dr. Deshpande, Dr. Bhonde, Geeta and Mary in NCL, Entomology Section. Thanks are also due to Mrs. Das and Mrs. Ahirebai for timely offered tea to me.

I acknowledge the sincere help by Dr. Angshuman Sarkar during my work in Biotechnoloy Laboratory, University of Pune, to overcome and discuss the problems and to extend his expertise assistance in my experimental work on protein analysis. I am thankful to Dhanashri and Abhijeet for their help during my work in Biotechnology Laboratory. I am also thankful to Vir, Shimi, Reza, Srijeeth and Sangeeta for their friendliness.

I express my sincere thanks to Mrs. Sahasrabudhe, (Head, Department of Statistics, S. P. College) and her colleague Mrs. Acharya for statistical analysis of the data on biological studies.

I sincerely express my gratitude to Dr. Ghate (Head, Department of Zoology, Modern College) for permitting me to use the photography unit of his department. I am thankful to Dr. Padhye for his co-operation in my work at Biotechnology Laboratory, University of Pune.

I acknowledge the support extended by the Principal and my colleagues of Annasaheb Magar College, Hadapser.

My acknowledgement will be incomplete without mentioning the moral support my father offered throughout when both of us were going through the loss of my mother. My special gratitude is to my daughter, Manasi, who managed all her own during her crucial examinations. It is my loving daughter's understanding and support that I could complete my research.

Salokhe S. G.

ABSTRACT

The effect of sub-lethal concentrations 0.00141% (LC_{20}), 0.00251% (LC_{30}) and 0.00336% (LC_{40}) of a dispersible concentrated formulation of the insect growth regulator flufenoxuron *Cascade*®, on larval growth and development, adult reproductive potential and egg hatchability of the red flour beetle, *Tribolium castaneum*, was investigated. When neonates were subjected to the sub-lethal concentrations of flufenoxuron in diet for 24h, there was a dose-dependent effect with respect to larval weight on the 7^{th} and 10^{th} day, percent pupation, percent adult emergence as well as time taken for adult emergence. A small proportion of larval-pupal as well as pupal-adult intermediates were observed at all the concentrations. Adults emerging from the LC_{20} and LC_{30} concentrations laid mostly non-viable eggs and the few larvae, which emerged from viable eggs died at the first instar stage. At the LC_{40} concentration, all the adults that emerged were deformed and subsequently died. Flufenoxuron exhibited transovarial ovicidal activity resulting in the production of non-viable eggs upon exposure of adults of different ages (2 day old, 3 day old and 4 day old) to treated diet. It was observed that in 2 days old adults, fecundity decreased with an increased concentration. In case of 3 days old adults, there was no difference in fecundity with respect to the concentrations tested, although it was significantly less from the control. In case of 4 day old adults there was a drastic reduction in fecundity at LC_{40} and the eggs laid were abnormal at all concentrations.

Topical application of sub-lethal concentrations of flufenoxuron to adults of either sex reduced the fecundity in a dose-dependent manner. Furthermore, the fecundity was reduced drastically in pairs where both the sexes were treated as compared to the pairs where only one sex was treated. Eggs showed a decrease in hatching percentage with increasing concentrations of flufenoxuron mixed with diet to which the eggs were exposed.

The effect of sub-lethal concentrations flufenoxuron (Cascade®) on certain biochemical parameters in the larvae of *Tribolium castaneum* was investigated. When neonates were fed on diet treated with sub-lethal concentrations for 24h, it was observed that at all concentrations tested, there was a significant reduction in chitin content on the 15^{th} day of development. Total soluble protein content at LC_{20} and LC_{30} decreased with increasing age of the larvae. At LC_{20} and LC_{40} concentrations there was a progressive increase in the protein:chitin ratio as a function of increase in age of the larvae. SDS-PAGE analysis of the larval tissue extracts indicated gross quantitative changes in some of the protein bands (MW 50-97 kDa). Western blot analysis revealed significant level of HSP70 in the extracts of larvae fed on LC_{30} treated diet, on the 7^{th} day of development. Interestingly, anti-HSP70 antibody detected a doublet of polypeptides of around 28-29 kDa which varied significantly both in quantity and proportion during the development. So was the case of the cyclin dependant kinase $p34^{cdc2}$ whose quantity increased during early development. Thus, sub-lethal concentrations of flufenoxuron alter expression of developmentally regulated proteins, HSP70 and $p34^{cdc2}$ and chitin formation in a stage-specific manner.

CONTENTS

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1. Gujar M. M., Gaikwad S. M, **Salokhe S. G.**, Mukherjee S. N. and Khan M. I. (2000). Growth inhibition and total loss of reproductive potential in *Tribolium castaneum* by *Atrocarpus hirsuta* lectin. *Invert. Repro. Dev.* 38:2 95-98.

2. Salokhe S. G., Pal J. K. and Mukherjee S. N. (2003) Effect of sub-lethal concentrations of flufenoxuron on growth, development and reproductive performance of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Invert. Repro. Dev.* 43:2 141-150**..**

3. Shaila Salokhe, Angshuman Sarkar, Samindranath Mukherjee and Jayanta K. Pal. 2005. Flufenoxuron, an acylurea insect growth regulator, alters development of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) by modulating levels of chitin, soluble protein content and hsp70 and $p34^{cdc2}$ in the larval tissues. (C*omm*) *Pestic. Biochem. Physiol.*

PAPERS/POSTERS PRESENTED IN SYMPOSIA AND CONFERENCES.

1. Gujar M. M., Gaikwad S. M., Rao K. N., Suresh C. G., **Salokhe S. G.** Mukherjee S. N. and Khan M. I. 2000. Structure and biological studies on *Atrocarpus hirsuta* lectin. Poster presented at the 5th IUPAC International Symposium on Bio-organic Chemistry, at National Chemical Laboratory, Pune, India from 30th Jan. to 4th Feb. 2000.
2. Teke S. P., Sahasrabudhe N. M., **Salokhe S. G.**, J. K. Pal and Mukherjee S. N. 2003. Designing the experiment to analyze the effectiveness of sub-lethal concentrations of insecticide. Paper presented at the 5th International Triennial Calcutta Symposium on Probability and Statistics, at Calcutta University from 28th-31st Dec. 2003.
3. **Salokhe S. G.**, Sarkar A., Mukherjee S. N. and Pal J. K. 2004. Influence of sub-lethal concentrations of an insect growth regulator flufenoxuron (Cascade®) on certain biological and molecular phenotypes in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Poster presented at XXVII All India Cell Biology Conference and International Symposium, at University of Pune from 7th-10th Jan. 2004.

ABBREVIATIONS

APS	**Ammonium persulphate**
BCIP/NBT	**5-bromo-4-chloro-3indolyl phosphate/nitroblue tetrazolium**
Bis-Acrylamide	N, N'-Methylenebisacrylamide
BPB	Bromophenol blue
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
DMAB	P-dimethyl aminobenzaldehyde
EDTA	Ethylenediamine tetra acetic acid
Glc NAC	N-acetyl-D-glucoseamine
HSP	**Heat shock protein**
KDa	Kilo Dalton
LC	Lethal concentration
mAb	Monoclonal antibody
MW	Molecular weight
PBS	**Phosphate buffered saline**
PMSF	**Phenylmethane sulfonyl fluroide**
SDS	Sodium dodecyl sulfate

TBS	Tris buffered saline
TEMED	N, N, N’ N’-tetramethyl ethylene diamine
Tween 20	Polyoxyethylenesorbitan monolaurate
UV	**Ultra violet**
µg	Microgram

OVERVIEW OF PESTICIDES

First and second generation pesticides:

A pesticide is any substance or a mixture of substances intended for preventing, destroying, repelling or mitigating any pest. The pesticide revolution dates back to early 1940s when DDT was first used as an insecticide. Since that time chlorinated hydrocarbons were used as "second generation pesticides" (Bowen and Hall, 1952) to follow the "first generation inorganic pesticides" such as lead arsenate (Carter, 1952). The development of the organophosphorus insecticides like parathion, tetraethyl pyrophosphate (TEPP) or octamethyl pyrophosphoramide (OMPA/schradan) resulted from the research of Schrader in the early 1940s, in Germany. Schradan is of historic importance for it was the first organophosphorus compound to be studied for use as a systemic insecticide. Effects of organophosphorus compounds are usually sharp, localized and short term. In 1962, Rachel Carson in her book "Silent Spring" brought into focus the adverse effects of indiscriminate use of DDT. Later on it was found that DDT exceeds maximum residue levels leading to health hazards (Mukherjee et al., 1980; Kalra and Chawal, 1983). As a result marketing and use of DDT was prohibited from 1st Jan. 1981 by European Commission's Directive.

In a variety of pest control activities, around the world chemicals continue to play a significant role. Pest control is a continuous warfare between the pesticide and the pest, since development and application of newer pesticides also leads to the problem of pests developing resistance. New insecticides continue to appear and create new problems since the pests develop resistance to them. As a result alternative methods of pest control were developed. Insecticides of organic origin were obtained through two main sources. Some are naturally occurring and the active ingredients are extracted from them, while some, which are of recent discovery are synthesized. Naturally occurring organic compounds include plant products e.g. pyrethroids (Casida, 1973; Casida and Quistad, 1995), rotenoids (Haley, 1978; Hayes, 1982; Matsumura, 1985), sabadilla (Allen et al., 1944; Hayes, 1982), neem oil (Saxena et al., 1981; Heyde et al., 1984; Schmatterer, 1990) etc., animal products e.g. Neris toxin isolated from marine annelids and mineral oils such as tar and petroleum have insecticidal properties. The synthetic organic compounds are broadly classified as organochlorines and organophosphorus compounds, carbamates and organic sulphur compounds. Conventional synthetic insecticides have been used worldwide in successfully controlling insect pests during the past five decades. Indiscriminate use of synthetic insecticides have caused ecological

disturbances as there occurs interaction between the pesticide and complex biological system of which the pest is only one component. Failure to recognize complexities that may be involved accounts for many problems in the use of insecticides. Compounds having other types of action such as attractant (Phelan and Baker, 1987; Vale et al., 1988; Park and Goh, 1992; James et al., 1996), repellents (Ntiamoah et al., 1996; Maganaga et al., 1996) and antifeedants (Schmutterer, 1990; Ascher, 1993; Mordue and Blackwell, 1993; Simmonds and Blaney, 1996) are also considered as behaviour modifying insecticides. Pest control can also be achieved by using biological control agents such as parasites (Sweetman, 1936; Doutt, 1964; Hall and Ehler, 1979; Van den Bosch et al., 1982; Greathead, 1986) and predators (DeBach, 1974). Out of the various possibilities, it was also thought to exploit and manipulate the exocrinological (external body secretions mediating intra-specific interactions) and endocrinological (hormonal body secretions regulating growth and development) environments of insects and to develop autocidal means of their control.

Second and third generation pesticides:

Williams (1956) gave a boost to the research on hormonal control of insect growth and postulated that juvenile hormone (JH) may be employed in insect control. His continuous research on various physiological effects of

JH led him to conclude that these compounds can also be used as insect-specific control agents to which pest species may not be able to develop resistance. Williams (1967) declared insect hormones as the "third generation pesticide". It was difficult to synthesize the natural JH and to use them as selective insecticide because of their environmental instability. Bower (1969), synthesized substituted aromatic terpenoid ethers that were more active than the natural hormones on *Tenebrio molitor* and *Oncopeltus fasciatus*. This opened up a new avenue of insect control by antihormones and the concept of "fourth generation pesticide" was born. In the past decades numerous JH analogues have been synthesized and tested (Grenier and Grenier, 1993; Horowitz and Ishhya, 1994; Kim and Krasfsur, 1995; O'Donnell and Klowden, 1997; Parkman and Frank, 1998). Since hormones regulate insect development and differentiation, their analogues are collectively called as insect growth regulators (IGRs). IGRs interfering with insect development are insect directed products, which are non-toxic to mammals. Recent IGRs include compounds that are non-hormonal but interfere with the metamorphosis of insects e.g. chitin synthesis inhibitors. IGRs include compounds that affect moulting and metamorphosis by mimicking juvenile hormone (JH agonists) or antagonizing JH activity (ecdysteroid agonists) or by interfering with cuticle formation (chitin

synthesis inhibitors), (Smet et al., 1990; Oberlander et al., 1997). Also the IGRs have antimetamorphic, larvicidal, ovicidal, diapause disrupting and embryogenesis inhibiting effects. Depending on their chemical nature hormone based IGRs are grouped as follows:

1.Moulting hormone analogues-MHA's

2.Anti-moulting hormone analogues-AMHA's

3.Juvenile hormone analogues-JHA's

4.Anti- juvenile hormone analogues-AJHA's

5.Neuropeptides.

The potential role of these IGRs in the insect pest management, are as follows:

1.Moulting hormone analogues (MHA's): Prothoracic gland secrets moulting hormone ecdysone that brings about normal moulting, growth and maturation of insects. It was observed that when MHA's were applied exogenously the ecdysone titre in the haemolymph increased causing moulting promotion and death of insects (Prakash et al, 1979; Hardman, 1987; Koolman, 1990). The use of MHA's in pest control is limited because of their inability to penetrate the insect cuticle and also due to their laborious and costlier sythesis.

2.Anti-moulting hormone analogues (AMHA's): These are compounds with antiecdysone activity resulting in inhibition or delay in the moulting cycle (Kubo et al., 1976; Warthen, 1979; Jacobson, 1986). However, these compounds were found to interfere with steroid hormone regulators in higher animals (Saxena, 1983) and therefore their use is limited.

3.Juvenile hormone analogues (JHA's): Studies on importance of JH titre in haemolymph for metamorphosis lead to the development of third generation pesticides called JHA's (Williams, 1967). Further studies revealed that JHA's act as ovicidal, larvicidal sterilants or terminators of diapause (DuRant et al., 1989; Nagai, 1990; Gokkes et al., 1990). Since JHA's have stage specific effect and they affect only the last larval instars, the earlier instars could continue to cause damage.

4.Anti-juvenile hormone analogues (AJHA's): These compounds induce a variety of physiological and behavioural changes including precocious metamorphosis of the immature stages, sterilization of adult females, induction of diapause and inhibition of sex pheromone production (Bowers, 1983).

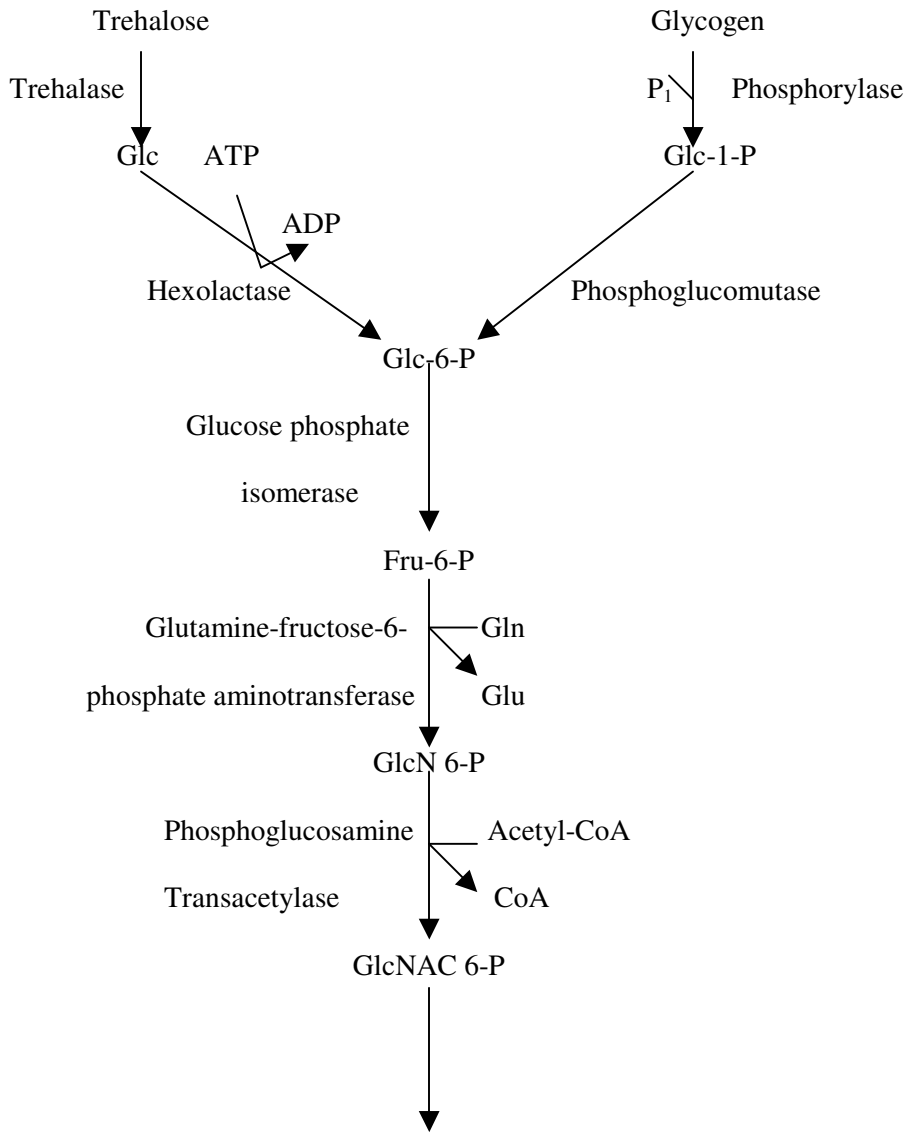
5.Neuropeptides: These are secreted by the brain and they stimulate ecdysone production. They regulate many physiological processes such as development, reproduction behaviour, homeostasis and metabolism of

muscle function (Menn et al., 1989). Kelly et al. (1990) found that the compounds that interfere with neuropeptide sythesis would lead to death of pest and can be used in integrated pest management practices.

Another major group of bioactive compounds, benzoylphenylureas has been found to possess growth-regulating activity. They differ from JHA's in their mode of action by inhibiting chitin synthesis, which is the important constituent in insect exoskeleton and has critical role at each stage in insect morphogenesis. Chemically chitin is N-acetylglucosamine (Purchase et al., 1946) and represented as, (1 - 4)–2--acetamido–2--deoxy–b–D--glucon.

In insects it is covalently linked with protein. It is synthesized as shown in Fig.1

Fig. 1


Trehalose
Glycogen
Trehalase
P_1
Phosphorylase
Glc
ATP
Glc-1-P
ADP
Hexolactase
Phosphoglucomutase
Glc-6-P
Glucose phosphate
isomerase
Fru-6-P
Glutamine-fructose-6-
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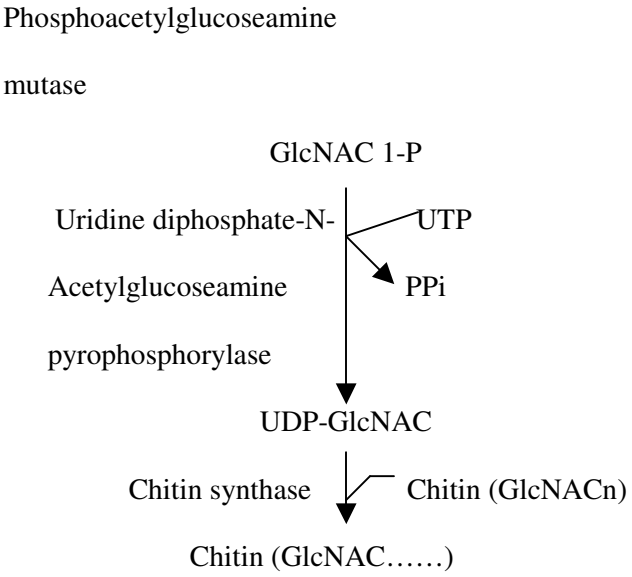
Transacetylase
CoA
GlcNAC 6-P



Deficiency or excess of chitin during any morphogenetic cycle in insect can produce deleterious and lethal effects. Therefore, chitin is an ideal target for pesticide development. Many compounds ranging from natural products such as plumbagin (Kubo et al., 1983) to antibiotics such as polyoxins and nikkomycin (Hori et al., 1971), insecticides such as benzimidazoles (Kuvano et al., 1982) and fungicides such as Captan® (Becker et al., 1978) have been shown to block chitin synthesis in insects. Several compounds of a new class of insecticides benzoylphenylureas, which are able to interfere with chitin synthesis, have been evaluated for their insecticidal action against wide range of insect pests. Different benzoylphenylureas are known to block chitin biosynthesis at different levels. The most widely used benzoylphenylurea, diflubenzuron, was found to induce the degradation of newly synthesized chitin in the insects

(Wellinga et al., 1973; Post et al., 1973, 1974 and Ishaaya and Casida, 1974). Moulting disruption in insects resulted because of the inhibitory action of benzoylphenylureas on ecdysone metabolizing enzymes, which leads to accumulation of ecdysone that stimulates chitinase production (Yu et al., 1975). However, in the larvae of *Musca domestica* it was observed that diflubenzuron disturbs the synthesis of chitin by reducing the rate of production of chitin during cuticle deposition (Grosscurt, 1976). Most of the physiological processes, such as feeding, oviposition, moulting are found to be affected due to disruption of chitin synthesis. In Orthopterans, the presence of diflubenzuron was found to reduce the amount of chitin deposited in the peritrophic membrane and gives it loose and fibrous texture (Clark et al., 1977; Becker, 1978). Conversion of glucose to fructose-6-phosphate, was found to be blocked by benzoylphenylureas, which results in inhibition of chitin synthesis (Saxena and Kumar, 1981). Benzoylphenyl urea, UD-19111 was found to prevent chitin synthesis by interfering with the proteolytic activation of chitin synthetase zymogen (Leighton et al., 1981). In cabbage armyworm, *Momestra brassicae* (L.) transport of 14c UDP – N-acetyl – glucosamine across the microvilli of midgut peritrophic membrane, was found to be inhibited by diflubenzuron (Mitusi, 1984). Retnakaran et al. (1985) observed that benzoylphenylurea inhibit the second polymerization

step in chitin synthesis, which involves formation of chitin microfibrils by oligosaccharides that covalently bound to specific receptor proteins. Chitin synthesis inhibitors also act by interrupting the synthesis and transport of specific proteins that are required for the assembly of Glc NAC monomers into polymeric chitin (Oberlander et al., 1999).

Organismal effects of benzoylphenylureas were observed at different levels from failure to feeding to delayed mortality. In diflubenzuron treated Gypsy moth larvae, ecdysis was completely prevented and the larvae wriggled in the unshed cuticle and died (Granett, 1974). Retnakaran et al. (1975) found that in diflubenzuron treated sixth instar larvae of Spruce bud worm, moult disruption resulted in the development of deformed pupa with a larval head and thorax or with the cast integument attached to the thorax i.e. larval-pupal intermediate. Benzoylphenylureas also have ovicidal effect as observed in Dipteran (Miura et al., 1976) and Lepidopterans (Earle et al., 1978; McLaughlin, 1977,1978; Moore et al., 1978).

Studies on environmental fate of benzoylphenylureas, reavealed that they are biodegradable and the degradation rate of diflubenzuron in soil was very fast and unrelated to soil type (Nimmo et al., 1984). Also they have residual activity on the eggs for a period of about 10-40 days after application (Hoying and Riedl, 1980; Lauren et

al., 1985). Detrimental effects seen with the use of benzoylphenylureas on non -target species were found to be minor and appeared to be temporary compared to the effects of conventional insecticides (Ali et al., 1978; Apperson et al., 1978; Anderson et al., 1982; Broadbent et al., 1984). It was also observed that the effect of benzoylphenylurea on important pollinators such as wild and domestic bees was negligible (Buckner et al., 1975; Johansen et al., 1977). The characteristics of novel mode of action, specificity to arthropods, safety towards vertebrates and lower ecological magnification have evoked considerable interest in the recent past to investigate the effect of different benzoylphenylureas on a wide range of insect pests.

Since the introduction of the first acylurea, Diflubenzuron a range of similar compounds has been introduced and studied for their insecticidal activity. Effects of two benzoylphenylureas, Alsystin and UC62644 on *Platynota stultana* larval, pupal survivorship, longevity and adult's fecundity was studied by Hejazi et al. (1986). According to them the pest population can be effectively controlled at minute concentrations relative to concentrations necessary to kill the pest. Apart from the morphogenetic effects of benzoylphenylureas the observations were also made on their influence on physiological

parameters of pests. Tiwari (1989) found that Diflubenzuron at sub-lethal concentrations affects the level of haemolymph proteins, which may disrupt the normal physiological function resulting in abnormal growth in *Diacrisia oblique*. Observations made by Clarke and Jewess (1990) on effect of benzoylphenylureas, Flufenoxuron, Teflubenzuron, and Diflubenzuron in *Spodoptera littoralis* larvae revealed that these insecticides are equally effective inhibitors of chitin synthesis. Furlong et al. (1994) found that Teflubenzuron is non-specific chitin synthesis inhibitor as it also prevents the formation of chitinous case of insect eggs. Mikolajczyk et al. (1994) showed that Teflubenzuron inhibits chitin synthesis by blocking N-acytyl-D-glucosamine incorporation in chitin.

Follas et al. (1995) studied the efficacy of Lufenuron against pests in apple and kiwifruit and found that it gave significant reduction in fruit damage on both apples and kiwifruit by leaf roller pests. Studies on Lufenuron by Emmanuel et al. (2000) on immature stages of potato tuber moth *Phthorimaea operculella* revealed that topical application of Lufenuron on eggs before larval hatch would reduce the amount of damage caused by potato tuber moth as part of IPM programme. Butter et al. (2003) tested Lufenuron for its toxicity to *Helicoverpa*

armigera **on cotton. They found that there is significant reduction in weight of treated larvae. Further they observed that, Lufenuron treatment at larval stage causes reduction in pupal and adult duration and pupal weight.**

The potential of Hexaflumuron for the control of ***Aubeonymus mariaefrunscae*** **population was evaluated by Marco and Castaenera (1996). They found a drastic reduction of egg hatching when adults were fed with Hexaflumuron treated leaves. Farinos et al. (1998) found that in** ***A. mariaefrunscae*** **adults treated with Hexaflumuron, impairment of chitin formation leads to embryo mortality in the egg - shell.**

Mechanism of action of the acylurea, Flufenoxuron on the larvae of ***Spodoptera littoralis*** **was studied by Sammur et al. (1996). They observed that along with the morphogenetic abnormalities due to flufenoxuron treatment of** ***S. litoiralis*** **larvae a significant decrease in chitin level and total protein content of the cuticle also took place. Rastegari et al. (2003) have studied sub-lethal effects of Flufenoxuron (at Lc_{10} to Lc_{25}) on** ***Spodoptera litura*** **and found that there was profound influence on larval development similar to that observed by Laecke et al. (1989) with Diflubenzuron, Chlorfluazuron and Hexafluron treated**

Spodoptera exigua. **It was also reported by Perveen et al. (2000) that sub-lethal doses of Chlorfluazuron influenced ovarian development and oogenesis of the common cutworm, *S. litura*.**

It is also a well established fact that living organisms respond at the cellular level to unfavourable conditions such as heat or other stressful situations including exposure to xenobiotics, UV, heavy metals, oxidizing agents, mutagens, carcinogens, insecticides and gene expression inhibitors by expression of specific sets of proteins called the heat shock/ stress proteins (hsps) (Lindquist, 1986; Nover, 1984, 1991; Feeder, 1996; Fiege et al., 1996; Delinger and Yocum, 1998). Recent studies indicate that stress proteins play a role in toxicity since they are induced as a result of damages caused to the cell by the toxicant (Hightower, 1991; Sanders, 1993). Similarly, it is also known that precise activation and inactivation of cyclin dependent kinases are necessary for normal cellular proliferation since they play a major role in controlling the activities of various proteins during the cell cycle by phosphorylating them. A review of literature revealed that there is paucity of information on IGR- induced stress and their effect on various biochemical parameters in insects. Therefore it was felt that it would be of interest to know about the response of *T. castaneum* to sub-

lethal concentrations of flufenoxuron at the molecular level with respect to some relavant biochemical parameters, which are directly related to the insect growth and development.

The present study, although supported by earlier observations, is significant with reference to the profound influence of sub-lethal concentrations of Flufenoxuron on the biological and certain biochemical parameters of stored grain pest *Tribolium castaneum* and to the best of our knowledge this is the first report of its kind on stored grain pest.

Present studies: *Tribolium castaneum* (Herbst) is an economically important pest of stored products worldwide feeding on a wide range of commodities (Arbogast, 1990). Amongst the various synthetic chemicals used to control *T. castaneum*, phosphine was the most widely used fumigant. Since late 70's it was reported that *T. castaneum* was developing resistance to phosphine and of late this has assumed serious proportions (Chaudhary, 2000). Another fumigant, methyl bromide is still used in controlling stored product pests including *T. castaneum*, for its rapid action and broad spectrum of activity. However, since methyl bromide is known to deplete the Earth's ozone layer it is being phased out in developed countries and is expected to be out by 2005 (MBTOC, 1998). It will be completely phased

out in developing countries as well by 2015 (MBTOC, 1998). As a replacement to methyl bromide, organophosphorus compounds were used as grain protectants (Snelson, J.T., 1987). Because of development of resistance to these insecticides in pests, they were no longer considered safe for marketing (EPA, 2000). Another class of insecticides, the synthetic pyrethroids, have greater flexibility with respect to environmental factors than other insecticides and have been also used to control stored product pests. But insects acquired resistance to them as well (Knight and Norton, 1989). More safe and effective insecticides than those described above are the IGRs that seems to be an ideal alternative. Several IGRs have been evaluated for their efficacy towards a variety of species of stored product pests, including the red flour beetle, *Tribolium castaneum*. Flufenoxuron, an acylurea IGR, acts on insects by reducing chitin incorporation in the cuticle (Clarke and Jewess, 1990). In the present study flufenoxuron was chosen to evaluate its efficacy on *T. castaneum* for its novel mode of action, specificity to arthropods, safety towards vertebrates and low ecological magnification. Flufenoxuron has been evaluated for its efficacy on a variety of pests of horticultural and ornamental plants. Although efficacy and toxicological effects of IGRs (Chitin synthesis inhibitors) have been extensively investigated (Oberlander et al., 1997), few studies have dealt with their sub-

lethal effects. Further, though these compounds work by inhibiting chitin synthesis, the precise mechanism of their inhibition remains elusive (Oberlander et al., 1991). Radwan et al. (1978) studied the effect of sub-lethal doses of Dimilin on the reproductive performance of *Spodoptera littoralis* Boisduval for three consecutive generations by treating the fourth instars of each generation. Biddinger and Hull (1999) reported on the sublethal effects of several classes of IGRs on the tufted apple bud moth *Platynota idaeusalis*. The effects of sub-lethal concentrations of flufenoxuron (Cascade®) on various life cycle and reproductive end points (viz. time to pupation, time to adult emergence, %pupation, % adult emergence, fecundity, fertilization and hatching success, and larval viability) are important for the assessment of overall ecological impact since non-target species in the periphery of the treated area often receive sub-lethal doses. The study reported here was undertaken to investigate the effect of sub-lethal doses (LC_{20}, LC_{30} and LC_{40}) of a dispersible concentrate formulation of flufenoxuron (Cascade®) against *Tribolium castaneum*, by exposing different developmental stages on treated diet as well as by topical application and observing the effect on larval growth and development, adult reproductive potential and egg hatchability.

The present study is also an attempt to understand the biochemical and molecular changes as an effect of sub-lethal concentration of flufenoxuron (Cascade®) on the larval tissues of *T. castaneum* (Herbst) with respect to chitin, total protein as well as a stress protein (HSP70) and a cell cycle regulatory protein ($p34^{cdc2}$), since these are considered to be general indicators of sub-lethal cellular protein damage.

SECTION I
BIOLOGICAL STUDIES

The effect of sub-lethal concentration of flufenoxuron on some biological parameters of *T. castaneum.*

INTRODUCTION

Juvenile hormone mimics, ecdysone agonists and chitin sythesis inhibitors are novel compounds affecting developmental process in insects. The benzoylphenylureas, insect growth regulators were the first compounds reported to interfere with chitin synthesis and the deposition of integumentory cuticle of insects (Mulder and Gigiswijit, 1973; Verloop and Ferrell, 1977; Van Eck, 1979; Marks et al., 1982). Following the discovery of the insecticidal properties of benzoylphenylureas, their potential had been recognized and were screened for their effects on various pests. Differential sensitivities of several benzoylphenylureas to Lepidopteran insects has been summerised by Retnakaran et al. (1985). Diflubenzuron was the most extensively studied benzoylphenylureas that is commercially available and finds wide application in forestry, some agricultural crop pests and fly and mosquito control (Maas et al., 1980).

The effects of benzoylphenylureas on organismal level (symptoms ranging from ovicidal, larvicidal to lethal effects on pupae and adults, as a whole) had been documented for a wide variety of insects (Mulder and Gigiswijit, 1973; Salama et al., 1976; Grosscurt, 1978; Schmidt and Dortlein, 1980; Ascher and Eliyahu, 1981; Haga et al., 1982). The

potential of insect growth regulators to suppress insect pests in stored commodities was first suggested by Thomas and Bhatnagar-Thomas (1968). Since then studies on stored product insects reared on laboratory diet (Strong and Diekmann, 1973; Losahiava, 1976) or on stored commodities such as corn, wheat and other grains (Bhatnagar-Thomas, 1973; Hoppe, 1974; Mc-Gregor and Kramer, 1975, 1976; Nickle, 1979) have advanced the possibility that insect growth regulators can be used in the management of stored product pests. Diflubenzuron was found to be effective against stored product pests, viz. *R. dominica, O. sunnamensis, S. granaries, S. oryza, T. castaneum and L. serricorne* (Carter, 1975; Mc-Gregor and Kramer, 1976). It was observed that chlorfluazuron was more effective than benzoylphenylureas viz. diflubenzuron, triflumuron or teflubenzuron in protecting wheat from infestations with *R. dominica, O. sunnamensis, S. oryza and T. castaneum* (Elek and Longstaff, 1994). Elek (1994) concluded that " the chitin synthesis inhibitors show promise as grain protectants, not only because they are effective at preventing progeny of those pests which develop inside grain, but also because they kill immature stages early in their development, thus minimizing damage to the commodity".

Most of the published research with insect growth regulators and stored-product pests had involved exposure of either adults or eggs on treated grain or diet and efficacy was assessed by inhibition of progeny development (Oberlander et al., 1997). Insect growth regulators do not kill adult insects, instead they eliminate the infestations by inhibiting development of immatures and reducing adult emergence (Williams and Amos, 1974; Amos et al., 1977). There is limited data regarding the effect of insect growth regulators on late instar larvae of stored grains (Hoppe, 1981; Arthur, 2003). Flufenoxuron, the insect growth regulator has shown to be toxic to some Coleopteran (Olszak et al., 1994), neuropteran (Vogt, 1992,1994; Carvalho, 1994b), Lepidopteran (Rastegari and Subrahmanyam, 2003) insects. Its effect on fecundity of stored product beetles was investigated by Elek and Longstaff (1994). However, sublethal effects of insect growth regulators on stored product pests have not been reported so far. The present studies was therefore undertaken to investigate the influence of sublethal concentration of insect growth regulator, flufenoxuron on the various stages in the life cycle (viz. time to pupation, time to adult emergence, %pupation, % adult emergence, fecundity, fertilization and hatching success, and larval viability) of the stored grain pest, *T. castaneum.*

MATERIALS AND METHODS

Insect: Red flour beetle, *Tribolium castaneum* (Herbst), a stored grain pest.

Source: From stock culture at N. C. L. Entomology laboratory, Pune.
Insect diet: Wheat flour, 5% Brewer's Yeast.

Culture: Culture of *T. castaneum* was maintained on a diet at a temperature of 29 ± 1ºC with 60 % relative humidity in a Remi's cooling incubator.

Insect growth regulator: Flufenoxuron (Cascade®)

Formulation: 10% DC (Dispersible concentrate).

CAS Number: 101463-69-8
US EPA PC Code: 108203

Source: Cynamide India Ltd., Bombay.

Use type; Insecticide

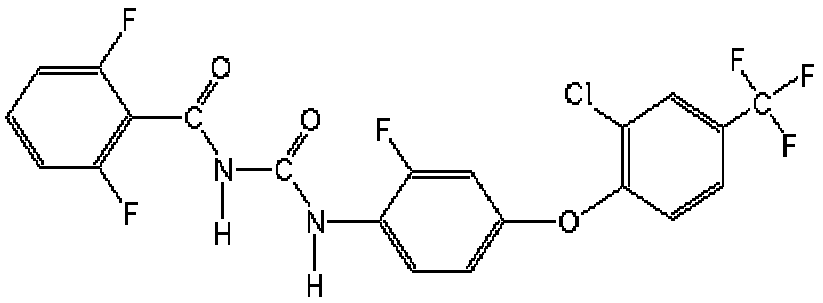
Chemical class: Benzoylphenylurea.

IUPAC Name: 1-{4-(2-chloro-alpha-trifluoro-p-tolyloxy)}-3-(2,6-diflurobenzoyl) urea.

Other designations: AC 811,678,WL 115110

Formula; $C_{21}H_{11}ClF_6N_2O_3$

Chemical structure:



METHODS

A stock culture of *T.castaneum* was maintained on a diet containing wheat flour and 5% Brewers yeast, at 29±1ºC and 60 % relative humidity. Eggs were collected by sieving, (sieve number 40) diet infested with adults. Newly emerged adults were obtained by collecting pupae and monitoring them for adult emergence. Flufenoxuron was thoroughly incorporated into diet using acetone as the carrier solvent. The treated flour was kept at room temperature for four hours, for complete evaporation of the solvent before use in the experiments. Determination of LC_{50} through diet was carried out by releasing newly hatched first instar larvae of *T. castaneum* in diet treated with various concentrations of flufenoxuron. Acetone mixed diet was used as control. For application of completely randomized design, homogenous experimental units were maintained by using the larvae of same age and keeping them in cooling incubator at 30˚C before and after the

treatment. For each concentration tested sets of five replicates of ten larvae each were prepared. The mortality count was taken after seven days. Subsequently, the sub-lethal doses used in the experiments (LC_{20}, LC_{30} and LC_{40}) were deduced by extrapolation from the regression line obtained by probit analysis and tested biologically to countercheck their validity.

Effect on larval development: **Effect of sub-lethal concentrations (LC_{20}, LC_{30} and LC_{40}) of flufenoxuron through diet on survival and metamorphosis of the larvae was examined by releasing ten newly hatched larvae in the treated diet. Acetone mixed diet was used as control. All experiments were replicated five times at each treatment. After 24 hours, the larvae were transferred to normal diet. On seventh and tenth day after the start of the experiment, the larvae were weighed five at a time and their survival was recorded. Once pupation had begun in any treatment, observations were made every day for adult emergence. Percentage pupation, time taken for pupation, percent adult emergence and time taken for adult emergence were recorded. Regression analysis was performed to determine dose dependent effects.**

Effect on fecundity: **Flufenoxuron mixed at various sub-lethal concentrations (LC_{20}, LC_{30} and LC_{40}) in diet was also used to determine**

the effect on fecundity of adults of different ages. *T. castaneum* pupae of the same age were kept separately and observed every day for adult emergence. The newly emerged adults were isolated and sexed after 24h. Each pair was kept in separate vials containing treated diet. Acetone treated diet was used as control. All experiments were replicated five times and the data obtained was analysed by ANOVA. When significant values were obtained it was proceeded for pair-wise comparison by t-test and Z-test.

Effect on hatching of eggs: Effect of sub-lethal concentrations (LC_{20}, LC_{30} and LC_{40}) of flufenoxuron through diet on the hatchability of eggs was determined by placing twenty eggs in treated diet and recording hatching of eggs every day till hatching in the control was completed and the data was confirmed three days later. All experiments were replicated five times. Regression analysis was performed to determine dose-dependent relationship.

Effect of topical treatment: To study the effects of sub-lethal concentrations on topical application to adults, the LC_{50} was determined by applying various concentrations of flufenoxuron to the ventral surface of the adult between mesothoracic and metahoracic legs using Hamilton microsyringe. Subsequently, the LC_{20} (0.1 µg/ µl), LC_{30} (0.4 µg/µl) and LC_{40}

(0.8 µg/µl) values were deduced by extrapolation of the probit mortality analysis. Effect of sub-lethal concentrations of flufenoxuron on fecundity of *T. castaneum* was monitored by topically applying different sub-lethal concentrations of flufenoxuron with a Hamilton syringe on the ventral surface of the adult between the mesothoracic and metathoracic legs. Adults treated similarly with acetone were used as control. The dispensing volume of solution at any concentration was always 1µl. Two hours after treatment the treated adults were sexed and transferred to normal diet. Crosses were performed as follows:

Treated males × untreated females

Treated females × untreated males

Treated males × treated females

Five replicates of each cross were made. Fecundity was observed for seven days and analysed by Student's t-test.

Determination of sub-lethal concentrations of flufenoxuron for *T. castaneum* larvae:

LC_{50} of flufenoxuron for the first instar larvae of *T. castaneum* through dietary treatment was 0.0042% (Fig. 2a). The sub-lethal concentrations of flufenoxuron through dietary treatment deduced by extrapolation of probit log analysis were LC_{20}-0.00141%, LC_{30}-0.0023% and LC_{40}-0.0033%.

Effects of sub-lethal concentratios of flufenoxuron on growth and development of *T. castaneum*:

Flufenoxuron at various sub-lethal dietary concentrations (LC_{20}, LC_{30} and LC_{40}) significantly reduced larval weight on seventh and tenth day of their growth period, compared to control (Table 1). Reduction in weight of the larvae on the tenth day was observed to be dependent on the weight of the larvae on the seventh day when compared to the control (Fig. 2b). Duration of normal development of *T. castaneum* adult from egg (Figs. 3-6) is around 25 days. When neonates were treated with sub-lethal concentrations of flufenoxuron through diet, it was observed that there was dose dependent effect on the reduction in weight of the larvae except on 7^{th} day when there was no significant reduction in weight at LC_{30} as compared to that in LC_{20} treated larvae (Table 1). In addition there was dose dependent

effect of flufenoxuron on time taken for pupation and adult emergence, which was significantly greater than that of the control (Table 1, Fig. 7). Further, a significant reduction in percent pupation and percent adult emergence was observed with increasing concentration of flufenoxuron (Table 1, Fig. 8). Sub-lethal concentrations of flufenoxuron through diet also adversely affected moulting of larvae resulting in the development of larval-pupal and pupal- adult intermediates (Table 1, Figs. 9-21). The percentage of larval-pupal and pupal- adult intermediates increased with concentration of flufenoxuron. Also some larvae died before moulting due to toxic effect of flufenoxuron, which was apparent in the form of blackening of the anterior part of the body (Figs 9, 18) or due to incomplete sclerotization of the larval body (Fig. 14). Larval-pupal intermediates with incompletely sclerotized wings (Figs. 10, 11, 15, 19, 20) were formed due to sub-lethal effects of flufenoxuron. The adult emergence was reduced because of the failure of the pupae to shed their cuticle, which remained attached to their body (Figs. 12, 13, 21). Further, adults emerging from the larvae fed on diet mixed with LC_{20} and LC_{30} of flufenoxuron laid non-viable eggs and those larvae, which emerged from viable eggs died at the first instar stage. At LC_{30} of flufenoxuron, treated larvae developed into adults with unsclerotised area between head and prothorax and prothoracic and mesothoracic sclerites

(Figs. 16, 17). While at LC_{40} of flufenoxuron, treated larvae developed into deformed adults that failed to retain their dorso-ventral posture. They were unable to ingest the food as their mandibles were abnormal with respect to chitinisation. These adults did not survive for longer durations.

Effect of sub-lethal concentrations of flufenoxuron on fecundity of *T. castaneum*:

Fecundity of *T.castaneum* adults of different ages was adversely affected when fed on the diet mixed with sublethal concentrations (LC_{20}, LC_{30} and LC_{40}) of flufenoxuron (Fig. 22). When the number of eggs laid by treated adults was compared with control using ANOVA test, it was observed that susceptibility to insecticide was age dependent and the average effect of different concentrations was not homogenous (Table 2). To enumerate the effective dose at each stage of the development, average fecundity per dose was compared pair-wise using T-test (Table 3). It was found that fecundity of two-day old adults was significantly reduced ($p<0.05$, $t_{18,\ 0.05}=1.74$) with increasing concentration of flufenoxuron. For three day-old adults it was found that all concentrations were equally effective in reducing fecundity, while for four-day old adults LC_{40} of flufenoxuron was found to be the most effective concentration for reducing fecundity (Tables 3, 5). Further, significant values were obtained when the

data of fecundity of adults of different ages at particular sub-lethal concentration of flufenoxuron was analysed by ANOVA (Table 4). To determine the susceptible stage for each sub-lethal concentration, average fecundity per stage of the adult was compared pair-wise using T-test (Table 5). It was found that the various sub-lethal concentrations of flufenoxuron had age specific effect on fecundity (Table 5). Also, older the adult more was the effect of flufenoxuron with reference to fecundity and percentage of abnormal eggs (Table 6). Abnormal eggs laid by the treated adults were observed to have no flour particles sticking to their surface (Fig. 23), which is generally found in the case of normal eggs (Fig. 3). Twin eggs were also observed in which there was lateral (Fig. 24) or vertical (Fig. 25) or at an angle of 90° (Fig. 26) fusion of the chorion, with small constriction between eggs. These eggs did not develop further but shrunk gradually and turned brown (Fig. 27). Further, the effect of LC_{20}, LC_{30} and LC_{40} concentrations on number of abnormal eggs laid by adults of different age was compared using Z-test (Tables 6, 7). It was observed that in case of two-day and three-day old adults, the percentage of abnormal eggs laid increased with increasing concentration of flufenoxuron. However, in four-day old adults, the percentage of abnormal eggs laid was higher at LC_{30} and LC_{40} as compared to LC_{20} (Table 6). While at LC_{20}, the percentage of abnormal eggs laid was

more in four-day old adults as compared to two-day and three-day old adults and at LC_{30} and LC_{40}, the percentage of abnormal eggs laid increased with increasing age of the treated adults (Table 6).

Effect of sub-lethal concentratios of flufenoxuron on hatching of eggs of *T. castaneum*:

Flufenoxuron at various sub-lethal concentrations (LC_{20}, LC_{30} and LC_{40}) significantly reduced the hatching of eggs as compared to control. The percentage hatching decreased with an increase in concentration of flufenoxuron (Fig. 28) in a dose dependent manner.

Effects of topical application of sub-lethal concentratios of flufenoxuron on *T. castaneum* adults:

LC_{50} of flufenoxuron for adults of *T. castaneum* through topical treatment was 1.6µg/ µ l (Fig. 29). The LC_{20}, LC_{30} and LC_{40} deduced from extrapolation of probit log analysis were LC_{20}- 0.4µg/ µl, LC_{30}-0.8 µg/ µl and LC_{40}-1µg/ µl. When adults of either sex of *T. castaneum* were topically treated with sub-lethal concentrations of flufenoxuron there was reduction in fecundity compared to control (Table 9). When both the sexes were treated and allowed to mate, the egg laying was significantly reduced ($p<0.05$, $t_{8,0.05}= 1.86$) at 0.4 µg/µl and 0.8 µg/µl of flufenoxuron as compared to that in control and in the pairs where only one sex was treated. At the above sub-

lethal concentrations of flufenoxuron when treated females crossed with normal males, number of eggs laid was lesser than that of the pair in which normal females were crossed with treated males. The fecundity of the normal females crossed with treated males was less than that of the control. At 1µg/µl of flufenoxuron the pair in which both the sexes were treated laid lesser eggs compared to the pair where only males were treated. There was no significant difference in fecundity between the pair where only females were treated and both the sexes were treated (Table 9).

Table 1

Effect of sublethal concentrations of flufenoxuron on growth and development of *T. castaneum*

Dose	% Larval survival X ± S.E.	Larval weight (mg.) X ± S.E.		% pupation X ± S.E.	Time taken for pupation (Days) X ± S.E.	% adult emergence X ± S.E.	Time taken for adult emergence (Days) X ± S.E.	% LPI	% PAI
		7^{th} day	10^{th}day						
0.00	100	1.66 ± 0.3	5.15 ± 1	100	17 ± 1	100	25 ± 0	0	0
LC_{20} 0.00014 %	88 ± 4.4*	1 ± 0.2	3.74 ± 0.8	95.5 ± 6	22 ± 2*	93.18 ± 6	27.4 ± 0.8*	2.27	4.54
LC_{30} 0.00025 %	70 ± 0*	1.03*	3.3*	90 ± 16.4	25.33 ± 57*	80 ± 16.4*	33.3 ± 1.1*	4.76	7.04
LC_{40} 0.00033 %	63.33 ± 5.7*	0.93 ± 0.2*	2.96 ±0.6 *	88.86 ± 9.6*	32 ± 1.7*	77.75 ± 9.6*	38 ± 2.6*	5.2	10.5

LPI: Larval-Pupal intermediates

PAI: Pupal-Adult intermediates

*Significant at 0.05% level.

Table 2

ANOVA for fixed age of adults for sub-lethal concentrations of flufenoxuron.

For 2 days old adults:					
Source	**D. f.**	**SS**	**MSS**	**F Ratio**	**F (3,16)**
Treatment	3	2843.8	947.93	24.49*	3.23
Error	16	619.20	38.7		
Total	19	3463			
For 3 days old adults:					
Source	**D. f.**	**SS**	**MSS**	**F Ratio**	**F (3,16)**
Treatment	3	5268.15	1756.05	151.38*	3.23
Error	16	185.6	11.6		
Total	19	5453.75			
For 4 days old adults:					
Source	**D. f.**	**SS**	**MSS**	**F Ratio**	**F (3,16)**
Treatment	3	7273	2424.33	244.265*	3.23
Error	16	158.79	9.9249		
Total	19	7431.8			

Table 3

Effect of sublethal concentrations of flufenoxuron on the fecundity of *T. castaneum* of different ages.

t-test values at fixed age of adult for sub-lethal concentrations of flufenoxuron.

$t_{18,0.05} = 1.74$.

Pair	2 day old adult	3 day old adult	4 day old adult
$\mu_1 - \mu_2$	3.50*	18.26*	21.47*
$\mu_1 - \mu_3$	6.11*	19.80*	21.81*
$\mu_1 - \mu_4$	8.31*	19.80*	28.48*
$\mu_2 - \mu_3$	2.61*	1.53 n. s.	0.3391 n. s.
$\mu_2 - \mu_4$	4.80*	0.92 n s.	7.008*
$\mu_3 - \mu_4$	2.19*	0.61 n.s.	6.6697*
Conclusion	$\mu_1 > \mu_2 > \mu_3 > \mu_4$	$\mu_2 = \mu_3 = \mu_4$	$\mu_2 = \mu 3 > \mu_4$

* significant value. n s. not significant.

$\mu 1$= Average fecundity in control, $\mu 2$ = Average fecundity in LC_{20} treated adults, $\mu 3$ = Average fecundity in LC_{30} treated adults, $\mu 4$ = Average fecundity in LC_{40} treated adults.

Table 4

ANOVA for fixed sub-lethal concentrations of flufenoxuron and different age of adults.

For LC_{20}:

Source	**D.f.**	**SS**	**MSS**	**F Ratio**	**F (3,16)**
Treatment	3	5487.75	1829.25	73.76*	3.23
Error	16	396.79	24.8		
Total	19	5884.55			

For LC_{30}:

Source	**D.f.**	**SS**	**MSS**	**F Ratio**	**F (3,16)**
Treatment	3	5037.4	1679.13	119.08*	3.23
Error	16	225.59	14.09		
Total	19	5263			

For LC_{40}:

Source	**D.f.**	**SS**	**MSS**	**F Ratio**	**F (3,16)**
Treatment	3	6930.55	2310.18	97.47*	3.23
Error	16	379.20	23.7		
Total	19	7309.75			

Table 5

Effect of flufenoxuron on the fecundity of *T. castaneum* of different ages at sub-lethal concentrations.

t-test values at fixed concentration for different age of adult.
$t_{18,0.05} = 1.74$.

Pairs	**Concentrations**		
	LC_{20}	LC_{30}	LC_{40}
μ_1 - μ_2	4.699*	16.02*	10.81*
μ_1 - μ_3	12.48*	26.89*	12.71*
μ_1 - μ_4	13.32*	26.89*	17.13*
μ_2 - μ_3	7.78*	10.86*	1.90*
μ_2 - μ_4	8.62*	10.86*	6.32*
μ_3 - μ_4	0.841 n.s.	0.0 n.s.	4.42*
Conclusion	$\mu_1 > \mu_2 > \mu_3$ $\mu_2 > \mu_4$ $\mu_3 = \mu_4$	$\mu_1 > \mu_2 > \mu_3$ $\mu_2 > \mu_4$ $\mu_3 = \mu_4$	$\mu_1 > \mu_2 > \mu_3 > \mu_4$

* significant value. n.s. not significant.

μ1= Average fecundity in control, μ2 = Average fecundity of 2 day old adult, μ3 = Average fecundity of 3 day old adult , μ4 = Average fecundity of 4 day old adult

Table 6

Z-test values for number of abnormal eggs laid by the adult of particular age treated with sublethal concentrations of flufenoxuron.

$Z_{0.05} = -1.64$.

Concentration	2 day old adult	3 day old adult	4 day old adult
$P_1 - P_2$	-2.0573*	-2.256*	-5.80*
$P_1 - P_3$	-1.22 n. s.	-3.638*	-2.49*
$P_2 - P_3$	0.672 n. s.	-1.51 n.s.	-0.757 n.s.
Conclusion	$P_1 < P_2 < P_3$	$P_1 < P_2 < P_3$	$P_1 < P_2 = P_3$

P_1: Value for LC_{20}, P_2: value for LC_{30}, P_3: Value for LC_{40}.

* significant value. n.s. not significant.

Table 7

Z-test value for number of abnormal eggs laid by adults of different ages treated with a particular sub-lethal concentration of flufenoxuron.

$Z_{0.05} = -1.64$.

Age of the adult.	Concentration LC_{20}	LC_{30}	LC_{40}
$P_1 - P_2$	-0.667n.s.	-1.77*	-3.75*
$P_1 - P_3$	-4.46 *	-9.30*	-5.56*
$P_2 - P_3$	-2.69*	-6.06 *	-1.96 n.s.
Conclusion	$P_1 = P_2 < P_3$	$P_1 < P_2 < P_3$	$P_1 < P_2 < P_3$

P1: Value for 2 day old adult, P2: value for 3 day old adult

P3: Value for 4 day old adult. *significant value. n.s. not significant.

Table 8

Percent abnormal eggs laid by the adults of different ages of *T. castaneum* treated with sub-lethal concentrations of flufenoxuron.

Control	2 day old adult LC_{20} LC_{30} LC_{40}	3 day old adult LC_{20} LC_{30} LC_{40}	4 day old adult LC_{20} LC_{30} LC_{40}
0	0.67 3.66 2.38	2.2 9.52 20	13.15 63.88 100

Table 9

Effect of topical application of sub-lethal concentrations of flufenoxuron on the fecundity of *T. castaneum* of different sexes.

Fecundity for seven days (X ± S.E.)

Dose of flufenoxuron (µg/µl)	Treated male X Normal female (μ_1)	Treated female X Normal male (μ_2)	Treated male X Treated female (μ_3)	Control (μ_4)
0.4	56.75±1.66	46.42±0.16	19.97±1.16	73.8±2.46
0.8	27±2.3	11.8±0.8	7.1±0.33	
1.0	10.65±0.6	9.1±2.4	4.25±2	

Table 10

Effect of topical application of sub-lethal concentrations of flufenoxuron on the fecundity of *T. castaneum* (when different sexes were treated and mated).

t-test values for different concentrations of flufenoxuron.

$t_{8,0.05} = 1.86$.

Pairs	**Dose of flufenoxuron** 0.4 (µg/µl)	0.8 (µg/µl)	1 (µg/µl)
$\mu_1 - \mu_2$	5.54*	5.58*	0.56 n.s.
$\mu_1 - \mu_3$	16.24*	7.66*	2.74*
$\mu_1 - \mu_4$	5.138*	12.42*	22.30*
$\mu_2 - \mu_3$	20.203*	4.85*	1.38 n.s.
$\mu_2 - \mu_4$	9.934*	21.43*	16.83*
$\mu_3 - \mu_4$	17.7*	24.036*	19.62*
Conclusion	µ4> µ1>µ2 >µ3	µ4> µ1>µ2 >µ3	µ1=µ2,µ4>µ1>µ3 µ4>µ2 =µ3

* significant value. n.s. not significant.

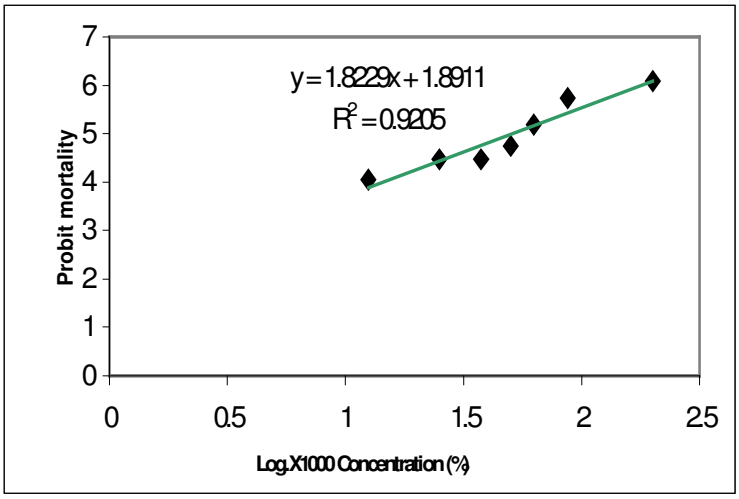
Table 11

Effect of sub-lethal concentrations of flufenoxuron on hatching percentage of eggs of *T. castaneum*.

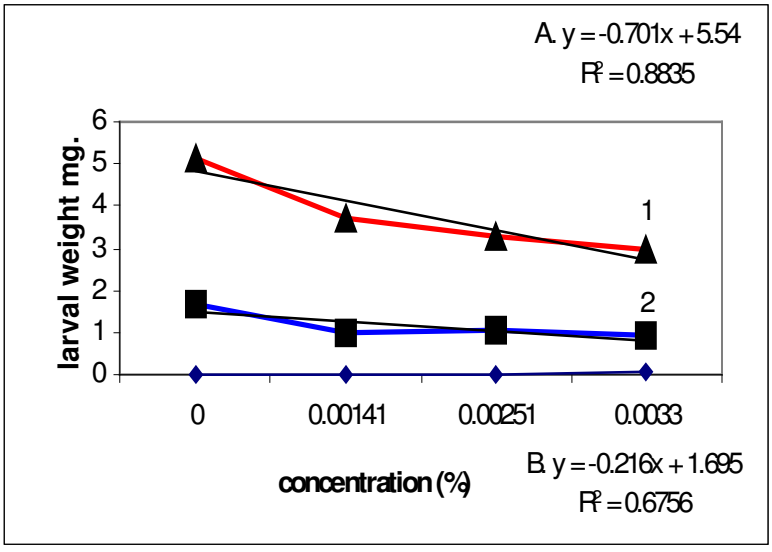
Day after treatment	Control	Percentage Hatching X ± S.E. LC_{20}	LC_{30}	LC_{40}
4th	22 ± 1.78	12.5 ± 6.0	11.25 ± 1.0	11.25± 3.4
5th	52 ± 3.3	27.5 ± 7.7	28.75 ± 1.0	25 ± 4.8
6th	56 ± 4.9	30 ± 7.2	28.75 ± 1.0	27.5 ± 5.2
7th	57 ± 5	32.5 ± 6.0	30 ± 1.6	27.5 ± 5.0

Fig. 2

a) Regression graph of dose-response for flufenoxuron on mortality of first instar larvae of *T. castaneum*.



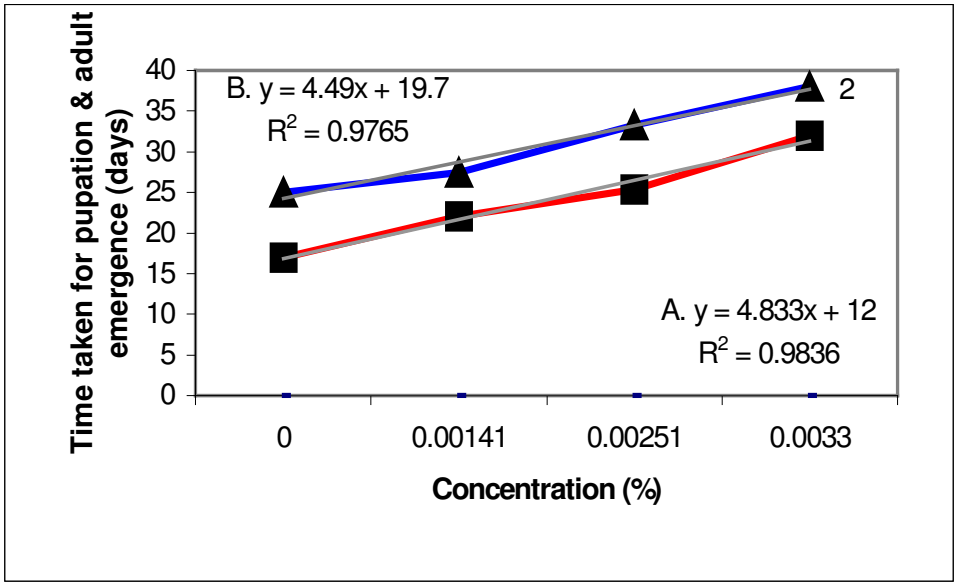
b) Regression graph of dose-response for flufenoxuron on weight of the larvae of *T. castaneum*.



1.Trendline for graph showing dose-response for flufenoxuron on weight of the 7th day old larvae. 2. Trendline for graph showing dose-response for flufenoxuron on weight of the 10th day old larvae. A. Equation for trendline 1. B. Equation for trendline 2.

Fig. 7

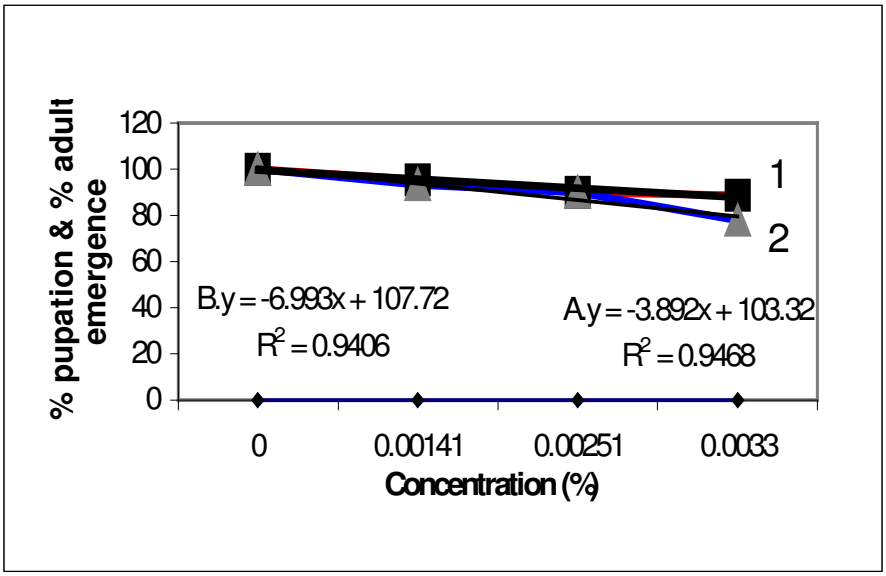
Regression graph of dose-response for flufenoxuron on time taken for pupation and adult emergence of *T. castaneum*.



1.Trendline for graph showing dose-response for flufenoxuron on time taken for pupation. 2. Trendline for graph showing dose-response for flufenoxuron on time taken for adult emergence. A. Equation for trendline 1. B. Equation for trendline 2.

Fig. 8

Regression graph of dose-response for flufenoxuron on %pupation and %adult emergence of *T. castaneum*



1.Trendline for graph showing dose-response for flufenoxuron on %pupation. 2. Trendline for graph showing dose-response for flufenoxuron on %adult emargence. A. Equation for trendline 1. B. Equation for trendline 2.

Fig. 28

Regression graph of dose response for flufenoxuron on % hatching of eggs of *T. castaneum*

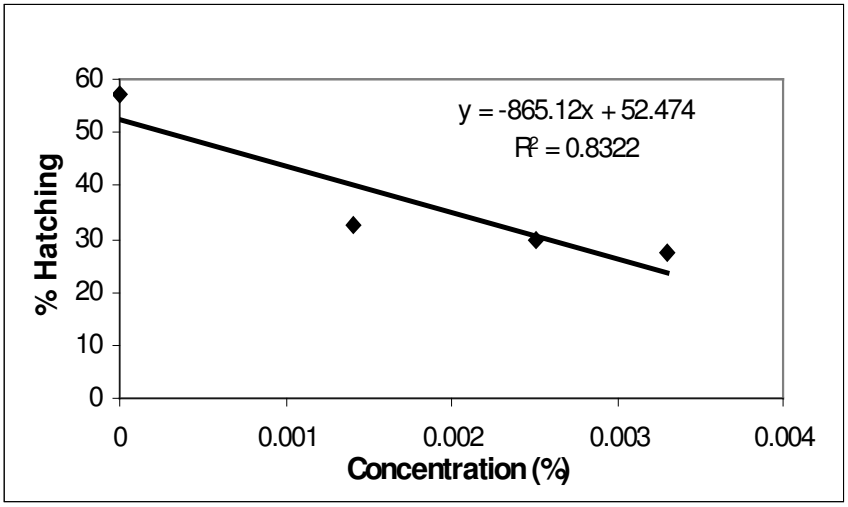
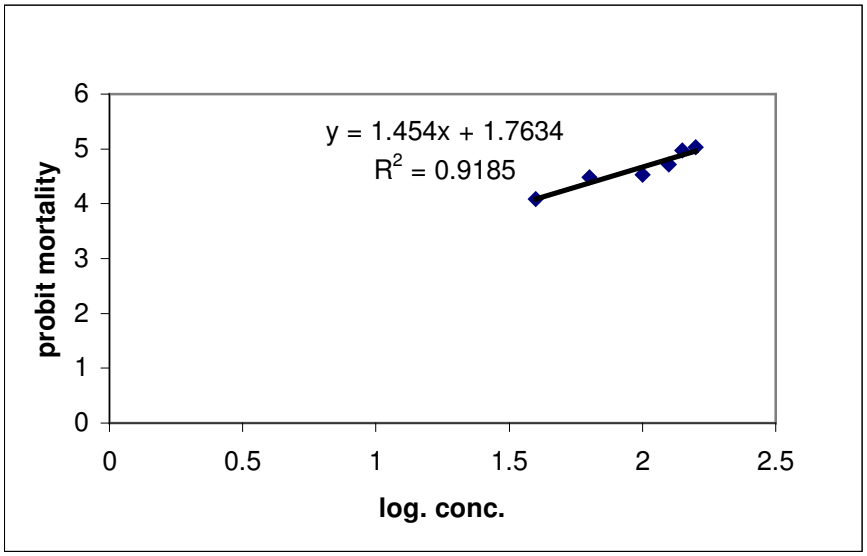


Fig. 29

Regression graph of dose response for flufenoxuron on mortality of topically treated adults of *T. castaneum*.



A major structural element of insect cuticle, chitin, has been considered as a desired target site for selective pesticides, chitin synthesis inhibitors, which act on insects of different orders causing abnormal endocuticular deposition and abortive molting (Mulder and Gijswijt, 1973). Following the discovery of the insecticidal properties of benzoylphenylurea by Philips-Dupher company, their great potential as chitin synthesis inhibitors has been recognized (Hajjar and Casida, 1979). Diflubenzuron (Dimilin) was one of the first chitin synthesis inhibitor reported, causing reduction in chitin content in the endocuticle of several insect species (Post and Vincent, 1974; Deul et al, 1978; Mitsui et al, 1984). Dimilin is the widely registered and practically used benzoylphenylurea. The main uses of dimilin are against pests in forestry, horticulture, field crops and in home (Retnakaran and Wright, 1987). The search for more potent acylurea has led to the development of new compounds such as chlorfluazuron (Haga et al, 1982), teflubenzuron (Becher et al, 1983) and hexaflumuron (Sbragia et al, 1983). These compounds are very potent against

Lepidopteran pests and ornamentals (Ishaaya et al, 1986; Ishaaya and Klein, 1990). The recently introduced benzoylphenylurea, flufenoxuron, exhibit enhanced insecticidal activity compared to diflubenzuron (Ascher and Nemny, 1984; Anderson, 1986) although having the same mode of action. Also, it was reported that diflubenzuron was less effective at low dose rates than flufenoxuron (Clarke and Jewess, 1990). Flufenoxuron was evaluated for the control of Lepidopteran crop pests in field and laboratory (Santiago et al., 1997).

When considering the impact of pesticide on pests, we must examine not only the effect of direct mortality but also those sub-lethal effects that might ultimately result in reduced population number. While measuring levels of mortality from direct insecticide exposure is a common screening method, possible sub-lethal effects as a result of either direct or indirect insecticide exposure remain relatively unknown. Sub-lethal dosage of insecticides, not only favor the beneficial insects but also slow down insect pest resisting the insecticides. Sub-lethal doses of

chitin synthesis inhibitors diflubenzuron, chlorfluazon and hexaflumeron had been found to influence *S. exigua* larval development (Laecke et al., 1989). The present study is thus an attempt to understand the effect of flufenoxuron at sub-lethal concentrations on certain important biological parameters of stored product pest *T. castaneum* under laboratory conditions.

Sub-lethal concentrations (LC_{20}, LC_{30} & LC_{40}) of flufenoxuron incorporated in the diet and fed for 24h to newly hatched first instar larvae of *T. castaneum* were shown to affect growth, development and fecundity. Subsequent feeding on a flufenoxuron-free diet did allow the larvae to gain sufficient weight to achieve growth and moulting. However, significant growth retardation was reflected by lower larval weight and a delay in both the time taken for pupation and adult emergence. Significant reduction in pupation as well as adult emergence was also observed. At lower concentrations (LC_{20} and LC_{30}) more larval-pupal and pupal-adult intermediates were observed, although no abnormal adults were observed. At the highest concentration tested (LC_{40}) deformed adults were encountered, one type had deformed wings only whereas the other had undeveloped and deformed body parts and unsclerotized patches on the

exoskeleton of the thorax, in addition to wing deformities. Similar observations were reported by Arthur (2001) on both *T. castaneum* and *Tribolium confusum* on exposure to hydroprene. Developmental abnormalities such as larval-pupal and pupal-adult intermediates as well as deformed adults similar to those found with the use of JH analogues, suggest that flufenoxuron may influence reproduction by causing hormonal imbalance (Bull, 1986; Deecher et al., 1989; Deecher et al., 1990 a,b). The severity of adult deformity was so great that such adults could not retain their dorso-ventral posture. Furthermore, due to incomplete chitinisation of the mandible they could not feed and as a result, died within a week after emergence. Our findings support those of Neumann and Guyer (1987) and Clarke and Jewess (1990) who observed such effects of benzoylphenylureas on *Heliothis virescens* and *Spodoptera littoralis* respectively.

Adults emerging from lower concentrations (LC_{20} and LC_{30}) laid mostly non-viable eggs and those eggs, which hatched resulted in larval mortality during the first instar. This is possibly due to impairment of cuticle secretion in the affected embryos, as has been reported in case of *Leptinotarsa decemlineata* as a result of treatment with diflubenzuron (Grosscurt, 1978). This is the first report on the effects of varying sub-lethal

concentrations of flufenoxuron on the reproduction of the surviving adults of *T. castaneum.*

There was considerable variability in the response of adults of *T. castaneum* of different ages with respect to fecundity, when fed on diet treated with sub-lethal concentrations of flufenoxuron. Two day old adults showed a dose-dependent effect, whereas, in three day old adults all the concentrations were equally effective. However, in case of four day old adults the highest sub-lethal concentration (LC_{40}) was the most effective. Furthermore, in the younger adults there was a dose-dependent effect on the number of abnormal eggs laid. In most insects the egg is covered by a sticky "shell" of protein secreted by the accessory glands. Abnormal eggs lacked the sticky layer as reflected by the lack of flour sticking to their surface and this may be due to the absence of the accessory gland secretion. The overall trend, however, revealed an age-dependant effect on fecundity in *T. castaneum* adults with sub-lethal concentrations of flufenoxuron.

The reduction in the fecundity of treated females and reproductive potential of males as a result of topical application of sub-lethal concentrations of flufenoxuron may be as a result of egg sterilization in RH-5849 (non-steroidal ecdysone agonist) treated Lepidopteran females through the disruption of oogenesis as reported by Wing et al. (1988) and by

interference with spermatogenesis in males, respectively. Smagghe and Degheele (1992b) found that RH-5849, a precursor of the ecdysone agonist tebufenozide, reduced fecundity by causing the resorption of oocytes in adult female of *Spodoptera littoralis* It is apparent from the present study that sub-lethal concentrations of flufenoxuron exhibit transovarial ovicidal activity as described previously in different flies (Wright and Harris 1976; Wright and Spates 1976; Ivie and Wright, 1978; Chang, 1979), the boll weevil, *Anthonomus grandis* (Moore and Taft,1975) and the codling moth, *Cydia pomonella* (Moffitt et al.,1983). Chlorfluazuron and pyriproxyfen have a similar effect on *H..virescens* females (Neumann and Guyer, 1987) and white flies (.Ishaaya and Horowits, 1992; Ishaaya et al., 1994), respectively.

The present study reveals that flufenoxuron is more effective through ingestion compared to topical application, in case of *T. castaneum*, in contrast to the findings of Clarke and Jewess (1990), according to which in *Spodoptera littoralis*, sub-lethal concentrations of flufenoxuron were ten times more effective by topical application than by ingestion. This differential activity may be due to penetration differences (Biddinger and Hull, 1999), since *T. castaneum* and *S. littoralis* belong to different insect orders and the cuticle in case of *T. castaneum* is far sturdier than that of S. littoralis. Since, it is known that hard cuticles (as in case of *T. castaneum*),

the proteins are stabilized (sclerotized, cross-linked) by phenolic and quinone compounds that form covalent bonds and cross-link proteins to each other (and possibly to chitin), forming a very hard, rigid structure. This might possibly be the reason as to why suscesptibility of adult *T. castaneum* on topical application of flufenoxuron is much less compared to *S. littoralis*, which posses a soft cuticle, with some degree of stabilization, but probably with relatively few cross-links, thus allowing the penetration of flufenoxuron more effectively.

Eggs placed in diet treated with sub-lethal concentrations of flufenoxuron exhibited a dose-dependent, inverse relationship with respect to hatchability. Similar findings have been reported for *S. littoralis* where the eggs were dipped in either PH-6040 (Ascher and Nemny, 1974) or diflubenzuron and BAY SIR 8514 (Ascher et al., 1979).

Thus, in the present investigations on sub-lethal effects of flufenoxuron on biological parameters of *T. castaneum*, it was found that sub-lethal concentrations of flufenoxuron exhibited a dose-dependant inverse relationship with respect to growth, development and reproduction of *T. castaneum.* Further, the effective sub-lethal concentration of flufenoxuron was found to be dependent on the stage in the life cycle of *T. castaneum*.

Effect of sub-lethal concentrations of flufenoxuron on some biochemical parameters of T. castaneum.

INTRODUCTION

Insect cuticle is a polymeric network in which chitin occurs as micrifibrils surrounded by and bound to protein by covalent and non-covalent bonds. This complex is immersed in a matrix of loosely bound proteins (Hackman, 1976; Richard, 1978). Various biochemical changes occure during the development of insects. Of the changes, which occur in the physiology of insects, those involving chitin and protein should yield the most information since they are very closely associated with growth and reproductive processes (Muzzarelli, 1977). It was shown using chitin synthesis inhibitors that disruption of the polysaccharide formation resulted in malformed cuticles, lacking their normal exoskeletal properties and unable to withstand increased internal pressure during moulting (Mulder and Gijswijt, 1973). Benzoylphenylureas affect a cascade event involved in chitin biosynthesis (Post et al., 1974; Marks and Sowa, 1974). The disturbance of the formation of cuticular tissue by benzoylphenylureas led to

biochemical studies on the possible effects on the chitin and protein constituents of the cuticle.

A correlation between toxicity to *Oncopeitus fasciatus* and the extent of chitin synthesis inhibition for a large number of benzoylphenylureas was reported (Hajjar and Casida, 1979). The benzoylphenylurea action in insects may be indirect by altering ecdysone or JH levels (Verloop and Ferrell, 1977; Yu and Terriere, 1977) or direct by inhibiting a critical step in chitin formation (Post et al., 1974). In diflubenzuron treated insects, based on accumulation of UDP-Glc NAC, it was concluded that benzoylphenylureas inhibit chitin synthatase (Post et al., 1974; Deul et al., 1978; Cohen and Casida, 1982). Further, it was observed that diflubenzuron inhibits the transport of UDP-Glc NAC across biomembranes in *Momestra brassicae* (Mitsui et al., 1984). Benzoylphenylureas were shown to block conversion of glucose to fructose-6-phosphate resulting in inhibition of chitin synthesis (Saxene and Kumar, 1981).

It was observed that specific receptor proteins are to be laid first to which oligosaccharides are linked and polymerized to form microfibrils, then the inhibition of synthesis of these specific receptor proteins would result in feedback inhibition of chitin biosynthetic pathway (Stevenson and Tung, 1971; Oberlander et al., 1980). It was reported that in *Calliphora*

erythrocephala, larval haemolymph protein presumably in the form of one or more its constituent subunits was incorporated into the cuticle (Scheller et al., 1080). It was observed that larval haemolymph proteins are secreted in haemolymph during larval growth and feeding (Thomson, 1975). Robert and Brock (1981) called thes as storage proteins and found that they are in the range of 50,000 and are involved in cuticle formation, metamorphosis and vitellogenesis process in *Drosophila melanogaster*. Larval storage protein acts as a low affinity carrier protein for ecdysteroids (Reum et al., 1982).

Generally, benzoylphenylureas were found to inhibit chitin synthesis (Retnakaran, 1986; Retnakaran and Wright, 1987). However, the benzoylphenylurea, teflubenzuron inhibited the synthesis of the protein associated with chitin (Porcheron et al., 1991). Significant decrease in chitin content of the cuticle was found due to treatment of *Spodoptera littoralis* larvae with flufenoxuron (Sammour and EL-Ansary, 1996). Similar observations were made by Ishaaya and Casida (1974) in housefly larval cuticle after treatment of larvae with TH 6040. The ratio of protein:chitin was found to increased with increasing concentration of benzoylphenylureas TH 6040 housefly larva (Ishaaya and Casida, 1974) and flufenoxuron in *T. castaneum* (Sammour and Ansary, 1996).

The organisms vary in the biochemical, physiological, morphological and behavioral mechanisms that underlie stress tolerance. The expression of stress genes is a potentially sensitive indicator of any chemical or physical assault.The cellular defence mechanism involves the induction of heat shock proteins (hsps) or stress proteins (Atkinson and Walden, 1985). The increased expression of stress proteins play related, protective and reparative fuctions to reduce protein aggregation and non-native conformation caused by environmental perturbations (Craig et al., 1983; Linquist, 1986; Welch, 1993; Feder, 1996). Organisms exposed to pesticides had been shown the increased level of hsp70 in the cell (Chowdhuri et al., 1999; Nazir et al., 2001). Induction of hsp70 can be used as a biomarker of exposure against pesticides (Sanders, 1990, 1993; Bierkens et al., 1997). The relative levels of this proteins are important as too little or too much hsp70 can result in developmental malformations and aberrant growth control (Feder et al., 1992; Elefant and Palter, 1999).

The animal cell cycle consists of S phase (chromosomal DNA replication) followed by M phase (segregation of the replicated chromosome into two daughter nuclei) and G1 & G2 phases between M & S and S & M respectively. Cdc2/ cyclin B is a central regulator of the transition through G2/M phase of the cell cycle. Precise activation and inactivation of cdc2 are

necessary for normal cellular proliferation. Environmental stresses like UV light, toxins etc. induce delays in the cell cycle that commonly results from inhibition of cdc2/ cyclinB kinase and arrest in the G2/M phase of the cell cycle (Herzinger et al., 1995; poon et al., 1996).

In the present studies, in order to investigate the biochemical effects of flufenoxuron, *T. castaneum* neonates exposed to various sub-lethal concentrations of flufenoxuron in the diet were subjected to assays of chitin and protein levels, expression of stress protein hsp70 and cell cycle regulatory protein $p34^{cdc2}$ during growth and development.

Materials

Chemicals for Bioassay:

All reagents were either purchased from Sigma Chemical Co., USA or Life Technologies (Gibco BRL, USA). The sources of specific chemicals are as follows:

P-dimethyl aminobenzaldehyde (DMAB) was purchased from Qualigens® fine chemicals. Potasium tetraborate was purchased from Sigma Chemical Co. Coomassie brilliant blue G (CBB) and Bovine Serum Albumen (BSA) were purchased from Sigma Chemical Co. Protein molecular weight marker was purchased from Bangalore Genei Pvt. Ltd., India. Anti-HSP70 and Anti-$p34^{cdc2}$ monoclonal antibodies were purchased from Sigma Chemical Co., USA. Secondary antibodies (Anti mouse IgG- Alkaline phosphatases) were purchased from Sigma Chemical Co., USA or from Bangalore Genei Pvt. Ltd., India. Nitrocellulose membrane was purchased from Schleicher and Schuell Inc.,USA and Life Technologies (Gibco BRL).

Routinely used chemicals were purchased from Hi-Media and MERCK India Ltd

A) Chitin analysis:

i) Homogenizing buffer: 5mM Tris, 38mM Glycine, pH 8.4

ii) Acetic anhydride: 1.5% (v/v) prepared in acetone.

iii) N-acetyl glucosamine: 1mg/ml.

iv) Potassium tetraborate: 100 mM

v) P-dimethylaminobenzaldehyde (DMAB): 10% DMAB prepared in acetic acid containing 12.5% 10 N HCl (v/v)

B) Protein analysis:

i) Protein extraction buffer (PEB)
20mM Tris-HCl, pH 8.0;

1mM EDTA, 0.1% Triton X-100,

1mM PMSF (added just before use).

1X Protease Inhibitor Coctail (PI).

Protein estimation:

i)) Bradford reagent:

50mg Coomassie brilliant blue (CBB) (G-250)

50ml H_3PO_4

Add distilled water to make volume 100 ml. (Added drop by drop with constant gentle stirring).

ii) Bovine Serum Albumen (BSA):

1mg /1ml BSA (stored at –20 ° C)

(Diluted 10 times before use).

SDS Polyacrylamide gel electrophoresis:

i) Gel solution

29.2gm Acrylamide

0.8 gm N –N' Methylene Bis Acrylamide

Distilled water to make volume 100ml

Stirred well to dissolve & then filtered the solution through Whatman filter paper No.1 or 0.2μm membrane filter by vacuum pump. Stored at 4° C in amber coloured bottle.

ii) Tris buffers:

a) 1.5M Tris –HCl (pH 8.8)

18.17 gm Tris base

Distilled water 60 ml

Dissolved & adjusted pH to 8.8 with HCI & then distilled water was added

to make the volume 100 ml

b) 0.5M Tris –HCl (pH 6.8):

6.06 gm Tris base

Distilled water 60 ml

Dissolved & adjusted pH to 8.8 with HCl & then distilled water was added

to make the volume 100 ml.

iii) 10% Running gel (SDS-PAGE):

Distilled water	3.98ml
1.5M Tris HCl (pH 8.8)	2.5ml

Gel solution	3.33ml
10% SDS	100µl
10% APS	75µl
TEMED	7.5µl

iv) Stacking gel:

Distilled water	6.1ml
0.5M Tris HCl (pH 6.8)	2.5ml
Gel solution	1.3ml
10% SDS	100µl
10% APS	75µl
TEMED	15µl

v) 10% SDS:

2gm SDS dissolved in Distilled water to make final volume 20 ml.

Dissolved by gentle stirring.

vi) 10 % Ammonium per sulphate (APS):

100 mg APS dissolved in 1ml distilled water & stored at 4° C (Freshly prepared solution was always used)

vii) TEMED (N, N, N' N'-tetramethylethylenediamine)

viii) Sample Buffer /Laemmli Buffer (LB):

LB was prepared without β- Mercaptoethanol & divided into 10 equal aliquots.Stored at -10° C. β- Mercaptoethanol was added to an aliquot (100 µl / aliquot for 2X LB & 250 µl /aliquot for 5X LB) just before use.

LB (2X)	**LB (5X)**
2.5ml 0 .5 M Tris –HCl pH 6.8.	6.25ml 0.5 M Tris – HCl pH 6.8
4 ml 10 % SDS	1 gm SDS
2ml Glycerol	2gm Sucrose
0. 5 ml 1% Bromophenol Blue	0.012 gm Bromophenol Blue
1 ml β- Mercaptoethanol	2.5 ml β-Mercaptoethanol
1.25 ml distilled water	1.25 ml distilled water

ix) Protein Molecular Weight Marker:

5µl Molecular weight Marker

25µl Sample buffer (2X)

x) Electrode / tank buffer (5X):

15gm Tris base, 72 gm Glycine, 5gm SDS. Distilled water to dissolve & make volume 1000ml. Stored at room temperature.

xi) Coomassie brilliant blue (CBB) stain-R- 250:

a) Stock solution: 2.5gm CBB (R-250)

250ml distilled water. Dissolved in distilled water, filtered with Whatman filter paper (No.1) and stored at room temperature.

b) Working solution: 62.5ml CBB stock solution, 250 ml Methanol, 50ml. Glacial Acetic Acid. Distilled water to make volume 500ml

xii) Destainer:

	I	II
Methanol	50%	10%
Glacial acetic acid	10%	10%
Distilled water	40%	80%

xiii) Gel storage solution: 7% Glacial acetic acid in distilled water.

Western Blot:

i) Transfer Buffer

a) Stock solution (5X): 15gm Tris Base, 72gm Glycine dissolved in 700ml distilled water and then add distilled water to make the final volume 1000ml. Stored at 4°C.

b) Working Solution (1X): 200ml 5X Transfer Buffer, 40ml Methanol, 760ml distilled water.

ii) Ponceau red-S.

iii) Phosphate Buffered Saline (PBS) 10X, pH 7.5:

20gm NaCl, 0.5gm KCl, 3.6gm Na_2HPO_4, 0.6gm KH_2PO_4. Dissolved in distilled water, pH was adjusted to 7.5 & then final volume was adjusted to 250ml. Stored at room temperature.

iv) PBST (0.01%): 100ml PBS (1X), 10µl Tween-20.

v) Blocking reagent:

a) 3gm Bovine serum albumen (BSA)

100ml PBS (1X)

Dissolve and stored at -20^0C

b) 10ml 10% blocking reagent, 90ml distilled water.

vi) Primary antibodies:

a) Mouse anti-HSP70 antibody.

b) Mouse anti- P34 ^{cdc2} antibody.

ix) Secondary antibodies:

Goat anti-mouse/ anti-rabbit IgG AP conjugate.

x) Color Detection of Western Blot:

Color Developing solution (prepared just before use).

For Alkaline Phosphatase Conjugate

10ml DIG Detection Buffer (1X)

200µl NBT/BCIP

METHODS

Chitin Extraction and Estimation:

The effects of sub-lethal concentrations of flufenoxuron on chitin content of the larvae were determined by releasing the neonates in the treated diet for 24h. Subsequently the larvae were transferred to normal diet. Diet mixed with appropriate quantity of acetone was used as control. Five replicates of 250 larvae each were prepared. On 7^{th}, 10^{th}, 15^{th} days after the treatment, 200 larvae on 7^{th} day, 150 larvae on 10^{th} day and 100 larvae on 15^{th} day were weighed and homogenized in homogenizing buffer (1gm/10ml), containing 5mM Tris, 38mM Glycine, pH 8.4. The homogenate was centrifuged at 10,000 rpm for 15min and the precipitate was dissolved in 6 N HCl. The dissolved precipitate was transferred into hydrolysis tubes, which were sealed under vacuum and the material was hydrolysed at 100°C for 16h in a temperature controlled heating block. On completion of hydrolysis, the solution was centrifuged at 10,000 rpm for 10min. The supernantant was collected and neutralized with 30% NaOH. N-acetylglucosamine content was estimated according to the method of Ressig et al. (1955).

Glucosamine 0.1-1µmole was taken in capped test tubes. To this was added 1.5% acetic anhydride (v/v) prepared in acetone and 1ml potassium tetraborate (100mM, pH 8.5).The tubes were sealed tightly, incubated in boiling water bath for 3min and cooled immediately in an ice bath. Subsequently 3ml of p-dimethyl aminobenzaldehyde (DMAB) [10% DMAB prepared in acetic acid containing 12.5% 10 N HCl (v/v)] diluted with acetic acid (1:9) was added to the solutions in the tubes. The tubes were incubated at 37℃ for 20 minutes. Absorbance of the mixture was measured at 585 nm in a Jasco V-550 UV /visible spectrophotometer.

Protein Extraction and Estimation:

Neonates of *T. castaneum* were fed on control and treated diet (with sub-lethal concentrations of flufenoxuron LC_{20}, LC_{30} and LC_{40}) for 24h and transferred to normal diet. Five replications of 250 larvae each were made for each concentration. After the treatment 200 larvae on 7^{th} day, 150 larvae on 10^{th} day and 100 larvae on 15^{th} day were homogenized in protein extraction buffer (1gm/4ml) containing 20mM Tris-HCl (pH 8.0), 1mM EDTA, 1mM PMSF and 0.1% Triton X-100, followed by centrifugation at 10,000rpm at 4℃ for 20 minutes. The supernatant was removed and stored at -80℃. Protein content of the supernatant was determined by Bradford's

microestimation method (Bradford, 1976). The entire experiment was repeated thrice on different occasions.

SDS PAGE:

Samples containing equal amount of protein, as determined by Bradford's method from control and treated larval tissue extracts were denatured in sample buffer (Laemmli, 1970) for 3-5 minutes at 100℃ and analysed on 10% SDS PAGE (Laemmli, 1970) along with molecular weight marker protein. Electrophoresis was carried out at a constant current (25 mA) at room temperature, and the gel was stained overnight with 0.125% Coomassie brilliant blue-R250, followed by destaining in destainer I and destainer II till the background became clear.

Western Blotting:

Following SDS PAGE, proteins were electrophoretically transferred to a nitrocellulose membrane at a constant current (70 mA) for 12-14h at 4℃ (Towbin et al., 1979). After transfer, blot was stained with Ponceau red S to check the efficiency of transfer. Blots were then processed for immunoreaction using anti-HSP70 and anti-$p34^{cdc2}$ monoclonal antibodies.

Color detection method for Western Blot:

The blots were destained and equlibrated by washing thrice (10min each) in PBS at room temperature with gentle shaking. Then blots were saturated with 3% BSA in PBS for 4h at room temperature with gentle shaking. Blots were incubated overnight with primary antibody in phosphate buffered saline (PBS, pH 7.4) at 4℃ and then with alkaline phosphatase conjugated secondary antibody for 4h at room temperature with gentle shaking. Following each antibody incubation blots were washed thrice (15 min each) in PBS. Blots were developed for color reaction using NBT-BCIP, as the substrates. Reaction was stopped, by washing the blot with distilled water. The results were analyzed using Biorad gel documentation system.

Effect of flufenoxuron on total chitin and protein content during development of *T. castaneum*:

During normal development of *T. castaneum* larvae from neonates to late instars, it was observed that there was reduction in chitin content on 10^{th} day as compared to 7^{th} and 15^{th} day (Fig. 30), while protein content of the larvae increased with time (Fig. 31). The effects of sub-lethal concentrations of flufenoxuron on chitin and protein content of the larvae and their analysis are presented in Figs. 30 & 31. The results are summarized as follows:

On 7^{th} day chitin content of the larvae was reduced in a dose dependent manner when treated with sub-lethal concentrations of flufenoxuron (Fig. 30). There was a significant reduction in soluble protein content in LC_{20} and LC_{30} treated larvae as compared to that in control (Fig. 31). At LC_{40} concentration, chitin content of treated larvae was significantly reduced (Fig. 30), however there was no significant reduction in protein content as compared to that in control larvae (Fig. 31).

On 10^{th} day there was significant reduction in both chitin content (Fig. 30) and protein content (Fig. 31) in LC_{20} and LC_{30} treated larvae as compared to that in control larvae. Whereas, in LC_{40} treated larvae, though there was significant reduction in chitin content (Fig. 30) protein content was not significantly reduced as compared to that in control larvae (Fig. 31).

On 15th day, in LC_{20} and LC_{30} treated larvae, it was observed that the chitin as well as protein content was significantly reduced (Figs. 30, 31), while in LC_{40} treated larvae the chitin content although was reduced (Fig. 30), protein content was not reduced significantly as compared to that in control larvae (Fig. 31).

When the results are expressed as the protein:chitin ratio, it was highest on 11th day as compared to 7th and 15th day old controlled larvae (Table 1). There was progressive increase in the ratio from 7th to 15th day after treatment with sub-lethal concentrations of flufenoxuron.except at LC_{40} (Table 1).

Effect of flufenoxuron on protein profiles (SDS PAGE) during development of *T. castaneum*.

In order to determine the effect of flufenoxuron on protein profiles, protein extracts from control and flufenoxuron treated (LC_{20}, LC_{30} and LC_{40}) samples were analysed by SDS-PAGE. Although no significant difference was observed between the protein profiles of control and treated samples in general, there were quantitative differences in some subunits (molecular weight 50-97 kDa) in a stage specific manner. A protein band of 97kDa decreased considerably in treated samples of 10th day old larvae in particular, as compared to that in the control sample (densitometric scan

profile for this particular band is shown in Figs. 32b, 33c and 34b). Further, a protein band of slightly lower molecular weight (80 kDa, indicated by star) increased in quantity during development of the larvae (Fig. 32a, lanes 2, 3 & 5; Fig. 32c). Interestingly, this band was particularly affected in flufenoxuron treated samples of 10^{th} day oid larvae (lanes 3 vs 4). Another protein band 9molecular weight 50 kDa) appeared reduced in quantity in all stages (7^{th}, 10^{th} and 15^{th} day old larvae) as compared to the respected control larvae (Figs. 32d, 33b & 34 c). However, more specific analysis would be required to identify these proteins and thereby conclude on the effect of such changes on development per se of the larvae. These results have been repeatedly observed in a few independent analysis (standard deviations are indicated).

Effect of flufenoxuron on specific proteins (Western blot analysis):

HSP70: The level of HSP70 in the larvae treated with sub-lethal concentrations of flufenoxuron was found significant only in LC_{30} treated larvae on seventh day after the treatment (Fig. 35a, lane 2). Further, no detectable level of HSP70 was found in the control as well as treated larvae on 10^{th} and 15^{th} day. However, as seen in the Western blot anti- HSP70 antibody detected a doublet of polypeptide of around 28kDa (Fig. 35a, lanes 1, 4, 5, 6). A significant variation of the quantities and proportions of the

doublet were observed in the control and treated larvae during development (Fig. 35a and b). In control larvae the higher molecular weight (29kDa) polypeptide of doublet was detected only on 7th day (with barely detectable amount later) (Fig. 35a, lane 1) while in LC_{30} treated larvae it was detected only on 10th day after treatment (Fig. 35a, lane 4). However the lower molecular weight (28kDa) polypeptide was detected in all the stages in the control with a gradual decrease in the quantity during development, except on 10th day when it increased (Fig. 35a, and b).

P34^{cdc2}: In control and LC_{30} treated larvae two forms of the protein p34^{cdc2} (phosphorylated and non-phosphorylated) were detected using anti- p34^{cdc2} antibody. These two forms showed significant changes during development (Fig. 36a). In treated larvae, there occurred increased phosphorylation of p34^{cdc2} as indicated by about one and half fold increase in higher molecular weight forms as was observed on 7th day after the treatment (Fig. 36a, b, lane 2). Further, it was observed that there was a substantial decrease in the basal level of expression of p34^{cdc2} from 7th to 15th day in control larvae. However, the amount (both forms) of p34^{cdc2} was reduced in quantity at 10th day after treatment as compared to the control (Fig. 36a, b, lanes 3 vs 4 and lanes 5 vs 6).

Fig. 30

Effect of sub-lethal concentration of flufenoxuron on chitin content per larva during development of *T. castaneum*

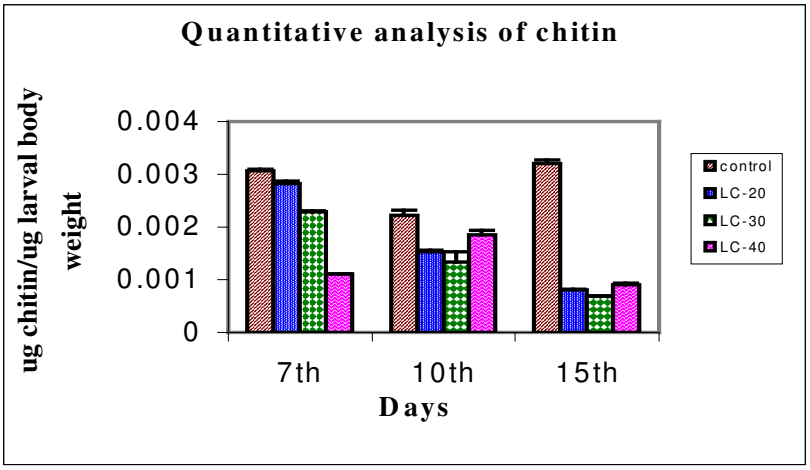


Fig. 31

Effect of the sub-lethal concentration of flufenoxuron on total soluble protein content per larva during development of *T. castaneum*.

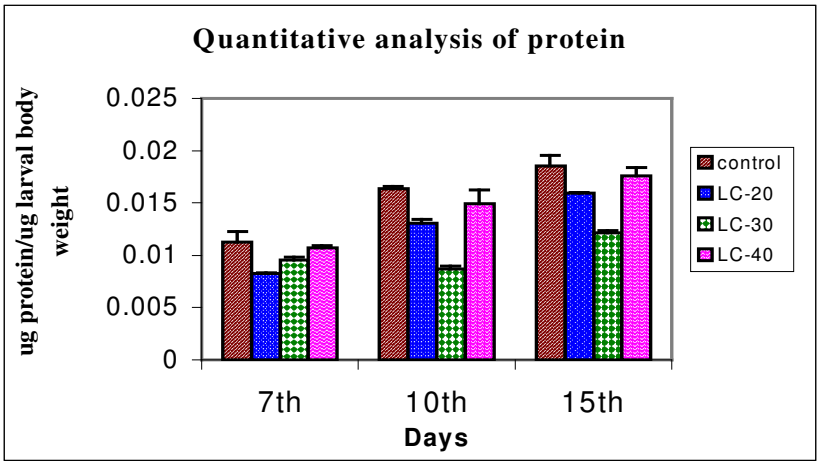


Table 12

Ratio of µg of soluble protein and chitin content per µg of larval body weight of *T. castaneum* larvae treated with sub-lethal concentrations of flufenoxuron.

Dose	Days after treatment		
	7th day	10th day	15th day
Control	3.69	7.349	5.77
LC_{20}	2.92	8.5	19.8
LC_{30}	4.166	6.49	17.6
LC_{40}	9.58	8.08	19.41

T.castaneum neonates fed on diet treated with sub-lethal concentrations of flufenoxuron for 24hrs show overall reduction in total protein content at all concentrations treated during their development. SDS-PAGE protein analysis revealed the induction of some new proteins in response to oral administration of sub-lethal concentrations of flufenoxuron through diet. Inspite of the appearance of a few new proteins, the total protein concentration decreased with increasing concentrations of flufenoxuron. Similar results were reported by Kulkarni and Mehrotra (1975) in *Schistocerca gregaria* due to Sumithion treatment. Also, Tiwari (1989) found that Diflubenzuron at sub-lethal concentrations affects the level of haemolymph proteins, which may disrupt the normal physiological function resulting in abnormal growth in *Diacrisia obliqua*. Proteins of molecular weight 50kDa and 80kDa were expressed more in control than in treated larvae. Such proteins are associated with enzymatic activities in cuticle formation (sclerotization, melanin formation, catecholamine metabolism), deficiency of which interferes with the digestion of cuticle during moulting as observed in *Drosophila melanogaster* and *Manduca sexta* by Marcu and Locke (1998). The quantitative reduction of these proteins in treated larvae possibly result in larval-pupal and pupal-adult intermediates as reported in the present study.

Observations on total chitin content of the larvae treated with sub-lethal concentrations of flufenoxuron revealed that reduction in chitin content was more significant on 15^{th} day after treatment. Further, protein: chitin ratio was higher on 11^{th} day as compared to 7^{th} and 15^{th} day in control larvae whereas in treated larvae the ratio increased with increasing concentrations of flufenoxuron from 7^{th} to 15^{th} day, except at LC_{30} where there was a decrease in the ratio on 15^{th} day. Our results are in agreement with those observed by Ishaaya and Casida (1974) and Clarke and Jewess (1989). Similar dose-dependent effects of flufenoxuron on protein: chitin ratio with time elapsing after flufenoxuron treatment was recently reported by Sammour et al. (1996) in case of *Spodoptera littoralis* larvae. Cross-linking of chitin and protein to form cuticle plays a crucial role in the development of insect. Variations in protein: chitin ratio due to treatment with sub-lethal concentrations of flufenoxuron was reflected on growth and development of *T. castaneum* by decrease in larval weight, delay in the development of pupal and adult stages as well as formation of abnormal adults.

It was interesting to note that at higher concentration of flufenoxuron (LC_{40}), the total soluble protein and chitin content of the treated larvae were not much affected. This may be due to activation of the detoxification

process as observed by Wood et al. (1986). Further, involvement of some low molecular weight (23 kDa) proteins in vitellogenesis and embryogenesis was observed by Robert and Brock (1981). Quantitative changes in the proteins of low molecular weight (28-30kDa) were observed in LC_{40} treated larvae of *T. castaneum*. This may be the reason for loss in posture and reduction in fecundity in LC_{40} treated larvae as reported in the present study.

Expression of one of the major stress proteins (HSP70) in the larvae treated with sub-lethal concentration of flufenoxuron was significant in LC_{30} treated larvae on 7^{th} day after the treatment. Also, it was repeatedly observed that there was cross reactivity of HSP70 with some low molecular weight (28 kDa and 29 kDa) proteins, which seemed to resolve in the gel near mobility of the small heat shock proteins. Alternatively, these low molecular weight peptides are possibly proteolytic fragments of HSP70 as observed by Robert and Gilbert (2000) in *Manduca sexta*. These low molecular weight proteins were found in the early developmental period (7 day old larvae) and their expression reduced gradually, during subsequent stages of development. Proteins of low molecular weight (29kDa) were found to be the major haemolymph proteins (MHP) in *Bombyx mori* by Bosquet et al. (1985). Further, Plantevin et al. (1987) found that the rates of synthesis of these MHP showed quantitative changes during development in *Bombyx*

mori and their synthesis was regulated by juvenile hormone (JH) titre. In LC_{30} treated larvae the induction of 29kDa protein expression on 11^{th} day coincides with the prolongation of the larval period. However, it is not known as to how flufenoxuron influences JH titre, which in turn modulates expression of such proteins. There appears to be correlation between the expression of these low molecular weight proteins (small HSPs of 28 and 29kDa) and the phosphorylation state of $p34^{cdc2}$. However, further investigations are required to explain the significance of such observations. Absence of expression of HSP70 at higher concentration (LC_{40}) is more likely to be related to collapse of the cellular machinery, rather than its inability to induce HSP70 as observed by Bierkens et al., (1997) in *Raphidocelis subcapitula*. Thus, it appears that HSP 70 induced on 7^{th} day provides the main protection against insect growth regulatory stress at LC_{30} concentration of flufenoxuron.

A key step in regulating the entry of eukaryotic cells into mitosis is the activation of protein kinase cdc2 by selective phosphorylation of some sites. Therefore, it was interesting to observe the expression of cdc2 during the developmental process of *T. castaneum* larvae treated with sub-lethal concentrations of flufenoxuron. $P34^{cdc2}$ from treated and control tissue was observed by immunoblotting as two bands whose expression changed during

different developmental stages. The highest level of both the phosphorylated and non-phosphorylated forms were observed in the LC_{30} treated larvae on 7^{th} day after the treatment. The abundance of the phosphorylated forms suggest cell cycle blockage at G2/M phase. These data support those observed by Goss et al. (2003) in HeLa cell due to anisomycin induced cell stress. Thus, the data suggest that p34 ^{cdc2} become rapidly phosphorylated during the treatment and at the same time it also shows the variability in the expression of this protein during the developmental stages. This clearly indicates that the effect of sub-lethal concentrations of flufenoxuron has developmental stage specificity.

Fig. 1

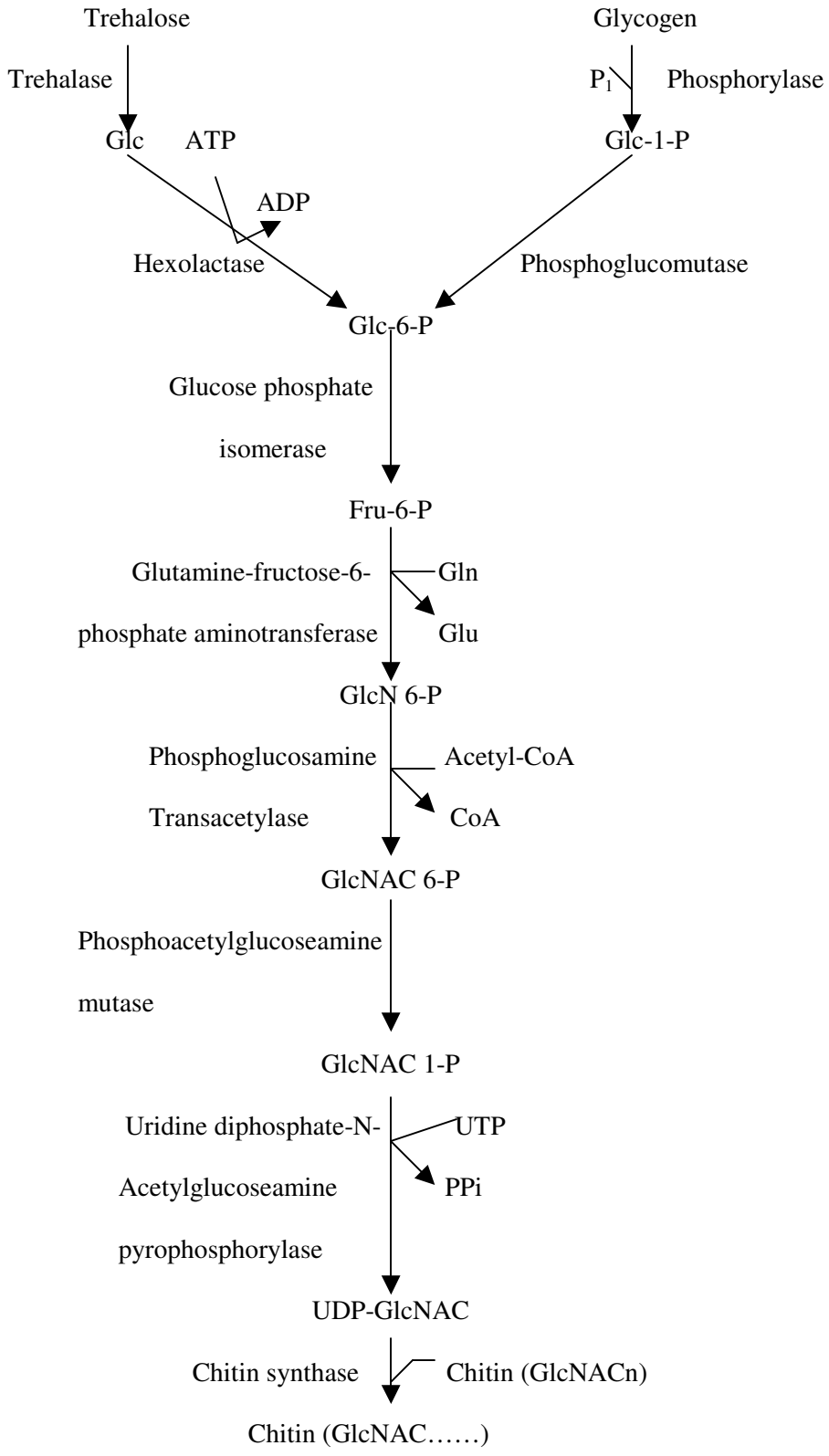
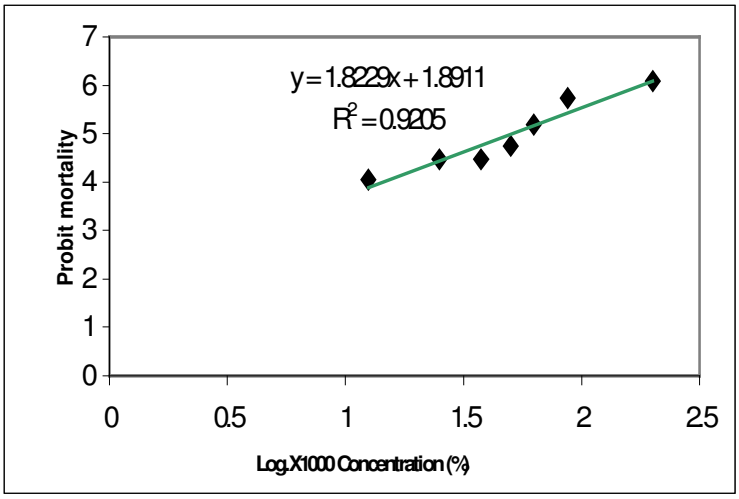
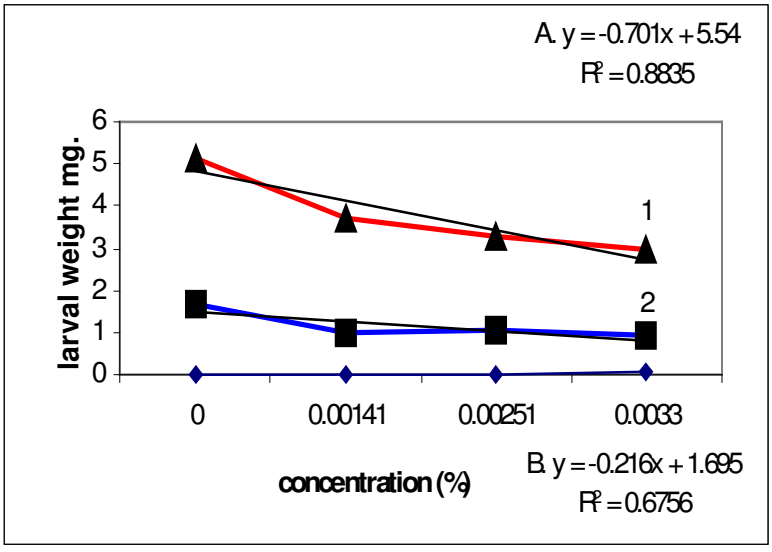

Trehalose
Glycogen
Trehalase
P_1
Phosphorylase
Glc
ATP
Glc-1-P
ADP
Hexolactase
Phosphoglucomutase
Glc-6-P
Glucose phosphate
isomerase
Fru-6-P
Glutamine-fructose-6-
Gln
phosphate aminotransferase
Glu
GlcN 6-P
Phosphoglucosamine
Acetyl-CoA
Transacetylase
CoA
GlcNAC 6-P
Phosphoacetylglucoseamine
mutase
GlcNAC 1-P
Uridine diphosphate-N-
UTP
Acetylglucoseamine
PPi
pyrophosphorylase
UDP-GlcNAC
Chitin synthase
Chitin (GlcNACn)
Chitin (GlcNAC……)

Fig. 2

a) Regression graph of dose-response for flufenoxuron on mortality of first instar larvae of *T. castaneum*.



b) Regression graph of dose-response for flufenoxuron on weight of the larvae of *T. castaneum*.



1.Trendline for graph showing dose-response for flufenoxuron on weight of the 7th day old larvae. 2. Trendline for graph showing dose-response for flufenoxuron on weight of the 10th day old larvae. A. Equation for trendline 1. B. Equation for trendline 2.

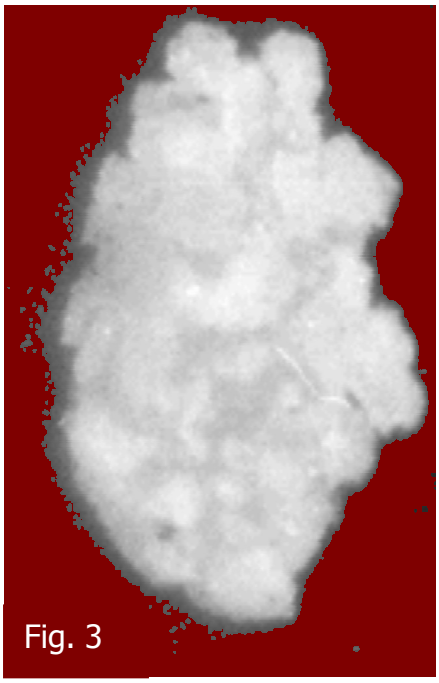


Fig. 3

Fig. 4

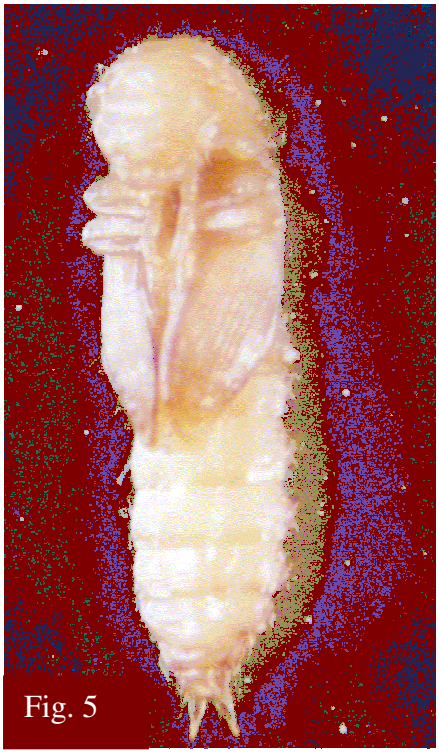


Fig. 5

Fig. 6

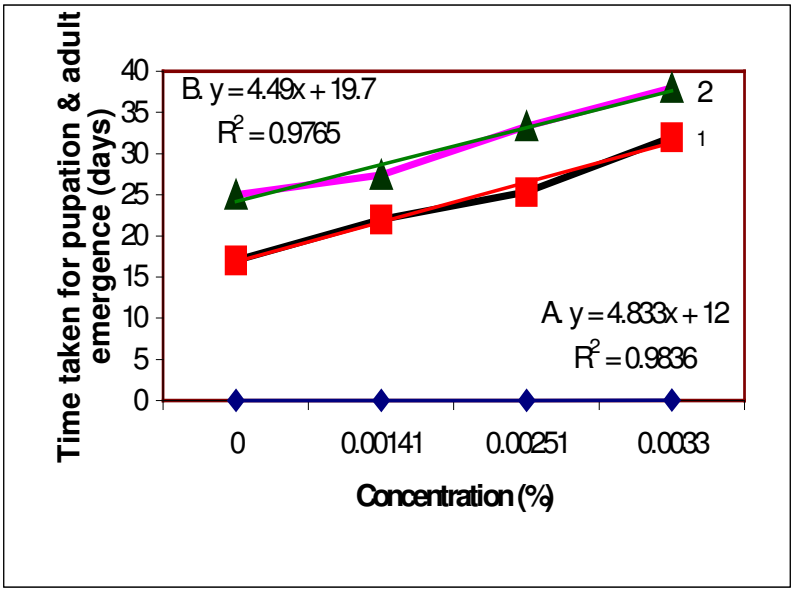

Time taken for pupation & adult emergence (days)
40
35
30
25
20
15
10
5
0
B. y = 4.49x + 19.7
R² = 0.9765
2
1
A. y = 4.833x + 12
R² = 0.9836
0
0.00141
0.00251
0.0033
Concentration (%)

Fig. 7

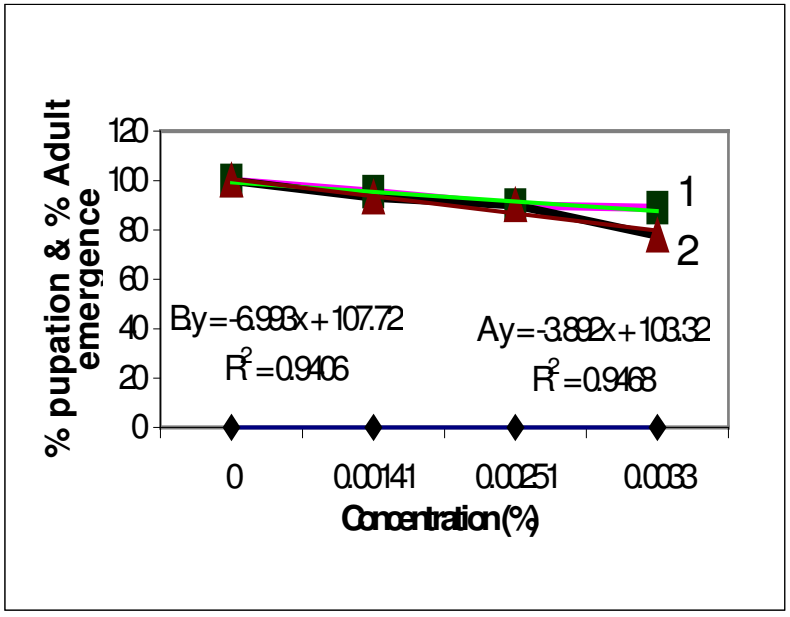

% pupation & % Adult emergence
120
100
80
60
40
20
0
1
2
By = -6.993x + 107.72
R² = 0.9406
Ay = -3.892x + 103.32
R² = 0.9468
0
0.00141
0.00251
0.0033
Concentration (%)

Fig. 8

Fig. 9

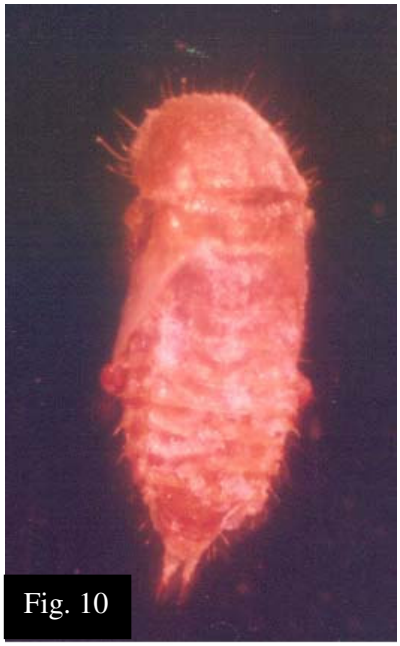


Fig. 10

Fig. 11

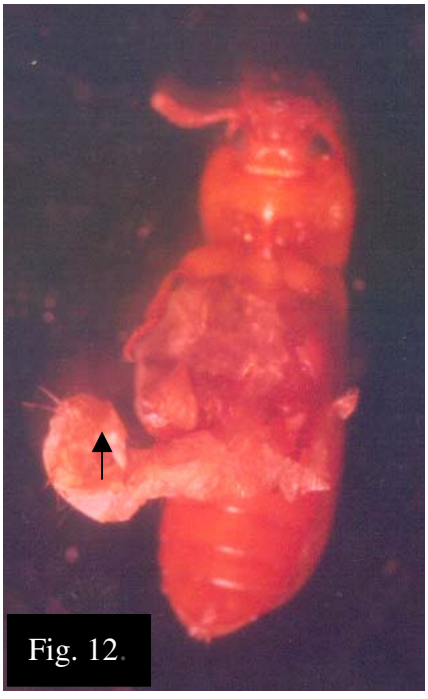


Fig. 12.

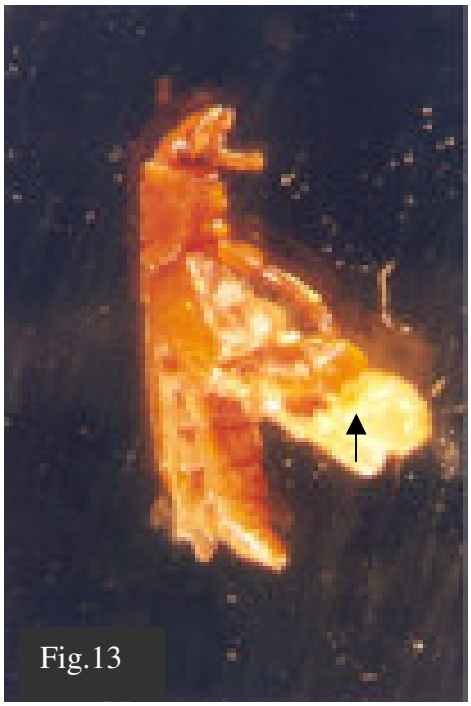


Fig.13

Fig. 14

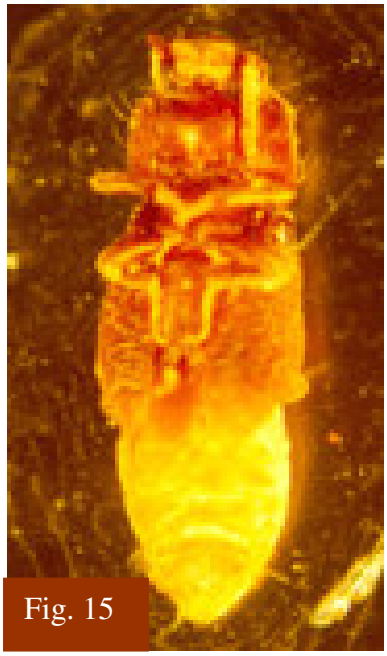


Fig. 15

Fig. 16

Fig. 17

Fig. 18

Fig. 19

Fig. 20

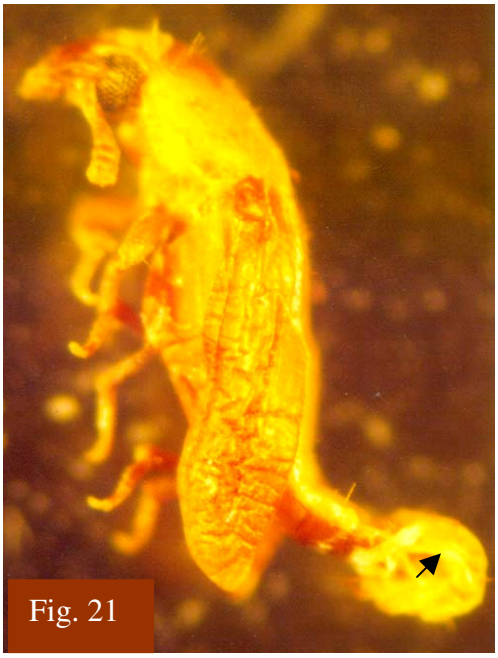


Fig. 21

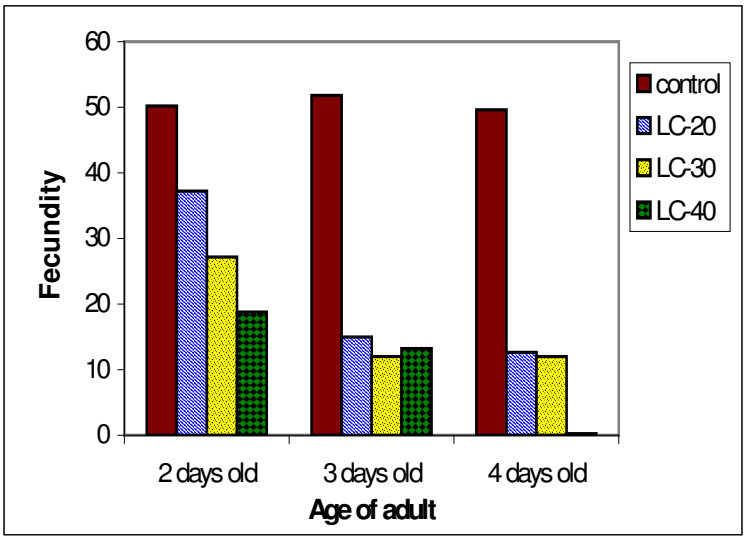

60
50
40
30
20
10
0
Fecundity
2 days old
3 days old
4 days old
Age of adult
control
LC-20
LC-30
LC-40

Fig. 22

Fig. 23

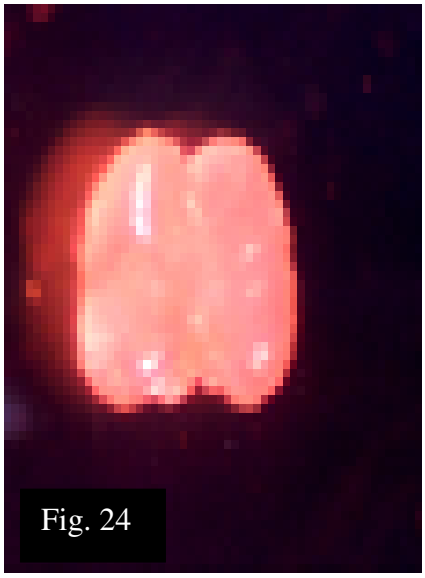


Fig. 24

Fig. 25

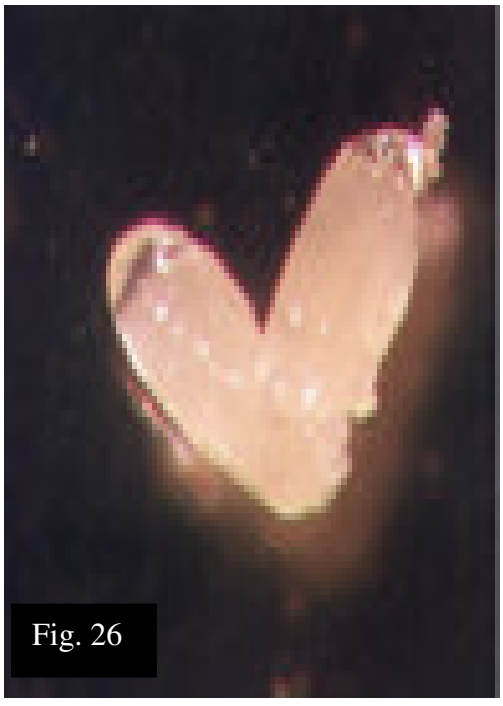


Fig. 26

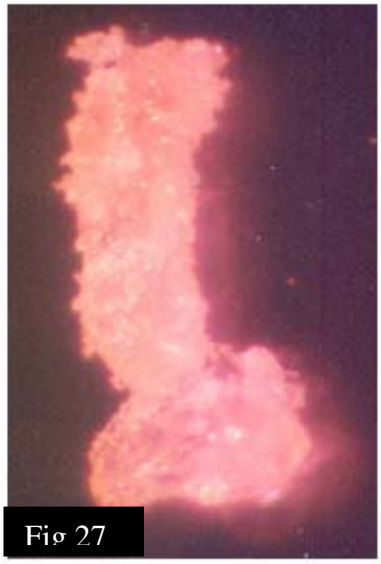


Fig 27

Fig. 28

Regression graph of dose response for flufenoxuron on % hatching of eggs of *T. castaneum*

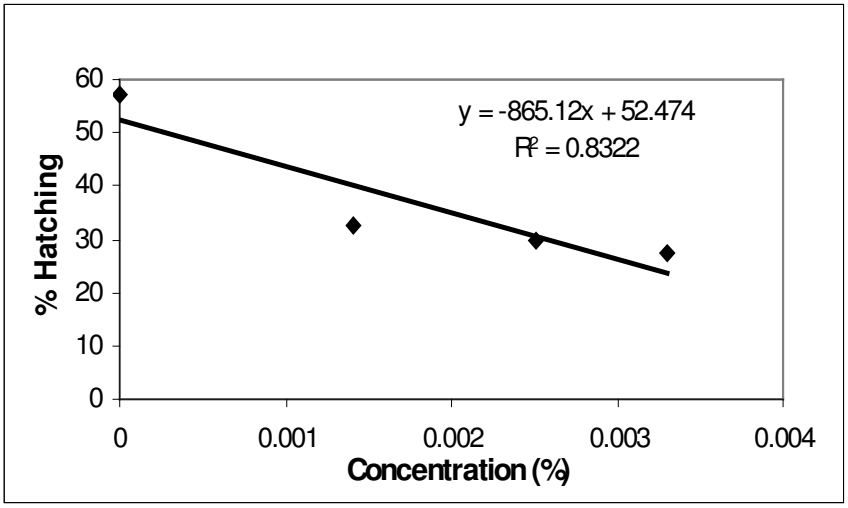


Fig. 29

Regression graph of dose response for flufenoxuron on mortality of topically treated adults of *T. castaneum*.

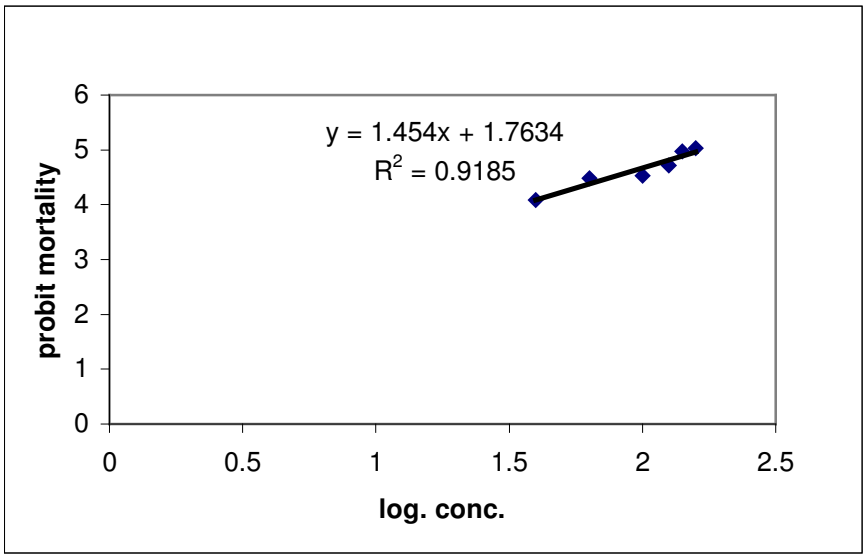


Fig. 30

Effect of sub-lethal concentration of flufenoxuron on chitin content per larva during development of *T. castaneum*

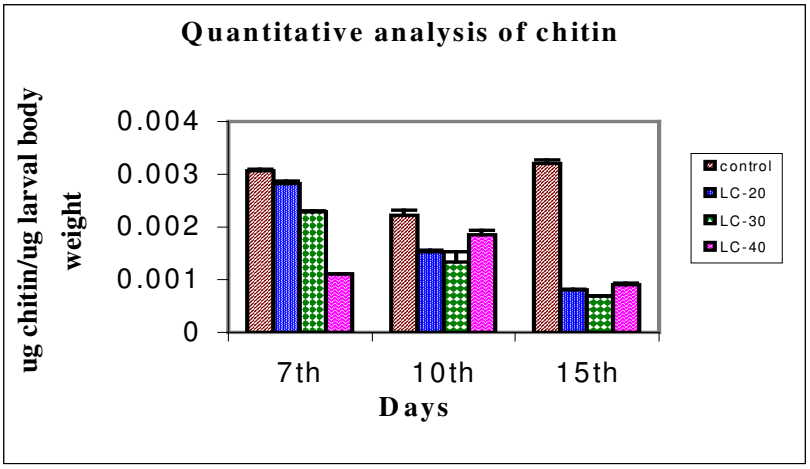


Fig. 31

Effect of the sub-lethal concentration of flufenoxuron on total soluble protein content per larva during development of *T. castaneum*.

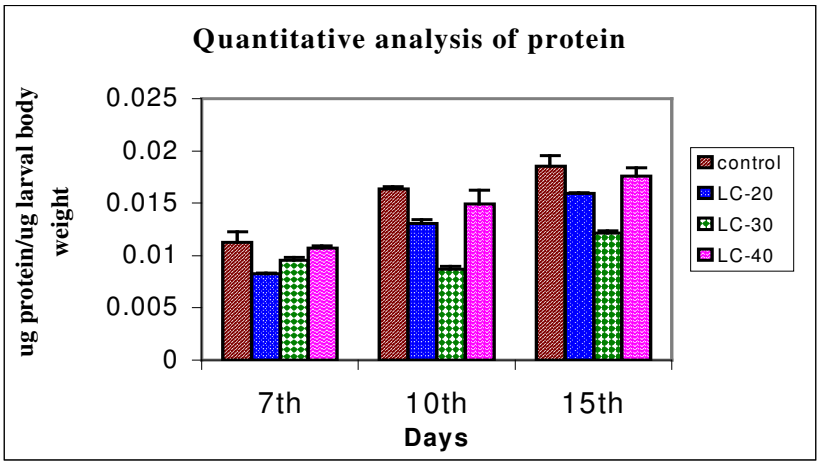
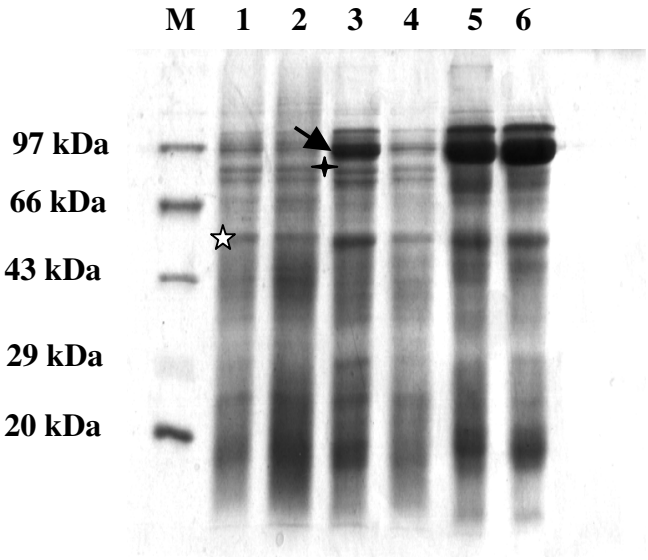
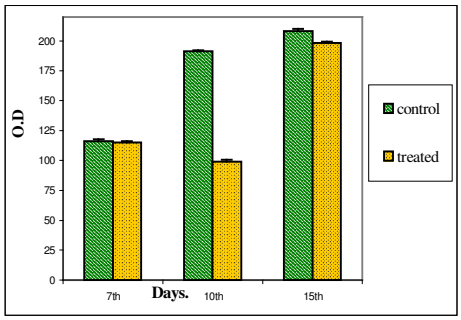


Fig. 32

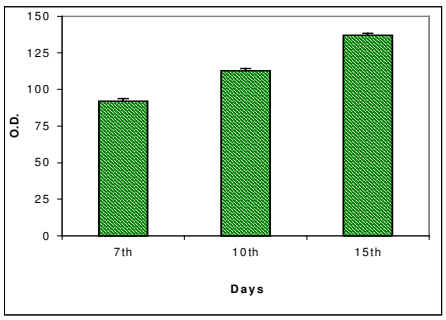
(a)


M 1 2 3 4 5 6
97 kDa
66 kDa
43 kDa
29 kDa
20 kDa

(b)


O.D
200
175
150
125
100
75
50
25
0
7th
Days.
10th
15th
control
treated

(c)


O.D.
150
125
100
75
50
25
0
7th
10th
15th
Days

(d)

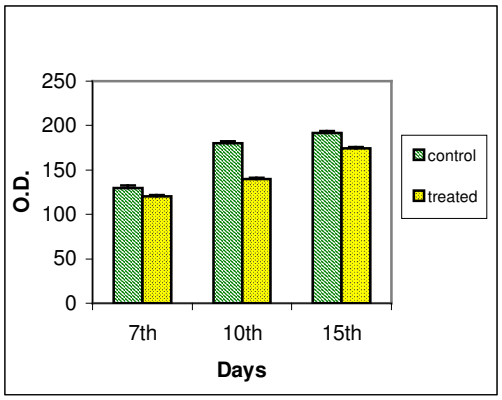

O.D.
250
200
150
100
50
0
7th
10th
15th
Days
control
treated

Fig. 33

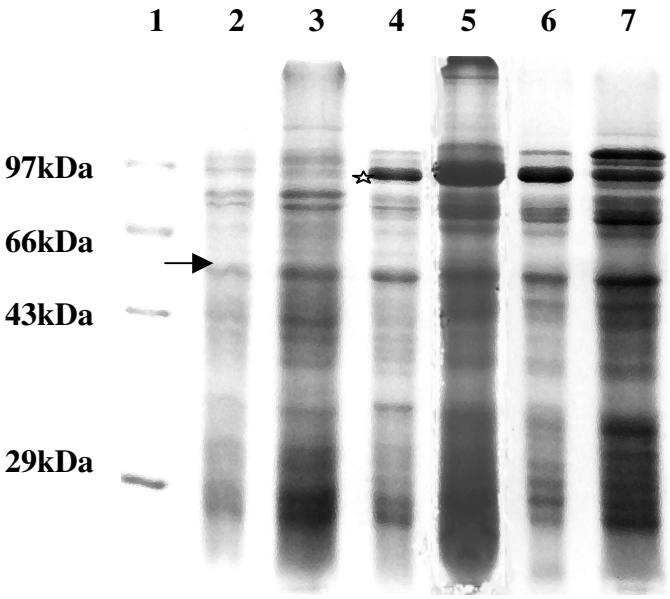


Fig. 34

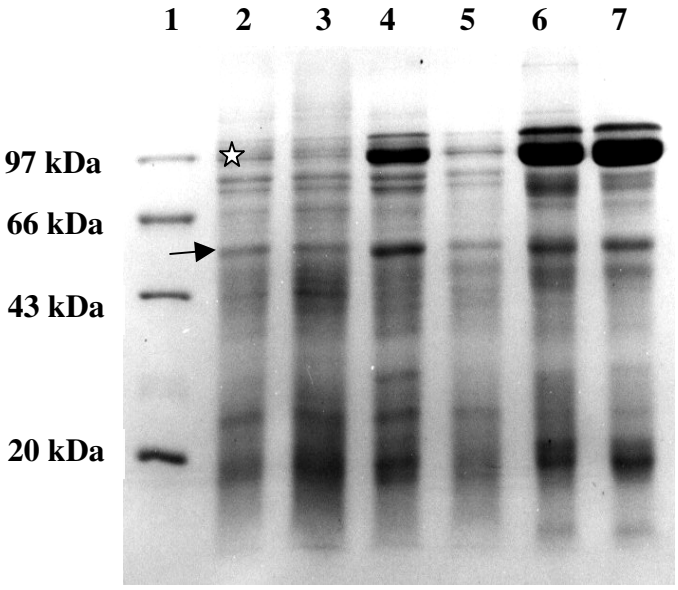
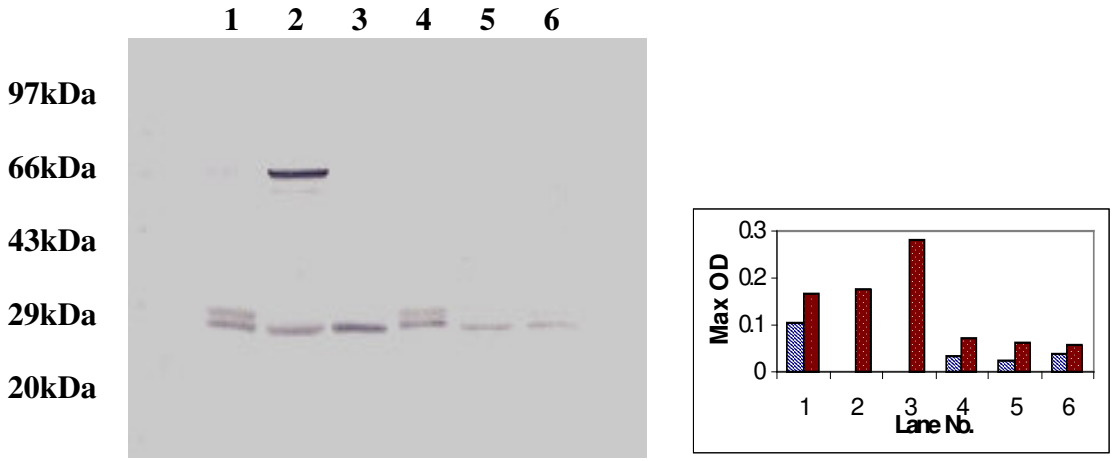
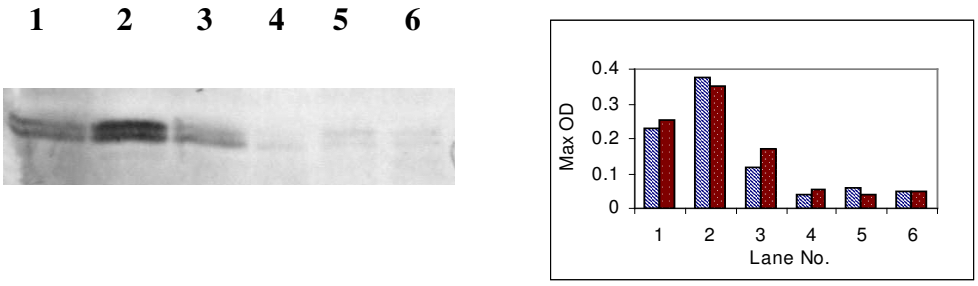


Fig. 35



(a) **(b)**

Fig. 36



(a) **(b)**

Figure legends:

Fig. 1: Scheme of chitin biosynthesis

Fig.3-6: Photographs showing developmental stages of *T. castaneum.*

Fig.3: Egg

Fig.4: Larva

Fig.5: Pupa

Fig.6: Adult

Fig.7: Regression graph of dose-response for flufenoxuron on time taken for pupation and adult emergence of *T. castaneum*. 1.Trendline for graph showing dose-response for flufenoxuron on time taken for pupation. 2. Trendline for graph showing dose-response for flufenoxuron on time taken for adult emergence. A. Equation for trendline 1. B. Equation for trendline 2.

Fig.8: 1. Regression graph of dose-response for flufenoxuron on %pupation and %adult emergence of *T. castaneum*. 1.Trendline for graph showing dose-response for flufenoxuron on %pupation. 2. Trendline for graph showing dose-response for flufenoxuron on %adult emargence. A. Equation for trendline 1. B. Equation for trendline 2.

Fig.9-12: Photographs showing effect of LC20 of flufenoxuron on the development of T. castaneum.

Fig.9: Larva with blackened anterior part due to toxic effect of flufenoxuron.

Fig.10: Larval-pupal intermediate- Dorsal view.

Fig.11: Larval-pupal intermediate-ventral view.

Fig.12: Pupal-adult intermediate showing incomplete shedding of pupal case.

Fig.13-16: Photographs showing effect of LC30 of flufenoxuron on the development of T. castaneum.

Fig.13: Larva with blackened anterior part due to toxic effect of flufenoxuron.

Fig.14: Larval-pupal intermediate- Dorsal view.

Fig.15: Larval-pupal intermediate-ventral view.

Fig.16: Pupal-adult intermediate showing incomplete shedding of pupal case.

Fig.17-20: Photographs showing effect of LC40 of flufenoxuron on the development of T. castaneum.

Fig.17: Larva with blackened anterior part due to toxic effect of flufenoxuron.

Fig.18: Larval-pupal intermediate- Dorsal view.

Fig.19: Larval-pupal intermediate-ventral view.

Fig.20: Pupal-adult intermediate showing incomplete shedding of pupal case.

Fig.21: Photograph showing pupal-adult intermediate with incomplete shedding of pupal case (indicated by arrow) due to treatment of neonates with LC_{40} of flufenoxuron .

Fig.22: Diagram showing effect of sublethal concentrations ($LC_{20,}$ LC_{30} & LC_{40}) of flufenoxuron on fecundity of *T. castaneum*.

Photographs showing abnormal eggs laid by adults of different ages treated with sublethal concentrations of flufenoxuron.

Fig.23: Abnormal egg without flour particles attached to the surface.

Fig.24: Twin eggs with lateral fusion of chorion.

Photographs showing abnormal eggs laid by adults of different ages treated with sublethal concentrations of flufenoxuron.

Fig.25: Twin eggs with vertical fusion of chorion.

Fig.26: Twin eggs with fusion of chorion at an angle of 90°.

Fig. 32 (a): SDS PAGE Analysis of total soluble protein from control and LC_{20} treated larvae of different developmental stages of *T. castaneum*. 30µg of total soluble proteins (in each lane) were used for electrophoresis. Although loading for 10^{th} day treated sample appeared less.Samples were loaded as follows: Lanes 1, 3 and 5 are of control for 7^{th}, 10^{th} and 15^{th} day old larvae and lanes 2, 4 and 6 are of LC_{20} treated for 7^{th}, 10^{th} and 15^{th} day old larvae. M is molecular weight marker protein. Arrow, asterisk and star indicate the polypeptide showing the variation in their expression pattern. **(b)** Quantitative profile for 97kDa protein (arrow) for all the lanes. **(c)** Quantitative profile for 80kDa protein subunit (asterisk) for lanes 1, 3 and 5. **(d)** Quantitative profile for 50kDa protein subunit (star) for all the lanes.

Fig. 33: SDS PAGE Analysis of total soluble protein from control and LC_{30} treated larvae of different developmental stages of *T. castaneum*. 30µg of total soluble proteins (in each lane) were used for electrophoresis. Samples were loaded as follows: Lanes 2, 4 and 6 are of LC_{30} treated for 7^{th}, 10^{th} and 15^{th} day old larvae and lanes 3, 5 and 7 are of control for 7^{th}, 10^{th} and 15^{th} day old larvae. Lane 1 contained molecular weight marker protein. Asterisk and arrow indicate the polypeptide showing the variation in their expression pattern. Comparison of precise quantitative variation between control and treated samples could not be done due to unequal loading.

Fig. 34 (a): SDS PAGE Analysis of total soluble protein from control and LC_{40} treated larvae of different developmental stages of *T. castaneum*. 30µg of total soluble proteins (in each lane) were used for electrophoresis. Although loading for 10^{th} day treated sample appeared less. Samples were loaded as follows: Lanes 2, 4 and 6 are of control for 7^{th}, 10^{th} and 15^{th} day old larvae and lanes 3, 5 and 7 are of LC_{40} treated for 7^{th}, 10^{th} and 15^{th} day old larvae. M is molecular weight marker protein. Arrow, asterisk and star indicate the polypeptide showing the variation in their expression pattern. **(b)** Quantitative profile for 50kDa protein (arrow) for all the lanes. **(c)** Quantitative profile for 97kDa protein subunit (asterisk) for all the lanes.

Fig. 35(a): Western blot analysis (anti- HSP70) of different developmental stages of *T. castaneum* during flufenoxuron treatment (LC_{30}). Lanes 1, 3 and 5 are control larvae for 7^{th}, 10^{th} and 15^{th} day old, whereas lanes 2, 4 and 6 are of treated larvae of the same stages respectively. **(b)** Quantification profile (low molecular weight bands) of the same blot.

Fig. 36(a): Western blot analysis (anti- p34 ^{cdc2}) of different developmental stages of *T. castaneum* during flufenoxuron treatment (LC_{30}). Lanes 1, 3 and 5 are control larvae for 7^{th}, 10^{th} and 15^{th} day old whereas lanes 2, 4 and 6 are of treated larvae of the same stages respectively. **(b)** Quantification profile of the same blot.

1.LC_{50} of flufenoxuron through diet for *T. castaneum* larvae was calculated by probit –log analysis and found to be 0.0042. LC_{20}, LC_{30} and LC_{40} values calculated and confirmed experimentally and found to be 0.00141%, 0.0025% and 0.00336% respectively.

2.When neonates of *T. castaneum* were subjected to sub-lethal concentrations of flufenoxuron in artificial diet for 24 h there was a dose dependent inverse relationship with respect to larval weight on 7^{th} and 10^{th} day, % pupation, % adult emergence as well as time taken for pupation and adult emergence.

3.At all concentrations tested a small proportion of larval- pupal and pupal-adult intermediates were formed.

4.The fecundity of adults emerging from the LC_{20} and LC_{30}concentrations was greatly reduced. They laid non-viable eggs. At higher concentration (LC_{40}) deformed adults with short life span were encountered.

5.Adults of different ages (2 day-old, 3 day-old, 4 day-old) when fed on flufenoxuron treated diet showed transovarial ovocidal activity. Also, there was dose dependent effect on the number of abnormal eggs laid.

6.LC_{50} on topical treatment of adult *T. castaneum* was found to be 0.8 ug/ ul

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6.Topical treatment of sublethal concentration of flufenoxuron to adults of *T. castaneum* of either sex reduced the fecundity in a dose dependent manner.

7.The fecundity was reduced drastically in pairs where both the sexes were treated as compared to the pairs were either sex was treated.

8.*T. castaneum* eggs exposed to diet treated with sub-lethal concentration of flufenoxuron exhibited a dose dependent inverse relationship with respect to hatchability.

9. Flufenoxuron was found to be more effective through ingestion compared to topical treatment for suppressing the population of *T. castaneum*

10.At the molecular level the two important components, chitin and total soluble proteins, which are involved in the growth and developmental process of the insect were significantly affected due to treatment with sub-lethal concentrations of flufenoxuron, which coincided with developmental as well as reproductive impairments in *T. castaneum.*

11. The cellular response in the form of induction of HSP70 was observed on 7^{th} day after treatment in case of *T. castaneum* larvae fed on LC_{30} treated diet along with the expression of cell cycle regulatory protein, $p34^{cdc2}$.

12.The absence of expression of $p34^{cdc2}$ in case of larvae fed on LC_{40} concentration of flufenoxuron was also reflected by the effects produced on life cycle as well as reproductive endpoints. Biochemical analysis revealed that the effect of sub-lethal concentrations of flufenoxuron on fecundity, early embryonic abnormalities and later processes such as moulting, formation of pupae and adults may be mediated through perturbation of cell cycle as well as altered expression of developmentally regulated proteins involved in cuticle formation in a stage specific manner.

13.This information about the sub-lethal effects on pest species, as well as on beneficial arthropods, is needed to fully integrate compounds such as IGR's into future IPM programs. How these sub-lethal responses affect subsequent populations in the field has been poorly investigated and are

extremely difficult to ascertain. Thus care should be taken when making assumptions based on laboratory data.

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SYNOPSIS

Title of the thesis: Studies on the effect of a new Insect Growth Regulator on the biology and biochemical parameters of *Tribolium castaneum*.

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INTRODUCTION:

In a variety of pest control activities around the world synthetic chemicals continue to play a significant role. The use of conventional organic insecticides to control pests has given rise to the problem of development of resistant strains of insects besides environmental repercussion. As a result search for safer alternatives for pest control continues. New insecticides continue to appear and create new problems. Extensive efforts have been made during the last few decades to develop compounds interfering with normal growth and development of insects. Such insecticides with novel modes of action are called insect growth regulators (IGRs). They affect molting and metamorphosis by mimicking Juvenile hormone (JH, Juvenile hormone agonists) or antagonizing JH activity (ecdysteroid agonists) or by interfering with cuticle formation (Chitin synthesis inhibitors) (Smet et al., 1990; Oberlander et al., 1997). In addition to their short environmental persistance and low toxicity to vertebrates IGRs have several characteristics that make them potentially successful alternative to conventional insecticides. The discovery of benzoylphenylureas during the early 70's was an important step towards the development of new group of insecticides, which exert their action on the insect integument. Subsequently several compounds of this type have been evaluated for their insecticidal action against a wide range of insect pests.

Tribolium castaneum (Herbst) is an economically important pest of stored products, worldwide, feeding on wide range of stored commodities (Arbogast,1991). Amongst the

various synthetic chemicals used to control *T. castaneum*, phosphine was the most widely used fumigant. Since late 70's it was reported that *T. castaneum* was developing resistance to phosphine and of late this has assumed serious proportion (Chaudhary, 2000). Another fumigant, Methyl bromide is still used in controlling stored product pests including *T. castaneum*, for its rapid action and broad spectrum of activity. However, since methyl bromide was found to deplete Earth's ozone layer it is being phased out in developed countries and is expected to be out by 2005 (MBTOC, 1998). It will be completely phased out in developing countries as well by 2015 (MBTOC, 1998). As a replacement to methyl bromide, organophosphorus compounds were used as grain protectants (Snelson, JT, 1987). Besides development of resistance to these insecticides in pests, they were no longer considered safe for marketing (EPA, 2000). Another class of insecticides, the synthetic pyrethroids, has greater flexibility with respect to environmental factors than other insecticides and has been also used to control stored product pests. But insects acquired resistance to them (Knight and Norton, 1989). More safe and effective insecticides than those described above are IGRs that seems to be an ideal alternative. Flufenoxuron an acylurea IGR, acts on insects by reducing chitin incorporation in the cuticle (Clarke and Jewess, 1990). In the present study it was chosen to evaluate its efficacy on *T. castaneum* for its novel mode of action, specificity to arthropods, safety towards vertebrates and low ecological magnification. It has been evaluated for its efficacy on a variety of pests of horticultural and ornamental plants. However, there are very few reports of studies on sub-lethal effects of IGRs. Further, there is paucity of information on IGR induced stress and their effect on various biochemical parameters in insects.

The present research is an investigation on the effect of sub-lethal doses of a dispersible concentrate formulation of the flufenoxuron on certain biological and biochemical parameters of *T. castaneum*. The effects of sub-lethal concentrations of flufenoxuron on the various stages in the life cycle and reproductive end points (viz. time to pupation, time to adult emergence, % pupation, % adult emergence, fecundity and hatching success and larval viability) were investigated in *T. castaneum*. Studies on such effects are important for the assessment of overall ecological impact of IGRs or pesticides in general, since non-target species in the periphery of the treated area often receive sub-lethal doses. Moreover studies with sub-lethal concentrations also provide insight into the mode of action of insecticides.

Chitin synthesis inhibitors act by interrupting the synthesis and transport of specific proteins, that are required for the assembly of N-acetyl-D-glucosamine (Glc NAC) monomers into polymeric chitin (Oberlander et al., 1998). A review of literature reveals that there is paucity of information on IGR-induced stress and their effect on various biochemical parameters in insects. Thus the sub-lethal effects of flufenoxuron was determined on chitin, total soluble protein as well as a stress protein (HSP70) and a cell cycle regulatory protein ($p34^{cdc2}$), since these are considered to be general indicators of sub-lethal cellular protein damage (Werner et al., 2002).

METHODOLOGY

A stock culture of *T. castaneum* was maintained on a diet containing wheat flour and 5% Brewers yeast, at 29±1ºC and 60 % relative humidity. Eggs were collected by sieving (sieve number 40), diet infested with adults. Flufenoxuron (Cascade®) belonging to the benzoylphenylurea family was mixed in diet using acetone as a carrier solvent and

the neonates of *T. castaneum* were released in the treated diet. An acetone treated diet was used as a control. The mortality count was taken after 7 days. The sub-lethal doses (LC_{20}, LC_{30} and LC_{40}) used in the experiments were deduced by extrapolation of the probit mortality analysis.

To study the effects of flufenoxuron on biological parameters:

1. Growth and development: Neonates of *T. castaneum* were subjected to the sub-lethal concentration of flufenoxuron through diet for 24hrs and observations were made on larval weight, %pupation, %adult emergence, time taken for pupation and adult emergence and morphological deformities. Regression analysis was performed to determine dose dependent effects.

2. Fecundity: Adults of different ages (2 days old, 3 days old, 4 days old) were exposed to diet treated with sub-lethal concentrations of flufenoxuron and their fecundity was observed. The data was compared by ANOVA, student's t-test and Z-test.

3. Hatching of eggs: The effects of sub-lethal concentrations of flufenoxuron through diet on the hatchability of eggs were determined by placing 20 eggs in treated diet and recording hatching of eggs every day till hatching in the control was completed. Regression analysis was performed to determine dose dependent effects.

4. Topical application: Adults were treated topically with sub-lethal concentrations, LC20 (0.4µg/µl), LC30 (0.8µg/µl) and LC40 (1µg/µl) of flufenoxuron deduced by experimental observations. Acetone treated adults were used as control. Crosses were performed as follows: treated males x untreated females, treated females x untreated males, treated males x treated females. Fecundity was observed for 7 days and analysed by a t-test.

To study the effects of flufenoxuron on biochemical parameters:

1. Chitin extraction and estimation: *T. castaneum* neonates treated with sub-lethal concentrations of flufenoxuron through diet were collected on 7th, 10th and 15th day after the treatment, weighed and homogenized in homogenizing buffer containing Tris (5 mM) Glycine (38 mM), pH 8.4. The homogenate was centrifused at 10,000 rpm for 15min and the precipitate was dissolved in 6N HCl, transferred into hydrolysis tubes which were sealed under vacuum and the material was hydrolysed at 100° C for 16h in a temperature controlled heating block. After hydrolysis the solution was centrifuged at 10,000 rpm for 10min. The supernatant was collected and neutralized with 30% NaOH. N-acetyglucosamine contant was estimated according to the method of Ressig et al. (1955).

2. Protein extraction and estimation: *T. castaneum* neonates treated with sub-lethal concentrations of flufenoxuron through diet were collected on 7th, 10th and 15th day after the treatment, weighed and homogenized in protein extraction buffer containing 20mM Tris-HCl (pH 8), 1mM EDTA, 1mM PMSF and 0.1% Triton X-100, followed by centrifugation at 10,000 rpm at 4°C for 20min.The supernatant was removed and stored at –80°C. Protein content of the supernatant was determined by Bradford's method (Bradford, 1976).

3. SDS PAGE: Samples containing equal amount of protein, as determined by Bradford's method from control and treated larval tissue extracts were denatured in sample buffer (Laemmli, 1970) for 3-5 min at 100℃ and analyzed on 10% SDS PAGE (Laemmli,1970) along with molecular weight marker proteins. Electrophoresis was carried out at a constant current (25 mA) at room temperature and the gel was stained overnight with 0.125% Coomassie brilliant blue-R250.

4. Western Blotting: Following SDS PAGE, proteins were electrophoretically transferred to a nitrocellulose membrane at a constant current (70 mA) for 12-14h at 4℃ (Towbin et al., 1979). Blots were then processed for immunoreaction using anti-HSP70 and anti-p34 ^{cdc2} monoclonal antibodies. In brief, blots were saturated with 3% BSA for 4 h at room temperature and incubated overnight with primary antibody in phosphate buffered saline (PBS, pH 7.4) at 4℃ and then with alkaline phosphatase-conjugated secondary antibody for 4h at room temperature. Following each antibody incubation, blots were washed thrice (15 min each) in PBS. Blots were developed for color reaction using NBT-BCIP, as substrates. The results were analyzed using Biorad gel documentation system.

RESULTS AND DISCUSSION:

EFFECTS OF FLUFENOXURON ON BIOLOGICAL PARAMETERS OF *T. castaneum*.

Flufenoxuron at sub-lethal concentrations has dose dependent effects on larval weight, %pupation, %adult emergence, time taken for pupation and adult emergence. It affects molting and results in the development of larval-pupal and pupal-adult intermediates. Similar results were obtained with the use of JH analogues (Bull, 1986; Deecher et al., 1990a, 1990b) which suggests that flufenoxuron may influence reproduction by causing hormonal imbalance. Adults emerging from the larvae fed on diet mixed with LC-20 and LC-30 of flufenoxuron laid non-viable eggs and those eggs, which hatched resulted in larval mortality during the first instar. There was considerable variability in the response of adults of *T. castaneum* of different ages with respect to fecundity when fed on a diet treated with sub-lethal concentrations of flufenoxuron. The

overall trend, however, was an age-dependent effect on fecundity in *T. castaneum* adults of sub-lethal concentration of flufenoxuron. Also, adults laid abnormal eggs, which lacked the sticky layer as reflected by the lack of flour sticking to their surface. This may be due to absence of accessory gland secretion. Topical application of sub-lethal concentrations of flufenoxuron to adults of either sex reduced the fecundity in dose-dependent manner. Furthermore, the fecundity was reduced drastically in a pair where both the sexes were treated as compared to the pairs where only one sex was treated. Hatching percentage of eggs decreases with increasing concentration of flufenoxuron mixed with diet to which eggs were exposed.

EFFECTS OF FLUFENOXURON ON BIOCHEMICAL PARAMETERS OF *T. castaneum.*

When neonates were fed on diet treated with sub-lethal concentrations of flufenoxuron for 24hrs it was observed that at all concentrations tested, there was a significant reduction in chitin content on the 15^{th} day of development. Total soluble protein content at LC_{20} and LC_{30} decreased with increasing age of the larvae. At LC_{20} and LC_{30} concentrations there was a progressive increase in the chitin: protein ratio as a function of increase in age of the larvae. Similar results were reported by Kulkarni and Mehrotra (1975) in *Schistocerca gregaria* due to Sumithion treatment. SDS-PAGE analysis of the larval tissue extracts indicated gross quantitative changes in some protein bands (MW 50-97 kDa). Western blot analysis revealed significant levels of HSP70 in the extracts of larvae fed on LC_{30} treated diet, on the 7^{th} day of development. Interestingly, anti-HSP70 antibody detected a doublet of polypeptides of around 28-29 kDa which varied significantly both in quantity and proportion during development.

Proteins of low molecular weight (29kDa) were found to be the major haemolymph proteins (MHP) in *Bombyx mori* by Bosquet et al. (1985). Further, Plantevin et al. (1987) found that the rates of synthesis of these MHP showed quantitative changes during development in *Bombyx mori* and their synthesis was regulated by juvenile hormone (JH) titre. However, it is not known as to how flufenoxuron influences JH titre, which in turn modulates expression of such proteins. The cyclin dependent kinase, $P34^{cdc2}$ from treated and control tissue was observed by immunoblotting as two bands whose expression changed during different developmental stages. The abundance of the phosphorylated forms suggest cell cycle blockage at G2/M phase. These data support those observed by Goss et al. (2003) in HeLa cell due to anisomycin induced cell stress. Thus, sub-lethal concentrations of flufenoxuron alter expression of developmentally regulated proteins, HSP70 and $P34^{cdc2}$ and chitin formation in a stage-specific manner.

CONCLUSION:

In addition to direct mortality, the sub-lethal effects of flufenoxuron on development and fertility of surviving individuals should help in suppressing subsequent generations. Biochemical data suggest that the effect of flufenoxuron on fecundity, early embryonic abnormalities and later processes such as moulting, formation of pupae and adults may be mediated through perturbation of cell cycle as well as altered expression of developmentally regulated proteins involved in cuticle formation in a stage specific manner. The responses given by life cycle stages of *T. castaneum* to the sub-lethal concentration of flufenoxuron can be helpful in suggesting the incorporation of this IGR in IPM programs for the control of field population of this pest. Also, this can be used as

a model system for studies on sub-lethal effects of insecticides on insects and to understand the mode of action of insecticides belonging to the benzoylphenylurea family.

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