GENERAL

INTRODUCTION
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; Schmutterer, 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 (Schmutzer, 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 secretes 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 synthesis.
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 synthesis would lead to death of pest and can be used in integrated pest management practices.

**Insect growth regulators (Chitin synthesis inhibitors):**

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. 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
Figure legend:

Fig. 1: Scheme of chitin biosynthesis
Fig. 1

Trehalose → Trehalase → Glc → ATP → ADP → Hexolactase → Glc-6-P → Glucose phosphate isomerase → Fru-6-P → Glutamine-fructose-6-phosphate aminotransferase → Gln → Glu

GlcN 6-P → Phosphoglucomutase → GlcNAc 6-P → Phosphoglucoaminyltransferase → Acetyl-CoA → CoA

GlcNAc 6-P → Transacetylase → CoA

GlcNAc 1-P → Uridine diphosphate-N-acetylglucosamine pyrophosphorylase → UDP-GlcNA

Chitin synthase → Chitin (GlcNAc)n

Chitin (GlcNAc... ...
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). Benzoylphenylurea, 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 benzoylephynylurea 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., 1998).

Organismal effects of benzoylephynylureas 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 budworm, 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 oxicidal 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 benzoylephynylureas, revealed 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 benzoylephynylureas 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 benzoylephynylurea 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 benzoylephynylureas 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 benzoylephynylureas, Alsythin 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 benzoylephynnureas 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 benzoylephynnureas, 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-acetyl-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 *Aubeonium mariae frunscae* population was evaluated by Marco and Castaenedera (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. mariae frunscae* 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. littoralis* 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 LC10 to LC25) 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 a 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
relevant 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 [Methyl Bromide Technical Options Committee (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. Biddingger 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\textsuperscript{cdc2}), since these are considered to be general indicators of sub-lethal cellular protein damage.