

**FLUFENOXURON-INDUCED MALE REPRODUCTIVE
TOXICITY IN *SPODOPTERA MAURITIA* BOISD.
(LEPIDOPTERA: NOCTUIDAE)**

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by
RESHMA V



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DEPARTMENT OF ZOOLOGY UNIVERSITY OF CALICUT

Reaccredited by NAAC “A+” grade

Ph: 0494 2407419

Mob: 9895325917

E-mail: manogemvinod@gmail.com

Calicut University (P.O.) 673 635

Kerala

Dr. E. M. Manogem

Associate Professor



CERTIFICATE

This is to certify that this thesis entitled “**Flufenoxuron-induced male reproductive toxicity in *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae)**” submitted by **Ms. Reshma. V** to the University of Calicut during the period of 2016-2023 for the award of the degree of **Doctor of Philosophy in Zoology**, is the result of the bonafide research work carried out at the Department of Zoology, University of Calicut under my guidance and supervision. The contents of the thesis have been checked for plagiarism using the software ‘iThenticate’, and the similarity index falls under the permissible limit. I further certify that the topic discussed in this thesis has not been previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles of any other University or Institute.

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26.12.2024

Dr. E. M. Manogem
Associate Professor

DECLARATION

I hereby declare that this dissertation, work entitled “**Flufenoxuron-induced male reproductive toxicity in *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae)**” is the authentic record of research work originally carried out by me under the supervision and guidance of **Dr. E.M. Manogem**, Associate Professor, Department of Zoology, University of Calicut, and that no part of this has been published previously or submitted for the award of any other Degree / Diploma. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents

University of Calicut
26.12.2024

Reshma V

Dr. E.M. Manogem
Associate Professor
Department of Zoology
University of Calicut

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എന്റെ അച്ഛൻ

ABSTRACT

Insect pest infestations pose a significant challenge to global agricultural cultivations, particularly in rice production systems, necessitating effective pest management techniques to minimize losses and crop protection. Armyworm (*Spodoptera mauritia* Boisduval) is a sporadic pest that has emerged as a major pest in India, causing significant losses in wet-season rice production. Environmentally friendly pesticides, such as insect growth regulators (IGRs), interfere with insect growth and development and are used as an alternative to chemical pesticides. Disruption of insect growth and reproduction are the key mechanisms of different IGRs; among them, chitin synthesis inhibitors have acquired attention due to their mode of action by targeting the insect chitin. Flufenoxuron, a third-generation chitin synthesis inhibitor, is a viable alternative against the control of several insects and mites. Therefore, in this context, the present study explored the effects of the insecticide chitin synthesis inhibitor, flufenoxuron, against the rice-swarming caterpillar and evaluated the effect on spermiogenesis as a model to illustrate male reproductive development.

The current study showed that flufenoxuron adversely impacted the growth parameters required for male reproductive development. The sub-lethal effects of flufenoxuron significantly impaired the hatchability rate of eggs and lowered the number of fertile, normal adult insects. Moreover, flufenoxuron-treated sets showed a high sensitivity in biochemical indices to the impact of this pesticide. The

treatment of the compound significantly depleted proteins, lipids, and carbohydrates in the testis and haemolymph, producing degenerated spermatozoa that may not produce healthy offspring. The results obtained from histo-morphological analysis and confocal image analysis confirmed that flufenoxuron suppresses differentiation of spermatozoa. Metabolomics analysis of *S. mauritia* haemolymph suggests that flufenoxuron can interfere with carbohydrate and lipid metabolic pathways and the chitin biosynthetic pathway, subsequently impacting the growth and reproduction of *S. mauritia*. The study aims to improve understanding of the regulation of reproduction, specifically male reproductive development triggered by chitin synthesis inhibitors, and promote the creation of endocrine disruptors as potential insecticides for controlling male insects.

സംഗ്രഹം

ആഗോള കാർഷിക രംഗത്ത്, പ്രത്യേകിച്ചും നെല്ല്പാദന രംഗത്ത് കീടങ്ങൾ ഉണ്ടാക്കുന്ന പ്രയാസങ്ങൾ നിരവധി ആണ്. ഇത് വിള നഷ്ടം കുറയ്ക്കാനും വിള സംരക്ഷണത്തിനും ഫലവത്തായുള്ള കീട നിയന്ത്രണ സാങ്കേതിക വിദ്യയുടെ ആവശ്യകത അനിവാര്യമാക്കുന്നു. ഇന്ത്യയിൽ മഴക്കാല നെല്ല്പാദനത്തിന് ഗണ്യമായ നാശനഷ്ടം വിതയ്ക്കുന്നതിൽ മുഖ്യ പങ്ക് വഹിക്കുന്ന സ്പോറോഡിക് പെസ്റ്റ് ആയി മാറിയിരിക്കുകയാണ് പട്ടാള പുഴ (സ്പോറോഡിക്റ്റാ മൗറീഷ്യ ബോയ്സ്ഡ്). രാസ കീടനാശിനികൾക്ക് പകരം ഉപയോഗിക്കുന്ന ഇൻസക്റ്റ് ഗ്രോത്ത് റെഗുലേറ്റർ പോലുള്ള പരിസ്ഥിതി സൗഹൃദ കീടനാശിനികൾ, കീടങ്ങളുടെ വളർച്ചയിലും വികാസത്തിലും കാര്യമായ ഇടപെടൽ നടത്തുന്നവയാണ്. കീടങ്ങളുടെ വളർച്ചയെയും പ്രത്യുല്പാദനത്തെയും തടസ്സപ്പെടുത്തുക എന്നതാണ് വിവിധ ഇൻസക്റ്റ് ഗ്രോത്ത് റെഗുലേറ്ററുകളുടെ പ്രധാന പ്രവർത്തന രീതി. ഇതിൽ തന്നെയുള്ള കൈറ്റിൻ സിന്തസിസ് ഇൻഹിബിറ്റർ എന്ന ഇൻസക്റ്റ് ഗ്രോത്ത് റെഗുലേറ്റർ പ്രാണികളിൽ കാണപ്പെടുന്ന കൈറ്റിനെ ലക്ഷ്യം വെച്ചുകൊണ്ടുള്ള അതിന്റെ പ്രവർത്തന രീതി കൊണ്ട് ശ്രദ്ധ നേടിയിട്ടുള്ളതാണ്. ഫ്ലൂഫെനോക്സുറോൺ, എന്ന മൂന്നാം തലമുറയിൽ പെടുന്ന കൈറ്റിൻ സിന്തസിസ് ഇൻഹിബിറ്റർ ധാരാളം പ്രാണികൾക്കും ചെളികൾക്കും എതിരായി ഉപയോഗിക്കാവുന്ന വിജയപ്രദമായ കീട നിയന്ത്രണ ഉപാധിയാണ്. ഈ പശ്ചാത്തലത്തിൽ പട്ടാള പുഴവിന്റെ മേൽ ഫ്ലൂഫെനോക്സുറോൺ എന്ന കീടനാശിനിയുടെ ഫലത്തിനെ കുറിച്ചും, ബീജോൽപാദനത്തിനെ പുരുഷ പ്രത്യുല്പാദന വികാസത്തിന്റെ ഒരു ഉദാഹരണമായി വിലയിരുത്തുകയുമാണ് ഈ പഠനം മുന്നോട്ട് വെക്കുന്ന ആശയം. പുരുഷ പ്രത്യുല്പാദന വികാസത്തിന് ആവശ്യമായ വളർച്ചാ ഘടകങ്ങളെ ഫ്ലൂഫെനോക്സുറോൺ പ്രതികൂലമായി ബാധിച്ചതായി നിലവിലെ പഠനം ചൂണ്ടി കാണിക്കുന്നു.

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പ്ലൈനോക്സറോൺ പരീക്ഷിച്ച ലിംഗഭേദത്തിലുള്ള കീടങ്ങളിൽ വലിയ തോതിലുള്ള ബയോ കെമിക്കൽ സൂചികാ സംവേദനക്ഷമത കാണാൻ സാധിച്ചിട്ടുണ്ട്. പ്രസ്തുത സംയുക്തത്തിന്റെ ഉപയോഗം കീടങ്ങളുടെ വൃഷണങ്ങളിലും, രക്തത്തിലുമുള്ള മാംസ്യം, കൊഴുപ്പ്, അന്നജം എന്നിവയുടെ ഗണ്യമായ കുറവിനു കാരണമാകുന്നു. തൽഫലമായി നിർജീവമായ സ്പേർമാറ്റോസോവ ഉൽപ്പാദിപ്പിക്കപ്പെടുകയും അവയ്ക്ക് ആരോഗ്യമുള്ള സന്തതികളെ ഉൽപ്പാദിപ്പിക്കാനുള്ള ശേഷി ഇല്ലാതായിത്തീരുകയും ചെയ്യുന്നു. ഹിസ്റ്റോ മോർഫോളജിക്കൽ അനാലിസിസിന്റെയും കോൺഫോക്കൽ ഇമേജ് അനാലിസിസിന്റെയും ഫലങ്ങൾ ശേഖരിച്ചതിൽ നിന്ന് പ്ലൈനോക്സറോൺ, കീടങ്ങളുടെ വിവിധ തരം സ്പേർമാറ്റോസോവയുടെ ഉൽപാദനം അമർച്ച ചെയ്യുന്നതായി കണ്ടെത്തി. സ്പോഡോപ്റ്റെറ മൗറീഷ്യയുടെ രക്തത്തിന്റെ മെറ്റബോളോമിക്സ് വിശകലനം ചെയ്യുന്നതിലൂടെ പ്ലൈനോക്സറോണിനു അന്നജത്തിന്റെയും കൊഴുപ്പിന്റെയും പോഷണ പാതയെയും, കൈറ്റിനിന്റെ ബയോ സിന്തറ്റിക് പാതയെയും തടസ്സപ്പെടുത്താൻ സാധിക്കും എന്ന് മനസ്സിലാക്കാൻ കഴിഞ്ഞിട്ടുണ്ട്. തൽഫലമായി സ്പോഡോപ്റ്റെറ മൗറീഷ്യയുടെ വളർച്ചയെയും പ്രത്യുല്പാദനത്തെയും ഇത് ദോഷകരമായി ബാധിക്കുന്നു. സ്പോഡോപ്റ്റെറ മൗറീഷ്യയുടെ പ്രത്യുല്പാദനത്തിന്റെ നിയന്ത്രണത്തെ, വിശേഷാൽ കൈറ്റിൻ സിന്തസിസ് ഇൻഹിബിറ്റർ മൂലമുള്ള പുരുഷ പ്രത്യുല്പാദന വികാസത്തെ പറ്റിയുള്ള ധാരണ വർദ്ധിപ്പിക്കുവാനും, പുരുഷ പ്രാണികളെ നിയന്ത്രിക്കാൻ ഉള്ള പ്രബലമായ കീടനാശിനികളായി എൻഡോക്രെയിൻ ഡിസ്റപ്റ്ററുകളെ നിർമ്മിക്കാൻ പ്രോത്സാഹനം നൽകുക എന്നതുമാണ് ഈ പഠനം ലക്ഷ്യം വെക്കുന്നത്

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LIST OF ABBREVIATIONS

<i>S. mauritia</i>	:	<i>Spodoptera mauritia</i>
IGR	:	Insect Growth Regulator
BPU	:	Benzoyl Phenyl Urea
CSI	:	Chitin Synthesis Inhibitor
JH	:	Juvenile Hormones
JHA	:	Juvenile Hormones Analogues
MI	:	Moult Inhibitor
DFB	:	Diflubenzuron
CHS	:	Chitin synthases
IPM	:	Integrated Pest Management
SUR	:	Sulfonylurea Receptor
ED	:	Effective Dose
PBS	:	Phosphate Buffered Saline
GSI	:	Gonadosomatic Index
SE	:	Standard Error
ANOVA	:	Analysis of Variance
DMRT	:	Duncan's Multiple Range Test
μM	:	Micromolar
μl	:	Microlitre
μg	:	Microgram
SDS-PAGE	:	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TCA	:	Tricarboxylic Acid
BSA	:	Bovine Serum Albumin
TMRM	:	Tetramethyl Rhodamine Methyl Ester
DAPI	:	4',6-diamidino-2-phenylindole
CBP	:	Chitin-Binding Proteins
GC-MS	:	Gas Chromatography-Mass Spectrometry
PM	:	Peritrophic Matrix

MoA	:	Modes of Action
ATP	:	Adenosine Triphosphate
ABC transporters	:	ATP-Binding Cassette transporters
AMPK	:	AMP-Activated Protein Kinase
UDP-GlcNAc	:	Uridine diphosphate-N-acetylglucosamine
BSTFA	:	N,O-bis-(trimethylsilyl)trifluoroacetamide
TMCS	:	Trimethylchlorosilane
OPLS-DA	:	Orthogonal Partial Least Squares Discriminant Analysis
PLS-DA	:	Partial Least Squares Discriminant Analysis
PCA	:	Principal Component Analysis
VIP	:	Importance for the Projection
DSPC	:	Debiased Sparse Partial Correlation
QEA	:	Quantitative Enrichment Analysis
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
TIC	:	Total Ion Chromatograms
UAP	:	UDP-N-acetylglucosamine pyrophosphorylase
TRE	:	Trehalase
TPS	:	Trehalose-6-phosphate synthase
MIPS	:	Myo-Inositol-3-Phosphate Synthase

General Introduction

The advancements in agriculture have significantly contributed to global socioeconomic growth, with crop and livestock farming playing a pivotal role in the development. These sectors are the foundation for new agricultural practices that improve crop yields and enhance production, distribution, and storage. Adopting innovative farming techniques, including pest management strategies, has been a key factor in this progress (Matteson et al., 1984; Yudelman et al., 1998; Way & Van Emden, 2000). Over the past few decades, farmers have employed various insect pest control approaches using synthetic pesticides, biopesticides, integrated pest management (IPM), and non-chemical methods. However, the search for alternatives to synthetic pesticides has gained momentum due to the development of pest resistance to chemicals and the potential health and environmental risks (Eze & Echezona, 2012; Chowanski et al., 2014; Rani et al., 2021; Ayilara et al., 2023).

Pesticides with specific targets are considered more efficient and environmentally safer, while biological control methods, though eco-friendly, often produce slower results. Farmers frequently turn to chemical control methods for quicker outcomes. Although insect-specific pesticides and physical control measures are widely accessible, insect-specific biological control methods are less available. Insect-targeting strategies effectively manage specific pests

while minimizing risks to non-target insects and mammals, promoting safer and more sustainable pest control practices.

Insect-specific pesticide formulations play a crucial role in promoting safe agricultural growth by targeting and controlling various insect species through different modes of action. These insecticides can disrupt the nervous system, damage exoskeletons, or act as repellents. They are classified based on their method of entry as ingested, inhaled, or absorbed through the integument. They are typically applied as sprays or powders to plants and surfaces, making them effective across a range of species. The primary synthetic pesticide groups include carbamates, organophosphates, and chlorinated hydrocarbons (Plimmer, 1979; Karami-Mohajeri & Abdollahi, 2011; Abubakar et al., 2020; Araújo et al., 2023). However, the widespread use of these chemicals has led to significant environmental contamination and the emergence of resistant pest species. Reducing pesticide use to mitigate environmental harm and combat insecticide resistance is essential to tackle these issues. A promising, environmentally acceptable solution lies in discovering synergistic agents which could enhance pesticide effectiveness while minimizing toxicity and overall usage (Subbanna et al., 2019).

Insect growth regulators (IGRs), are compounds that interfere insect development, are emerging as a potential alternative to traditional pesticides due to their environmental safety and selective mode of action (Bennett & Reid, 1995; Mondal & Parween, 2000; Masih & Ahmad, 2019; Sarwar, 2020). IGRs are currently used commercially in forestry, agriculture, and public health, as well as to protect stored

products and manage insect pests (Sarwar, 2020). These regulators affect insect behaviour, reproduction, mortality, and both embryonic and post-embryonic development by disrupting key biochemical pathways essential for growth (Ghoneim & Bakr, 2018; Sarwar, 2020). IGRs can lead to abnormal morphogenesis and are more effective in controlling eggs than modern insecticides. Unlike conventional pesticides, which quickly kill insects, IGRs work through prolonged exposure, limiting population growth by affecting adult insects and their offspring (Mondal & Parween, 2000). This gradual but comprehensive impact makes IGRs a promising tool for sustainable pest management.

Insect growth regulators (IGRs) are classified into three groups based on their mechanisms of action: juvenoids, also known as juvenile hormones (JHs) and their analogues (JHAs); ecdysone agonists; and chitin synthesis inhibitors (CSIs), also referred to as moult inhibitors (MIs) (Bitsadze et al., 2013; Tripathi & Tiwari, 2014). In insects, juvenile hormones, ecdysones, and moulting hormones regulate critical physiological and behavioural processes and control the morphogenetic changes during metamorphosis (Gilbert et al., 1980). Advancements in structural analysis and synthesis have made synthetic analogues and mimics of these hormones readily available, offering effective tools for pest control by targeting the key developmental pathways.

Chitin is the second most abundant renewable polymer in nature after cellulose. It plays a crucial role in lower invertebrates, contributing to structures such as egg shells, mollusc shells, fungal cell walls, and

arthropod exoskeletons. Targeting chitin for pest management is particularly appealing as it is essential for forming the insect exoskeleton, which protects against physical damage, infections, and dehydration. Chitin is composed primarily of N-acetyl-D-glucosamine and is found not only in the exoskeleton but also in the trachea, eggs, and ovarioles of insects, as well as in the peritrophic matrix of the midgut. The widespread presence of critical structures makes it an attractive target for disrupting insect development and controlling pest populations.

Chitin synthesis inhibitors (CSIs) are a class of compounds that disrupt insect moulting and result in defective cuticle formation by interfering with chitin biosynthesis (Cohen, 1987). Chitin synthesis inhibitors (CSIs) function by inhibiting the enzyme chitin synthase, which is crucial for the formation of chitin in insects (Merzendorfer, 2006). These inhibitors have been successfully used to control agricultural pests of the orders Lepidoptera, Diptera, and Coleoptera (Apolinário & Feder, 2021), eventually effectively suppressing different life stages of insect pests.

Several compounds, including captan and sulphenimide fungicides, triazine herbicides, chlorinated hydrocarbons, phenylcarbamates, kitazin P, tunamycins, avermectins, plumbagin, and benzoylphenyl ureas (BPUs), hinder chitin formation in living organisms (Hajjar, 1985). Among these, BPU, also known as benzoylureas and acyl ureas, are gaining prominence in pest control due to their effectiveness, ease of detection through chemical analysis, and demonstrated anti-tumoral activity in animal studies (Jenkins et al., 1984; Mayer et al., 1984).

Additionally, BPUs pose minimal threat to beneficial natural enemies, making them an attractive option for pest management.

Benzoylphenyl ureas (BPUs) primarily target the sites where epidermal cells produce chitin. Their unique mode of action distinguishes BPUs from other insecticides; by inhibiting chitin synthesis, these synthetic insect growth regulators effectively prevent chitin production in the epidermis (Doucet & Retnakaran, 2012). This targeted approach is believed to reduce the risk of cross-resistance among insect pests. In the insects exposed to BPUs, the endocuticle is damaged and loses flexibility, impairing their ability to moult and survive. Additionally, BPUs are considered relatively safe for higher animals and plants, as these organisms do not contain chitin in their bodies, making them a suitable option for environmentally reliable pest management.

In the early 1970s, Philips-Duphar developed the first chitin synthesis inhibitor (CSI), diflubenzuron (DFB; marketed as Dimilin, Adept, Vigilante), that features a benzoylphenyl urea (BPU) structure. The accidental discovery of diflubenzuron led to extensive research and the commercialization of nearly fifteen BPU insecticides. The discovery of BPUs can be categorized into three generations: the first began with diflubenzuron, the second triflumuron, and the third flufenoxuron (Ganguly et al., 2020). Since then, various active compounds have been introduced to the market, including flufenoxuron, fluxyloxuron, chlorfluazuron, lufenuron, triflumuron, fluzuron, teflubenzuron, hexaflumuron, novaluron, and noviflumuron (Smagghe et al., 2019).

The third-generation BPU's exhibited increased topical activity and a broader range of efficacy. These CSIs disrupt chitin formation, resulting in the development of an aberrant cuticle that ultimately leads to the death of the exposed insects or shortly after their subsequent moult. Additionally, CSIs affect eggs, potentially reducing insect fecundity (Acheuk et al., 2012). Chitin synthesis inhibitors can disrupt egg hatching by preventing embryos from breaking through the vitelline membrane. This blockage renders the eggs non-viable, making these inhibitors valuable in pest control strategies (Muthukrishnan et al., 2012). Furthermore, ultrastructural studies on the effects of acyl ureas on *Drosophila* embryogenesis revealed that when female flies were exposed to high doses of lufenuron and mated with untreated males, egg hatching was inhibited entirely (Gangishetti et al., 2009). High doses of chitin synthesis inhibitors also induce genetic-level regulation, with some genes being upregulated to suggest involvement in detoxification processes (Muthukrishnan et al., 2012).

Flufenoxuron, a chitin synthesis inhibitor (CSI), is widely used as an insecticide in agriculture to disrupt the normal development of insects by inhibiting chitin production, ultimately leading to death. It has been shown to effectively suppress pest species of Lepidoptera, Coleoptera, and Diptera, making it a valuable tool in the management of ornamental plants, forestry, and fruit and vegetable crop production (Yasir et al., 2012; Nasr et al., 2020; Tanani et al., 2022).

Sexual reproduction is essential for sustaining life, and an organism's reproductive health can be influenced by many factors, including age, stress, xenobiotics, diet, lifestyle, and behaviour (Hoyer, 2001). Insects

possess a highly successful reproductive system that contributes significantly to their diversity and population dynamics. However, certain insect species, such as caterpillars, beetles, and aphids, can cause considerable harm to forestry, livestock, and human activities, leading to substantial economic impacts. Targeting the reproductive capabilities of these pests offers a strategic approach to control population growth.

Insect reproduction is affected by several factors, including food availability, photoperiod, and humidity (Drake, 1994). In addition, substances like chitin synthesis inhibitors can disrupt the peritrophic matrix in an insect's midgut, impairing its ability to acquire essential nutrients for reproduction (Cohen, 2001). Consequently, the nutritional quality of the insect and the stage of gamete maturation become critical factors that can decrease the reproductive potential of insects, thereby reducing the population of subsequent generations (Costa et al., 2017).

The disruption of the chitin synthesis process can have significant negative impacts on the reproductive systems of insects. Research has demonstrated that chitin synthesis inhibitors (CSIs) can influence insect reproduction in several ways, including a reduction in the size and number of eggs produced by female insects, decreasing the viability of both eggs and sperm and disrupting mating behaviours (Ghoneim, 2018; Smagghe et al., 2019). These adverse effects can ultimately lead to a decline in the populations of targeted pest species. Moreover, the specific impacts of CSIs on an insect's reproductive system can vary based on factors such as the insect species, the dosage and timing of pesticide application, and prevailing environmental

conditions. Understanding these variables is crucial for developing effective pest management strategies that leverage the reproductive vulnerabilities of pest insects.

The expression of various traits essential for reproduction plays a significant role in regulating individual variation in male reproductive success over a lifetime. Natural selection favours phenotypes that give males a reproductive advantage, enabling them to sire more offspring. Numerous studies have highlighted the importance of environmental factors in mediating these selection (Filice & Long, 2018; Joseph et al., 2018; Mandi et al., 2020). Males contribute equally to reproduction by providing sperm necessary for fertilization and the production of offspring. Any disruptions in sperm quality can have adverse effects on future generations. Exposure to toxicants has been associated with reduced sperm quality by disrupting spermatogenesis and affecting reproductive development (Wedell et al., 2002).

Ngoula et al. (2012) reviewed that insecticides can impact the male reproductive system at various sites, including the neuroendocrine system, central nervous system, and accessory sex glands. Insecticides may directly damage sperm, alter spermatogenesis function, or disrupt any stage of endocrine regulation, such as hormone synthesis, their release, storage, transport, receptor recognition and binding, or central nervous system activity. Despite these aspects, the available information reveals that the potential for male reproductive failure due to exposure to chitin synthesis inhibitors (CSIs) in insects has received little attention.

The moth species *Spodoptera mauritia* belongs to the Noctuidae family and is commonly known as the armyworm (Brown & Dewhurst, 1975). The larvae of *S. mauritia* are considered agricultural pests, as they feed on paddy and are responsible for significant economic losses in many countries (Grant, 1982). These larvae can cause severe crop damage, with outbreaks leading to substantial economic loss for farmers. The paddy armyworm, *S. mauritia*, is indigenous to Sub-Saharan Africa; it has also been reported in India, Pakistan, and Australia. Epidemics of this migratory moth are often associated with climatic changes and seasonal fluctuations. It is prevalent across the rice-growing regions of Southern India, Tamil Nadu, and Kerala, particularly in the Delta and West Coast areas. In certain states of India, it is considered a serious paddy pest, causing significant damage, whereas in other areas, it only sporadically leads to significant crop damage (Reddy et al., 2020; Adak & Rath, 2021).

The outbreaks of *S. mauritia* typically occur after prolonged dry conditions, followed by heavy rainfall. An alternate host creates favourable conditions for the outbreak to flourish. Swarming caterpillars can severely impact paddy, damaging immature seedlings, leaves and the base of rice panicles (Sarma & Salam, 2018). Due to their nocturnal nature, the larvae primarily feed at night or on overcast days, usually targeting the upper part of the rice canopy. Older larvae feed voraciously, almost entirely defoliating the plants, while younger larvae cause the plants to appear sickly, with wilted tips and clipped leaves. The rapid movement from field to field often leads to significant crop loss within a week.

The rice armyworm has been controlled using a variety of control measures, including physical, biological, and chemical methods. Physical methods include using light traps for mass collection of adult moths. Biological control involves use of egg and larval parasitoids, birds, and other natural predators. Chemical control, using organophosphate and organochlorine pesticides, is highly effective, producing visible results in a short period. However, the use of chemical pesticides has harmful environmental consequences.

Insect growth regulators, particularly chitin synthesis inhibitors (CSIs), are highly effective in controlling lepidopteran pests. However, the impact of CSIs on reproductive suppression has not been extensively studied. Though few studies suggest that chitin synthesis inhibitors may influence reproductive development, detailed information on their indirect effects remains limited. Specifically, there is no comprehensive data on the effects of flufenoxuron, a CSI, on the rice swarming caterpillar *Spodoptera mauritia*. Therefore, the primary goal of this research is to investigate whether flufenoxuron affects the growth, development, and reproduction of the sporadic paddy pest, *Spodoptera mauritia*.

Objectives of the investigation

The effectiveness of insect growth regulators in field trials and laboratory investigations on a variety of insects has raised awareness of the significance of managing agricultural pests. Among them, the complex molecular mechanism of action, metabolism, degradation, and toxicology of chitin synthesis inhibitors have all been well described in

several studies. Such thorough information highlights how the deliberate application of IGRs and CSIs in modest dosages can significantly disrupt the typical development and life cycle of targeted insects, establishing a significant and beneficial connection to the discoveries reported during fieldwork. From the extensive literature survey conducted for the present study, it becomes strikingly evident that there is a notable lack of comprehensive information and investigations regarding the efficacy of the chitin synthesis inhibitor, flufenoxuron, specifically on male reproductive development of *Spodoptera mauritia*. Consequently, the primary objective of the present research problem has been carefully designated to highlight and address this in detail.

The research problem itself entails examination and exploration of the study involves conducting toxicity experiments to elucidate efficacy of flufenoxuron on sixth instar stage of the *S. mauritia*. Through these experiments, larval growth, metamorphosis, and male reproductive capabilities, the spermatogenesis of this pest will be analysed. In addition to these experiments, the study has been methodically constructed to explore into the complexities of the action of chitin synthesis inhibitor specifically on the metabolic pathway of *S. mauritia*. The thorough understanding of the complex molecular mechanisms, toxicity, and impacts on male gonadal development, this research study aims to significantly contribute valuable information and potential strategies for effective agricultural pest control. The present research was, therefore, undertaken to gain insights into the following aspects:

- Examine the effect of the chitin synthesis inhibitor, flufenoxuron, in the male reproductive development of the late larval stage of *Spodoptera mauritia*.
- Examine the impact of flufenoxuron on the biochemical and histopathological analysis of the testes of *S. mauritia*.
- Live cell examination of the effect of flufenoxuron on dichotomous spermiogenesis of *S. mauritia*.
- Impact of flufenoxuron on metabolomics profiling and chitin synthesis pathway analysis in *S. mauritia*.

Acute toxicity of flufenoxuron on the male reproductive development of *Spodoptera mauritia*

1.1 INTRODUCTION

The intensive use of synthetic pesticides has led to insecticide resistance, environmental pollution, and health hazards to humans and non-target organisms. As a result, eco-friendly safe compounds, like Insect Growth Regulators, were developed as alternative pest control agents (Li & Fantke, 2023). Over the past few decades, investigation has concentrated on identifying chemicals that impede insect development and reproduction that target the endocrine regulating systems. Since then, several substances known as "insect growth regulators" (IGRs) have been in use that negatively affect the development and reproduction of insects. Their mode of action is highly selective and species-specific (Tunaz & Uygun, 2004). IGRs are classified as juvenile hormone analogues (methoprene, pyriproxifen, and fenoxycarb), ecdysone agonists (methoxyfenozide, tebufenozide), and chitin synthesis inhibitors (diflubenzuron, lufenuron, flufenoxuron) (Oberlander & Silhacek, 2000; Poppenga & Oehme, 2010).

Rice is a major cereal crop grown in diverse ecosystems (Pathak et al., 2018), from rain-fed uplands to irrigated lowlands, thriving in warm, tropical climates with ample water. However, rice crops face

significant threats from pests, especially insects, which can cause substantial yield losses and economic damage. Over 800 insect pests have been identified, with about 100 species attacking rice. Among these, nearly 20 insects, including defoliators, stem borers, gall midges, planthoppers and leafhoppers, directly harm rice crops and spread diseases (Iqbal,2020). Effective management techniques, including agronomic approaches, biological control, and chemical control, are essential to reduce the damage caused by these pests (Baker et al., 2020).

The rice swarming caterpillar, *Spodoptera mauritia* is a sporadic pest that causes significant losses to rice crops. Upland rice is the insect's primary host but also attacks weeds and graminaceous plants. It is widely distributed throughout Southeast and East Asia. In recent years, the rice-swarming caterpillar has become a serious pest in Southern and Eastern India, severely affecting wet-season rice production (Tanwar et al., 2010). It is common in all rice-growing areas of South India, especially along the West Coast and Delta regions (Sarma et al., 2021). In some parts of India, it is a major pest causing serious damage; in others, it occasionally causes severe damage. Reports have indicated that in Kerala, the rice swarming caterpillars devastated 1304 hectares of land, destroying paddy crops, particularly in the Alappuzha district (Jena et al., 2017). The pest outbreaks are triggered by a prolonged dry period followed by significant rainfall.

Controlling the population growth of devastating pests like armyworms is crucial. The most common method for managing rice armyworms has been using chemical insecticides, which have proven effective (Deshmukh et al., 2020). In India, rice swarming caterpillars are typically controlled using insecticides such as chlorpyrifos,

quinalphos, triazophos, or dichlorvos. Studies have shown that exposure to these pesticides, particularly organophosphates and neonicotinoids, is associated with endocrine disruption and reproductive abnormalities, primarily in mammals (Suwannarin et al., 2021).

However, widespread insecticide use poses risks to the environment and food safety within agroecosystems (Chagnon et al., 2015). As an alternative, various methods have been developed for pest management programs. To improve strategies for controlling larval populations, efforts have been made to explore the impact of insect growth regulators on insect population control. Insect growth regulators (IGRs) are synthetic insect hormones which can be used as insecticides to regulate the populations of harmful insect pests (Tunaz & Uygun, 2004). IGRs prevent an insect from reaching maturity by interfering with the moulting process. Due to the perceived safety, IGR insecticides have been used to reduce adverse effects on the environment and beneficial species that are not their intended targets. Among the benzophenylureas, several insect growth regulators work effectively against *Spodoptera* species through contact and ingestion inhibition of the biochemical processes that lead to the formation of chitin synthetase, resulting in abnormal endocuticle deposition and interrupting moulting (Clarke & Jewess, 1990; Tabozada et al., 2014; Mead & Khedr, 2018).

Insect development is a complex process regulated by various physiological mechanisms, including chitin synthesis, which plays a crucial role in forming the exoskeleton and other essential structures (Muthukrishnan et al., 2012). Intricate hormonal, genetic, and environmental factors orchestrate the precise regulation of insect

development. Hormones, such as ecdysteroids and juvenile hormones, play crucial roles in coordinating developmental transitions and triggering specific physiological changes at each stage (Nijhout, 2021). Chitin, a polysaccharide composed of N-acetylglucosamine units, is vital in providing structural integrity to various insect tissues and organs.

Chitin synthesis inhibitors (CSIs) disrupt chitin production, leading to insect developmental abnormalities. CSIs act by targeting enzymes involved in chitin synthesis, impairing the moulting process and subsequently causing the death of lepidopteran pests at various stages of their life cycle (Liu et al., 2019). Lepidopteran pests pose a significant threat to global agriculture by causing substantial yield losses and crop damage (Maish, 2019). In recent years, innovative and sustainable pest control methods have gained significant attention (Nawaz et al., 2019). One promising approach is the utilization of chitin synthesis inhibitors (CSIs) as pesticides to combat lepidopteran pests. They offer significant environmental benefits by targeting lepidopteran pests, reducing the need for broad-spectrum pesticides that harm beneficial insects and other non-target organisms. CSIs have lower toxicity to mammals, birds, and aquatic life and are readily biodegradable, reducing environmental persistence and bioaccumulation (Sun et al., 2015). These properties make CSIs a more environmentally friendly alternative to conventional pesticides for insect pest control.

Chitin Synthesis Inhibitors (CSIs) are highly effective against a broad spectrum of lepidopteran pests, including armyworms, cutworms, and

corn borers (Clarke & Jewess, 1990; Tabozada et al., 2014; Mead & Khedr, 2018). CSIs suppress insect populations by disrupting chitin synthesis, interfering with moulting and preventing pests from advancing to the next life stage. This targeted action lowers the likelihood of resistance development compared to conventional pesticides. Additionally, some CSIs have residual effects (Fontoura et al., 2012), providing prolonged protection and reducing the need for frequent applications.

Flufenoxuron is a benzoylurea insecticide that inhibits chitin biosynthesis (MOA Group 15) in nymphal mites and lepidopteran larvae (ACP, 1994). It blocks the enzyme chitin synthase, which disrupts insect moulting by preventing proper exoskeleton formation. This interference with chitin synthesis affects the growth and development of immature stages, such as eggs, larvae, and pupae, ultimately reducing pest populations and enhancing control effectively. Flufenoxuron has been employed for the control of insect pests belonging to the orders Diptera (Matsuda, 2022), Coleoptera (Ali et al., 2022), and Lepidoptera (Clarke & Jewess, 1990; Bakr et al., 2013; Tabozada et al., 2014; Amein & Mohammad, 2022). Flufenoxuron is an effective stomach and contact insecticide (Hsiao et al., 2013), inhibiting chitin synthesis during insect development. Additionally, flufenoxuron has been used as an insecticide for public hygiene, primarily to control fleas and cockroaches (Clarke & Jewess, 1990). Nevertheless, earlier research has indicated that flufenoxuron harms insects' reproduction ability (Salokhe et al., 2003; Yasir et al., 2012; Shaurub et al., 2018). Flufenoxuron provides long-lasting protection

due to its residual effects, maintaining efficacy even after application. Its selectivity for insects dependent on chitin synthesis, especially lepidopteran pests, minimizes impact on non-target organisms and reduces ecological risks.

Pesticides can negatively impact insect reproductive organs and systems in various ways. They disrupt hormonal balances, impair reproductive organ development and function, or cause genetic damage, reducing fertility, sterility, or death (Bretveld et al., 2006). Additionally, pesticides can affect mating behaviour by interfering with pheromone production, altering mating signals, or impairing the ability to find and recognize mates, resulting in decreased mating success and lower reproductive rates (Muller, 2018).

Moreover, a thorough knowledge of the impacts of chitin synthesis inhibitors (CSIs) is essential for studying insect development and reproduction. Limited research exists on the reproductive toxicity of flufenoxuron, leaving the underlying processes not fully understood. Examining the effects of these substances on insect physiology and reproduction is crucial for gaining deeper insights and enabling their more effective application. Though the effects of chitin synthesis inhibitors (CSIs) on the exoskeleton and moulting process are well-documented (Doucet & Retnakaran, 2012), their impact on testis development remains largely unexplored. Hence, the present study aims to investigate the specific effects of flufenoxuron on testis development in *Spodoptera mauritia*, shedding light on the potential implications for insect reproductive success and population dynamics.

1.2 REVIEW OF LITERATURE

1.2.1 Insect growth regulators

Chemical pesticides are effective tools for managing insect populations, but their excessive use can negatively impact beneficial species and create significant environmental risks (Marx, 1977; Pimental, 1983). To address the problems associated with the indiscriminate use of traditional insecticides, eco-friendly and cost-effective alternatives for pest management are being explored (Ghoneim & Bakr, 2018). Modern insect pest management seeks to minimize the harmful effects of hazardous insecticides on both the food chain and the environment, leading to a growing focus on alternative pest control strategies (Way & Van Emden, 2000). Therefore, innovative and sustainable solutions are crucial to developing inclusive approaches that target species-specific pests and promote the overall health of ecosystems. The increasing demand for less harmful insecticides and the rise of pesticide-resistant species has heightened interest in alternative solutions such as insect growth regulators (IGRs). Advances in understanding insect endocrine regulation have facilitated the practical application of insect hormones and their analogues as insect growth regulators (IGRs) (Keeley et al., 1990; Riddiford, 1994). The term IGR was first introduced by Staal (1975) to describe bio-rational chemical substances that act as “third-generation pesticides,” disrupting insect development. This concept builds on Williams's (1967) and Henrick et al. (1973) research.

The chemicals negatively impacting insect growth and development are a novel method employed as a successful substitute for managing insect pests and vectors (Fansiri et al., 2022). The compounds target specific physiological regulatory mechanisms necessary for the normal development of insects, earning them the classifications of "insect hormone mimics" or "insect growth regulators" (IGRs). IGRs have proven effective against pests resistant to traditional insecticides, such as pyrethroids or organophosphates (Ahmad & Arif, 2010; Zhou et al., 2011). These substances regulate key physiological functions in insects, and rather than being directly toxic, they alter hormonal pathways related to moulting, metamorphosis, and reproduction (Siddall, 1976; Tunaz & Uygun, 2004; Aravindarajan et al., 2017). Moreover, IGR compounds have been extensively tested and shown to be effective chemical agents for controlling several insects such as *Aedes aegypti*, *Spodoptera spp.*, and *Heliothis spp* (Kodandaram et al., 2010; Fansiri et al., 2022).

The development of adult insects with specific morphogenetic abnormalities, such as sterility or malformed genitalia that prevent mating or the production of fertile offspring, is an example of how certain compounds are designed to disrupt metamorphosis, ensuring no reproductively viable adults are formed (Grenier & Grenier, 1993; Tunaz & Uygun, 2004). Ideally, insecticides should exhibit selective toxicity, targeting physiological mechanisms, as many conventional insecticides have target sites similar to insects and mammals. Insect growth regulators (IGRs) fit into this category of selective insecticides. They are classified based on their mode of action: compounds that

inhibit the activity of insect hormones (such as juvenile hormones and ecdysteroids) and chitin synthesis inhibitors, which block cuticle formation (Oberlander & Silhacek, 2000; Tunaz & Uygun, 2004; Ghoneim & Bakr, 2018).

Insect growth regulators (IGRs) act on insect-specific systems and are unlikely to affect non-target organisms. Two commonly used types of IGRs are juvenile hormone analogues (JHAs) and chitin synthesis inhibitors. These regulators are highly effective at very low application rates, pose minimal risk to mammals, and exhibit strong selectivity for arthropods. Due to their low ecotoxicological profile and short environmental persistence, IGRs play a crucial role in integrated pest management. Chitin synthesis inhibitors work by disrupting the normal formation of chitin during moulting (Chen et al., 2019; Sarwar, 2020). On the other hand, juvenile hormone analogues mimic the effects of naturally occurring juvenile hormones in insects, thereby inhibiting their synthesis and interfering with development (Fansiri et al., 2022).

1.2.2 Chitin synthesis inhibitor: Chemistry and mode of action

The insect cuticle, composed primarily of chitin and proteins, serves multiple vital functions beyond forming the exoskeleton. It supports the linings of the digestive tract (peritrophic matrix), respiratory systems (trachea), reproductive ducts (including egg shells), muscle attachment sites, and gland ducts (such as salivary glands) and acts as a barrier between the insect and its environment (Moussian, 2010; Muthukrishnan et al., 2012). Chitin, a key structural component, is synthesized by the enzyme chitin synthase, which is critical in forming

the cuticle (Merzendorfer, 2006). Chitin is a β -(1,4) glycoside polymer of N-acetyl-D-glucosamine, and it exists in three forms: α , β , and γ chitin (Tunaz & Uygun, 2004). Chitin is found in the cuticle of insects and crustaceans, the cell walls of fungi and protozoa, but it is notably absent in vertebrates and higher plants (Sun et al., 2015).

In insects, two genes, CHS1 and CHS2, encode chitin synthases, the enzymes responsible for chitin production. CHS1 generates chitin microfibrils for the linings of the foregut, hindgut, and the serosal, epidermal, and tracheal cuticles. At the same time, CHS2 is essential for developing the peritrophic membrane in the midgut (Merzendorfer, 2013). Chitin synthesis is carried out by the extracellular enzyme chitin synthase, which is linked to the plasma membrane. However, for chitin synthesis to occur, proteases must first activate the enzyme, as it is initially produced in an inactive form (zymogen) within the endoplasmic reticulum of the epidermis (Tunaz & Uygun, 2004; Meissner, 2010). These enzymes and other key regulatory processes involved in chitin synthesis present valuable targets for regulation by specific chemicals (Cohen, 1993; Ruiz-Herrera & San-Blas, 2003; Tunaz & Uygun, 2004). Moreover, chitin is absent in vertebrates and higher plants, and the biosynthesis pathway of chitin offers an ideal target for insect-specific pesticides (Sun et al., 2015; Wang et al., 2020).

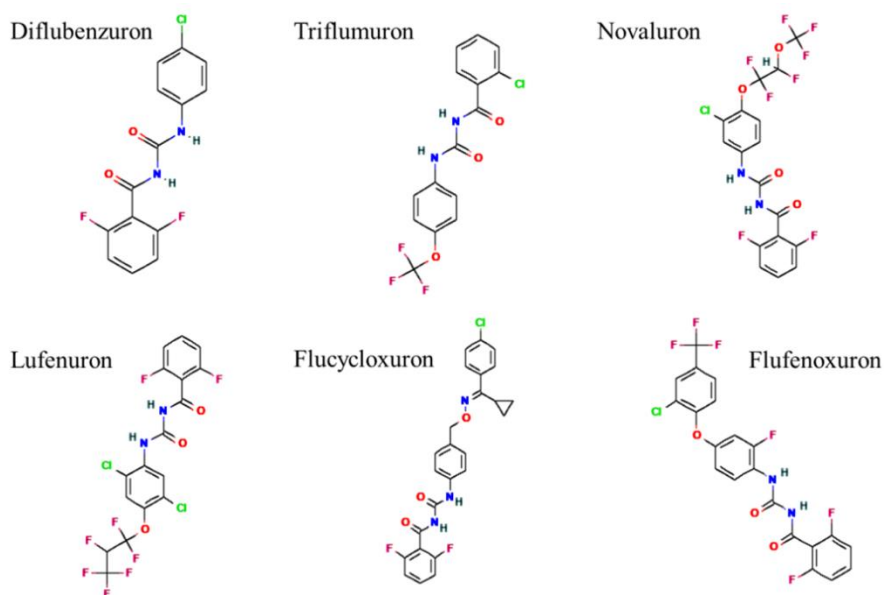
Chitin synthesis inhibitors (CSIs) represent an important class of chemicals in integrated pest management (IPM) as they prevent insects from synthesizing chitin, and they are safe for most non-target organisms (Doucet & Retnakaran, 2012; Khater, 2012; Ali et al.,

2020). Among these, benzoylurea CSIs have garnered significant attention due to their unique mode of action, low toxicity to humans, birds, and bees, and biological solid efficacy against pests (Muzzarelli & Marks, 1986; Khoushab & Yamabhai, 2010; Sun et al., 2015). Studies have shown that benzoylphenyl urea can disrupt hormonal balance in insects, leading to physiological impairments such as inhibition of DNA synthesis, alteration of enzyme activity, and suppression of microsomal oxidase activity (Ishaaya & Horowitz, 1998).

The development of benzoylurea CSIs began with the formulation of 1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)-urea (DU19111) by Muder and his team at Philip-Duphar Co. Originally intended as herbicide, DU19111 unexpectedly showed insecticidal properties. It was effective against larvae of various pests, including lepidopterans, mosquitoes, houseflies, desert locusts, and cotton strainers. Later, further research on compounds similar to DU19111 led to the development of diflubenzuron and the first commercial chitin synthesis inhibitor introduced to the market as Dimilin in 1975 (Sun et al., 2015; Gad et al., 2021). Consequently, diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea] was considered the most effective inhibitor of chitin production (Aravindarajan et al., 2017), with specific structural derivatives exhibiting even greater activity than the parent compound (Bull & Ivie, 1980; Tunaz & Uygün, 2004; Kumari, 2015).

Subsequently, three generations of benzoylurea chitin synthesis inhibitors were developed. The first generation includes dichlorbenzuron and diflubenzuron, the second triflumuron, novaluron,

and lufenuron, and the third flucycloxuron and flufenoxuron (Fig 1). The third-generation benzoyl phenyl ureas (BPUs) showed enhanced topical insecticidal activity and broader-spectrum larvicidal efficacy (Retnakaran & Wright, 1987; Retnakaran & Smagghe, 2010; Sun et al., 2015).



Chemical structure of Chitin synthesis inhibitors

Over the past 40 years, benzoyl phenyl urea (BPU) insecticides have acquired significant attention due to their remarkable efficacy (Tian et al., 2017). The structure of BPUs has three main components: the urea bridge, the aniline ring, and the benzoyl ring. These components can be analysed separately to study their structure-activity relationships, as each plays a distinct role in the overall expression of the compound's larvicidal activity (Sun et al., 2015).

BPU are characterized by a urea centre, with a benzoyl group attached to one nitrogen atom of the urea bridge and a phenyl group attached to the other nitrogen atom. For example, in diflubenzuron, two ortho-fluorine substitutions are present on the benzoyl ring, while a para-chlorine substitution is found on the phenyl ring. Across BPUs, the benzoyl portion typically lacks para substitutions, while the phenyl portion features more complex substitutions. Despite these variations, the core structure of BPUs remains consistent (Doucet & Retnakaran, 2012).

Benzoyl phenyl ureas (BPUs) have been shown to cause abnormalities in insect larvae of many insect orders during moulting (Oberlander & Silhacek, 1998), which are attributed to disruptions in chitin synthesis and its assembly within the cuticle. These disruptions impact the effectiveness of insecticides and the reproduction and hatching of eggs (Subramanian & Shankarganesh, 2016; Martínez et al., 2021). In addition, delayed adverse effects are observed in pupae and adults of different insect spp. While earlier studies attributed these deformities to hormonal imbalances, impaired chitin formation is now believed to be a key factor (Eisa et al., 1991; Khan & Qamar, 2011; Lopez-Mondejar et al., 2012).

The precise mechanism of action of BPU analogues remains unclear, though several theories have been proposed. These include the involvement of enzymes in chitin degradation and cuticular sclerotization, the hormonal regulation of transcriptional activity, and indirect effects on the transport of uridine diphosphate N-acetylglucosamine (UDP-N-acetylglucosamine) across membranes

(Aravindarajan et al., 2017). A study by Nakagawa et al. (1991) on synthesized BPU with larvicidal activity against rice stem borer larvae found that mono- or di substitutions with single chlorine or two fluorine at the ortho-position of the benzoyl ring were optimal, enhancing the electron-withdrawing and hydrophobic properties of the compound. The ability to allow for various substitutions, the phenyl ring maintained high hydrophobicity and electron-withdrawing potential, leading to high activity in compounds such as chlorfluazuron and teflubenzuron.

In general, BPU analogues target the immature or larval stages of insects. Three speculative target sites have been proposed: inhibition of UDP-N-acetylglucosamine transport through membranes, inhibition of proteases or their biosynthesis, and inhibition of chitin synthase or its biosynthesis (Tunaz & Uygun, 2004). More recent studies suggest that BPUs bind to a class of ATP binding cassette (ABC) transporters known as sulfonylurea receptors (SURs), which are associated with the inhibition of chitin cuticle formation (Tian et al., 2017). Inhibitors of chitin synthesis interfere with the normal development of various cuticle types and peritrophic matrices, leading to severe physiological dysfunctions (Merzendorfer, 2013).

The process of chitin formation begins with the entry of chitin precursors from the haemolymph into the epidermal cells through the basal lamina. In these cells, vesicles containing the chitin biosynthetic machinery are believed to be formed in the endoplasmic reticulum and the Golgi apparatus, where they undergo maturation. Once matured, the vesicles migrate to the plasma membrane, where they release chitin

microfibrils at the tips of microvilli. However, exposure to compounds like diflubenzuron may cause the vesicles to release their contents prematurely before the chitin is fully synthesized, halting the entire process. Some studies suggest that diflubenzuron may inhibit the transport of chitin synthase, further disrupting chitin formation (Eto, 1990).

1.2.3 Toxicity effects of chitin synthesis inhibitors on insects

For past several decades, diflubenzuron and other acylurea derivatives have been successfully used to control insect populations. When administered orally or topically, acylureas have been shown to cause several adverse effects on insect growth, development, and fecundity. These effects include reduced cuticle thickness, disruption of laminar organization, and decreased chitin content, which can lead to moulting failure and death (Mulder & Gijswijt, 1973; Gangishetti et al., 2009; Merzendorfer et al., 2012). In studies with *Drosophila* females, mature flies laid a typical number of eggs when fed a diet containing diflubenzuron, but the eggs failed to hatch (Wilson & Cryan, 1977; Merzendorfer et al., 2012; Mansur et al., 2010).

Abortive moulting and egg-hatching problems are the hallmarks of different chitin synthesis inhibitors, and they appear to be a direct result of a disruption of chitin deposition that arises from abnormal cuticle development (Merzendorfer, 2013; Liu et al., 2019; Tunckol, 2019). These substances have demonstrated efficacy in combatting several insect pest species (Pineda et al., 2007; Gelbic et al., 2011; Khajepour et al., 2012; Tabozada et al., 2014).

Benzoylphenylurea (BPU) pesticides are particularly effective against leaf-feeding insects and their larvae. These insecticides primarily exert their effects through ingestion; however, some species also exhibit ovicidal and contact toxicity and reduced fertility (Ishaaya & Horowitz, 1998; Tian et al, 2017). CSIs typically lead to malformations in the cuticle and significantly reduced chitin content (Merzendorfer, 2012). They have varying degrees of impact on the development and reproduction of organisms that synthesize chitin, resulting in specific symptoms such as abnormalities in egg hatching and abortive moulting. These issues are closely linked to structural alterations in different types of cuticles (Merzendorfer, 2013).

The effects of benzoylphenyl ureas (BPUs) on the physiology of insect moulting were thoroughly reviewed by Retnakaran and Wright in 1987. BPUs exhibit potent activity against the immature stages of lepidopteran insects (Fahmy, 2014; Ganguly et al., 2020). BPUs are particularly effective in controlling various pests, including bollworms, diamondback moths, *Spodoptera litura*, and *Spodoptera exigua* (Ishaaya et al., 1996). In addition to their efficacy against lepidopterans, BPUs are also effective against a range of non-lepidopteran insect species, including Hemiptera, Diptera, and Coleoptera (Perez-Farinos et al., 1998; Farnesi et al., 2012; Fontoura et al., 2012). The study demonstrated significant activity against Coleopteran larvae and are potent toxins against the early stages of leaf miners and whiteflies, including *Bemisia tabaci* and *Trialeurodes vaporariorum* (Ishaaya et al., 1996). Furthermore, BPUs have

applications in public health for controlling the larvae of flies and mosquitoes (Hsiao et al., 2013).

Insect growth regulators (IGRs) are primarily used as larvicides but also significantly reduce oviposition in many insect species. This reduction can function as an ovicide, decreasing the egg-laying rate or preventing the hatching process by inhibiting embryonic development (Perez-Farinos et al., 1998; Smagghe et al., 2019). According to Mondal & Parween (2000), IGRs affect various aspects of insect physiology, including embryonic and post-embryonic development, reproduction, behaviour, and mortality, particularly in treated stored product pests.

Diflubenzuron, the precursor to all benzoylureas, has been extensively researched and globally utilized. Diflubenzuron is widely used for controlling leaf-feeding larvae, locusts, grasshoppers, fruit flies, and cockroaches (Weiland et al., 2002; Abo-Elghar et al., 2004). Flucycloxuron, a second-generation benzoylphenyl urea (BPU) enhanced as an acaricide, was developed to control mites. The compound is a dispersible concentrate formulation with specialized surfactants for topical contact efficacy (Ahn et al., 1996; Doucet & Retnakaran, 2012). Lufenuron, considered more effective than diflubenzuron, is widely employed to manage the mushroom sciarid fly, *Lycoriella ingenua*, a common pest in the cultivation of *Agaricus bisporus* (Erler et al., 2011). Additionally, it is used as a chemosterilant against *Ceratitis capitata* (Navarro- Llopis et al., 2004).

Treatment with CSI hexaflumuron led to a significant and comparable decrease in the lifespan of both males and females *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Vasuki & Rajavel, 1992). Previous studies have documented decreased oviposition among CSI-treated females across different insect orders (Wilson & Cryan, 1997; Sáenz-de-Cabezón et al., 2006). Novaluron, a well-known benzoylphenylurea, works by inhibiting the biochemical processes involved in chitin synthetase production. The inhibition occurs through contact and ingestion, disrupting normal chitin formation and resulting in abnormal endocuticle deposition, which halts the moulting process (Tunaz & Uygun, 2004; Dhadialla et al., 2005; Cutler & Scott-Dupree, 2007). Novaluron interferes with cuticular synthesis in larval insects, leading to premature apolysis. The disruption causes the insect to become trapped mid-moult, eventually dying from starvation and desiccation (Djeghader et al., 2014).

Furthermore, chitin synthesis inhibitors, such as chlorfluazuron, have been shown to disrupt reproductive development in insects significantly. In *Spodoptera litura*, the administration of chlorfluazuron led to impaired testis growth and development, as evidenced by reduced testis size, weight, and sheath thickness (Perveen, 2012b). Research also indicated that these inhibitors affect spermatogenesis by a reduction in the number and size of eupyrene and apyrene sperm bundles in the testes of *S. litura*. Additionally, they significantly lowered the protein content in the testes, with unchanged carbohydrate and lipid levels (Perveen, 2000). A similar effect was observed in female *Agrotis ipsilon*, as chitin production inhibitors

reduced the levels of yolk proteins, carbohydrates, and lipids (Khatter, 2014).

The disruption of ovarian and testicular development is believed to contribute to reduced fecundity, fertility, and hatchability following sublethal exposure to chlorfluazuron (Perveen, 2000). Furthermore, novaluron impair tissue and cell mitosis in *Schistocerca gregaria*, leading to delayed egg-laying and decreased oviposition in larvae fed on treated diets (Ghoneim et al., 2015). Likewise, exposure to triflumuron in *Tribolium castaneum* caused distortions in the growth and development of male and female gonads. The treatment also reduced egg viability, delayed embryo development, and extended the incubation period, even at low concentrations (Parween et al., 2001).

1.2.4 Flufenoxuron

Flufenoxuron, a benzoylurea insect growth regulator, is known for its potent acaricidal activity. The primary mode of action involves disrupting chitin synthesis during the developmental stages of mites and insects, ultimately causing death. However, it has little to no effect on adult pests. Approved for use in a wide range of crops globally, flufenoxuron is known for several other names, including WL-115110, SKI-8503, AC-811678, CL-811678, and BAS-3071. It is marketed under the trade names Cascade™ and Floxate™. Originally developed by Shell Research Ltd., the compound was first reported in a study by Anderson et al. in 1986 (Pener & Dhadialla, 2012). Beyond agricultural applications, flufenoxuron is also used as an insecticide in

public health, particularly for controlling fleas and cockroaches (Clarke & Jewess, 1990).

Flufenoxuron is non-volatile and has low water solubility. The compound is only slightly persistent in soil but degrades rapidly in aquatic environments when exposed to sunlight. Though it poses minimal risk to mammals, it has a high potential for bioaccumulation (Ganguly et al., 2020). It is highly toxic to most aquatic organisms but only moderately toxic to birds, honeybees, and earthworms (Ganguly et al., 2020).

The insecticidal activity of flufenoxuron is due to its ability to inhibit chitin biosynthesis, impacting the CHS1 enzyme. This leads to developmental disruptions in insects through contact and stomach action. Inhibiting chitin production affects multiple physiological systems, including reproduction, as the insect's peritrophic membrane, trachea, and cuticle are compromised. Exposure to flufenoxuron may interfere with various metabolic pathways, ultimately impairing nutrient absorption and causing nutritional deficiencies in the insect. Reproductive success in insects is influenced by several factors, including habitat and diet, and flufenoxuron's impact on chitin production may exacerbate these effects.

1.2.5 Impact of flufenoxuron toxicity

The toxicity effects of flufenoxuron on several insect species have been extensively studied and documented such as *Spodoptera littoralis* and *Agrotis ipsilon* (El-Sheikh, 2002; Tabouzada et al., 2006), *Trichogramma evanescens*, and *Bracon brevicornis* (Tabouzada et al.,

2014), *Tribolium castaneum* (Salokhe et al., 2006), *Spodoptera littoralis* (Clarke & Jewess, 1990; Mostafa, 1993; Sammour & El-Ansary, 1996; Tabozada et al., 2014), *Musca domestica* (Nasr et al., 2020), *Schistocerca gregaria* (Bakr & Ghoneim, 2008), and *Lobesia botrana* (Bressan et al., 2002). Flufenoxuron has been used to control Lepidopteran larvae on fruits, vegetables, cotton, and grain crops, although with mixed results. Earlier studies have indicated its effectiveness against pests such as cockroaches, including *Blattella germanica* (Reid et al., 1992). Additionally, flufenoxuron, along with diflubenzuron and teflubenzuron, has proven effective against *Spodoptera littoralis* (Clarke & Jewess, 1990). However, a study found that flufenoxuron was less effective than lufenuron in controlling the cotton leafworm, *Spodoptera littoralis* (El-Sheikh & Aamir, 2011). Reda et al. (2010) demonstrated the effects of flufenoxuron on the biological and biochemical aspects of *Spodoptera littoralis*. Despite this, flufenoxuron outperformed other insecticides, such as novaluron, diflubenzuron, and teflubenzuron, in controlling the mushroom sciarid fly, *Lycoriella ingenua* (Erler et al., 2011).

Earlier studies have reported that flufenoxuron induces developmental abnormalities, such as larval-pupal and pupal-adult intermediates and malformed adults. These findings suggest that flufenoxuron may disrupt reproduction by causing hormonal imbalances (Bull, 1986; Deecher et al., 1989; Deecher et al., 1990a, 1990b). Further, the effects of sublethal concentrations of flufenoxuron on life cycle stages and reproductive endpoints in *Tribolium castaneum* showed drastic changes (Salokhe et al., 2003). In *Spodoptera littoralis*, flufenoxuron

was found to negatively impact pupation rates, adult emergence, fecundity, and fertility. Additionally, enzyme activities of acid phosphatase and α , β esterases significantly decreased, highlighting the compound's influence on biochemical processes (Reda et al., 2010).

Similarly, in *Schistocerca gregaria*, flufenoxuron inhibited protein content (Hamadah, 2014). Flufenoxuron notably affected the total content of carbohydrates, proteins, lipids, and cholesterol in *Musca domestica*, further supporting its broad biological impact (Tanani et al., 2022). Moreover, Mageed et al. (2018) demonstrated that flufenoxuron treatment in *S. littoralis* increased the activity of alkaline phosphatase, alpha and beta esterases, chitinase, and phenol oxidase while decreasing the activity of acid phosphatase suggesting a complex interaction with the insect's enzymatic systems.

Although flufenoxuron is primarily recognized as a chitin synthesis inhibitor, several studies have shown its impact on reproductive performance beyond its direct mortality effects. Salokhe et al. (2003) noted that the sub-lethal effects of flufenoxuron on the development and fertility of surviving individuals may contribute to suppressing future generations. In *Agrotis ipsilon* females, flufenoxuron treatment significantly reduced the oviposition period, leading to fewer eggs laid and decreased egg hatchability. Additionally, flufenoxuron caused a significant decrease in the mean total protein, carbohydrate, and lipid content in the ovaries, along with a noticeable reduction in overall ovariole length (Shaurub et al., 2018). Similarly, in *Tribolium castaneum*, treatment with flufenoxuron resulted in significantly reduced fecundity and egg hatchability (Yasir et al., 2012). Rastegari

and Subrahmanyam (2003) reported that flufenoxuron has a more pronounced long-term effect on reproductive indices, particularly fecundity. Furthermore, El-Bokl et al. (2014) investigated the effects of sublethal doses of flufenoxuron on the ultrastructure of *Rhynchophorus ferrugineus* testicles. The study revealed that the compound interfered with spermatogenesis, affecting one or more stages of the male maturation pathway and altering cellular processes during spermiogenesis.

1.3 MATERIALS AND METHODS

1.3.1 Experimental organism

The rice swarming caterpillar, *Spodoptera mauritia* Boisduval (Lepidoptera: Noctuidae), is a major sporadic pest of paddy, *Oryza sativa*, in Kerala, India. The larvae are voracious feeders and completely devastate the paddy fields, which gorge themselves on paddy leaves or an alternative host plant *Ischaemum aristatum*. The armyworm is prevalent across Asia, the Pacific region, and Africa. The media reports documented significant and minor outbreaks in Kerala, India, in 2017 (Jena et al., 2017) and 2022 (“Pattalapuzhu”; Nov. 3, 2022). Moreover, in our laboratory over the past few years, *Spodoptera mauritia* has been utilized as a model organism for several endocrinological and reproductive studies (Manogem & Krishnan Nair, 2013; Banu et al., 2019, 2022; Banu & Manogem, 2022).

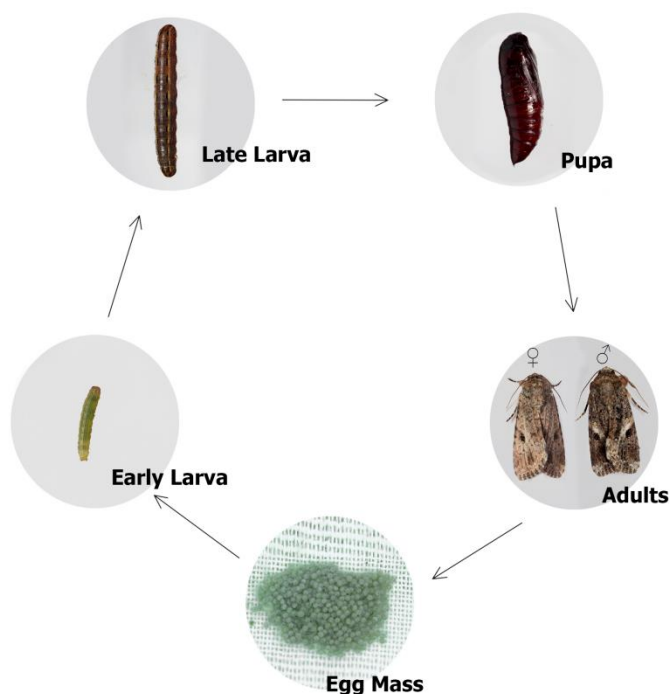
Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Lepidoptera
Superfamily	:	Noctuoidea
Family	:	Noctuidae
Genus	:	<i>Spodoptera</i>
Species	:	<i>mauritica</i>

1.3.2 Collection and rearing of the insect colony

The fertilized female moths from the paddy field were collected using light traps. The moths were then transferred to a glass beaker, covered with the muslin cloth, and fed a honey solution (1:1). After one or two days, the females laid eggs on the cloth and sides of the beaker. The eggs hatch into larvae after three days. Hatchlings were moved to a clean glass beaker for maintenance and upkeep. The alternative host plant, *Ischaemum aristatum*, was used to feed the larvae. Larval and pupal stages were also collected manually from organic paddy fields where an outbreak had been detected. The entire colony was maintained in a laboratory condition at room temperature (RH 90±3% and 10:14 light: dark photoperiod).

1.3.3 Biology of *Spodoptera mauritia*

Under standard rearing conditions, the life cycle of *S. mauritia* takes approximately 38.37±4.62 days to complete. The insect undergoes six larval instars and follows a consistent rate of development.



Life cycle of *S. mauritia*.

Eggs

The eggs were laid in mass covered with greyish hairs and displayed an average fecundity of 1013.50 ± 21.92 . Eggs are characterized as spherical in shape and green in colour. A few hours before hatching, eggs change from green to dark black. The incubation phase lasted for 3.00 ± 0.00 days.

First instar

The first instar larvae last about 2-3 days. The hatchlings measured about 1mm in length and 0.5mm in width. The larvae have sizeable black head shields and light green-coloured bodies. Setigerous, small, wart-like pigmented tubercles are present all over the body. The larvae descend through silken threads to feed onto the tender leaves supplied.

Second instar

The second instar lasts about 2- 3 days. The length measured about 2.5mm, and the width measured about 0.5mm. In this stage, the larvae bear three white longitudinal strips on the dorsal surface of the green-coloured body. The larvae also possess two pairs of white longitudinal and lateral stripes, with one prominent. In addition, each segment has setigerous, small, wart-like tubercles. The larvae hang down using silken threads for feeding.

Third instar

The larval duration is about 2-3 days. Larval length measured about 6mm and width about 1mm. The green-coloured larval body bears three white stripes on the dorsal side and two pairs on the lateral side of the body, and a pair of characteristic reddish black supra spiracular lines extend from the anterior to the posterior end of the body. The larval body has cervical and anal shields and three markings in continuation with the longitudinal stripes. From this stage onwards, silken threads were lacking.

Fourth instar

The larval instar lasts about 2-3 days. The larvae measured about 11.5mm in length and 2mm in width. The body's colour changed from green to grey, possessing three dull white longitudinal stripes. Each lateral side of the body has reddish-black lines. Their dorsal part was paler than the supra spiracular area, and intermittent black dots appeared on each segment dorsolaterally.

Fifth instar

The larval instar lasts about three days. The length measured approximately 22mm, and the width was about 4.5mm. In this stage, larvae become black. A double row of black, triangular markings is bordered with narrow white stripes on the dorsolateral side. Paired supra spiracular strips become transparent and pink in colour. The dorsal longitudinal stripes and the integument become partially translucent in this stage.

Sixth instar

The sixth instar larvae last about six days. Larvae measure length of about 30mm and width of about 6mm. The larval body is greyish-black. Larvae are identified by conspicuous triangular markings on the dorsal side. On the third day of the sixth instar, the larvae attain their maximum length of 35mm, and in this stage, larvae feed voraciously. Eventually, the larvae enter the wandering stage, where they do not take any food and move speedily. The stage lasts for the next 24 hours, and afterwards, the gut is emptied, and body size is reduced to a length of about 25.5mm. On the fifth day, the larvae becomes a wrinkled stage, known as prepupa, measuring approximately 20mm.

Pupal instar

The pupal stage exhibits an obtect form characterized by a reddish-black colouration. Female pupae last up to seven days, while male pupae last up to eight days. The pupal length was measured at about 16mm and the width at about 5mm.

Adult

Adults are medium-sized moths with distinct sexual dimorphism. The forewings of the adult moth are dark grey or brown in colour with a noticeable triangular black spot, and the hind wings are brownish-white with black margins. The moth is about 15 mm long, with an average wing span of 30 to 35 mm. Additional white markings on the forewings and large tufts of hair on the forelegs distinguish the male moth. Adult females lack these characteristics, which can be distinguished by the greyish-brown forewing having a black spot on it. The average lifespan of adults lasts up to one week. A female lays between 100 and 300 eggs in a single oviposition.

1.3.4 Test animal selection

Healthy, sixth instar day zero larvae of *Spodoptera mauritia* were selected and collected from the laboratory culture for all experimental analysis.

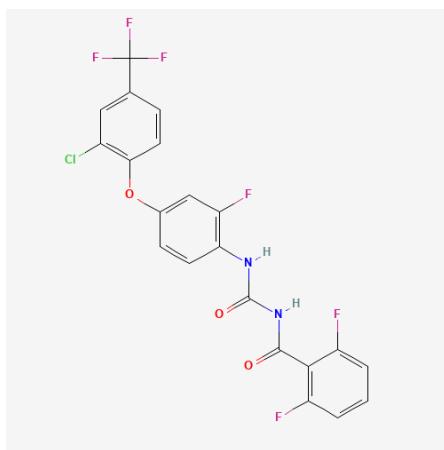
1.3.5 Chemical

The flufenoxuron (N-[[4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-fluorophenyl]carbamoyl]-2,6-difluorobenzamide) (purity >98%, Product Number: 31594, CAS No. 101463-69-8) analytical standard was procured from Sigma Aldrich and used for all the experiments.

Properties: Flufenoxuron is a benzoylurea insecticide that belongs to the monochlorobenzene, (trifluoromethyl)benzene, monofluorobenzene, and difluorobenzene chemical families. Flufenoxuron belongs to the benzoylurea chitin synthesis inhibitor

category, including triflumuron, diflubenzuron, and lufenuron. It is commonly used as acaricide and insecticide. It is comparable to a diphenyl ether in terms of function. It is an inhibitor of chitin production. Flufenoxuron is a colourless or white crystal and odourless. It decomposes at 169-172⁰C temperature. Highly soluble in volatile compounds like acetone, dimethyl formamide and dimethyl sulfoxide, poorly soluble in water. It shows a relative density of 1.57g/L and vapor pressure of 6.52×10^{-9} mPa at 20⁰C. When heated to decomposition, it emits toxic nitrogen oxides, fluoride, and chloride vapours (National Centre for Biotechnology Information, 2023).

Molecular Formula: C₂₁H₁₁ClF₆N₂O₃



Chemical structure of Flufenoxuron

1.3.6 Acute toxicity bioassay

Determination of effective dose

The acute toxicity of flufenoxuron was tested to determine the median effective dose (144-h-ED₅₀). Based on the previously reported LC₅₀ value (Praseeja, 2017), multiple dosages ranging from 0.01 to 5 µM of flufenoxuron were prepared through serial dilution in acetone. To find the ED₅₀ inducing 50% moult inhibition, approximately five microliters of each dose were topically administrated using a Hamilton micro syringe on the newly moulted healthy sixth instar larvae's dorsal side of the abdomen. Prior to chemical treatment, the selected larvae were starved for two hours. Flufenoxuron at different concentrations i.e., 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 µM, were topically applied to the selected larvae. An equivalent volume of acetone was employed as vehicle control, and chemical- and solvent-free groups were also maintained. For every concentration, four replicates were used (10 larvae in each replicate) and maintained under the same conditions. After the treatment, the experimental and control groups were air-dried and transferred to a new diet (*Ischaemum aristatum*). The larvae after the treatment were regularly monitored at every 24 h interval up to pupation. The morphological anomalies of flufenoxuron-treated larvae of different dosages were observed and recorded. To determine the effective dose, Schneider-Orelli's formula was applied to correct the concentration at which 50% moulting inhibition of larvae, representing the median effective dose. This correction was subsequently confirmed using the Probit regression analysis tool (Finney, 1971).

$$\text{Corrected Moulting inhibition} = \frac{P - P_o}{100 - P_o} \times 100$$

P - Moulting inhibition percentage of the treated group

P_o - Moulting inhibition percentage of the control group

Selection of doses for toxicological analysis

Based on the median effective dose for 144h duration, two doses, median effective dose-ED₅₀ (0.00068µg), and ½ of the median effective dose-ED₂₅ (0.00015µg) were chosen as treatment doses for further toxicological and developmental analysis. Newly moulted sixth instar larvae were grouped into four groups: control group (without solvent and toxicant), vehicle control group (Acetone), flufenoxuron ED₅₀ and ED₂₅ exposed for 48, 96 and 144h.

1.3.7 Effect of flufenoxuron on the development of *Spodoptera mauritia*

1.3.7.1 Morphological abnormalities

After treatment with two doses of flufenoxuron, newly moulted sixth instar larvae were continuously observed until the adult emergence and the findings were recorded. Each treatment was reproduced four times using different insect batches (with ten larvae per replicate) and with control sets.

1.3.7.2 Effect on larval weight

The newly moulted sixth instar larvae were exposed to the median effective dose and ½ of median effective dose of flufenoxuron for 48, 96 and 144h durations. At the end of each treatment period, larvae

were taken from the experimental set-up, weighed using a digital weighing balance (Shimadzu), and tabulated for statistical analysis.

1.3.7.3 Effect on fecundity and fertility

To determine fecundity and fertility, the adults that survived treatment of freshly moulted sixth instar larvae of *S. mauritia* with the ED₂₅ of flufenoxuron were allowed to mate with normal individuals. The females and males were transferred in pairs in 0.5-litre glass containers on the day of emergence to allow mating to measure fecundity. One treated male and one untreated female, one untreated male and one treated female, and one treated male and one treated female were the three mating combinations used for the study. One untreated (normal) male and one (normal) untreated female were used as a parallel control. The females were allowed to ovulate until they died.

Three replicates were carried out for each combination using different batches at various periods. Fecundity (total number of eggs laid by a female) and fertility (% of eggs hatch successfully) were calculated.

1.3.8 Effect of flufenoxuron on the male reproductive development of *Spodoptera mauritia*

1.3.8.1 Effect on testis morphology

After treatment with selected doses of flufenoxuron, newly moulted sixth instar larvae were dissected at 48h, 96h and 144h intervals. Immediately after the dissection, the testis was transferred to 50mM PBS to observe for morphological modifications in treated and control groups. Observations are recorded and photographed with a

stereomicroscope (Zeiss Discovery V20 attached with a Zeiss 506 camera).

1.3.8.2 Effect on testis weight

After treatment with the effective doses, newly moulted sixth instar larvae were dissected at 48h, 96h and 144h time intervals. After that, the testis was rinsed with 50 PBS to remove fat bodies, dried with filter paper, and weighed. The weight of the dissected testis from the experimental and control sets was determined using a digital weighing balance (Shimadzu). Observed the data and tabulated for statistical analysis.

1.3.8.3 Effect on testis volume

The newly moulted sixth instar larvae were exposed to the median effective dose (ED₅₀) and ½ of the median effective dose (ED₂₅) of flufenoxuron for 48, 96 and 144 h durations. Treated and untreated male larval testes were dissected in insect ringer solution, and the length (L) and width (W) were measured with the aid of stereomicroscope (Zeiss Discovery V20 attached with a Zeiss 506 camera). The testes were photographed using the ZEN application, and appropriate measurements were taken. The testis volume was determined for 48h and 96h (larval stage) according to Hiroyoshi et al., (2021), and testis volume was calculated using the following formula: $\text{Volume} = \pi/6 \times L \times W^2$, assuming that the testis is an ellipsoid. The paired testis was examined and tabulated for statistical analysis. After 144h (pupal stage), the testis is fused into a single spherical organ; the radius was determined using the mean diameter from 2 measurements across the organ's central axis divided by two and calculated the testis

volume (size) using formula $\frac{4}{3}\pi r^3$, r = radius of the testis (Liu et al., 2022).

1.3.8.4 Gonadosomatic Index (GSI)

The weight of the dissected testis from the experimental and control sets was determined using a digital weighing balance (Shimadzu). The testis was rinsed with insect ringer solution to remove fat bodies, then dried with filter paper and weighed. The gonado-somatic index was computed as per (Strum,1978).

$$GSI = \frac{\text{Weight of Gonad}}{\text{Weight of Insect}} \times 100$$

The studies were performed in triplicate, and the mean GSI value of each experimental group was determined and statistically analysed.

1.3.9 Statistical analysis

Statistical analyses of all experiments were carried out using the SPSS 24.0 statistical software. Replicates were kept in each group to reduce statistical errors. All data are provided as Mean $\pm$ Standard Error (SE) for each group. The differences between the control and treatment groups were examined using one-way ANOVA, followed by Duncan's Multiple Range as a post-hoc test. The significance level was chosen at $p > 0.05$ compared to the control groups. Statistical analysis for developmental parameters was performed using R version 4.2.3 (Eye Holes). Differences were evaluated using a one-way ANOVA after confirming equal variance (Levene test, $p = 0.5$) and normality (Shapiro test, $p > 0.5$ for all groups).

1.4 RESULTS

1.4.1 Effect of flufenoxuron acute toxicity on sixth instar larvae of *Spodoptera mauritia*

The present study examines the impact of the chitin synthesis inhibitor, flufenoxuron, on the sixth instar larvae of *Spodoptera mauritia* morphogenic abnormalities and developmental stages. To analyse the acute toxicity effect of flufenoxuron, the median effective dose (ED₅₀-144h) of flufenoxuron for 144 hours, which induces 50% moult inhibition, was performed. Further, the study mainly targets to find the minimal exposure level to flufenoxuron that adversely affects the male reproductive development of *S. mauritia*.

The data from the present investigation in Table 1 demonstrates that the median effective dose analysis showed negligible or minimal moult inhibition in both the control and vehicle control groups. Our findings indicate that the impact of flufenoxuron on the moulting process of sixth-instar larvae varies with dosage, demonstrating an apparent dose-dependent effect. The compound also showed a dose-dependent decrease in pupation and adult emergence. The results demonstrate a dose-dependent effect, where larvae exposed to a higher concentration (5 µM) showed complete inhibition of moulting and metamorphosis. A relatively lesser rate of moult inhibition was observed when a lower dose of flufenoxuron (0.025 µM) was administered. The findings suggest an inverse relationship between the dose and its effect on sixth-instar larvae. The median effective dose of flufenoxuron, ED₅₀ for 144 h, was calculated by probit analysis (Table 1). The obtained ED₅₀ value for the flufenoxuron treatment on the sixth instar larvae was 0.00068µg. Regression analysis showed a dose-dependent with a significant correlation of a value $R^2 = 0.964$ and slope value of 0.81

(Fig. 1). The chi-square test was employed to assess the model's goodness of fit, and a significant departure from the data from the model was defined as a p -value of less than 0.05.

Based on the value obtained, two doses, the median effective dose (ED_{50} -0.00068 μ g) and $\frac{1}{2}$ of the median effective dose (ED_{25} -0.00015 μ g), were selected to evaluate the toxicity of flufenoxuron on male reproductive development.

Table 1: Effect of flufenoxuron on the median effective dose for 144h (ED_{50} -144 h) in *Spodoptera mauritia* by Probit analysis.

Dose (μ M)	Moult inhibition (%) ($\bar{X} \pm SE$)		%CM
Control	2 $\pm$ 0.25		0
V control	2 $\pm$ 0.25		0
0.025	16 $\pm$ 0.4		14.28
0.05	32 $\pm$ 0.4		30.61
0.1	40 $\pm$ 0.7 ^a		38.77
0.25	48 $\pm$ 1 ^b		46.93
0.5	52 $\pm$ 0.86 ^c		51.02
1.0	64 $\pm$ 1.7 ^d		63.26
2.5	80 $\pm$ 2.3 ^d		79.59
5.0	100 $\pm$ 2.5 ^d		100
ED_{25} (μ M)	0.061		
95% Confidence Limits for Dose	LB	0.025	
	UB	0.111	
ED_{25} (μ g)	0.00015		
ED_{50} (μ M)	0.266		
95% Confidence Limits for Dose	LB	0.152	
	UB	0.473	
ED_{50} (μ g)	0.00068		
χ^2 (df=7)	16.724		
Variance (F)	7.61		
Slope value	0.81		
R ²	0.964		
P value	0.019		

CM-Corrected mortality, $\bar{X} \pm SE$ = Mean $\pm$ Standard error, χ^2 -Chi-square value, df-degrees of freedom, LB- Lower Bound and UB- Upper Bound. The mean in the same column followed by the same superscript does not differ significantly at $p < 0.05$ (Duncan test). Four replicates (10 larvae in each set) were treated at each concentration.

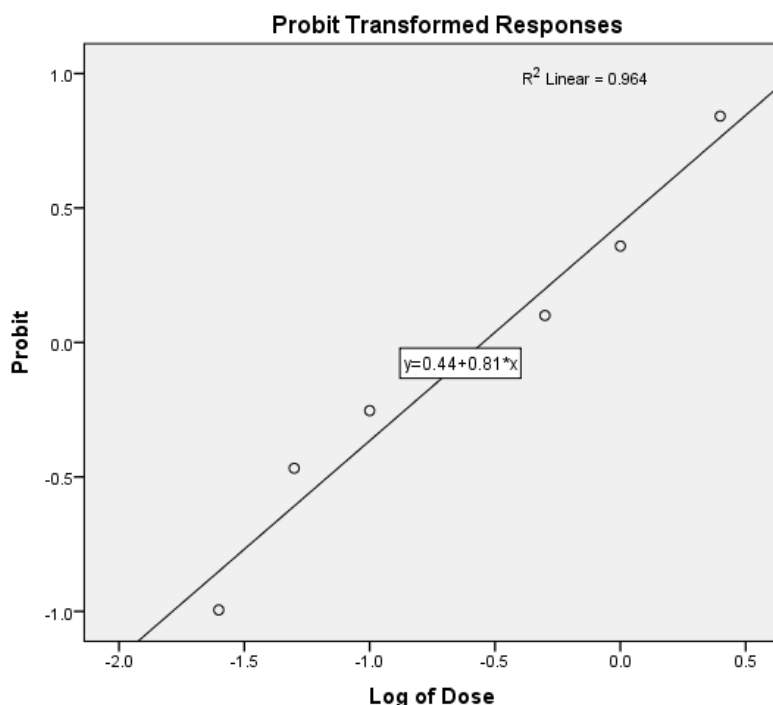


Figure 1: Plot illustrates the experimental result of regression analysis to determine the median effective dose of flufenoxuron.

1.4.2 Effect of flufenoxuron on developmental stages of *Spodoptera mauritia*

The toxicity bioassay findings revealed that application of flufenoxuron led to the developmental arrest by inhibition of chitin synthesis (Table 1). Morphological examinations of sixth instar larvae of *S. mauritia* on post-treatment with different concentrations of flufenoxuron (0.025 μM - 5.0 μM) indicated varying degrees of abnormalities, such as an elevated percentage of larval pupal mosaics/intermediates, abnormal pupae, and malformed adults. However, only a few pupae that survived and emerged as adults exhibited normal adults. The data further indicates a significant reduction in the pupation rate

with the dose administered, highlighting the pronounced moulting inhibition in sixth-instar larvae of *S. mauritia* (Fig. 2).

The results from the flufenoxuron treatment indicate that observable effects were noted at a concentration of 0.025µM, leading to around 14.28% moulting inhibition. In contrast, a dose of 5µM led to a complete cessation of pupal formation, as illustrated in Table 1. At higher concentrations of flufenoxuron, the effect of the chitin synthesis inhibitor becomes apparent through incomplete moulting, resulting in the formation of larval-pupal intermediates or mosaics. The results in Figure 2 indicate that doses of 2.5µM and 5µM resulted in approximately 76% and 100% of larval-pupal intermediate formation, suggesting a notable reduction or absence of normal pupal formation. In the toxicity analysis, after 144 h of observation, severe morphological anomalies were noticed compared to control pupae (Plate I: Fig. A), such as the formation of larval pupal intermediates and abnormal pupae, followed by further developmental arrest (Plate I: Fig. B, C, D, E & F). The larval-pupal intermediates were noted with different degrees of morphological aberrations, characterized by retaining parts of their last larval integument at the anterior end, sclerotized forelegs and pupal abdominal case (Plate I: Fig. B & D).

Figure 2 indicates a clear correlation between higher concentrations of flufenoxuron and increased pupal mortality and malformations, a decrease in pupation rates, followed by an increase in the percentage of larval-pupal intermediates. Moreover, the diverse degree of pupal malformations was evidenced as a highly sclerotized thoracic region that failed to shed larval integument, inverted shape and a malformed

head region, and pupae with reduced size and shrunken cuticle (Plate I: Fig. E & F). In higher concentrations of flufenoxuron treatment, a complete blockage of adult emergence was observed. The study highlighted that the defects in the moulting processes hindered the normal metamorphosis of larvae into adults. Many of these abnormalities were only apparent after metamorphosis, stemming from the inhibition of chitin synthesis, leading to irregular pupal formation. As the dosages increased, the insecticidal effectiveness became more pronounced.

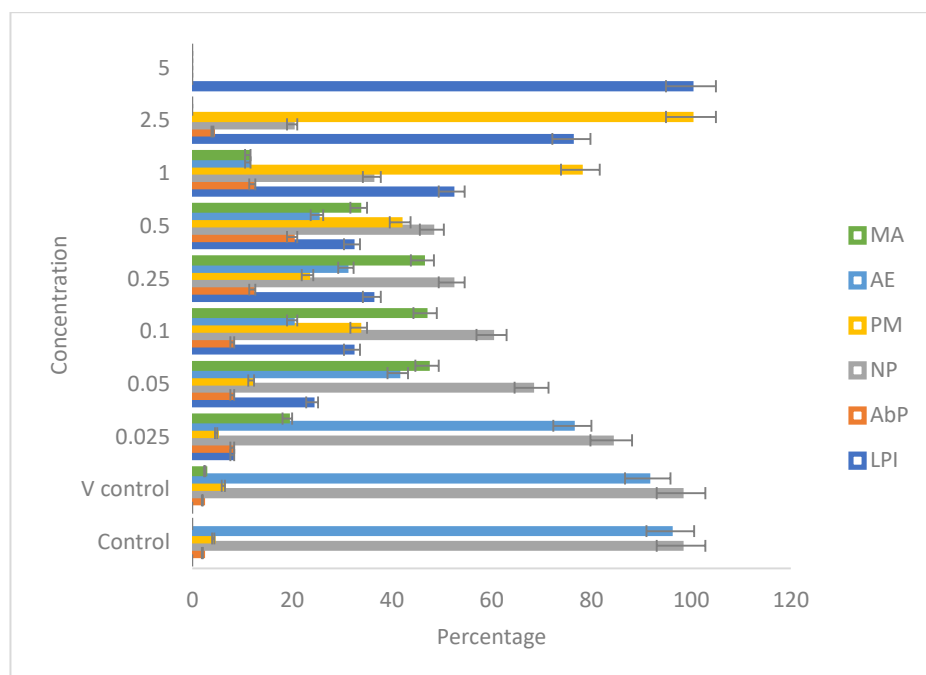


Figure 2: Effect of flufenoxuron on developmental stages of *Spodoptera mauritia*. The data expressed in the percentage mean values of four independent experiments (N=10). LPI- larval pupal intermediate, AbP- Abnormal pupa, NP- Normal pupa, PM- Pupal mortality, AE-Adult emergence, MA- Malformed adult. The data expressed in the mean $\pm$ SE values of four independent experiments (N=10). Statistical differences were considered significantly by one-way ANOVA



Plate I Figure A-F: Morphological malformations of pupal instar of *S. mauritia* post-treatment with 0.025 μM – 5.00 μM concentrations of flufenoxuron on the sixth instar larvae. A-Normal (Control) pupa; B & D-larval pupal intermediates containing larval body part and sclerotized body; C- Abnormal pupae with malformed thoracic region; E- Abnormal pupae with highly sclerotized thorax and failed to moult larval integument; F- Abnormal pupae with malformed head region.

1.4.3 Toxicity effect on adult emergence

To assess the impact of flufenoxuron on adult emergence and pupal survival, sixth-instar day zero larvae were subjected to treatment with the median effective dose (ED_{50} -0.00068 μg) and $\frac{1}{2}$ of the median effective dose (ED_{25} -0.00015 μg). The treatment group was closely monitored until adult emergence, and a parallel control group was employed for comparative analysis.

The data obtained showed that the sixth instar larvae of *S. mauritia* revealed a significant variation in adult emergence when treated with flufenoxuron (Table 2). There was a significant reduction in the number of adults emerged after the treatment with the ED_{25} dose when compared to the control ($96.66 \pm 0.33\%$) with 23.33 ± 0.33 per cent of

adults to emerge. Conversely, adult emergence was blocked entirely with the treatment of ED₅₀ concentration, and the survival rate of pupae was highly reduced. Moreover, the corresponding formation rate of larval pupal intermediate with ED₂₅ and ED₅₀ significantly increased to 66.67±0.88 % and 96.7±0.33 %, respectively.

Table 2: Effect of flufenoxuron on adult emergence of *S. mauritia*.

Experimental group	Larval-pupal intermediates (Mean%±SE)	No. of Normal Pupae (Mean%±SE)	Dead pupae (Mean%±SE)	Adult emerged (Mean%±SE)
Control	0	100±0	3.33±0.33	96.66±0.33
V Control	0	100±0	6.67±0.33	93.33±0.33
ED ₂₅	66.67±0.88*	33.33±0.88*	10±0.57	23.33±0.33*
ED ₅₀	96.7±0.33**	3.34±0.33**	3.33±0.33	0**

The data expressed in the mean ± SE values of three independent experiments (N=10). Statistical differences were considered significantly by one-way ANOVA (the same number of asterisks do not differ significant at $p < 0.05$).

1.4.4 Toxicity effect on instar body weight

The larvae exposed to the flufenoxuron treatment with four treated groups (control, vehicle control, ED₂₅ and ED₅₀) were weighed after different intervals. From the data obtained, the average weight of control and vehicle control larvae after 48hrs are 0.430±0.01g and 0.436±0.01g, respectively. Figure 3 shows that the weight after 48 h significantly decreased in treatment groups compared to the control and vehicle control groups. The weight of flufenoxuron-treated larvae is less than the control, 0.377±0.009g for ED₂₅ and 0.349±0.011g for ED₅₀. After 96 hours, the weight of instar in control and vehicle control was 0.241±0.005g and 0.240±0.005g; after the treatment, the weight of larvae significantly decreased to the treatment dose, 0.184±0.005g and

0.169±0.012g for ED₂₅ and ED₅₀, respectively. At the final interval (144h), the weight of pupae decreased considerably. In the case of high concentrations, the weight of larvae significantly decreased compared to the lower dose. Larval pupal intermediate weight for the ED₅₀ treated group was 0.102±0.005g, far less than the ED₂₅ treated group, 0.12±0.004g. These two values were significantly less than the value of the control group, 0.1364±0.003g. This indicates that the flufenoxuron affects the body weight of the larvae.

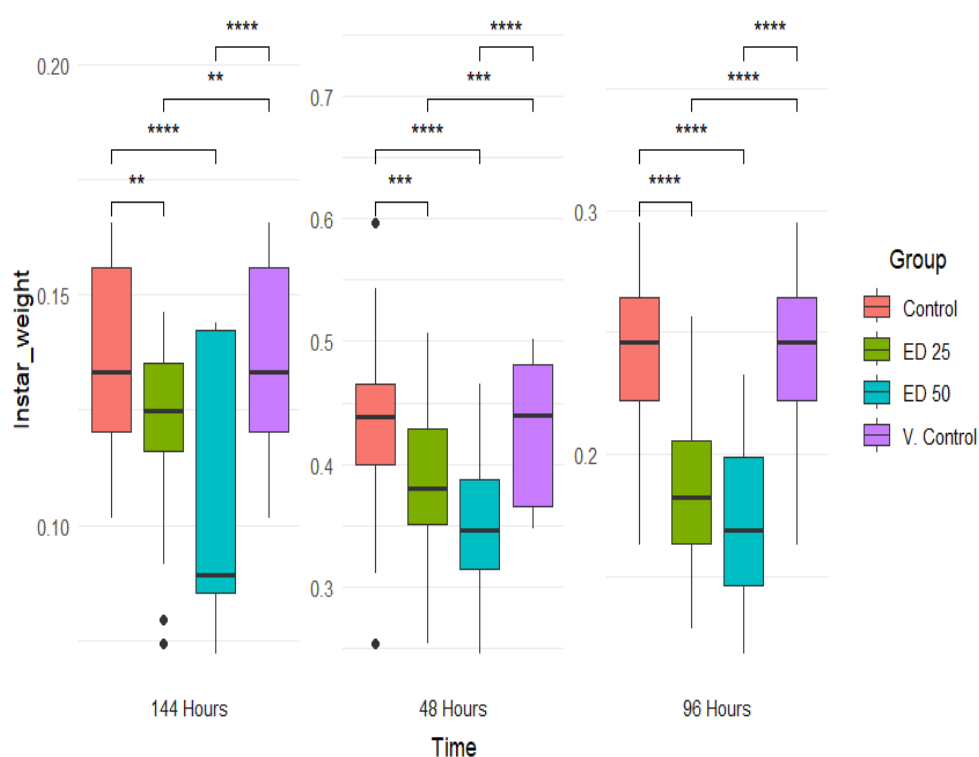


Figure 3: Instar body weight of flufenoxuron-treated sixth instar larvae of *Spodoptera mauritia* at different intervals.

Boxes represent the interquartile range, bars indicate the median, and whiskers span 1.5 times the interquartile range. Statistical differences were considered significant by one-way ANOVA (***) $p < 0.0001$, **** $p < 0.00001$).

1.4.5 Effect of flufenoxuron on the male reproductive development of *Spodoptera mauritia*

The current study explores the flufenoxuron exposure on *Spodoptera mauritia* larvae to investigate any potential damage caused to the male reproductive system. To assess the impact of flufenoxuron on the processes of testicular morphogenesis and spermatogenesis of *S. mauritia*, sixth instar day zero larvae were exposed to the median effective dose (ED₅₀-0.00068µg) and half of the median effective dose (ED₂₅-0.00015µg). After treatment, the recovered testes at different time intervals (48h, 96h & 144h) were analysed to observe the effects of flufenoxuron on the testicular morphology and development.

1.4.5.1 Testis morphology

The testes of *Spodoptera mauritia* larvae (48h & 96h) have a pair of yellow, kidney-shaped testes joined by a vas deferens to an ejaculatory duct. The testes are aerated by several tracheoles and located laterodorsally in the median region of the body. Each testis comprises four testicular follicles with distinct septa (Plate II: Fig. A). Each follicle consists of a smaller basal extremity on the concave side of the testis and a larger apical extremity on the convex side.

In the pupal stage (144h) of *S. mauritia*, the paired yellowish testes merge into spherical testicles separated into eight follicles connected to the vas deferens (Plate IV: Fig. A). Furthermore, the testis is bordered

by a smooth exterior surface called a cellular tunica, which comprises internal and external layers of cells and with numerous tracheae.

Microscopic examination of the *Spodoptera mauritia* larval testis after the post-treatment of flufenoxuron ED₂₅ and ED₅₀ doses at 48h and 96h exhibited relatively slight changes in the morphological features from the control. In contrast with the controls, flufenoxuron's application resulted in changes in the physical arrangements of four follicles (irregularly arranged follicles), which led to more structural changes in individual testis than in control (Plate II: Fig. C & D; Plate III: Fig. C & D). However, marked structural deformities in the testis were observed at 144h after flufenoxuron (ED₂₅, ED₅₀) treatment on the sixth instar larvae of *S. mauritia*. The treatment resulted in the lack of a testis spherical shape, and irregularity was noticed, possibly due to modest alterations in the tunic cells (Plate IV: Fig. C & D).

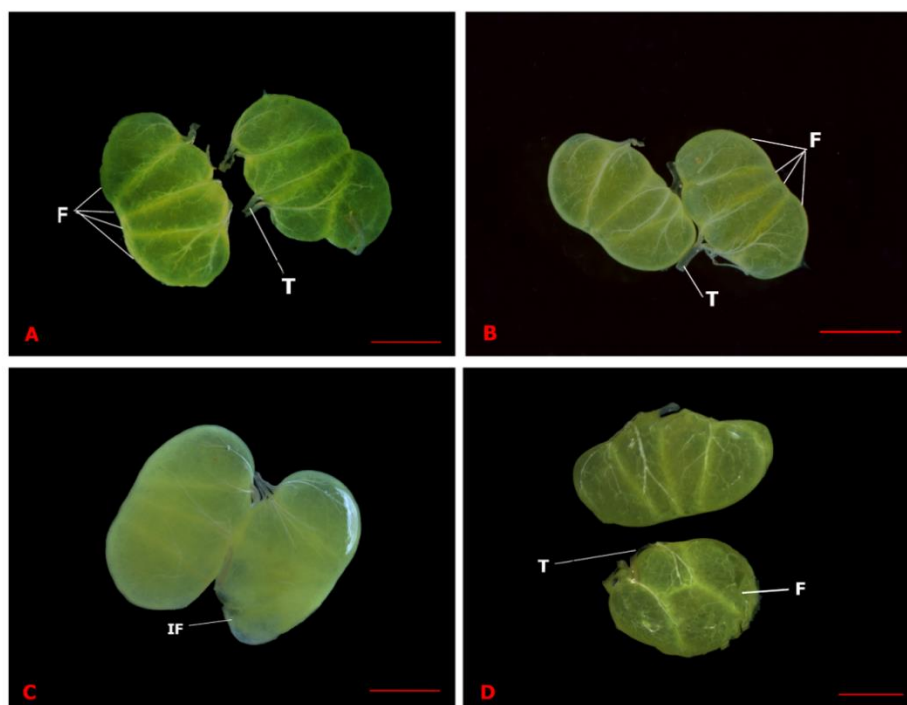


Plate II: Figure A-D Testis morphology of *Spodoptera mauritia* post-treatment with flufenoxuron (after 48h of treatment). (Scale Bar:500µm)

A & B: Control and Vehicle control set- Paired kidney-shaped testis of *Spodoptera mauritia* with four follicles (F).

C: Flufenoxuron treated (ED₂₅)- Paired kidney-shaped testis with slightly irregularly arranged follicles (IF).

D: Flufenoxuron treated (ED₅₀)- Testis Follicle (F) orientation alteration from normal kidney-shape.

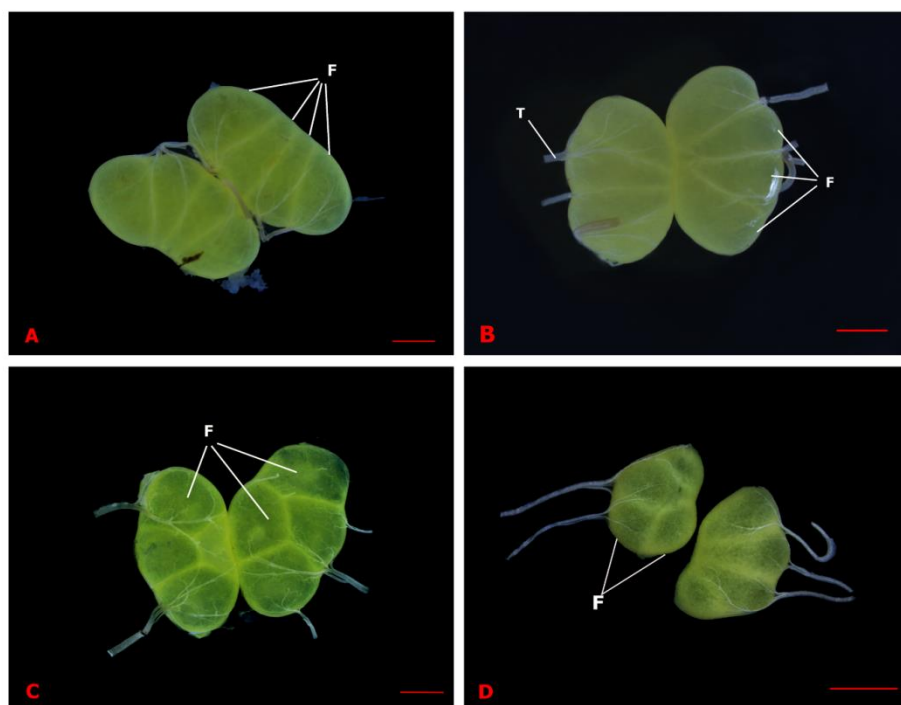


Plate III: Figure A-D Testis morphology of *Spodoptera mauritia* post-treatment with flufenoxuron (after 96h of treatment) (Scale Bar:500µm).

A & B: Control and Vehicle control- Paired kidney-shaped testis of *Spodoptera mauritia* with typically arranged regular four follicles (F)

C: Flufenoxuron treated (ED₂₅)- Paired testis with complete lack of normal kidney shape and differently oriented follicle (F)

D: Flufenoxuron treated (ED₅₀)- Reduced paired testis with differently oriented follicles.

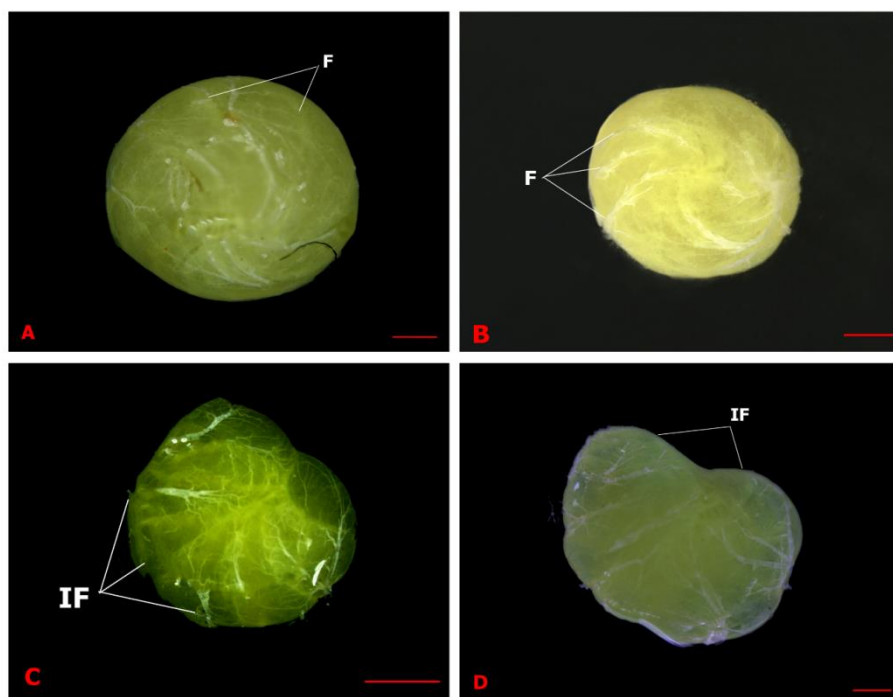


Plate IV: Figure A-D Testis morphology of *Spodoptera mauritia* on post-treatment with flufenoxuron (testis after 144h of treatment) (Scale Bar: 500 μ m)

A & B: Control and Vehicle control- Fused rounded testis of *Spodoptera mauritia*

C: Flufenoxuron treated (ED₂₅)- Abnormally fused testis with irregular edges.

D: Flufenoxuron treated (ED₅₀)- Abnormal fusion more prominent. Size enlarged due to irregular edges and defective fusion process.

1.4.5.2 Testis Weight

The development of the testes was negatively impacted when flufenoxuron was administered to sixth instar larvae of *S. mauritia*. This study determined the weight of the testis of *S. mauritia* larvae for two flufenoxuron treatment groups (ED₂₅ & ED₅₀) at different time intervals. The result indicated a gradual decrease in the weight of testis

in control groups at 48h to 144h. However, at 48h, the weight was higher by a negligible amount compared to 96h and 144h.

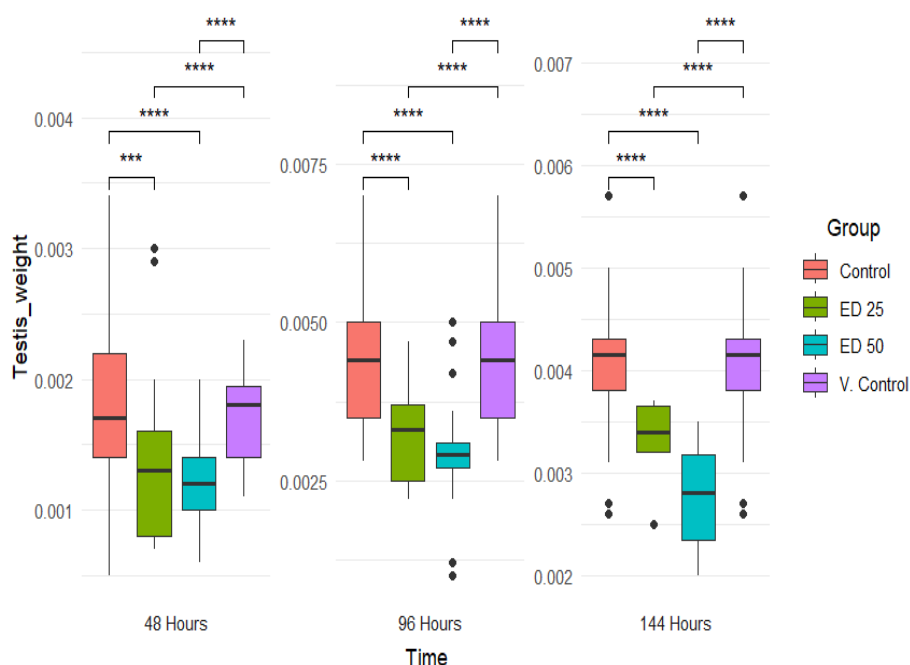


Figure 4: Testes weight of flufenoxuron treated sixth instar larvae of *Spodoptera mauritia*.

Boxes represent the interquartile range, bars indicate the median, and whiskers span 1.5 times the interquartile range. Statistical differences were considered significant by one-way ANOVA (*** $p < 0.0001$, **** $p < 0.00001$).

After flufenoxuron administration, the size of the larval testes was considerably reduced in a dose-dependent manner (Fig. 4). A decrease in testis weight was observed in response to the lower dose (ED₂₅) of flufenoxuron. The testis weight was significantly lower than the control groups at 48h, 96h, and 144h post-treatment with flufenoxuron. However, in ED₅₀ treatment groups, the testis mass drastically decreased after 48h, 96h, and 144h of administration. The weight of

testis at 48 hours was $0.0017\pm0.0001\text{g}$ and $0.0016\pm0.00008\text{g}$ in control and vehicle control samples, but in flufenoxuron, treated samples have a weight of $0.0013\pm0.00008\text{g}$ and $0.0011\pm0.00004\text{g}$ respectively in ED_{25} and ED_{50} doses. After 96 hours, testis weights were $0.0044\pm0.0002\text{g}$ and $0.0043\pm0.0002\text{g}$ in control and vehicle control; after treatment, weights were recorded as $0.0038\pm0.0002\text{g}$ and $0.0033\pm0.0001\text{g}$ in ED_{25} and ED_{50} respectively. The 144-hour pupal testis weighed about $0.0040\pm0.0001\text{g}$ and $0.0040\pm0.0001\text{g}$ in control and vehicle control, weight estimated after ED_{25} and ED_{50} dose treatment were $0.0034\pm0.00005\text{g}$ and $0.0032\pm0.00004\text{g}$ respectively.

1.4.5.3 Testis volume

Treatment of final instar larvae with two different effective doses of flufenoxuron adversely affected the subsequent development of the testes. The testes of ED_{50} -treated individuals were significantly reduced in volume in a dose-dependent way. The volume of the testes in the larval stage (after 48h of treatment) was $1.44\pm0.11\text{ mm}^3$ and $1.38\pm0.06\text{ mm}^3$ in control and vehicle control, and in ED_{50} doses of flufenoxuron administered exhibited a decrease of $1.35\pm0.087\text{ mm}^3$ compared to controls (Fig. 5), there was not much change occurred at dose ED_{25} , $1.38\pm0.10\text{ mm}^3$. At 96 hours post-treatment, control and vehicle control samples had testicular volumes of $3.21\pm0.21\text{ mm}^3$ and $3.10\pm0.21\text{ mm}^3$. However, the testicular volume decreased in the ED_{25} group to $3.02\pm0.28\text{ mm}^3$ but experienced a more substantial reduction of $1.51\pm0.051\text{ mm}^3$ after the application of ED_{50} . During the pupal stage (144 hours post-treatment), testicular volume was $3.95\pm0.61\text{ mm}^3$ and $3.93\pm0.062\text{ mm}^3$ in control and vehicle control, respectively; there was a slight decrease in the volume of the fused testes in the ED_{25} treated group ($3.76\pm0.25\text{ mm}^3$), whereas the higher dose treated group

showed a significant reduction of $3.26 \pm 0.27 \text{ mm}^3$ compared to the control volume of $3.95 \pm 0.61 \text{ mm}^3$. These effects were significant for all doses (Fig. 5).

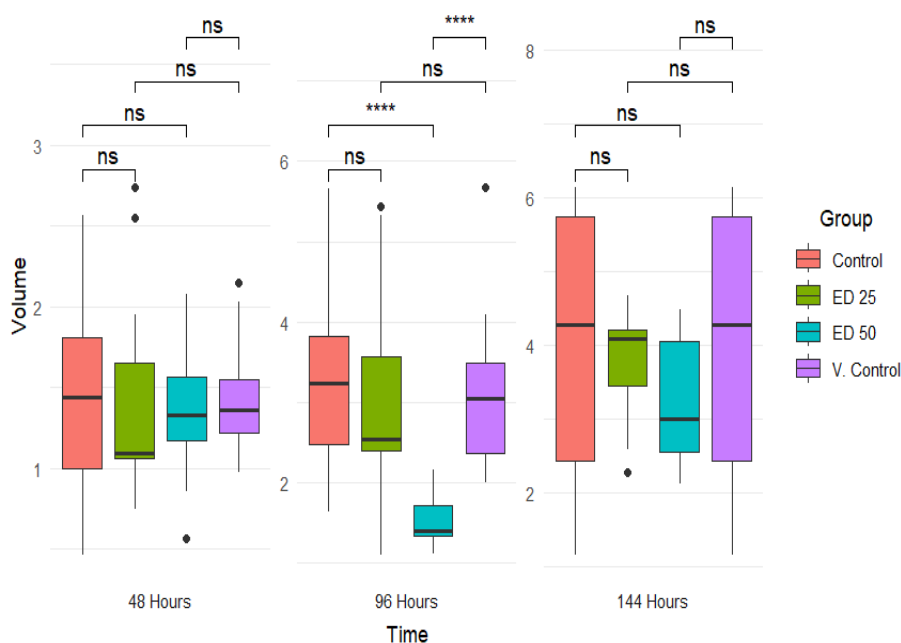


Figure 5. Testis volume of flufenoxuron treated sixth instar larvae of *Spodoptera mauritia*.

Boxes represent the interquartile range, bars indicate the median, and whiskers span 1.5 times the interquartile range. Statistical differences were considered significant by one-way ANOVA (**** $p < 0.00001$, ns- not significant).

1.4.5.4 Gonadosomatic index

In the present study, the sixth instar larvae treated with the median effective dose (ED₅₀) and sub-median effective dose (ED₂₅) of flufenoxuron showed that the testes reclaimed at different intervals revealed a distinct alteration in development. After 48 hours of treatments, the GSI values indicate that the ED₅₀ dose was more

effective than the control groups. There was a significant reduction in the value of the GSI of the ED₂₅ and ED₅₀ dose at 48h, 0.35 ± 0.021 and 0.346 ± 0.012 , compared to the control and vehicle control values, 0.407 ± 0.019 and 0.361 ± 0.023 , respectively. The GSI values gradually decreased after 96 hours of treatment and at the pupal period (144h) (Fig. 6). For control and vehicle control, the GSI values at 96 hours were 1.87 ± 0.11 and 1.9 ± 0.12 . In treated samples, GSI values for ED₂₅ and ED₅₀ treated larvae after the 96h were 1.83 ± 0.08 and 1.79 ± 0.10 , respectively. The GSI value at 144 hours was recorded as 3.01 ± 0.09 and 3.1 ± 0.17 in control and vehicle control. GSI estimated after treatment with ED₂₅ and ED₅₀, GSI reduced to 2.95 ± 0.13 and 2.88 ± 0.16 , respectively, in a dose-dependent manner. Data show a notable decrease in groups treated with high-dose flufenoxuron.

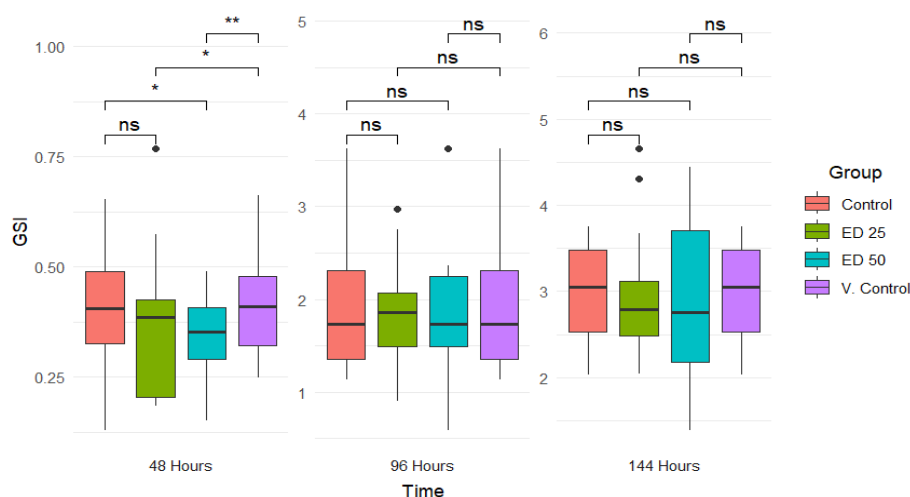


Figure 6. Gonado-somatic index (GSI) of flufenoxuron-treated sixth instar larvae of *Spodoptera mauritia*.

Boxes represent the interquartile range, bars indicate the median, and whiskers span 1.5 times the interquartile range. Statistical differences were considered significant by one-way ANOVA (* $p < 0.005$, ** $p < 0.001$, ns-not significant).

1.4.6 Effect on fecundity and fertility

The impact of flufenoxuron exposure on the sixth (final) instar larvae of *S. mauritia* on the reproductive efficiency of surviving adult males was evaluated by mating with untreated females. The study examined the reproductive efficiency of survived adult males post-treatment of ED₂₅ flufenoxuron on sixth instar larvae of *S. mauritia*. The findings revealed that the treatments disrupted the adult fecundity and fertility of *S. mauritia*. Flufenoxuron treatment significantly decreased the number of eggs laid and the percentage of egg hatchability compared to the control. The most significant reduction occurred in the treated group of both mated sexes, followed by a treated male mated with an untreated female. The adult female moths exposed to flufenoxuron with untreated males laid an average of 47 eggs per female (Table 4). The observations show that the treatment significantly reduced from the control mean value of 854.33 eggs per female. However, when treated males were paired with nontreated females, the mean fecundity decreased to 27.33 eggs per female. This mean value also significantly differed from the number of eggs laid from the treated female/nontreated male pairing and control group. An effective dose of flufenoxuron can negatively affect the total number of eggs laid by an untreated female that mates with a treated (exposed) male and the eggs laid by a treated female that mates with an untreated male.

Table 3: Effect of flufenoxuron on fecundity and egg hatchability of *S. mauritia*.

Fecundity (Total no. of eggs/♀) and Fertility (% Egg hatchability)							
Untreated male × Untreated female		Treated male × Untreated female		Untreated male × Treated female		Treated male × Treated female	
No. of eggs/♀	% Egg hatchability	No. of eggs/♀	% Egg hatchability	No. of eggs/♀	% Egg hatchability	No. of eggs/♀	% Egg hatchability
854.33±55.54	99.10±56.96	27.33±13.86*	85.36±12**	47±23.33*	97.16±22.92**	4±4*	0**

Means ± SE. Values followed by the number of stars in a column within each regime are significantly different ($p < 0.05$) (ANOVA followed by LSD post-test). The data expressed in the mean ± SE values of three independent experiments.

In the larvae exposed to flufenoxuron treatments, the percentage of egg hatchability found in the treated female with nontreated male pairing was 97.16 % (Table 3). This was not significantly different from the 85.36 % egg fertility found in the nontreated female with treated male pairing. However, both values were significantly less than the control hatchability. The percentage of egg hatchability in treated females paired with treated males was nil. Larval exposure to flufenoxuron by topical treatment significantly reduced the percentage of eggs that hatched, regardless of whether only the females or males were exposed. This suggests that flufenoxuron induces more of an effect on males than females.

The result suggested that the compound may have ovicidal activity, and the administration of flufenoxuron at ED₂₅ dose decreased the number of eggs laid by females in untreated male and treated female pairs (Table 4). The eggs laid by treated and untreated mating pairs of *S. mauritia* with ED₂₅ dose of flufenoxuron are depicted in (Plate V: Fig. D). It is also clearly evident that the eggs laid by treatment pairs did not hatch, and the dead or unhatched eggs had turned brownish-black in colour (Plate: V). It is also noted that the unfertilized eggs were found to be yellow.

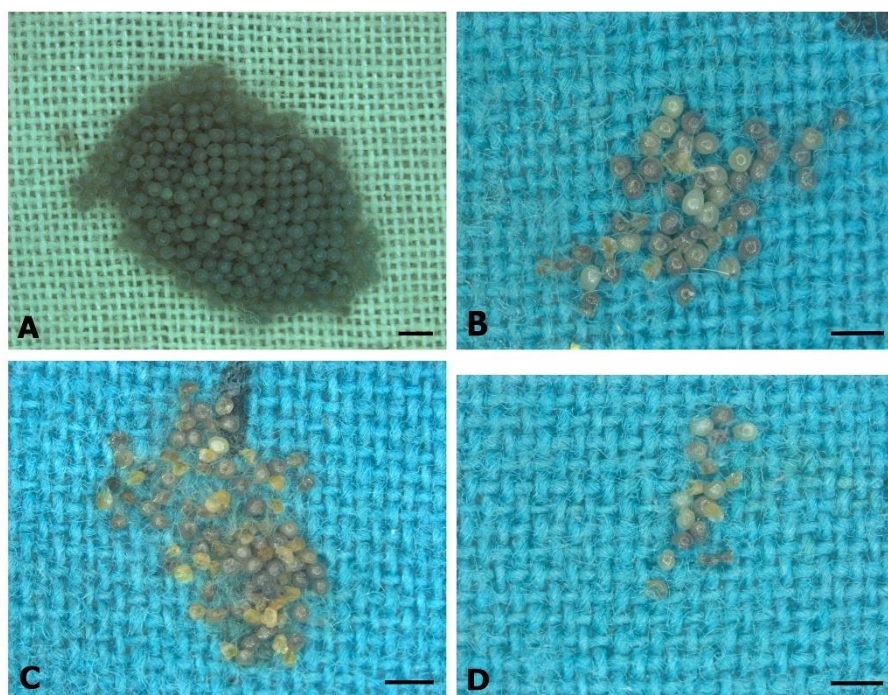


Plate V: Figure A-D Egg mass of *Spodoptera mauritia* treated with ED₂₅ dose A: Egg mass of *Spodoptera mauritia* (control), B: egg laid by treated male× untreated female pair, C: the egg laid by treated female× untreated male pairs, D: egg laid by treated male and treated female pair (treated with ED₂₅ dose). Yellow-coloured shrunk eggs are unfertilized. Brownish-Black colour eggs represent the dead or unhatched eggs (Scale Bar: 1000µm).

1.5 DISCUSSION

1.5.1 Acute toxicity of flufenoxuron

The chitin synthesis inhibitor, flufenoxuron, controls insect pest species by interfering with the chitin synthesis mechanism. The laboratory experiments conducted on the sixth instar larvae of *S. mauritia* with topical administration of the chitin synthesis inhibitor, flufenoxuron, of varying concentrations (0.025 μM -5 μM) exhibited significant toxicity. The study showed a dose-dependent response with high moulting inhibition in the treated groups. In the present investigation, the ED_{50} (median effective dose) of 0.266 μM (0.00068 μg) was determined on exposure of a series of concentrations (0.025 μM 5 μM) of flufenoxuron on sixth instar larvae *S. mauritia*. According to the findings, flufenoxuron at different doses led to mortality that resulted in moulting failures, leading to an incomplete life cycle and death within the cuticle.

The current investigation revealed that flufenoxuron had highly detrimental effects on the last instar larvae of *S. mauritia*. It is evident that the percentage of abnormal moulting into larval-pupal intermediates, pupal mortality and inhibition of adult emergence was high with higher doses of flufenoxuron. These findings align with the previous studies that topical application of chitin synthesis inhibitors blocks the successful moulting in many insects, *Spodoptera littoralis* (Clarke & Jewess, 1990; Bakr et al., 2013; Tabozada et al., 2014), *Drosophila melanogaster* and *Blattella germanica* (Abo-Elghar et al., 2004), *Conopomorpha sinensis* (Yao et al., 2021), *Aedes aegypti* (Seccacini et al., 2008), *Diaphorina citri* (Tiwari et al., 2012). Several

studies have highlighted the considerable impact of chitin synthesis inhibitors (CSIs) on lepidopteran pests, making them increasingly favourable for integration into agricultural practices. Assessments of CSI compounds, including flufenoxuron, lufenuron, and chlorfluazuron, have revealed both lethal and sublethal effects across diverse insect species. Notably, these effects were observed in *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Abdel-Aal, 2003; Reda et al., 2010); *Glyphodes pyloalis* (Lepidoptera: Pyralidae) (Aliabadi et al., 2016); *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) (Ahmed, & Moustafa, 2012); *Ephestia figulilella* (Lepidoptera: Pyralidae) (Khajepour et al., 2012) and *Spodoptera litura* (Lepidoptera: Noctuidae) (Zhu et al., 2012). These findings highlight the potential of CSIs as potent tools in agricultural pest management strategies.

Recent studies have demonstrated that topical application of exogenous CSIs can impede chitin production. The application of lufenuron, a CSI, to second-instar larvae of *Chrysoperla carnea* resulted in notable mortality rates (Suarez-Lopez et al., 2020). Studies have suggested that buprofezin, lufenuron, and pyriproxyfen effectively hinder the growth and development of okra jassids (Joarder et al., 2021). Likewise, high mortality rates were observed with the application of novaluron against the mosquito species *Culiseta longiareolata* (Bouaziz et al., 2011). In *Tribolium castaneum*, the dietary administration of lufenuron affected a decline in larval weight, pupation, and adult emergence (Arora et al., 2012).

The application of flufenoxuron on the sixth instar larvae of *S. mauritia* resulted in severe morphological defects in subsequent developmental stages. The treated larvae exhibited a complete inhibition of the pupal stage, leading to the formation of larval-pupal intermediates and ultimately culminating in death. In lower treatment doses, the larvae survived but formed pupae with defective pupal cases, preventing successful eclosion. The pupae that emerged into adults displayed acute morphological abnormalities. Similar findings were documented in various insect species. Reda et al. (2010) noted mortality in the second and fourth instars and observed morphological abnormalities in larvae, pupae, and adults of *S. littoralis* treated with flufenoxuron. Additionally, Khajepour et al. (2012) found that lufenuron, a chitin synthesis inhibitor, induced mortality with the formation of larval pupal intermediates, defective pupal and adults of *Ephesia figulilella*. Several studies reported similar outcomes for other lepidopteran species treated with IGRs, such as chlorfluazuron-treated in *S. littoralis* (Abdel-Aal 2003, 2012), methoprene-treated *Corcyra cephalonica* (Tripathi and Tiwari 2014), and hexaflumuron-treated in *Helicoverpa armigera* (Taleh et al., 2015).

In the present study, the chitin synthesis inhibitor flufenoxuron treatment revealed that the median effective dose of ED₂₅ slowed down the emergence of adults in *S. mauritia*. At high concentrations of ED₅₀, flufenoxuron completely inhibited the emergence of adults. These findings align with the report of Reda et al. (2010) on the reduction of adult emergence in *S. littoralis* with flufenoxuron treatment. Similar findings of a decline in adult emergence of *Helicoverpa armigera*

treated with lufenuron were reported by Butter et al. (2003). Similarly, comparable results were observed in *Tenebrio molitor* when treated with diflubenzuron, a chitin synthesis inhibitor that disrupted pupal adult development (Soltani et al., 1984). Likewise, the treatment of *Agrotis ipsilon* larvae with chlorfluazuron and flufenoxuron significantly decreased the adult emergence of both sexes (Shaurub et al., 2018).

In the current investigation, it was observed that the effective dose of flufenoxuron led to a reduction in larval weight at 48 hours, 96 hours, and 144 hours, as compared to the control groups. This decrease in weight is attributed to the physiological and metabolic disruptions induced by flufenoxuron in *S. mauritia*. Similar observations have been reported in other studies where the application of chitin synthesis inhibitors (CSIs) through direct or topical treatment decreased total body mass. Ahmed & Moustafa (2012) reported a considerable reduction in larval weight and a significant prolongation of the larval and pupal stages in *Pectinophora gossypiella* larvae at the LC₅₀ concentration of lufenuron. Zhu et al. (2012) found that sublethal doses of hexaflumuron on the third, fourth, and fifth instars of *S. litura* prolonged developmental time and decreased larval body weight. Consistent with these findings, Butter et al. (2003) observed that *Helicoverpa armigera* larvae treated with lufenuron had inflated heads and were substantially smaller than the controls across various instars. The observed reduction in weight across different instars may be attributed to decreased food consumption or reduced food utilization efficiency following flufenoxuron treatment. Abdel-Aal (2003) made

similar observations, noting that flufenoxuron and chlorfluazuron significantly reduced the efficiency of converting consumed and digested food into biomass in the last instars of *S. littoralis*.

1.5.2 Reproductive toxicity

Studies on the effects of chitin synthesis inhibitors on the reproductive aspects of insects are very sparse (Costa et al., 2017; Santorum et al., 2021). However, only a limited number of studies have explicitly delved into the morphological and ultrastructural alterations induced by chitin synthesis inhibitors in the reproductive organs of Lepidoptera (Santorum et al., 2021). These sporadic observations of changes in testicular development throughout an insect's life cycle provide a critical theoretical framework for comprehending the reproductive toxicity of chitin synthesis inhibitors in larval pests belonging to the Lepidopteran order. The current study observed that effective doses of flufenoxuron administered to sixth-instar larvae induced various abnormalities in the development and arrangement of follicles. Notably, alterations in the overall shape and arrangement of the testis were evident after treatment. Compared to the controls, the larval-paired testis, typically kidney-shaped with concave and convex extremities, underwent visible changes following flufenoxuron treatment. After 144 hours of treatment, the control testis maintained its round shape, while the flufenoxuron-treated group exhibited a modified shape due to an abnormal fusion process, potentially attributed to the physiological impact of flufenoxuron.

Similar effects were noted with the insecticide novaluron, a benzoylphenylurea class insect growth regulator, in *Bombyx mori*. The research demonstrated that novaluron induced ruptures in the cellular tunica and septa surrounding the testicular follicles (Santorum et al., 2021). Additionally, similar reports highlighted the action of azadirachtin, indicating a reduction in the size of testicular structures with internal morphological changes in *Spodoptera eridania* (Scudeler et al., 2023). The morphometric changes observed in the testicular region in our study suggest that flufenoxuron has a discernible impact on reproductive development during the latter stages of *S. mauritia* growth.

Based on earlier research (Dhadialla et al., 2005; Cutler & Scott-Dupree, 2007; Mommaerts et al., 2006), it is established that the benzoylphenylurea class of insecticides exerts control over insect development by inhibiting chitin synthesis and concurrently reducing reproductive capabilities. Perez-Farinos et al. (1998) detected a higher concentration of radioactive hexaflumuron in the gonads of younger males compared to older ones. This raised level of hexaflumuron was suggested to be sufficient to impact spermatogenesis, potentially leading to unsuccessful fertilization. As highlighted by Aliabadi et al. (2016), lufenuron has been shown to have detrimental effects on the reproductive processes of *Glyphodes pyloalis*.

The impact of chitin synthesis inhibitors on the testicular measurements of insects has been previously documented. According to Seth et al. (2004), treating *S. litura* last instar larvae with Insect Growth Regulators (IGRs) has detrimental effects on the development

of the male reproductive system. Sublethal dosages significantly reduce testicular size, volume, and length and width measures. Moreover, there is a decrease in the number of spermatozoa in the testicles following chitin synthesis inhibitor treatment, indicating an impact on spermiogenesis (Costa et al., 2017). Perveen (2000) also reported similar results, demonstrating that topical application of a sublethal dose of the chitin synthesis inhibitor chlorfluazuron negatively affects the development of testes by reducing their weight, volume, and sheath thickness. In our study, the administration of a chitin synthesis inhibitor to sixth-instar larvae revealed a significant decrease in the volume of the testis and gonadosomatic index. Recently, Banu et al., 2022 reported that the growth of the recovered adultoids ovaries was significantly reduced in the *S. mauritia* pupae treated with fenoxycarb.

The substance that exerts an adverse effect on the gonads of insects directly influences their reproductive capabilities. These findings highlight that flufenoxuron causes insect mortality and induces various detrimental outcomes. The larvae treated with these compounds experienced reduced fecundity and abnormalities in their testes, likely slowing from diminished nutrition. In effect, the disorders induced by flufenoxuron, altering chitin formation across multiple body areas, constrain and delay the absorption of nutrients, potentially linking to toxicological consequences and morphological modifications observed in the testis. Consequently, dietary disorders and nutritional inadequacies may adversely impact the quantity and quality of sperm in insects, contributing to a decline in overall reproductive success.

In earlier studies, exposure to Insect Growth Regulators (IGRs) in both adult and larval stages through topical application or ingestion of treated food decreased fecundity or fertility in several Lepidopteran pest species. The present study documented a reduction in fecundity in female and male moths treated with flufenoxuron in the sixth larval instar of *S. mauritia*. These observations are on par with the findings of other studies (Khebbeb et al., 2008; Wang and Tian, 2009; Reda et al., 2010). Treatment with flufenoxuron significantly lowered the number of eggs laid per *S. mauritia* and the percentage of egg hatchability compared to the control group. The most substantial reduction occurred when both mated sexes were treated, followed by a treated male mating with an untreated female. This suggests that flufenoxuron has a more pronounced effect on males than females. The current study is in agreement with previous reports on black cutworm (*Agrotis ipsilon*) (Shaurub et al., 2018), codling moth (*Cydia pomonella*) (Sun and Barrett, 1999), and *Spodoptera litura* treated with chlorfluazuron (Perveen & Miyata, 2000).

The toxicity assay findings indicate a correlation between exposure to toxicants during the larval stage and the fertility of eggs in *S. mauritia*. The negative impact of flufenoxuron on egg hatchability suggests a potential developmental effect on both male and female reproductive processes. Similar observations reported a significant reduction in the number of eggs deposited by *Pectinophora gossypiella* larvae treated with LC₅₀ concentrations of lufenuron (Ahmed & Moustafa, 2012). Shaurub et al. (2018) observed that the pre-oviposition period of *A. ipsilon* females, surviving treatment with chlorfluazuron and

Flufenoxuron, does not significantly decrease. However, oviposition time was markedly reduced, particularly with chlorfluazuron treatment. Similarly, Reda et al. (2010) demonstrated a dose-dependent reduction in fertility in *S. littoralis* following flufenoxuron treatment. Further, the reduced egg-hatch percentage observed in this study may be attributed to the sterilizing effect of eggs and sperm or the incapacity of sperm transfer during copulation (Ismail, 1980).

A recent study observed that novaluron decreased egg production and oviposition in *Bombyx mori* (Santorum et al., 2021). Likewise, in adult female *Oxya japonica* fed diflubenzuron, fertility and egg hatchability drastically decreased (Lim & Lee, 1982). Yao et al. (2021), noticed aberrant development in the egg-hatching of litchi fruit borer, reduced chitin content, and modified chitin distribution in freshly hatched larvae.

Electron microscopic observations on *Leptinotarsa decemlineata* embryos exposed to diflubenzuron revealed interference with cuticle formation (Grosscurt, 1978). The inhibition of chitin synthesis by diflubenzuron was proposed to weaken the embryo's exoskeleton and muscle attachments, preventing it from exerting the necessary pressure for eclosion from the egg (Moore et al., 1978). Another study reported that adults of *Cimex hemipterus* and *Urophorus humeralis* exposed to diets containing triflumuron experienced complete prevention of egg hatching (Blumberg et al., 1985). Joseph, (2020) found that novaluron reduced the populations of *Stephanitis pyrioides* (Hemiptera: Tingidae) by influencing egg hatching and disrupting nymphal development through abnormal ecdysis. These findings on the developmental

processes of embryos, particularly concerning egg hatching and nymphal development, serve as crucial insights that can contribute to a theoretical understanding of the reproductive toxicity of flufenoxuron in the target species.

1.6 CONCLUSION

The Chitin Synthesis Inhibitors (CSIs) had multiple effects on the morphology, metamorphosis, and development of lepidopterans. This study demonstrates that flufenoxuron adversely affects key life parameters of *Spodoptera mauritia*, including egg hatchability, larval duration, larval-pupal moulting, and adult emergence, by inhibiting chitin synthesis. The findings highlight the significant impact of flufenoxuron on the delicate balance of *S. mauritia*'s life cycle, emphasizing the need for further research and measures to mitigate its detrimental effects. Notably, flufenoxuron inhibited the growth and development of the male reproductive organs in *S. mauritia*. It may reduce the population growth of this insect species either through acute toxicity or by diminishing its reproductive capacity. However, further research is required to determine the precise impact of flufenoxuron at the cellular level on male organ development in *S. mauritia*. Nevertheless, this study suggests that the examined CSI, which are considered environmentally safer alternatives to synthetic chemical insecticides, could be integrated into insect pest management strategies

Sublethal effect of flufenoxuron on the testicular biochemical and histopathological profiling of *Spodoptera mauritia*

2.1 INTRODUCTION

Insects are considered among the most diverse and established species due to their extraordinary capacity to reproduce in all environmental conditions. Several factors, including humidity, photoperiod, and most importantly, the availability of food, affect an insect's capacity to reproduce (Costa et al., 2017). Reproductive success is essential to species survival (Li et al., 2018). Efficient reproductive systems are one of the primary reasons for insects' extraordinary success (Mari et al., 2018). These mechanisms generate gametes and, thus, an elevated number of eggs and juvenile individuals during every reproductive cycle (de Moraes et al., 2009). Insect reproduction and hormone modulation are possible targets for developing biorational pesticides. In this viewpoint, identifying chemicals that interfere with insect reproduction and development and target the corresponding endocrine regulating systems has been the core objective of successful research in the last few decades (Dhadialla et al., 2005).

Insect fertilization success is dependent on a number of variables, such as sperm competition and structure and function (Snook, 2005).

Nonetheless, insects show significant variation in sperm morphology and structure both within and between species (Snook, 2005; Werner and Simmons, 2008; Name et al., 2010). For instance, sperm polymorphism based on varying lengths and the generation of both fertilizing and non-fertilizing sperm within a species (Gottardo et al., 2016).

Spermatozoa are one of the cell types diversifying the fastest, despite their essential and conserved role in sexual reproduction. They display a remarkable spectrum of morphological and physiological variation. Thus, since its discovery approximately 350 years ago, spermatozoa have piqued scientific curiosity, and numerous comparative studies have substantially advanced our knowledge of sperm evolution (Pitnick et al., 2009). Unbelievably diverse sperm are found in insects, such as sperm gigantism in *Drosophilidae* (Pitnick et al., 1995), different types of sperm conjugation in *Hydradephagan* beetles (Higginson et al., 2015), and the generation of several different sperm types, like those in *Lepidoptera* (Friedlander et al., 2005). An intriguing example of testicular fusion and sperm polymorphism can be seen in *Lepidopteran* species. In particular, studying these traits is crucial to comprehending the reproductive biology of insects deemed as pests (Mari et al., 2018).

The interesting feature of *Lepidoptera* is the presence of a sperm polymorphism that produces two distinct kinds of gametes (Pereira & Santos, 2015; Mancini & Dolder, 2017). Two types of *Lepidoptera* sperm are regular nucleated sperm (eupyrene) and anucleated sperm. Both kinds of sperm are transported to the female and are necessary for

male reproduction. Apyrene sperm aids eupyrene sperm in accessing unfertilized eggs. The primary characteristic that separates these two kinds is whether or not they have a nucleus (Mari et al., 2018). It also presents itself as a strong substitute and a potential model for the evaluation of male reproductive toxicity due to its well-characterized developmental and reproductive events. Opportunities exist to develop insecticides that interfere with the endocrine system of insects.

Studies have shown that insect growth regulators, chitin synthesis inhibitors (CSIs) disrupt the formation of chitin, a key component of the insect exoskeleton. However, recent studies have shown that CSIs can also have detrimental effects on insect reproduction, specifically on spermatogenesis. Several CSIs like diflubenzuron, chlorfluazuron, lufenuron, and novaluron inhibit chitin synthase, the enzyme responsible for chitin biosynthesis, which indirectly affects spermatogenesis by disrupting testicular development, spermatocyte differentiation, and sperm maturation (Perveen, 2000; Cremonez et al., 2018). The study also showed that chitin is not only present in the exoskeleton but is also essential for the structural integrity of the male reproductive organs, including the spermatophore, which is required for successful mating and sperm transfer (McLay & Greco, 2011). Sharma & Sheiker (2001) reported that exposure to diflubenzuron at sublethal concentrations decreased fertility in *Helicoverpa armigera* males due to incomplete spermatogenesis, and the males were unable to successfully fertilize females, leading to a decrease in the number of viable offspring. A study has demonstrated that flufenoxuron exhibits reproductive inhibition properties in pest insect species (Yasir et al.,

2012). Earlier studies indicate that flufenoxuron may also exert toxic effects on the reproductive functions of lepidopteran species in *Agrotis ipsilon* (Shaurub et al., 2018).

Chitin, a polymer derived from amino sugars, is mostly made up of N-acetyl-D-glucosamine and is found in the peritrophic matrix of the insect midgut, the trachea, and the exoskeleton. In addition to forming the exoskeleton that supports the lining of the digestive tract, respiratory systems, reproductive ducts, and certain gland ducts, the cuticle of insects acts as an interface between living things and their surroundings (Tunaz & Uygun, 2004). As a result, modifications in chitin production following CSI administration may impact adult insects and consequently impact the histophysiology of several systems, including the reproductive system (Costa et al., 2017). Chitin synthesis inhibitors (CSIs) belong to IGRs that interfere with chitin biosynthesis, preventing insect moulting and the entire life cycle (Hammock & Quistad, 1981; Mondal & Parween, 2000). Among CSIs, benzoylphenylurea class has been used in the control of insect pests of the orders Lepidoptera, Coleoptera, Diptera and Hemiptera (Costa et al., 2017; Shaurub et al., 2018). Recent research indicates that BPUs interact with sulfonylurea receptors (SURs), a type of ABC-transporters that affect cuticle chitin production (Tian et al., 2017). Effectiveness of CSIs for management of several lepidopteran pest species has been examined recently (Ghoneim et al., 2015; Aliabadi et al., 2016; Kottaipalayam et al., 2022).

However, it is well recognized that CSIs have a major, sub-lethal impact on treated adults' ability to reproduce (Parween et al., 2001).

These substances work as efficient development suppressors for insects throughout their life cycle by altering the hormonal balance and causing physiological disruptions (Reda et al., 2010). An assessment by Yasir et al. (2012) proves that flufenoxuron has reproductive inhibition properties against pest insects. Previous studies have shown that flufenoxuron may also have toxic effects on reproductive functions in Lepidopteran species such as *Agrotis ipsilon*, (Shaurub et al., 2018). Agüero et al. (2014) conducted a study on using chitin biosynthesis inhibitors in the reproductive development of stink bug pests. The authors observed adverse effects on gonad formation in insects treated with diflubenzuron, based on representative results of effects on morphology in *Nezara viridula* L. (Hemiptera: Pentatomidae). Additionally, in a different study, CSIs had an impact on the male and female reproductive organs of *Dichelops melacanthus* (Hemiptera: Pentatomidae) (Cremonez et al., 2017). However, surprisingly, few studies examine how insecticides affect insect testicular accessory cells, particularly in adult *Euschistus heros* treated with CSIs. According to reports, CSIs hampered Lepidopteran, Orthopteran, and Coleopteran gametogenesis, particularly spermatogenesis (Acheuk et al., 2012; Costa et al., 2017; Santorum et al., 2021).

In order to understand the reproductive biology of insects, it is crucial to examine spermatogenesis and spermiogenesis. This knowledge can be a foundation for future research, particularly in pest control. In Lepidopteran pest insects, sperm development and behaviour are interesting targets for target-specific, environmentally safer biorational control techniques. Sperm dimorphism offers a broad range of

opportunities to modify sperm dynamics and functionality, reducing the pest species' reproductive fitness (Seth et al., 2023). Morphological analysis of the testes of *S. mauritia* throughout their development may reveal important alterations in the germ and somatic cells involved in spermatogenesis. Researching the male reproductive biology of pests can aid in the development of male sterility techniques and offer a theoretical framework for the creation of novel biological controls for pests (Ahmadi et al., 2018).

This study highlights the remarkable characteristics of male reproductive biology shared by all lepidopteran pests, particularly the presence of two unique forms of spermatozoa that serve as a fertility restriction. Our current understanding of Lepidopteran dichotomous spermatogenesis and the potential for targeted disruption of other associated processes are examined in light of this. A particular benefit of interfering with male reproduction would be to keep migratory Lepidopteran pest populations from growing. However, to date, no information is available on the effect of chitin synthesis inhibitor flufenoxuron on the testicular development and spermiogenesis of Lepidoptera. Therefore, the present study explores the impact of flufenoxuron on the testicular development, spermiogenesis and biochemical indices of *S. mauritia*.

2.2 REVIEW OF LITERATURE

2.2.1 Overview of insect's male reproductive development

Male reproductive development in insects is a highly coordinated process that begins during embryogenesis and continues through post-embryonic stages into adulthood. Primordial germ cells, set aside early in embryonic development, migrate to specific areas in the embryo where they form the testes (Pfister, 2008; Kitadate & Kobayashi, 2010). The reproductive system remains immature during the larval stages of holometabolous insects (Redei & Stys, 2016). However, the process of sperm production in adult male insects and its transfer to females requires a significant investment of biochemical resources and the precise coordination of various developmental processes. The maturation of the gonads and secondary reproductive organs are synchronized with the insect's metamorphic development, which includes adaptations in energy acquisition, sensory processing, motor function, and the central nervous system to ensure successful mating.

Hormones such as ecdysteroids and juvenile hormones regulate the maturation of the testes and other reproductive structures during the pupal stage (Nijhout & Laub, 2018). In the adult stage, the male reproductive organs fully develop, allowing active spermatogenesis and sperm transfer during mating. In addition to sperm, male accessory glands produce seminal fluid that supports sperm viability and motility, and in some species, they produce spermatophores, capsules or packets of sperm (Gillott, 2003). Hormonal cues also regulate post-mating reproductive behaviour and physiology, influencing sperm production

and accessory gland function (Vitecek et al., 2013; Isaac, 2019). The interaction of developmental biology, endocrinology, and reproductive anatomy ensures that male insects are reproductively competent as adults. Hormones that regulate cell expression during moulting cycles also influence primary and secondary reproductive systems (Happ, 1992). Although much research has traditionally focused on regulating reproductive maturation in female insects as a tool for pest management, recent studies have shifted toward investigating male reproductive systems. Several investigations aim to develop innovative strategies for pest management by targeting male reproductive functions, offering new potential avenues for controlling insect populations.

2.2.2 Lepidopteran testicular development

The male reproductive systems of most insects, including those in the order Lepidoptera, are composed of a single pair of testes, each containing several testicular tubes or follicles. These are connected to a seminal vesicle via accessory glands, the ductus ejaculatorius, and the vasa deferentia (Ozyurt et al., 2015). The larval testes of Lepidopteran species consist of a pair of kidney- or bean-shaped structures, with each testis composed of four testicular follicles (Friedlander et al., 2005; Pereira & Santos, 2015). Each follicle has two distinct ends: a larger, "free" distal blind end on the convex side of the testis and a smaller "open" proximal end on the concave side. The proximal end extends into a separate vas efferens, with the four vasa efferentia of each testis fusing to form one of the two paired vasa deferentia. At the distal end of each follicle is the germarium, which produces

spermatogonia throughout the insect's lifespan (Friedlander et al., 2005).

The most significant testicular growth occurs during the final larval instar, driven by an increase in the frequency of primary germ cell mitotic division (Friedlander et al., 2005). At this stage, each pair of testes consists of four follicles, each containing three distinct regions packed with gonial cells at different stages of development (Bebas et al., 2018). As the larvae enter the pre-pupal stage, the paired kidney-shaped testes fuse into a single organ, which undergoes torsion. This results in the formation of a single gonad with eight twisted chambers that develop into testicular follicles in pupae and adult males (Pereira & Santos, 2015; Bebas et al., 2018). The testicular growth is driven by a marked increase in the frequency of mitotic divisions in the primary germ cells (spermatogonia). As a result, the testes become increasingly packed with germ cells at various stages of development (Friedlander et al., 2005). Each testicular follicle is organized into three regions containing gonial cells at different developmental stages. This organization ensures the continuous production of sperm during the insect's reproductive life. By this stage, each pair of testes consists of four follicles actively producing spermatogonia, spermatocytes, and spermatids.

2.2.3 Spermatogenesis of insects

The process of germ cell differentiation during spermatogenesis in Lepidopteran testes is highly structured and similar across the species. In the last-instar larvae, progenitor cells for both somatic and germline

cells occupy the distal portion of the testicular follicle, forming a germarium. At the centre of the germarium lies a Verson's cell, an apical complex responsible for organizing the development of cysts containing germ cells. These cysts are the basic units within which spermatogenesis occurs (Phillips, 1970; Friedlander et al., 2005).

In various insect orders such as Lepidoptera, Hemiptera (Sternorrhyncha), and Diptera, germline cells develop synchronously within cysts, which are surrounded by somatic cystic cells (Mancini & Dolder, 2017; Vitale et al., 2011; Name et al., 2012; Pereira & Santos, 2015). A key feature of insect spermatogenesis is the division of sperm into two distinct types: eupyrene (nucleated, motile sperm) and apyrene (anucleated, non-motile sperm) spermatozoa. Notably, each cyst will exclusively contain either eupyrene or apyrene spermatozoa, never both simultaneously (Wolf & Traut, 1987; França & Bão, 2000; Pereira & Santos, 2015; Mancini & Dolder, 2017). This separation of sperm types is significant as both eupyrene and apyrene sperm have distinct roles in reproduction, with eupyrene sperm responsible for fertilization and apyrene sperm potentially assisting in sperm competition or influencing female reproductive physiology (Silberglied et al., 1984). Moreover, germline cells remain interconnected via cytoplasmic bridges, maintaining their connection throughout development (Friedländer et al., 2005; Vitale et al., 2011; Pereira & Santos, 2015). The synchronization of development is a critical aspect of efficient spermatogenesis, allowing insects to produce large numbers of sperm cells in a relatively short period.

2.2.4 Lepidopteran dichotomous spermatogenesis

Lepidopteran dichotomous spermatogenesis follows a consistent pattern of meiosis and spermatogenetic proliferation, beginning early in development. Spermatogonia start dividing even before larvae emerge from the egg, establishing the foundational process for spermatogenesis (Friedländer et al., 2005). In *Bombyx mori*, the testes in late embryonic stages already contain four developing follicles with mitotically dividing spermatogonia (Takeuchi, 1969). By the first larval instar, the testes display encysted spermatogonia, and their proliferation continues throughout the insect's lifespan (Marec et al., 1993).

According to Polanska et al. (2005), spermatogenesis in Lepidopteran testes occurs in three distinct zones within the follicles: the growth zone, the maturation/reduction zone, and the transformation zone. In the growth zone, spermatogonia divide and grow into spermatocytes. These spermatocytes undergo meiotic division in the maturation/reduction zone, forming spermatids. Finally, in the transformation zone, spermatids undergo spermiogenesis, leading to the formation of mature spermatozoa.

In Lepidoptera and other insects, the number of mitotic divisions during spermatogonial development determines the differentiation stages of these cells. Most Lepidopteran species have six generations of spermatogonia, resulting in a clone of 64 cells within each cyst. These cells undergo DNA replication and enter meiotic prophase, transforming into primary spermatocytes (Friedlander et al., 2005).

The spermatogonia are characterized by round cells with a prominent and conspicuous nucleolus, while spermatocytes have a significant cytoplasmic volume, as well as a developed spherical nucleus (Pereira & Santos, 2015; Pezenti et al., 2021). During meiosis in primary spermatocytes, two divisions occur without DNA replication. The decision to develop as eupyrene or apyrene sperm is shared among all cells in the cyst, possibly mediated by intercellular bridges or factors in the extracellular fluid. After meiosis, each cyst typically contains 256 spermatids that mature into spermatozoa (Friedlander et al., 2005). As the larvae mature, the frequency of spermatogonia decreases, and they are found in a more peripheral position within the testes. In the adult stage, the testes are largely composed of fully developed spermatozoa, ready for release, while fewer spermatogonia remain in the outer regions of the testis (Pezenti et al., 2021). This reflects the shift from active spermatogonial proliferation in earlier developmental stages to the maintenance of mature sperm in adult Lepidoptera.

In adult Lepidoptera, centripetal spermatogenesis is a commonly observed characteristic (Phillips, 1970; Pereira & Santos, 2015; Mancini & Dolder, 2017; Pezenti et al., 2021). Many species exhibit dichotomous spermatogenesis, producing two types of spermatozoa: eupyrene and apyrene (Garvey et al., 2000; Mancini & Dolder, 2017). Differences between eupyrene and apyrene spermatocytes become evident early in the zygotene stage of primary spermatocytes. Apyrene spermatocytes are distinguished by smaller cytoplasmic volume, fewer and smaller mitochondria, absence of specific chromosomal configurations, prematurely condensed chromatin, shorter pachytene

chromosomes, lack of chiasmata, and the presence of two separated nucleoli rather than one (Kawamura et al., 1998; Garvey et al., 2000; Reinholdt et al., 2002; Friedlander et al., 2005).

2.2.5 Eupyrene and Apyrene Spermiogenesis

Spermiogenesis is the process that transforms spermatids into fully developed spermatozoa. Differentiation begins before meiosis division. The presence of flagella indicates spermatid differentiation. The flagella originate from basal bodies and are evenly distributed among the resulting spermatids. The nucleus elongates and attaches laterally to the basal body and axoneme (Friedlander et al., 2005). In many moths, spermatogenesis, including spermiogenesis, the late stage at which elongated spermatids start to form spermatozoa, is usually completed by the end of metamorphosis (Friedlander et al., 2005; Bebas et al., 2018). Depending on the species, apyrene spermatogenesis starts soon or after pupation and continues in the imago, whereas eupyrene spermatogenesis starts in the larva and typically ends after pupation (Friedlander, 1997).

The developing eupyrene spermatids of Lepidoptera, possess a vesicle in front of the basal body, referred to as an 'acrosome,' and display structural similarities to acrosomes in other animals. However, its function in insect sperm remains uncertain, and there is no evidence that it acts as a lysosome containing hydrolytic enzymes, as is common in other species (Friedlander et al., 2005).

During spermiogenesis, the mitochondria of the developing eupyrene spermatid fuse to form a structure known as the 'Nebenkern'. This

Nebenkern later divides into two mitochondrial derivatives, essential for the energy supply of the spermatozoa. Initially, germ cells are enclosed by somatic cells forming the cyst wall. As spermiogenesis advances, these cyst wall cells extend pseudopodia between the maturing spermatozoa (Friedlander et al., 2005). Although the precise role of this invasion by the supporting cells remains unclear, it is believed to play a crucial role in ensuring the proper progression of spermiogenesis (Friedlander et al., 2005; White-Cooper & Bausek, 2010).

Apyrene spermiogenesis differs from eupyrene spermiogenesis in several key aspects. In apyrene spermatids, the basal body becomes separated from the micronuclei and attaches to the cell membrane at the anterior end of the elongating cell. Unlike eupyrene spermatids, there is no acrosome-like vesicle. Instead, an electron-opaque truncated cone of extracellular origin is observed at the anterior end. The mitochondrial derivatives in apyrene spermatids also exhibit distinct characteristics. They lack clear cristae in longitudinal sections and contain a rod of paracrystalline material, which may have metabolic significance, although its exact role remains unclear (Friedlander et al., 2005).

2.2.6 Control of male reproduction in insects

In insects, hormonal factors dictate the growth, development and reproduction. Ecdysteroids, particularly the active form 20-hydroxyecdysone (20E), and sesquiterpenoid hormones like juvenile hormones (JHs), are crucial for insect development. These hormones

regulate key processes such as moulting, metamorphosis, and reproductive functions (Pezenti et al., 2021). This combination of structural and hormonal factors highlights the complexity of Lepidopteran reproduction, which plays an important role in understanding insect biology and pest management.

The influence of insect growth regulators (IGRs) on insect reproduction extends beyond females, significantly affecting male reproductive physiology. In males, key processes in the reproductive system are predominantly regulated by juvenile hormones (JHs) and ecdysteroids (Raikhel et al., 2005). Ecdysteroids positively influence spermatogenesis by increasing the frequency of germ cell divisions and facilitating their differentiation into more advanced stages. This regulation also affects the development of testes and associated structures, such as seminal ducts (Happ, 1992; Friedlander, 1997; Raikhel, 2005).

Early studies highlighted the inhibitory effects of JHs on spermatogenesis. Mitsui et al. (1976) found that juvenile hormone application prevented spermatogenesis in *Bombyx mori*, with similar findings reported by Yagi and Fukushima (1975). More recent research by Bebas et al. (2018) on the testes of *Galleria mellonella* showed that the juvenile hormone analogue fenoxycarb caused significant degenerative changes, including hypertrophy of the testicular wall and disorganization of testicular follicles, leading to the destruction of germ cells in various stages of spermatogenesis. In *Bombyx mori*, fenoxycarb also suppressed ecdysone release in late-instar larvae, reducing the production of spermatogonia and mature sperm (Turgay-

izzetoglu et al., 2018). Moreover, studies on *Tribolium castaneum* have demonstrated that untreated females mated with males exposed to IGRs exhibited reduced egg production and failure of eggs to hatch. Males were found to be more vulnerable to treatment than females. Studies have shown that methoprene, a juvenile hormone analogue, can impair spermatogenesis during early development, increasing male susceptibility (Wijayaratne et al., 2012). Additionally, sublethal doses of bisacylhydrazine ecdysone agonist insecticides have been shown to disrupt male reproductive function in *Spodoptera litura*, potentially altering population dynamics by reducing male fertility (Seth et al., 2004).

2.2.7 Control of insect reproduction using chitin synthesis inhibitor

The reproductive processes of female and male insects have been extensively studied due to their importance in species propagation and their potential as targets for insect control strategies. Insect growth regulators (IGRs) are a class of compounds that interfere with insect growth, development, and reproduction by either inhibiting chitin synthesis or mimicking key hormones such as juvenile hormone (JH) and 20-hydroxyecdysone (20E) (Dhadialla et al., 1998, 2005). Among these regulators, chitin synthesis inhibitors (CHIs) have been explored for their potential to affect oogenesis and spermatogenesis in insects negatively, making them useful tools for pest management (Perveen & Miyata, 2000; Mansur et al., 2010; Cremonez et al., 2018; Catchot et al., 2020).

Adults that emerge from larvae exposed to chitin synthesis inhibitors (CSIs) often exhibit various physiological limitations, resulting in reduced physical and reproductive fitness (Mondal & Parween, 2000). Research has shown that insecticides from the benzoylphenylurea (BPU) class control insect growth by inhibiting chitin synthesis and, through their high ovicidal effects, reduce reproduction (Dhadialla et al., 2005; Cutler & Scott-Dupree, 2007; Mommaerts et al., 2006). Diflubenzuron, the first commercially available BPU, has been shown to affect adult female reproduction, leading to reductions in both fecundity and fertility (Lim & Lee, 1982; Soltani, 1987; Soltani & Soltani Mazouni, 1992).

Similarly, Belinato et al. (2009) found that sublethal doses of the CSI triflumuron impair various aspects of viability and reproductive capacity in adult mosquitoes. The presence of chitin in mosquito ovaries, as documented by Moreira et al. (2007), suggests that ovarian tissue could be a target for insect control. Moreover, the follicular epithelium plays an active role in synthesizing and transporting yolk and non-yolk proteins to the oocytes. It is reasonable to assume that it may also be involved in the chitin synthesis process, as chitin was detected in the inner layer of the follicular epithelium in oocytes (Mansur et al., 2010). The study also revealed that a modest suppression of oogenesis was observed in *Drosophila melanogaster* treated with lufenuron prior to vitellogenin development (Wilson & Cryan, 1997). Similarly, prior studies have reported the effects of novaluron on reproductive characteristics in target insect pests (Cloyd et al., 2004; Joseph, 2020).

According to Costa et al. (2017), lufenuron may have affected spermiogenesis in *Anthonomus grandis*, leading to pathological alterations in the midgut epithelium. Similarly, Santorum et al. (2021) confirmed that novaluron has detrimental effects on the testes and the spermatogenesis process in *Bombyx mori*. Their study demonstrated that even a brief exposure of 24 hours to this insecticide caused significant disruptions to the organization, distribution, and development of sperm cysts in *B. mori* larvae, resulting in severe alterations to the testicular cells. In addition to the changes observed in the testes, alterations were also detected in the ovaries of *B. mori*. The outer layer of the ovarioles showed modifications, including ruptures in the epithelial sheath and tunica propria. Furthermore, there was noticeable detachment and spacing between the cells of the sheath, germ cells, and follicular cells. Based on these findings, Santorum et al. (2021) speculated that chitin synthesis inhibitors (CSIs) may induce cell death in ovarian follicles. Additionally, research on *Schistocerca gregaria* revealed that lufenuron, a member of the benzoylphenylurea class, caused a decline and disintegration of the follicular epithelial cell layers in ovarioles, along with the disintegration of organelles (Ghazawy, 2012).

Inhibitors of chitin synthesis have demonstrated direct impacts on insect reproduction. Moreira et al. (2007) showed that lufenuron affects vitellogenesis in *Aedes aegypti*. Similarly, Acheuk et al. (2012) reported that teflubenzuron significantly impacts female reproduction in *Locusta migratoria* by reducing fecundity and fertility, and disrupting vitellogenesis, ovarian development, and ecdysteroid

production. A related study by Lim and Lee (1982) found that treatment with diflubenzuron decreased fecundity in *Oxya japonica*. This decrease was identified as an indirect consequence of the compound's inhibitory effect on food consumption. Additionally, Grosscurt (1978) and Medina et al. (2002) documented the ovicidal effects of diflubenzuron, attributing these effects to the suppression of chitin synthesis in insect embryos.

2.2.8 Haemolymph and testicular biochemistry

Haemolymph serves as the circulating fluid, or "blood", within the bodies of insects and flows through their open circulatory system, directly enveloping the organs and tissues. Water is the primary constituent of haemolymph, serving as a solvent for various molecules. Insect bodies contain approximately 20-50% of their total water content in haemolymph, with larval stages having a higher proportion than adults. Haemolymph acts as a reservoir of water for tissues during desiccation and stores other chemicals. It also contains haemocytes or circulating cells. Additionally, haemolymph functions as a hydraulic fluid and plays crucial roles in the immune system and transportation of hormones, nutrients, and metabolites (Kanost, 2009).

Haemolymph is composed of inorganic and organic components, with citric acid, other organic acids and phosphates making up a significant portion of the anion content in insect haemolymph (Pannabecker et al., 1992). The primary carbohydrate in haemolymph is trehalose, an essential energy source for tissues. Hormones regulate trehalose levels and can be synthesized from glucose phosphate stored in the fat body

(Tellis et al., 2023). Glycerol and sorbitol can also be synthesized in response to diapause or low temperatures, protecting the insects from freezing (Storey & Storey, 2013). Lipoidal compounds, such as diacylglycerol, are transported in haemolymph via specific proteins (Van der Horst, 2019). Haemolymph also contains pigments, amino acids, and tyrosine, which is used for sclerotization. Catecholamines derived from tyrosine are in haemolymph for pigmentation (Kanost, 2009).

Proteins are a major component of haemolymph plasma (Liu et al., 2022). Their concentration increases during each instar and decreases at each moult (Dean et al., 1980). The fat body synthesizes most plasma proteins, with contributions from the epidermis and haemocytes. Each species' plasma contains a few highly abundant proteins and over a hundred proteins at lower concentrations (Price, 1973). The most abundant proteins are hexamers; they are synthesized by the fat body and reach high concentrations in the last instar (Martins et al., 2008). During metamorphosis, they are broken down into amino acids for synthesizing proteins in the adult stage. Other storage proteins that differ from hexamerins have been identified in Lepidopterans (Kanost, 2009).

Several haemolymph proteins transport small molecules with low solubility in water. Insect plasma contains two key iron-binding proteins, ferritin and transferrin, essential in sequestering dietary iron and transporting ions between tissues (Whiten et al., 2018). The primary transport protein in haemolymph is lipophorin, which facilitates the transport of lipids between tissues. Lipophorin forms

complexes with lipids, effectively protecting them from exposure to water (Kanost, 2009). In insects that utilize lipids as fuel for flight muscles, diacylglycerol is released in response to signals from the adipokinetic hormone (Van der Horst, 2003). Additionally, juvenile hormone (JH), a sesquiterpenoid lipid, is transported in the haemolymph via specific carrier proteins. Different insect species possess distinct transport proteins that help maintain the solubility of JH and protect it from degradation (Kanost, 2009).

2.2.9 Effect of BPU on Biochemical Indices

The three main biochemical components of an insect's body tissues are carbohydrates, proteins, and lipids. Proteins play a crucial role in various physiological processes. They are essential for binding foreign substances (Wilkinson, 1976) and are necessary for body growth and reproduction. Several factors influence the regulation of protein synthesis in insects, including hormonal signalling, environmental conditions, and nutrient availability (Carlisle et al., 1987).

Reports indicate that protein concentrations have been observed to increase during different phases of development in *Muscina stabulans* larvae treated with chitin inhibitors IKI-7899 and XRD-473 (Basiouny, 2000). In contrast, Hamadah (2014) reported that the application of flufenoxuron reduced protein content in the haemolymph of *Schistocerca gregaria* and inhibited protein accumulation during both the nymphal and adult stages. Similarly, Ghoneim et al. (2012) found a depletion of protein content in the haemolymph of *S. gregaria* nymphs in their final instar following treatment with chlorfluazuron. Further,

chlorfluazuron and flufenoxuron (Cascade) were shown to suppress protein content in the Lepidopteran *Spodoptera littoralis* (Ghoneim et al., 1994; Zohry, 2006). Likewise, in fourth-instar larvae of the mosquito *Culiseta longiareolata*, treatment of novaluron caused total protein content to decrease significantly (Bouaziz et al., 2011). Conversely, Ghoneim et al. (2005) reported that lufenuron treatment caused *Musca domestica* pupae to acquire higher protein levels.

Saleh and Abdel-Gawad (2018) investigated the effects of two insect growth regulators, diflubenzuron and chromafenozide, on the biochemical composition of sixth instar larvae of *Spodoptera littoralis*. Their findings revealed variations in native protein, lipoprotein, glycoprotein, and protein band patterns on SDS-PAGE between control and treated larvae. Similarly, Salokhe et al. (2006) reported that the insect growth regulator flufenoxuron, administered at sublethal doses to *Tribolium castaneum* (Herbst) larvae, caused a reduction in total soluble protein. Although a few protein bands, particularly those in the molecular weight range of 50–97 kDa, were reduced in treated larvae, the overall protein profile between treated and control groups showed no significant differences.

The total protein content in the haemolymph of the silkworm *Bombyx mori* was inhibited by various stressors (Etebari & Matindoost, 2004). This inhibition may lead to the breakdown of proteins into amino acids, which subsequently enter the tricarboxylic acid (TCA) cycle as keto acids, providing the insect with an energy source. Thus, maintaining the levels of free amino acids in the haemolymph may serve as a physiological mechanism to counteract protein depletion in

tissues. This process likely contributes to compensatory mechanisms during insecticidal stress, as it provides essential intermediates for the Krebs cycle, supporting metabolic functions under adverse conditions (Nath et al., 1997; Hamadah, 2014).

Carbohydrates play a crucial role in the normal development of embryos, the shape and function of all tissues during metamorphosis, and the performance of male and female reproductive organs (Chippendale, 1978). Some researchers have hypothesized that the activity of various insect growth regulators (IGRs) can increase carbohydrate content in certain insect species, while others have reported a decline. These conflicting results may be attributed to differences in species sensitivity, the IGRs' potency, or the insects' developmental stage (Hamadah, 2014). For instance, disturbances in carbohydrate content in the haemolymph of *Schistocerca gregaria* have been documented following treatment with lufenuron and flufenoxuron (Tanani et al., 2012; Hamadah, 2014).

Chlorfluazuron and mevalonic acid, treated separately or synergistically, have been shown to promote increased carbohydrate levels in the haemolymph and fat body of the last instar larvae and pupae of *Spodoptera littoralis* (Ghoneim et al., 1994). Similarly, El-Gammal et al. (1993) reported that chlorfluazuron augmented carbohydrate content in *Schistocerca gregaria*. Additionally, pupae and adult females of *Tenebrio molitor* treated with diflubenzuron exhibited elevated carbohydrate levels (Soltani et al., 1987; Soltani-Mazouni et al., 1999). Likewise, the topical application of lufenuron or diofenolan on late third instar larvae of *Musca domestica* resulted in increased carbohydrate content throughout their pupal stage (Ghoneim et al., 2006). Furthermore, the fourth instar larvae of the mosquito *Culiseta*

longiareolata treated with novaluron showed a notable increase in carbohydrate levels beginning five days post-treatment (Bouaziz et al., 2011).

Lipids are crucial structural components in insects, forming the cuticle and cell membranes while also serving as a significant energy source. Insects obtain lipids through synthesis and dietary sources (Gilbert, 1967). Research suggests that a deficiency in juvenile hormones can lead to fat accumulation, indicating that neuroendocrine-controlled feedback loops regulate insect lipid turnover (Hill & Izatt, 1974; Downer, 1985). Studies have revealed that flufenoxuron treatments reduce lipid levels in the haemolymph and fat body of *Schistocerca gregaria* (Hamadah, 2014). In contrast, treatments with lufenuron and diofenolan resulted in decreased lipid content in the pupae of the red palm weevil, *Rhynchophorus ferrugineus*, at both the beginning and end of their life cycle. However, mid-aged pupae exhibited increased lipid levels (Ghoneim et al., 2003).

Additionally, an increase in lipid content in the haemolymph of *Spodoptera littoralis* larval and prepupal stages following chlorfluazuron treatment has been reported (Ghoneim et al., 1994). Similarly, lipid concentrations rose in the pupae and adult females of the mealworm *Tenebrio molitor* after diflubenzuron treatments (Soltani-Mazouni et al., 1999). However, high concentrations of insect growth regulators (IGRs) such as pyriproxyfen, tebufenozide, and lufenuron led to decreased lipid content in the haemolymph of early-aged nymphs of *S. gregaria* (Sh et al., 2012). In contrast, novaluron treatments on fourth-instar larvae of the mosquito *Culiseta longiareolata* resulted in increased lipid levels throughout the body starting on day 5 post-treatment (Bouaziz et al., 2011).

2.3 MATERIALS AND METHODS

2.3.1 Experimental organism

Our previous chapter demonstrated that flufenoxuron exhibits toxic potential in the male reproductive development of *Spodoptera mauritia*. Understanding the effects of flufenoxuron on the biochemical parameters and reproduction of *S. mauritia* is crucial for the informed and targeted application of these chemicals. Therefore, this chapter delves into the impacts of flufenoxuron on the biochemical profile, histopathology, and live cell imaging of male sixth instar larvae of *S. mauritia*. Previous studies have shown that spermiogenesis begins in the last instar larvae of *Spodoptera mauritia* (Manogem & Nair, 2013). Consequently, this study used newly ecdysed sixth instar larvae to investigate the effects of flufenoxuron on spermiogenesis in *S. mauritia*.

2.3.2 Treatment of flufenoxuron on sixth instar larvae

Laboratory stock culture was used to obtain newly ecdysed *S. mauritia* sixth instar larvae. Flufenoxuron, chitin synthesis inhibitor dissolved in acetone, was applied topically in two doses, ED₅₀ and ED₂₅ (0.00068µg and 0.00015µg), as described previously (Chapter 1). The experimental and control larvae were maintained in separate plastic tubs covered with muslin cloth. The larvae were collected at different intervals for further analysis (48h, 96h, and 144h).

2.3.3 Sample collection

Following treatment of the median effective dose and sub-median effective dose of flufenoxuron, the treated and control groups were sacrificed for extracting haemolymph and testis at specific intervals (48h, 96h and 144h) for biochemical and histopathological analysis. For biochemical and histopathological analysis, the larvae (48h and 96h) and pupae (144h) were sedated and pinned dorsally on a wax-lined Petri dish. Insect ringer's solution (NaCl - 0.65 gm, KCl - 0.025 gm, CaCl - 0.03 gm, NaHCO₃ - 0.025 gm dissolved in 100 ml distilled water) was used for dissections. A longitudinal cut was made on the dorsal side of the abdomen, followed by a vertical cut that opened the abdomen and removed the testes using fine forceps under a stereoscopic microscope. The isolated testis was rinsed with insect ringer solution and kept at -80°C in 50mM PBS for further analysis.

2.3.4 Effect of flufenoxuron on biochemical parameters of haemolymph and testis

The haemolymph and testis samples collected from sixth instar larvae of *S. mauritia* treated with ED₅₀ and ED₂₅ (0.00068µg and 0.00015µg) flufenoxuron and control groups at specific intervals were processed for biochemical analysis and protein profiling.

2.3.4.1 Haemolymph sample preparation

To extract the haemolymph, the ED₅₀ and ED₂₅ (0.00068µg and 0.00015µg) flufenoxuron-treated larvae and control groups at specific intervals of 48h, 96h and 144h were anaesthetized with diethyl ether.

One of its thoracic legs was amputated with sharp scissors, and the haemolymph oozed out immediately are drawn into a calibrated capillary tube. The haemolymph is transferred into a centrifuge tube containing phenylthiourea crystals. Kept on ice for a few minutes. Centrifuge the haemolymph to remove haemocytes and undissolved phenylthiourea. Pipet out the cell-free suspension and kept it at -80°C for further analysis. Collected and stored haemolymph samples from each treatment and control group were diluted according to the demand of each biochemical experiment.

2.3.4.2 Tissue sample preparation

After treatment with a median effective dose and sub-median effective dose ED_{50} and ED_{25} ($0.00068\mu\text{g}$ and $0.00015\mu\text{g}$) of flufenoxuron, the sixth instar larvae were dissected out testis at specific intervals (48h, 96h and 144h). The testis of treated and control samples of *Spodoptera mauritia* was homogenized in prechilled 10mM phosphate buffer pH 7, supplemented with protease inhibitor PMSF at a concentration of 1mM by using polytron homogenizer in cold conditions. The homogenate was centrifuged for 10 min in cold conditions (4°C) at 20,000 rpm, and the supernatant was collected and stored at -80°C till utilized.

2.3.4.3 Protein estimation

The protein assay of haemolymph and tissues was performed employing the Bradford method (Bradford, 1976), using bovine serum albumin as a standard. About 0.3 ml of protein solution and 0.3 ml of Bradford reagent were added and mixed well. The absorbance was read at 595nm in a Microplate reader (SynergyTM HT, Bio-Tek Instruments).

The absorbance measurements were corrected using a blank value. The protein concentration was determined by a standard curve using BSA values ($\mu\text{g}/\mu\text{l}$) on the X-axis and absorbance values on the Y-axis. The protein values were calculated in $\mu\text{g}/\mu\text{l}$.

2.3.4.4 Protein Profiling

The haemolymph and tissue homogenate were obtained from the treatment and control groups of larvae with 1% final SDS, and the mixture was centrifuged for 10 minutes at 10000 rpm. On a maxi vertical system (Genie), the samples were subjected to 10% SDS-PAGE analysis (Laemmli, 1970). Electrophoresis was performed at a constant current of 45 mA in stacking gel and 85 mA in separating gel. The stained gel was photographed and analysed using Gel analyzer software for band detection. Band intensity was calculated using Image J software. A hierarchical cluster was constructed based on treatment group and band intensity using Origin software.

2.3.4.5 Lipid estimation

The total amount of lipids in each treatment and control group was determined by the vanillin assay procedure (Van Handel, 1985) using cholesterol as the standard. Briefly, a 200 μl sample was mixed with chloroform: methanol mixture (1:1). After vigorous vortexing, each sample was centrifuged for 10min at 10,000rpm and 4 $^{\circ}\text{C}$. The supernatant was transferred to a new Eppendorf, and 100 μl supernatant was transferred to a glass vial and heated at 90 $^{\circ}\text{C}$ until complete solvent evaporation. Ten microlitres of concentrated sulphuric acid were then added to each sample. Then, incubated at 90 $^{\circ}\text{C}$ for 2 min in a water bath. After cooling to room temperature, 190 μl of vanillin

reagent was added to each vial. The vial was homogenized and incubated at room temperature for 15 minutes, and its absorbance was measured at 525nm in a microplate reader (SynergyTM HT, Bio-Tek Instruments).

2.3.4.6 Carbohydrate estimation

The haemolymph and tissue glucose levels were determined by the Phenol- Sulphuric acid method (Van Handel, 1985). A standard stock solution was prepared using D-glucose. Different dilutions of the working solution with a glucose concentration ranging from 0.05mg/ml- to 250mg/ml were prepared. 100µl sample was mixed with methanol: chloroform (1:1) and vortexed for 10 min. Samples were centrifuged at 10,000 rpm for 10 minutes, and the supernatant was collected. To 100µl of the supernatant, add 100µl chloroform and 75µl deionized water. Centrifuge and 40µl supernatant transfer to new Eppendorf, dilute with 180µl deionized water. Add 200µl 2% phenol and 500µl concentrated sulphuric acid. After cooling to room temperature, the absorbance was measured at 490 nm in a microplate reader (SynergyTM HT, Bio-Tek Instruments). The total glucose content was expressed in a µg/ml sample.

2.3.5 Effect of flufenoxuron on histology of testes

The flufenoxuron-exposed ED₅₀ and ED₂₅ (0.00068µg and 0.00015µg) sixth instar larval testis was reclaimed at 48h, 96h, and 114h, and the testes were fixed overnight in Bouin's fixative. The tissues were washed in distilled water, dehydrated in grades of ethanol, cleared in methyl benzoate, infiltrated, embedded in paraffin wax and sectioned in a Reichert precision rotary microtome with a thickness of 5µm. The

sections were stained using Harris haematoxylin-eosin and mounted with DPX. The control and treated tissue sections were observed and photographed under a Zeiss Axio-Scope A.1 microscope. Histopathological alterations from all experimental groups were subjected to the semiquantitative scoring system (Gibson-Corley et al., 2013).

2.3.6 Flufenoxuron-induced spermiogenesis dysfunction using confocal microscopy studies

Confocal microscopy examined the nuclear damage, apoptosis, and mitochondrial membrane potential of the testes of ED₅₀ and ED₂₅ (0.00068µg and 0.00015µg) flufenoxuron treated sixth instar larvae of *S. mauritia*. The analysis was performed after the pupation of treated and control larvae to analyze all developmental stages of spermiogenesis. Testis from treated and control groups were placed into a 1.5 ml sterile Eppendorf tube with 100µl of 50 mM PBS. The cell suspension was combined with 100µl of mitochondrial membrane potential-dependent dye, TMRM (50nM), and nuclear dye, Hoechst-33342 (5µM). Suspensions were then kept for about 20 min in the dark, followed by 2 min centrifugation at 2000 rpm. Discard the supernatant and resuspend in 50µl PBS. Hoechst-33342 staining of the nucleus helps to locate different stages of developing spermatozoa. The cells were imaged using a Nikon A1R confocal microscope outfitted with an argon and diode laser for laser scanning. A diode laser line at 405 nm was employed to obtain emission at 450± 25 for the Hoechst imaging. TMRM was conducted using 562 nm laser line for TMRM excitation, with emission measured at 600 ± 25. A Retiga Exi Camera (Q imaging) with NIS element software was used to acquire images.

2.3.7 Statistical analysis

IBM SPSS (24.0 versions) and Microsoft Excel were used for statistical analysis. The data are presented as the mean $\pm$ SE of separate experiments. ANOVA one-way F-test and the LSD test were determined to be statistically significant at $p < 0.05$.

2.4 RESULTS

2.4.1 Effect of flufenoxuron on biochemical contents of *S. mauritia*

The present study examined the variations in the biochemical indices of haemolymph and testis of the newly moulted treated sixth instar larvae of *S. mauritia* on post-treatment of flufenoxuron at 48, 96, and 144 hours. The concentrations ED₂₅ (0.00015 μ g) and ED₅₀ (0.00068 μ g) of flufenoxuron were utilized to determine their effects over varying periods on the biochemical components of the *S. mauritia* testicular and haemolymph profile.

2.4.1.1 Effect of flufenoxuron on haemolymph and testicular carbohydrate concentration of *S. mauritia*

Treatment of final instar larvae with two different effective doses of flufenoxuron adversely affected the total carbohydrate concentration of haemolymph and testis. There was a significant reduction in carbohydrate concentration in a dose-dependent way (Fig 1).

After 48 hours of treatment with ED₂₅ (0.00015 μ g) and ED₅₀ (0.00068 μ g) doses, there were notable effects on the haemolymph carbohydrate concentration. The concentrations dropped significantly to 8.75 ± 0.21 μ g/ μ l and 2.67 ± 0.39 μ g/ μ l respectively, compared to the

control group at $23.57 \pm 0.92 \mu\text{g}/\mu\text{l}$. Similar reductions were observed after 96 hours of treatment. The lower dose of flufenoxuron ($0.00015 \mu\text{g}$) led to a concentration of $13.57 \pm 0.82 \mu\text{g}/\mu\text{l}$, while the higher ED_{50} dose resulted in a more drastic decrease of $4.26 \pm 0.43 \mu\text{g}/\mu\text{l}$, which is highly significant compared to the control at $17.27 \pm 0.16 \mu\text{g}/\mu\text{l}$ (Fig 1). Further, at 144 hours, the total carbohydrate concentration increased as compared to both 48 hours and 96 hours. However, there were also significant reductions noted with ED_{25} and ED_{50} concentration, of carbohydrate $31.93 \pm 0.19 \mu\text{g}/\mu\text{l}$ and $26.42 \pm 0.25 \mu\text{g}/\mu\text{l}$ respectively, which was far less from the control groups, $48.80 \pm 1.8 \mu\text{g}/\mu\text{l}$ and $45.51 \pm 2.4 \mu\text{g}/\mu\text{l}$.

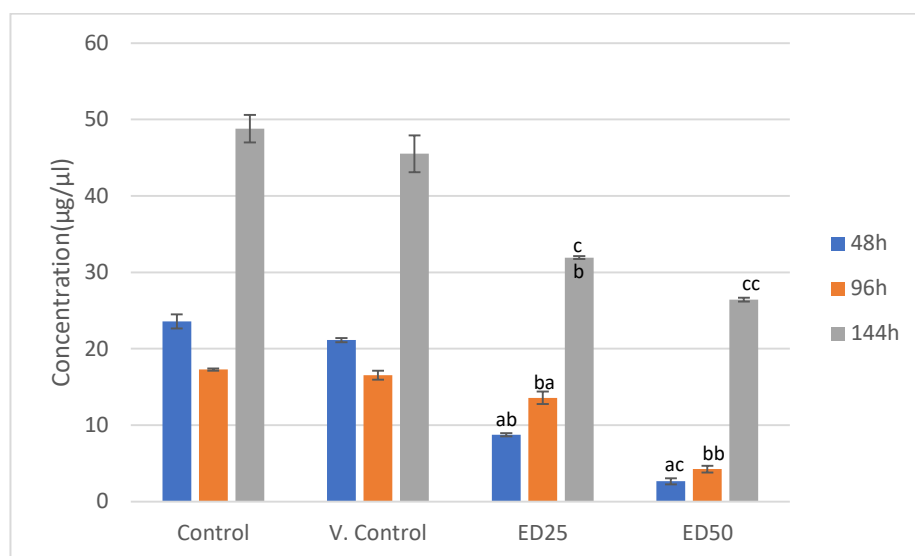


Figure 1: Haemolymph carbohydrate concentration of flufenoxuron treated sixth instar larvae of *S. mauritia*.

The data expressed in the mean $\pm$ SE values of four independent experiments. Statistical differences were considered significant by one-way ANOVA (the same letters do not differ significantly at $p < 0.05$).

The analysis of total carbohydrate concentration following treatment with two different concentrations of flufenoxuron reveals a significant reduction in testicular carbohydrate content (Fig 2). Marked differences were observed at 48-, 96-, and 144-hours post-treatment. Flufenoxuron ED₂₅ and ED₅₀ concentrations led to a notable decrease in carbohydrate concentration, with the effect being more pronounced with the higher ED₅₀ dose.

The data depicted in Figure 2, reveals that after 48 hours of treatment, the carbohydrate concentration in larval testes following administration of ED₂₅ and ED₅₀ doses of flufenoxuron decreased by 22.27 ± 1.25 µg/µl and 17.84 ± 3.87 µg/µl, respectively, compared to controls 31.36 ± 0.34 µg/µl and 30.79 ± 0.23 µg/µl. After 96 hours of exposure, two concentrations (ED₂₅ and ED₅₀) of flufenoxuron induced a significant decrease in testicular carbohydrate levels of 24.2 ± 0.68 µg/µl and 17.61 ± 0.68 µg/µl relative to controls.

At 144 hours of treatment, a rapid and considerable reduction in carbohydrate concentration was observed at 8.97 ± 0.9 µg/µl and 3.4 ± 1.2 µg/µl with ED₂₅ and ED₅₀ concentrations. In the present study, both effective doses of flufenoxuron in *S. mauritia* significantly reduced carbohydrate levels in haemolymph and testis.

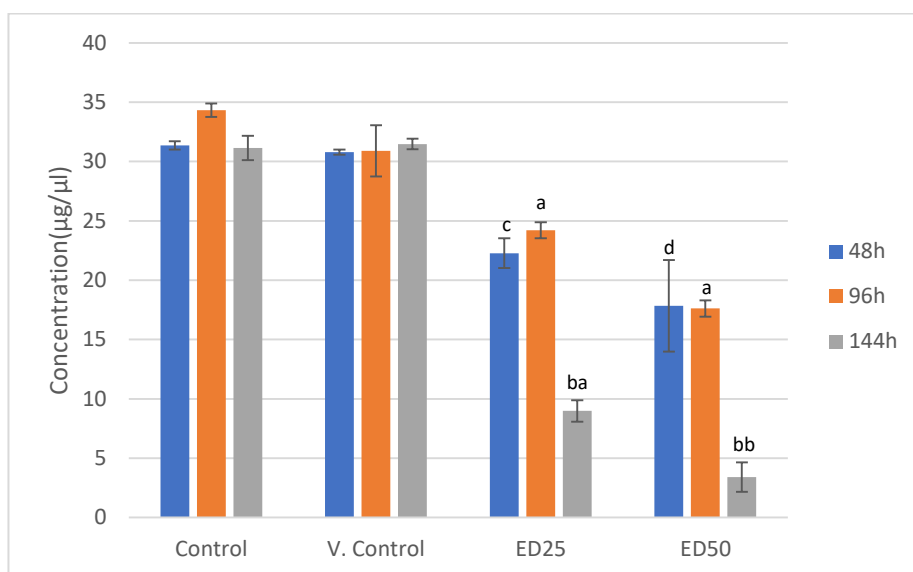


Figure 2: Testis carbohydrate concentration of flufenoxuron treated sixth instar larvae of *S. mauritia*.

The data expressed in the mean $\pm$ SE values of four independent experiments. Statistical differences were considered significant by one-way ANOVA (the same letters do not differ significantly at $p < 0.05$).

2.4.1.2 Effect of flufenoxuron on haemolymph and testicular lipid concentration of *S. mauritia*

The impact of flufenoxuron on the haemolymph and testicular lipid profile in *S. mauritia* was assessed following treatment with ED₂₅ (0.00015µg) and ED₅₀ (0.00068µg) concentrations at 48, 96, and 144-hours intervals. All treated concentrations resulted in a decrease in lipid levels compared to controls at each time interval. The data presented in Fig 3 reveal the total lipid concentration in the haemolymph after 48 hours was 295.17 ± 2.26 µg/µl and 292 ± 1.45 µg/µl in the control and vehicle control groups. Post-treatment with ED₂₅ and ED₅₀ concentrations of flufenoxuron, showed a significant

reduction in lipid concentration of $125.80 \pm 1.44 \mu\text{g}/\mu\text{l}$ and $93.58 \pm 5.1 \mu\text{g}/\mu\text{l}$.

Similarly, after 96 hours of treatment, a significant decrease in lipid concentration was noted in the treated groups compared to the control groups, with values of $189.3 \pm 0.75 \mu\text{g}/\mu\text{l}$ and $197.23 \pm 0.41 \mu\text{g}/\mu\text{l}$ with a decrease of $164.61 \pm 0.36 \mu\text{g}/\mu\text{l}$ and $126.12 \pm 2.1 \mu\text{g}/\mu\text{l}$, respectively. Notable variations are noted after 144 hours of ED₂₅ and ED₅₀ flufenoxuron treatment. The lipid levels significantly declined at $141.68 \pm 0.91 \mu\text{g}/\mu\text{l}$ and $110.96 \pm 0.52 \mu\text{g}/\mu\text{l}$, respectively, compared to $247.23 \pm 0.76 \mu\text{g}/\mu\text{l}$ and $267.63 \pm 1.37 \mu\text{g}/\mu\text{l}$ in the control and vehicle control group.

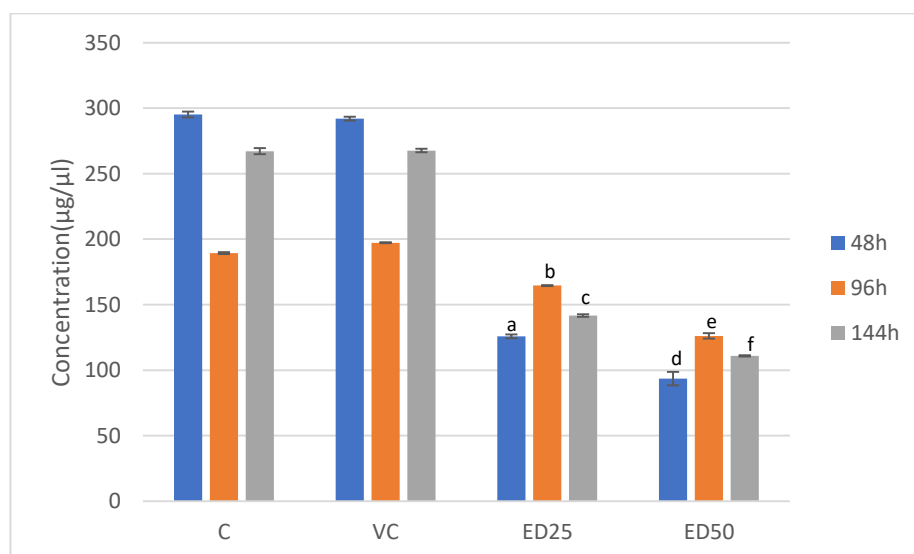


Figure 3: Haemolymph lipid concentration of flufenoxuron treated sixth instar larvae of *S. mauritia*.

The data expressed in mean $\pm$ SE values of four independent experiments. Statistical differences were considered significant by one-way ANOVA (the same letters do not differ significantly at $p < 0.05$).

The effective doses of flufenoxuron led to a significant decrease in lipid concentration in the testes of sixth instar larvae of *S. mauritia* after 48, 96, and 144 hours of treatment (Fig. 4). In the control groups, the total lipid concentration after 48 hours of treatment was 212.71 ± 0.25 and 218.54 ± 0.57 but significantly reduced following treatment with ED_{25} and ED_{50} to 173.84 ± 0.6 and 168.01 ± 0.56 , respectively.

Similarly, after 96 hours of treatment, testicular lipid concentration showed a notable decrease to 169.67 ± 1.13 and 138.13 ± 0.66 compared to the control (244.5 ± 1.57). A similar pattern was observed after 144 hours, where the lipid amount significantly decreased with ED_{25} concentration, with a more pronounced reduction at ED_{50} compared to the controls (Fig. 4).

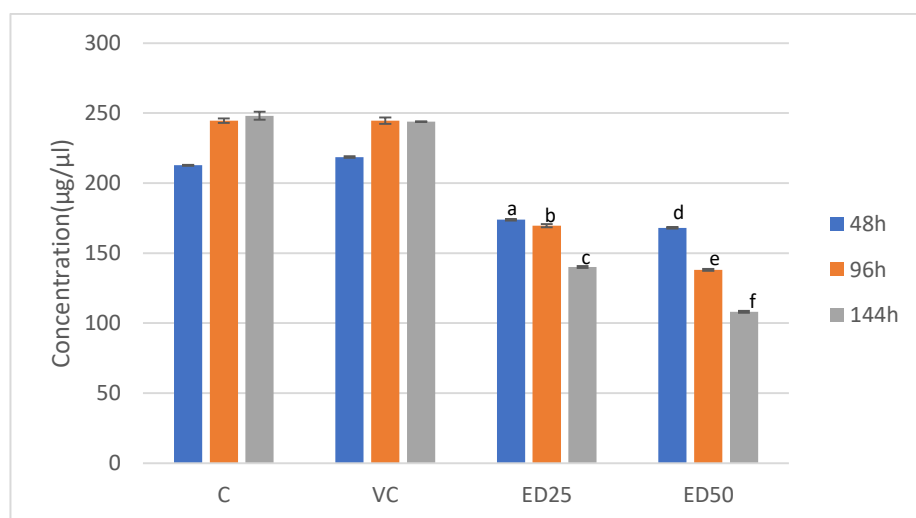


Figure 4: Testis lipid concentration of flufenoxuron treated sixth instar larvae of *S. mauritia*.

The data expressed in the mean $\pm$ SE values of four independent experiments. Statistical differences were considered significant by one-way ANOVA (the same letters do not differ significantly at $p < 0.05$).

2.4.1.3 Effect of flufenoxuron on haemolymph and testicular total protein concentration

The impact of ED₂₅ (0.00015µg) and ED₅₀ (0.00068µg) doses of flufenoxuron significantly affected the total protein concentration in the haemolymph and testicular protein levels of *S. mauritia* (Fig. 5 & 6). The total soluble protein levels in the haemolymph after 48 hours were 18.51±0.18 µg/µl and 19.45±0.22 µg/µl in the control and vehicle control groups. Following treatment with the chitin synthesis inhibitor, no significant difference in total protein levels was observed compared to controls. However, after 96 hours, a substantial reduction in protein levels was evident in larvae treated with ED₂₅ and ED₅₀ doses of flufenoxuron, measuring 15.43±0.07 µg/µl and 18.02±0.26 µg/µl, respectively.

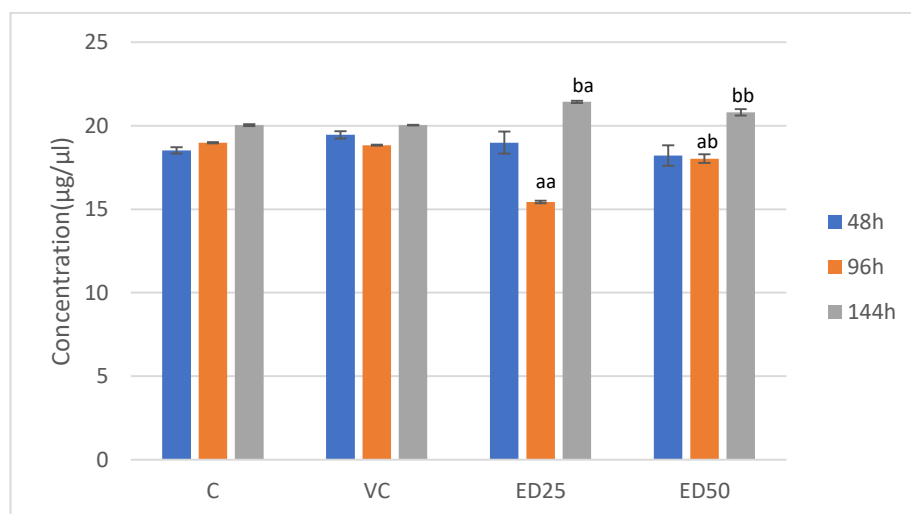


Figure 5: Haemolymph protein concentration of flufenoxuron treated sixth instar larvae of *S. mauritia*.

The data expressed in the mean ± SE values of four independent experiments. Statistical differences were considered significant by one-way ANOVA (the same letters do not differ significantly at $p < 0.05$).

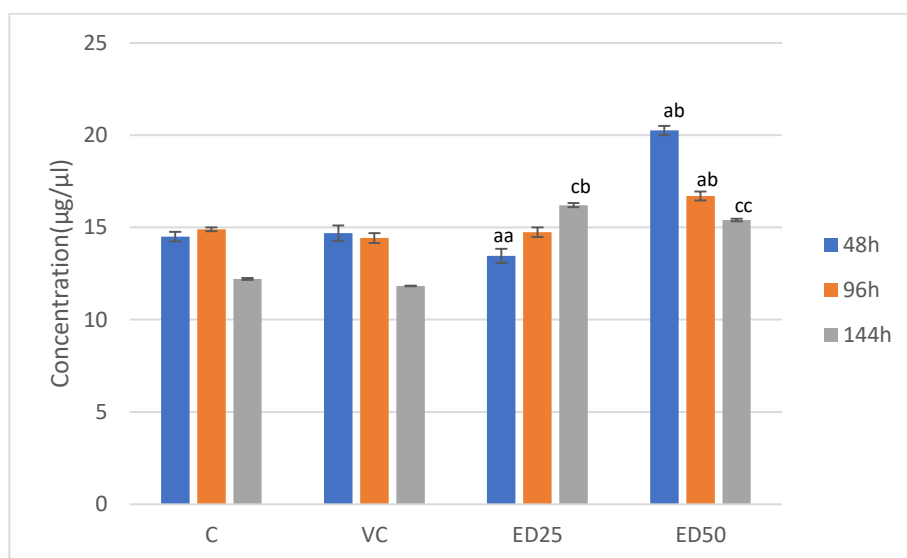


Figure 6: Testis protein concentration of flufenoxuron treated sixth instar larvae of *S. mauritia*.

The data expressed in the mean $\pm$ SE values of four independent experiments. Statistical differences were considered significant by one-way ANOVA (the same letters do not differ significantly at $p < 0.05$).

The observations at 144 hours post-treatment showed a reverse trend, with a significant increase in haemolymph protein concentration of 21.42 ± 0.06 $\mu\text{g}/\mu\text{l}$ and 20.78 ± 0.19 $\mu\text{g}/\mu\text{l}$ for ED₂₅ and ED₅₀ doses, respectively, compared to controls. Thus, flufenoxuron demonstrated a noticeable but slight impact on haemolymph protein concentration in *S. mauritia* larvae.

Quantitative analysis of testicular protein levels on post-treatment of flufenoxuron on sixth instar day 0 larvae at 48, 96, and 144 hours revealed that effective doses of flufenoxuron significantly increased the protein content compared to controls (Fig. 6). After 48 hours of treatment, the testicular protein concentration in the ED₂₅ group decreased significantly. In contrast, it led to a notable increase in the

ED₅₀ treated group. At 96 hours post-treatment, a significant change was observed in the ED₅₀ group, with a concentration of 16.69 ± 0.24 µg/µl compared to controls. Subsequently, after 144 hours of post-treatment, a significant increase in testicular protein levels was observed at 16.20 ± 0.11 µg/µl and 15.39 ± 0.06 µg/µl for the ED₂₅ and ED₅₀ groups, as compared to the control group 12.20 ± 0.05 µg/µl.

2.4.2 Electrophoretic SDS haemolymph protein profile of *S. mauritia*

The SDS-PAGE analysis of haemolymph proteins of ED₂₅ (0.00015µg) and ED₅₀ (0.00068µg) flufenoxuron-treated sixth instar larvae of *S. mauritia* after 48, 96 and 144 hours and control samples revealed distinct variations in the expression of different proteins (Fig. 7). The electrophoretogram reveals significant differences in the percentage of protein concentration and the bands within the middle-range proteins (61 kDa to 77 kDa) after 48 hours of flufenoxuron treatment. Notably, the expressions of the bands at 71 kDa and 77 kDa were inhibited, while the expressions at 61 kDa showed a higher rate. Additionally, the band at 48 kDa was not expressed.

At 96 hours, relatively less expressed haemolymph protein bands were observed at 182 kDa and 250 kDa and in the middle range (61 kDa to 77 kDa) in individuals treated with ED₂₅. Conversely, haemolymph from individuals treated with ED₅₀ showed a slight increase in protein band intensity compared to control (Fig. 8).

After 144 hours of treatment, bands corresponding to high-range proteins (182 kDa, 250 kDa) and middle-range proteins (55 kDa, 61

kDa, and 71 kDa,) displayed an increase in band intensity in Lane 11 and 12. Additionally, noticeable changes were observed in the other two bands (22 kDa, 35 kDa) compared to the control.

The intensity of each band of *S. mauritia* haemolymph is graphically represented in Figure 8. From the graph, we can clearly see the band intensity variations with respect to the concentration of flufenoxuron, exposure time of flufenoxuron and molecular weight of each band. There was a significant increase in band intensity of 250 kDa and 182 kDa at 48h after the treatment with ED₅₀ concentration. The expression of 77 kDa and 71 kDa was considerably reduced compared to the controls. After 96h, different grades of protein band intensity were observed with ED₂₅ and ED₅₀. The bands, 250 kDa, 182 kDa, 77 kDa, 71 kDa, 61 kDa and 55 kDa, decreased their intensity at ED₂₅ concentration and an increase at ED₅₀ concentration. At 144h after the flufenoxuron treatment, the band intensities of 250 kDa, 67 kDa, 65 kDa, 55 kDa and 12 kDa showed a significant increase in band intensity with both flufenoxuron concentrations.

The protein profiling analysis was validated using a dendrogram. The dendrogram represented the distance based on the band intensity of each band of haemolymph proteins (Fig 8). From the dendrogram, it is clear that the variations in band intensity with respect to the molecular weight of haemolymph protein were found to vary highly with the concentration of flufenoxuron applied. Clearcut variations exist in the flufenoxuron-treated and control/vehicle control groups of haemolymph protein samples. The dendrogram was initially divided into two major clades. The vehicle control and control groups showed

similar expression profiles, which come under the first major clade and vice versa. The second significant clades consist of flufenoxuron-treated groups. Further, few control and vehicle control groups show a close relationship with pesticide-treated groups, indicating that both control/vehicle control and pesticide-treated molecular markers show similar expressions (i.e., band intensity). Important to note that the pesticide concentrations were irregularly altering the band intensity of selected molecular weight. ED₂₅-250, ED₅₀-250, and ED₅₀-182 showed high levels of expression variations compared to other treated and control groups. Similarly, ED₂₅-182, ED₂₅-77, ED₂₅-71 and ED₂₅-61 showed variations in expression of protein bands compared to control groups. The general band intensity relationships of the individual protein band in the SDS-PAGE were noted in the dendrogram; in the case of 250 kDa, 77 kDa and 71 kDa protein bands separated based on their relative intensity, control and ED₅₀ branched separately from a common branch and ED₂₅ branched into differently. The distance in these bands is similar to the bar diagram representation of differently expressed band intensity. The control and ED₅₀ bands also branched separately from a shared branch, highlighting their unique characteristics. Interestingly, the ED₂₅ band displayed a distinct branching pattern, diverging into a separate cluster. This signifies the band intensity and expression level differences, indicating significant variance in the underlying protein composition. The dendrogram visually represents these differences in band intensity of *S. mauritia* haemolymph, similar to a bar diagram, illustrating the varying protein expression levels.

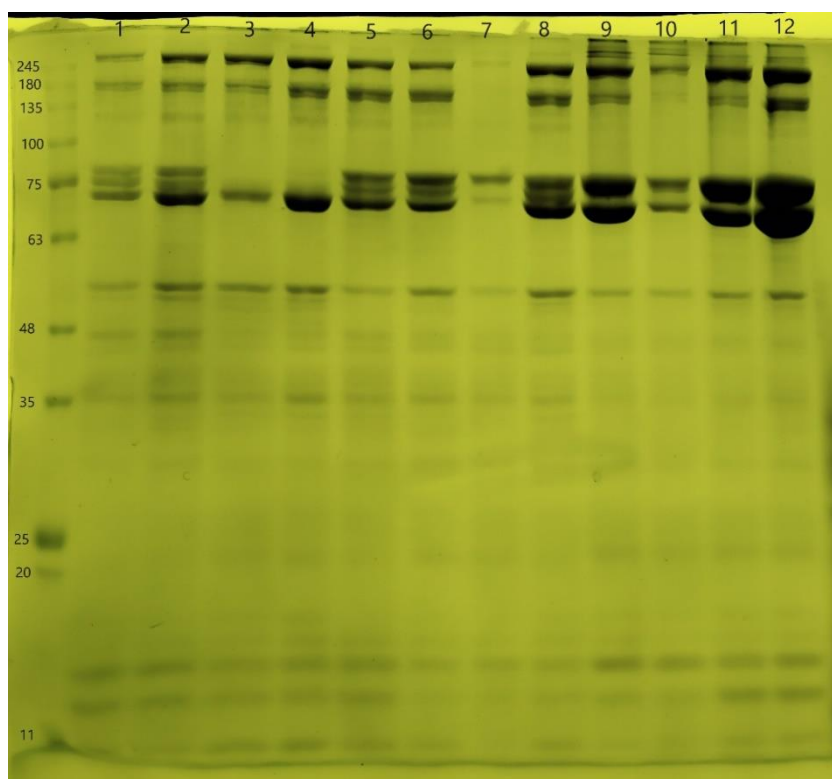


Figure 7: Electrophoretogram of SDS protein profile of *S. mauritia* control and flufenoxuron treated haemolymph samples. Pre-stained protein ladder(Lane 0), Haemolymph sample after 48h of treatment [Control (Lane 1), Vehicle control (Lane 2), Flufenoxuron treated ED₂₅ (Lane 3) and ED₅₀ (Lane 4)]; Haemolymph sample after 96h of treatment [Control (Lane 5), Vehicle control (Lane 6), Flufenoxuron treated ED₂₅ (Lane 7) and ED₅₀ (Lane 8)]; Haemolymph sample after 144h of treatment [Control (Lane 9), Vehicle control (Lane 10), Flufenoxuron treated ED₂₅ (Lane 11) and ED₅₀ (Lane 12)]

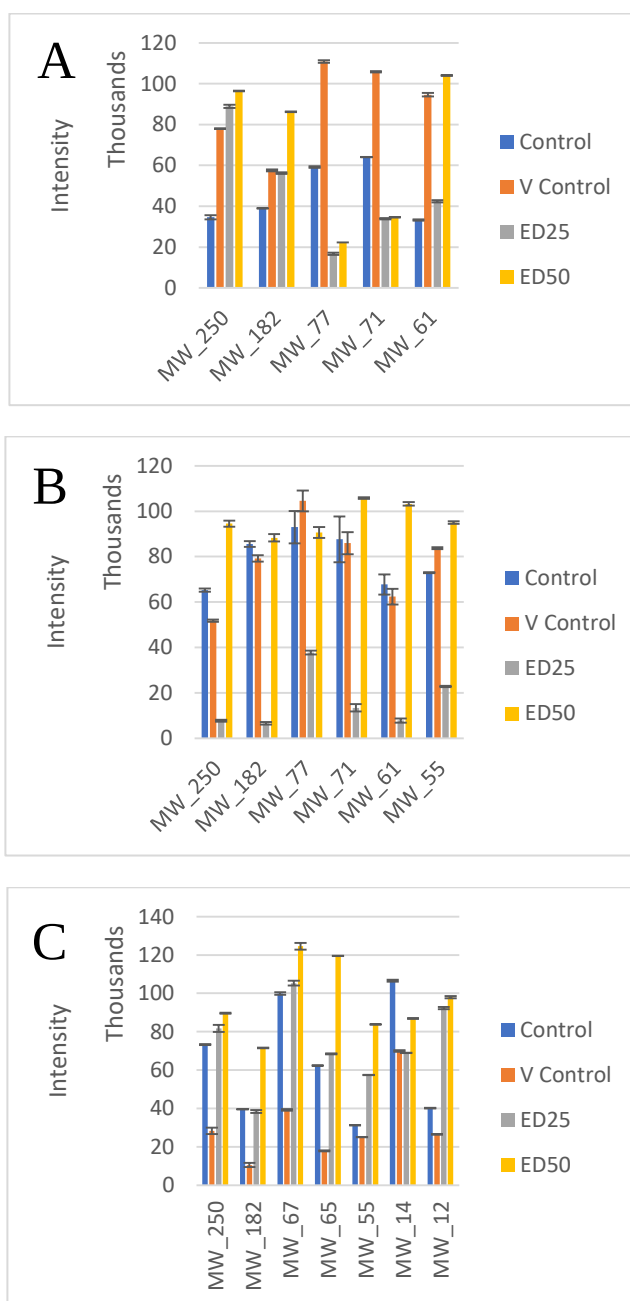


Figure 8: Protein intensity with a specific molecular weight in the control and treated haemolymph protein samples of *S. mauritia* after 48h (A), 96h (B), and 144h (C) of ED₂₅ and ED₅₀ flufenoxuron treatment. The data expressed in the mean $\pm$ SE values of three independent experiments.

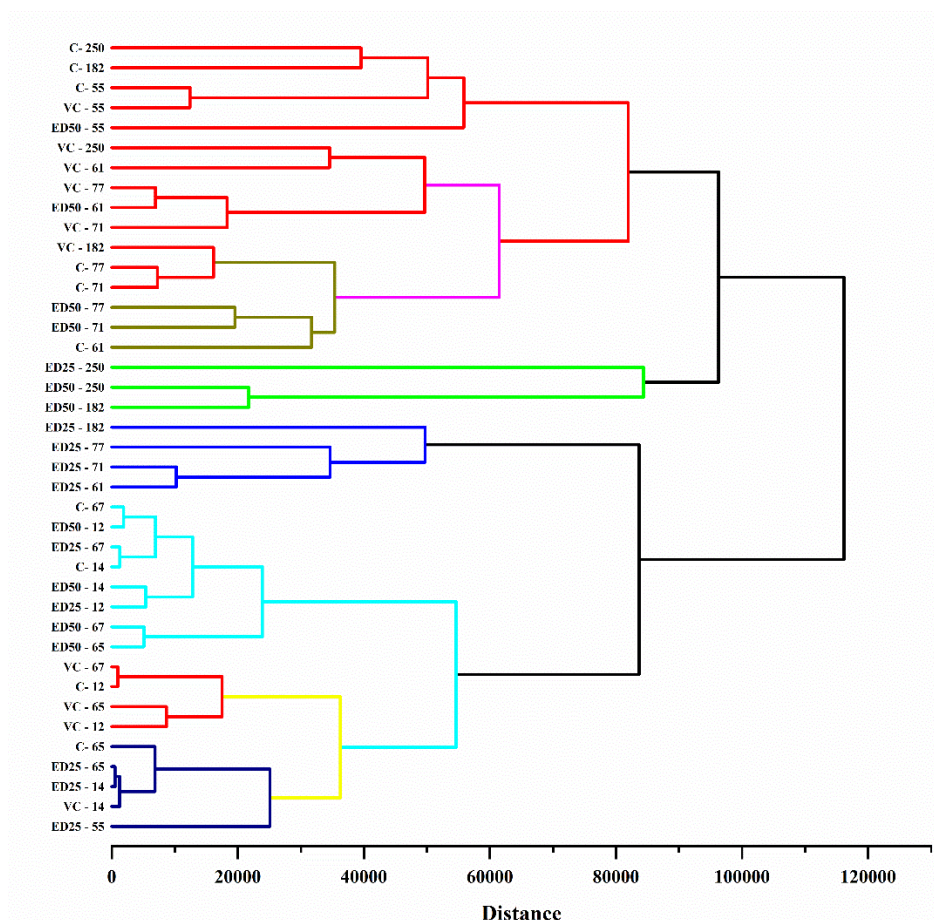


Figure 9: Dendrogram representation of haemolymph protein bands in the control and treated haemolymph protein samples of *S. mauritia* after 48h, 96h, and 144h of ED₂₅ and ED₅₀ flufenoxuron treatment.

2.4.3 Electrophoretic SDS testicular protein profile of *S. mauritia*

The SDS-PAGE analysis of testicular protein profiles following treatment with ED₂₅ and ED₅₀ flufenoxuron on sixth instar day zero larvae after exposure at 48 hours, 96 hours, and 144 hours displayed notable differences compared to control groups (Fig 10). Specifically, significant alterations were observed in testicular proteins, particularly in the range of 65 kDa to 79 kDa.

After 48 hours of treatment with flufenoxuron, a notable decrease in intensity was observed in protein bands with molecular weights of 65 kDa and 79 kDa. Other bands with faint intensity (34 kDa, 33 kDa, 32 kDa, and 28 kDa) were also detected compared to controls.

At 96 hours post-treatment, proteins in the middle range (65 kDa and 79 kDa) were noticed, and their intensity levels gradually increased from the ED₂₅ concentration to the ED₅₀ concentration of flufenoxuron. By 144 hours of treatment, these protein bands exhibited increased intensity, particularly at the ED₂₅ concentration, followed by a decrease at the ED₅₀ concentration. Additionally, in Lane 12, proteins in the molecular weight range of 28 kDa to 34 kDa were expressed compared to other control and treated lanes, with reduced intensity observed in Lane 11.

After the flufenoxuron treatment, the protein band intensity of the 48th hour was drastically decreased by two effective doses of flufenoxuron compared to control. A slight change occurred after 96h of flufenoxuron treatment; the protein band intensity increased with the ED₂₅ dose, and afterwards, the ED₅₀ dose reduced the testicular protein intensity. A similar pattern of intensity shifts was also found in 144h of flufenoxuron treatment (Fig 11).

The intensity of each band of *S. mauritia* testis is represented in Fig 11. From the graph, we can clearly see the band intensity variations in the concentration of flufenoxuron, exposure time of flufenoxuron and molecular weight of each band. After 48h, the 65 kDa protein band showed significant reduction after treatment with both flufenoxuron concentrations. After 96h and 144h, 65 kDa showed a significant increase in band intensity compared to control. 34 kDa, 32 kDa, 28

kDa, and 26 kDa protein bands showed a similar pattern of significant increase in both ED₂₅ and ED₅₀ concentration at 96h and 144h of treatment.

The dendrogram represented the distance between each band based on the molecular weight and band intensity of each band of testicular protein (Fig 12). Colour indicates the closely related group with respect to their molecular weight and band intensity. *S. mauritia* testis protein samples after treatment with flufenoxuron and control/vehicle control groups differ significantly. The dendrogram was initially separated into two major clades; a clear separation was noted between the control and treated groups. The control and vehicle control groups displayed expression profiles that fit into the first major clade and vice versa. A subset of the vehicle control and control groups strongly correlated with the flufenoxuron-treated groups. This suggests that the molecular markers for the pesticide-treated and control vehicle control groups exhibit comparable expression patterns in terms of band intensity. It's important to remember that the band intensity of the chosen molecular weight protein was sporadically changing due to flufenoxuron concentrations. ED₅₀-52, ED₂₅-52, and ED₂₅-47 all exhibited significant expression variances, and they are highly separated from others of the second major clade. Similarly, the band intensity of ED₂₅-79, ED₂₅-65, ED₅₀-79 and ED₂₅-34 clearly separated from the group. Moreover, it was observed that the protein bands with a molecular weight of 65 kDa and 79 kDa demonstrated a significant variance in intensity when compared to the control and treated groups. This distinction in intensity allowed for their clear separation within the dendrogram, illustrating the hierarchical relationship between the bands. Additionally, both the control and the ED₅₀ and ED₂₅ bands

were found to branch separately from a shared branch, further emphasizing their unique characteristics. This observation highlights the variance in band intensity and expression levels, indicative of significant differences in the underlying protein composition. The representation of these differences in band intensity within the dendrogram is comparable to a bar diagram, offering a visual representation of the contrasting levels of protein expression.

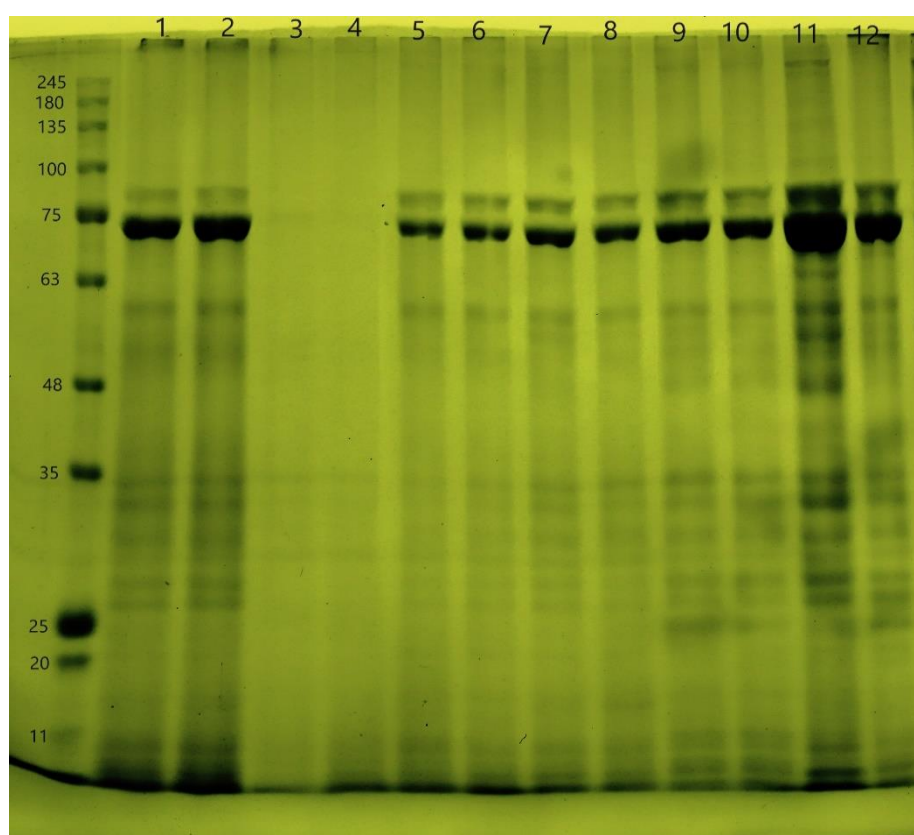


Figure 10: Electrophoretogram of SDS protein profile of *S. mauritia* control and flufenoxuron treated testis samples. Pre-stained protein ladder (0), Haemolymph sample after 48h of treatment [Control (1), Vehicle control (2), Flufenoxuron treated ED₂₅(3) and ED₅₀ (4)]; Haemolymph sample after 96h of treatment [Control (5), Vehicle control (6), Flufenoxuron treated ED₂₅ (7) and ED₅₀ (8)]; Haemolymph sample after 144h of treatment [Control (9), Vehicle control (10), Flufenoxuron treated ED₂₅ (11) and ED₅₀ (12)]

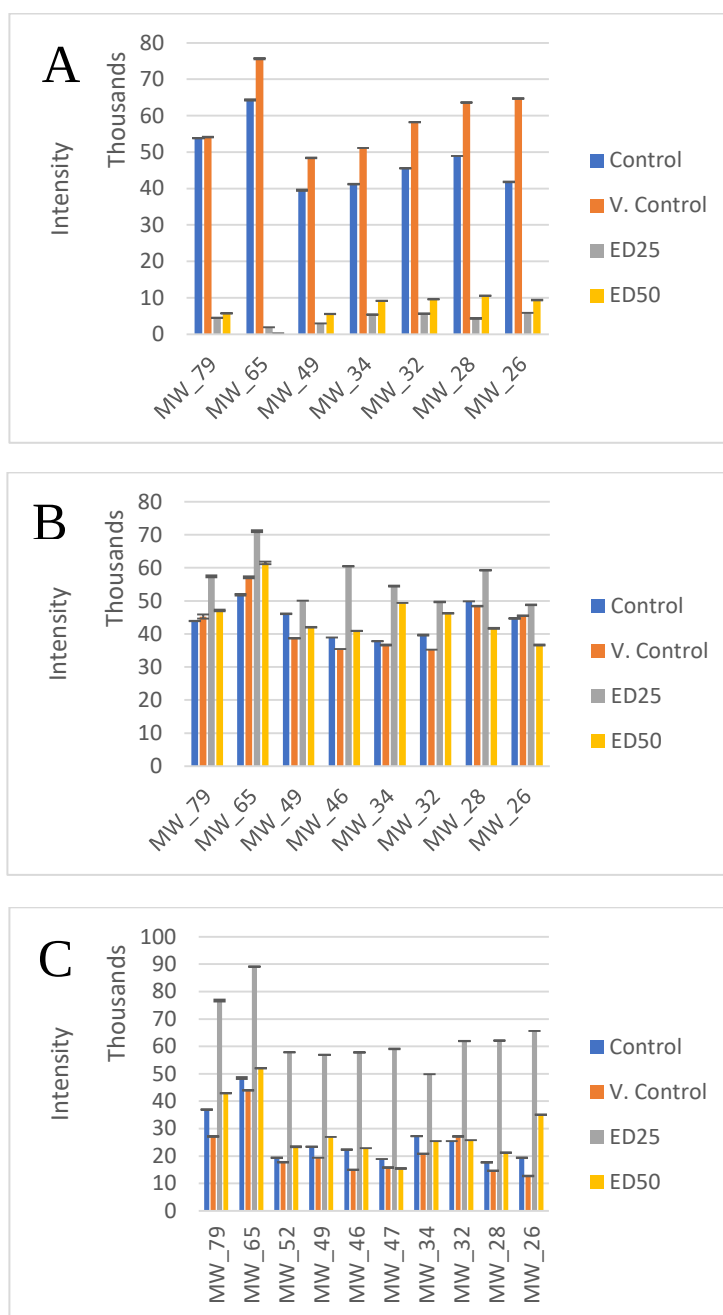


Figure 11: Protein intensity with a specific molecular weight in control and treated testicular protein samples of *S. mauritia* after 48h (A), 96h(B) and 144h(C) of treatment. The data expressed in the mean $\pm$ SE values of three independent experiments.

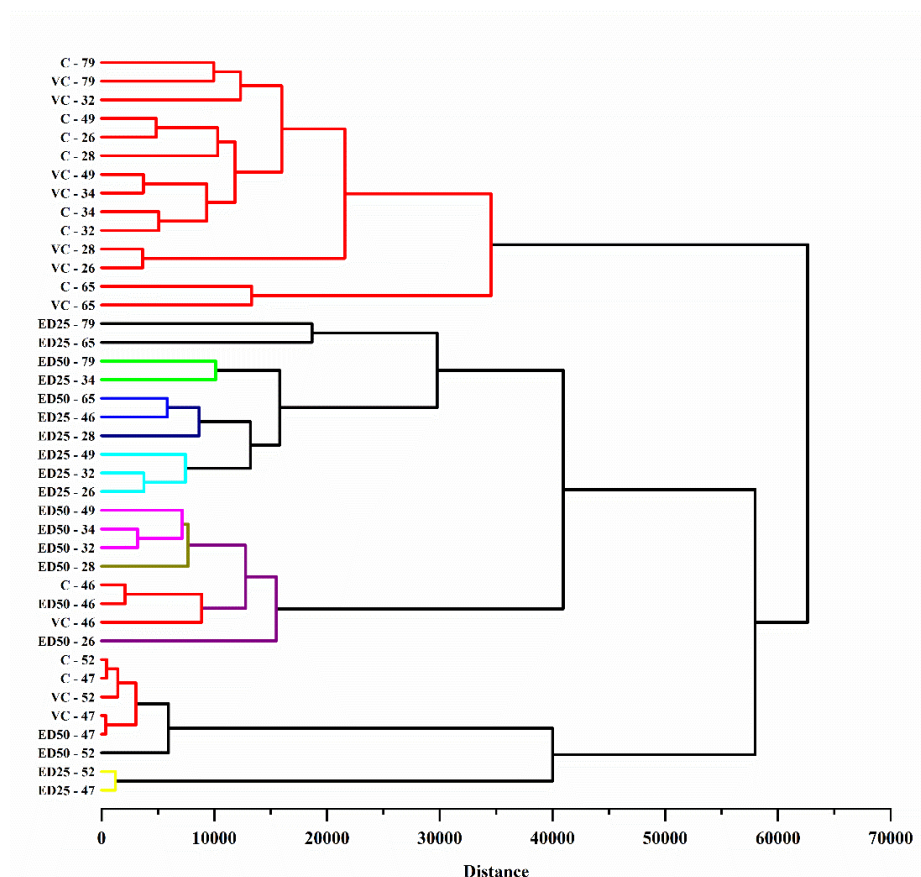


Figure 12: Dendrogram representation of testis protein bands in control and treated testicular protein samples of *S. mauritia* after 48h, 96h, and 144h of ED₂₅ and ED₅₀ flufenoxuron treatment.

2.4.4 Testicular histomorphology of sixth instar larvae of *Spodoptera mauritia*

The testes of *Spodoptera mauritia* larvae (Control) at 48h (day 2), 96h (day 4) and 144h (Pupa) showed different stages of developing germline cysts. In day 2 (48h) and day 4 (96h) sixth instar larvae of *S. mauritia* typically contain four follicles (F1, F2, F3, and F4) in each pair of testes (Plate I: Fig A & B). Each testis contains internal tunica

cells that emit septa. Exterior tunica cells are closely packed, forming an outer epithelium that divides each testis into four testicular follicles (Plate I: Fig A & B). Each follicle has a smaller basal extremity on the concave side and a larger apical extremity on the convex side. Spermatogenesis occurs along the length of the follicle, from the apical to the basal extremity.

The cysts' maturation process during spermatogenesis occurs at the distal-proximal direction within each follicle and is present at different developmental stages of developing sperm cysts. Germ line cells are located at the follicle's distal part, and the proximal part is recognized by spermatocyte cyst cells. Each follicle consists of germ cells and sperm-producing cyst cells, and each cyst is formed by somatic cells. In 48h of the sixth instar larval testis, spermatogonial cysts, spermatocytes cysts, and spermatidal cysts are present, and few elongation spermatids are evident (Plate III: Fig A & B). After 96h, eupyrene spermatids and spermatozoa dichotomy are visible (Plate IV: Fig A & B). In each cyst, only one type of spermatozoa is present, either apyrene or eupyrene, but never both types simultaneously. The spermatid cysts and sperm bundles are placed at the basal follicle extremity, spermatogonia and spermatocyte cysts are found at the apical follicle extremities.

In the prepupal stage (144h), the testes begin to fuse a single yellow organ, and the fusion process is completed in the pupal stage, containing eight follicles coated with a double-layered granular epithelium. The germ cells are organized in cysts in the testis follicular lumen and different positions according to the degree of maturation.

The maturation occurs centripetally. Spermatogonia (SG) is observed in the testis's peripheral region, followed by spermatocytes (SC). There is also observed spermatid cyst (S) next to the spermatocyte, which forms two different cysts that elongate and form specific spermatozoa.

2.4.5 Effect of flufenoxuron on testicular histopathology of sixth instar larvae of *Spodoptera mauritia*

The current study demonstrated that treatment of ED₂₅ (0.00015µg) and ED₅₀(0.00068µg) flufenoxuron after 48, 96, and 144 hours of exposure, on the sixth instar day zero larvae of *S. mauritia*, induced significant histological alterations in the testis. After administering flufenoxuron, the testicular morphology showed structural disorganization in the granular epithelium (E), resulting in altered arrangements of follicles (F) and reduction in follicles (Plate I: Fig: C and D). The findings revealed a significant alteration in testicular follicle formation, characterized by uneven distribution of follicular epithelium. Moreover, several areas exhibited thinning of the epithelial layer, while others showed increased thickening, accompanied by large vacuoles within epithelial cells. Furthermore, fewer follicles were observed in larval stage flufenoxuron-treated ED₂₅ and ED₅₀ (Plate I: Fig C & D) compared to the control groups.

In day 2 (48h) and day 4 (96h) sixth instar larvae of *S. mauritia* each pair of testes typically contain four follicles (F1, F2, F3, and F4) (Plate I: Fig A & B). A noticeable disorganization was observed in the apical and basal extremities (AE & BE) in the treated testes compared to the control group (Plate I: Fig C). Additionally, it was observed that

spermatogonial cells were present within a single follicle (Plate I: Fig D). Furthermore, the chitin synthesis inhibitor disrupted the tunica's organization, as evident in the results compared to the controls. Following treatment with the ED₅₀ dose, shape distortion was observed, rendering it difficult to distinguish the shape and size of follicles. In the control group, spermatogonia were exclusively found at the apical extremity, whereas noticeable changes were evident in the ED₅₀ flufenoxuron-treated larvae (Plate I: Fig D).

Plate II shows that flufenoxuron significantly influences the fusion of the testes and disrupts the formation of the spherical shape (Plate II: Fig: C & D). The epithelium is irregularly formed with varying thickness across different regions, resulting in noticeable structural differences in the treated group. The inner arrangement of follicles is completely disrupted, making it difficult to distinguish individual follicles within the testes. Numerous follicular septa run parallel to the epithelium, trapping spermatogonial cells between them and forming a highly thickened epithelial area (Plate II: Fig D). The testes exhibit the follicular septa containing many vacuolated tunica cells, and the regular fusion of follicular septa is disrupted at the central portion. Additionally, many undifferentiated spermatogonial cells are visible.

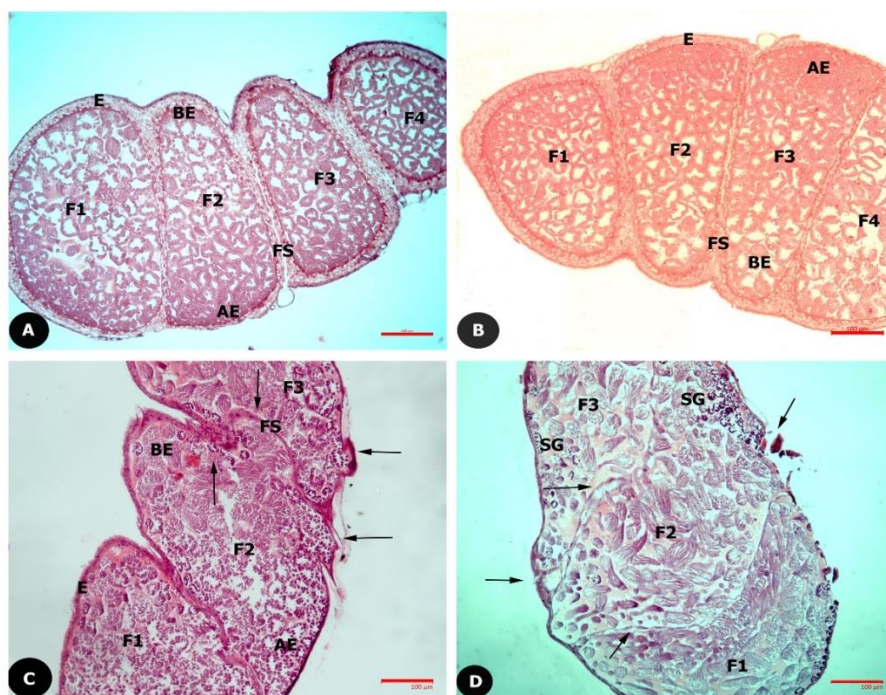


Plate I: Fig: A -D Histo-morphology of larval testis (Scale Bar: 100 μ m): The kidney shape of the testes composed of testicular tunica with Epithelium (E); Four follicles (F1, F2, F3 and F4); Follicular septum (FS); Apical extremity (AE); Basal extremity (BE). Arrow indicates the damage in epithelium and follicular septum.

A: Control

B: Vehicle control

C: Flufenoxuron treated (ED_{25}) after 96 hours: Malformed and reduced number of testicular follicles (F1, F2 & F3) with irregular arrangement in testicular tunica/Epithelium. Irregularly arranged and damaged follicular septum (FS).

D: Flufenoxuron treated (ED_{50}) after 96 hours: Shape distortion in testicular follicles and testicular tunica. Changes are noted in the apical and basal extremities, and spermatogonial cells (SG) are found in both areas.

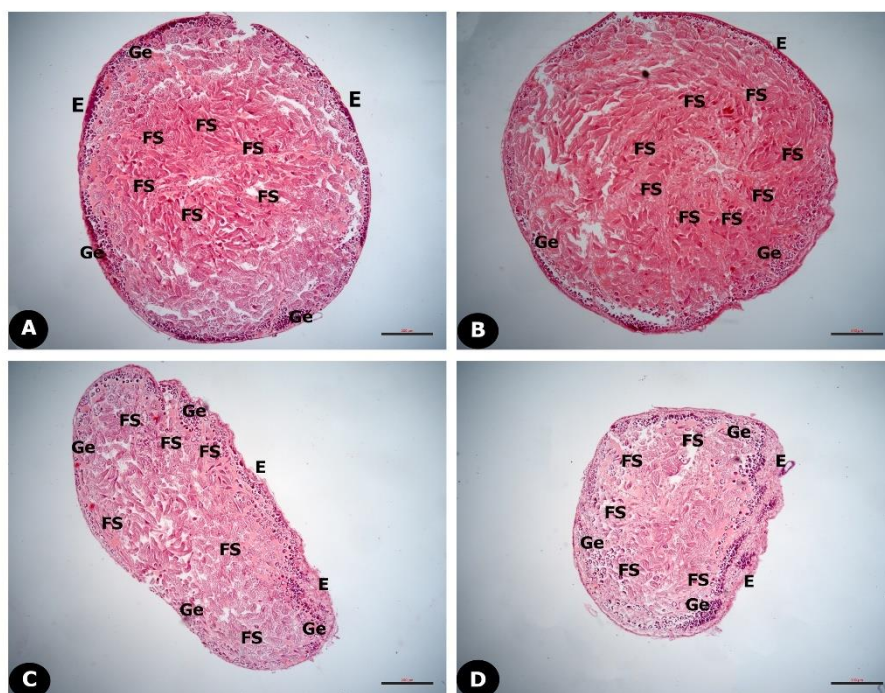


Plate II: Fig: A- D Histo-morphology of pupal testis (Scale Bar: 200 μ m): note the spherical shape of the testes composed of testicular tunica with Epithelium(E); follicular septum (FS); region of germarium (Ge) with spermatogonia (SG); spermatocytes (C) spermatids (T) and spermatozoa (Es)

A: Control

B: Vehicle control

C: Flufenoxuron treated (ED₂₅) after 144 hours: irregularly arranged testicular tunica and Epithelium (E) with uneven thickening and vacuolation found within and vacuolated follicular septa (FS); due to their irregular arrangement, the follicular area is undistinguished.

D: Flufenoxuron treated (ED₅₀): Malformed and abnormally arranged follicle (F) with damaged and highly vacuolated follicular septa (FS). Unevenly thickened testicular tunica and epithelium. Underdeveloped germarium (Ge) and some spermatogonia trapped between abnormally organized follicular septum

The flufenoxuron exposure resulted in pronounced alteration in the testicular cysts with disorganization in the arrangement within the cells. After 48h of treatment (Plate III: Fig C, D, E & F), highly vacuolated germarium are evident in the treated groups. Further, a large number of disorganized spermatocysts and spermatids were also present. Slight malformation was found in eupyrene spermatocyst (Plate III: Fig C, E, and F). Moreover, a noticeable void was seen inside the spermatocyst cells and unevenly arranged germ cells. Elongating spermatocyst also had a highly vacuolated area due to degeneration in the tail portion of developing cells. The follicular septum and outer epithelium were also found with vacuolation due to the irregular arrangement and damage within the epithelial cells.

The damages were more pronounced with higher concentrations of flufenoxuron. Within 96h of flufenoxuron treatment, these damages were more pronounced, and disruption or degeneration inside the spermatid cells was more evident (Plate IV: Fig C, D, E and F). The typical size of both eupyrene and apyrene spermatids significantly decreased, creating space between the cells within the spermatid and the surrounding cyst cells.

The exposure to flufenoxuron resulted in striking alterations in the spermatocyst and spermatozoa, with extreme necrosis and disorganization in the arrangement of the cyst cells. Spermatid cyst cells have a large space between the developing cystic cells and the septa. Significant tissue reduction was also visible inside the spermatids. A reduction in connective tissue also represents the area between each spermatid cyst. Cells in the spermatocyst lost their

normal shape due to damage in the cytoplasm of cells. The result showed a significant decrease in the number of developing spermatids in the proximal region, exhibiting a large amount of cyst-free cellular region and making the uneven distribution of cysts as compared to the controls. The follicular septum contains many vacuoles, which induces structural disorganization of cells within the septa (Plate IV: Fig D). The application of a higher dose (ED_{50}), it was evident that there was an extensive degenerated and necrotic spermatid (Plate IV: Fig E), with malformed eupyrene sperm bundle (EUS).

The examination of the testis revealed the number of degenerated spermatozoa and highly vacuolated follicular septum were evident after 144h of flufenoxuron treatment (Plate V: Fig C, D, E and F). ED_{25} -treated groups were more pronounced with the necrosis of spermatozoa and spermatocytes (Plate V: Fig. C and D). Highly degenerated spermatids (eupyrene) with nuclear heads are noticed, which blocks the further development of spermatozoa. Flufenoxuron post-treatment with ED_{50} evidenced eupyrene and apyrene spermatozoa with severe degeneration and necrosis; therefore, they cannot be easily distinguished (Plate V: Fig E). Moreover, it was evident that few immature spermatogonia lie between the follicular septum and epithelium, which forms a large epithelial area. The epithelium with many vacuoles was obvious in this stage (Plate V: Fig D).

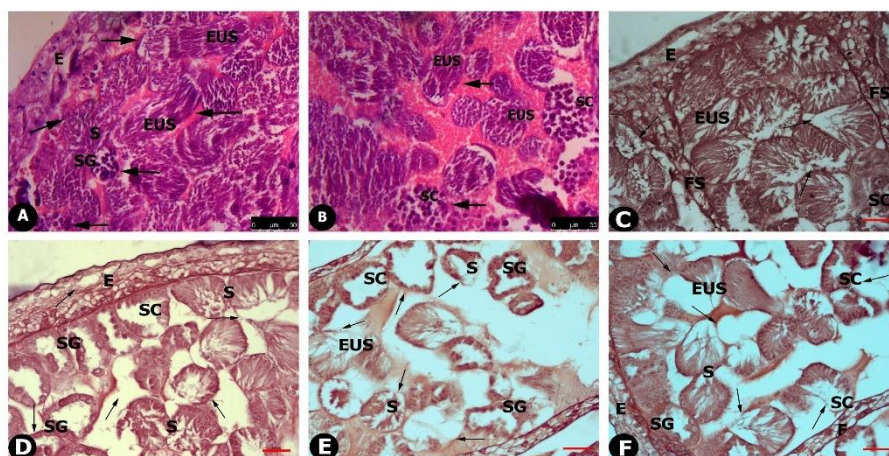


Plate III: Fig A- F. Histological sections of *S. mauritia* testis after 48h of flufenoxuron treatment. Arrow indicates the damage in epithelium, follicular septum, and spermatocyst.

A & B: Control & Vehicle control (Sections of *S. mauritia* testis after 48h)

C & D: Flufenoxuron treated (ED_{25}): note the rupture in tunica cell and septa (FS), with marked irregularity; necrotic eupyrene spermatid (EUS) and spermatocyst (SC) with irregularly arranged cellular components and large cavity.

E & F: Flufenoxuron treated (ED_{50}): necrotic spermatocytes (SC) with a large cavity inside; visible damage in spermatocyte cyst cells; large vacuolated area developed within the elongating spermatid cyst (S) due to degeneration of tail portion; Epithelium (E) and follicular septa (FS) found with vacuolated cells and degenerated spermatogonia (SG)

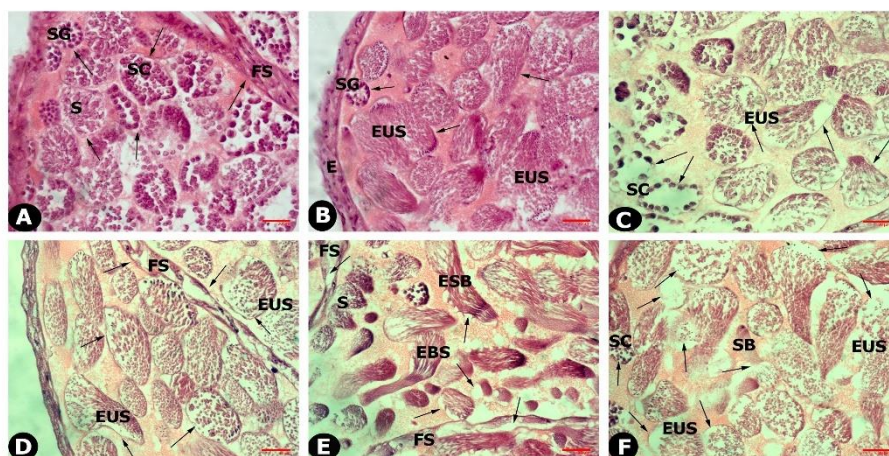


Plate IV: Fig A- F. Histological sections of *S. mauritia* testis after 96h of flufenoxuron treatment.

A & B: Control & Vehicle control (*S. mauritia* testis section at 96 hours)

C & D: Flufenoxuron treated (ED_{25}): Malformed arrangement within developing spermatids (S); visible gaps found with different intensity; eupyrene spermatid (EUS) with vacuolated tail end; number and arrangement of cells within spermatocyte cyst cells were found defective; follicular septum (FS) with vacuolated cells.

E & F Flufenoxuron treated (ED_{50}): Highly vacuolated spermatids(S), spermatocyte (SC) and eupyrene spermatids and degenerated cellular components are visible; vacuolated area with degeneration of cells within the cyst; thin and vacuolated follicular septum (FS) formed.

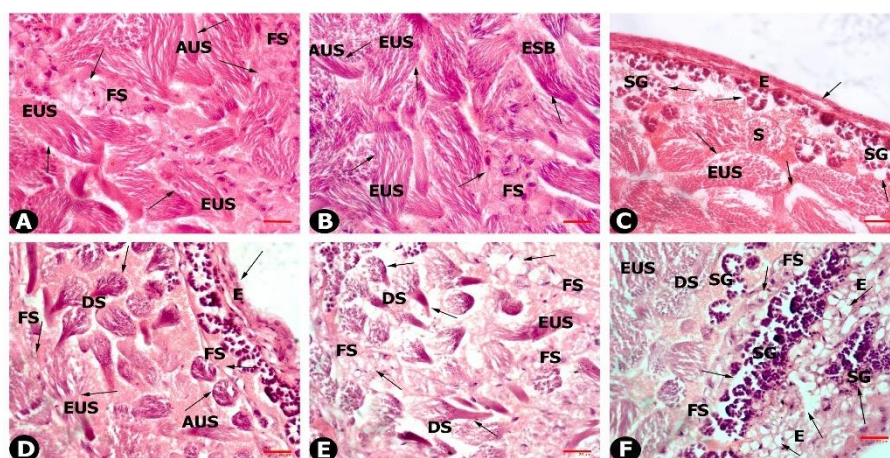


Plate V: Fig A-F. Histological sections of *S. mauritia* testis after 144h of flufenoxuron treatment.

A & B: Control & Vehicle control

C & D: Flufenoxuron treated (ED_{25}): Degenerated spermatids (DS) with large vacuolated area; necrotic eupyrene and apyrene spermatids (EUS & AUS) are evident; narrowing of follicular septa (FS) and that run nearby the epithelium, spermtogonial cysts trapped in between.

E & F: Flufenoxuron treated (ED_{50}): Highly degenerated spermatids (DS) and spermatozoa (EUS) with many empty spaces within the cyst. Vacuolated Epithelium and necrotic spermatogonia (SG) present between the vacuolated epithelium; follicular septum (FS) highly vacuolated.

2.4.6 Semiquantitative scoring of histopathological examinations of testis treated with flufenoxuron

The histopathological studies data revealed no notable changes in the control and vehicle control groups of the sixth instar larval testis of *S. mauritia*. In contrast, tissue degeneration in the *S. mauritia* testis was observed on post-treatment of two different doses (ED_{25} , ED_{50}) of flufenoxuron. Table 1 depicts the semiquantitative scoring of histopathological examinations from different groups (controls and

treated) of testicular tissue associated with flufenoxuron application in the sixth instar stage of *S. mauritia*.

Table 1: Semiquantitative scoring of histopathological examinations

	(Hours)	Treatment groups			
		Control	V. Control	ED ₂₅	ED ₅₀
Irregular spermatogonia with cavity	48	-	-	++	+++
	96	-	-	+++	+++
	144	-	-	+	++
Early Spermatocyst with necrosis	48	-	-	+	++
	96	-	-	++	+++
	144	-	-	+	+++
Late spermatocyte with necrosis	48	-	-	++	+++
	96	-	-	+	++
	144	-	-	+	++
Vacuolation in outer epithelium	48	-	-	+	++
	96	-	-	++	+++
	144	-	-	+	+++
Vacuolation in follicular epithelium	48	-	-	++	++
	96	-	-	+++	+++
	144	-	-	++	+++
Early spermatid cyst with necrosis	48	-	-	+	+
	96	-	-	+	++
	144	-	-	+	++
Elongating spermatid cyst with necrosis	48	-	-	+	+
	96	-	-	+	++
	144	-	-	++	+++
Degenerated apyrene sperm bundle	48	-	-	-	-
	96	-	-	+	++
	144	-	-	++	+++
Degenerated eupyrene sperm bundle	48	-	-	-	-
	96	-	-	+	++
	144	-	-	+++	+++

Note: -none, +mild, ++moderate, +++severe

2.4.7. Effect of Flufenoxuron on impairment of dimorphic spermiogenesis of *S. mauritia*

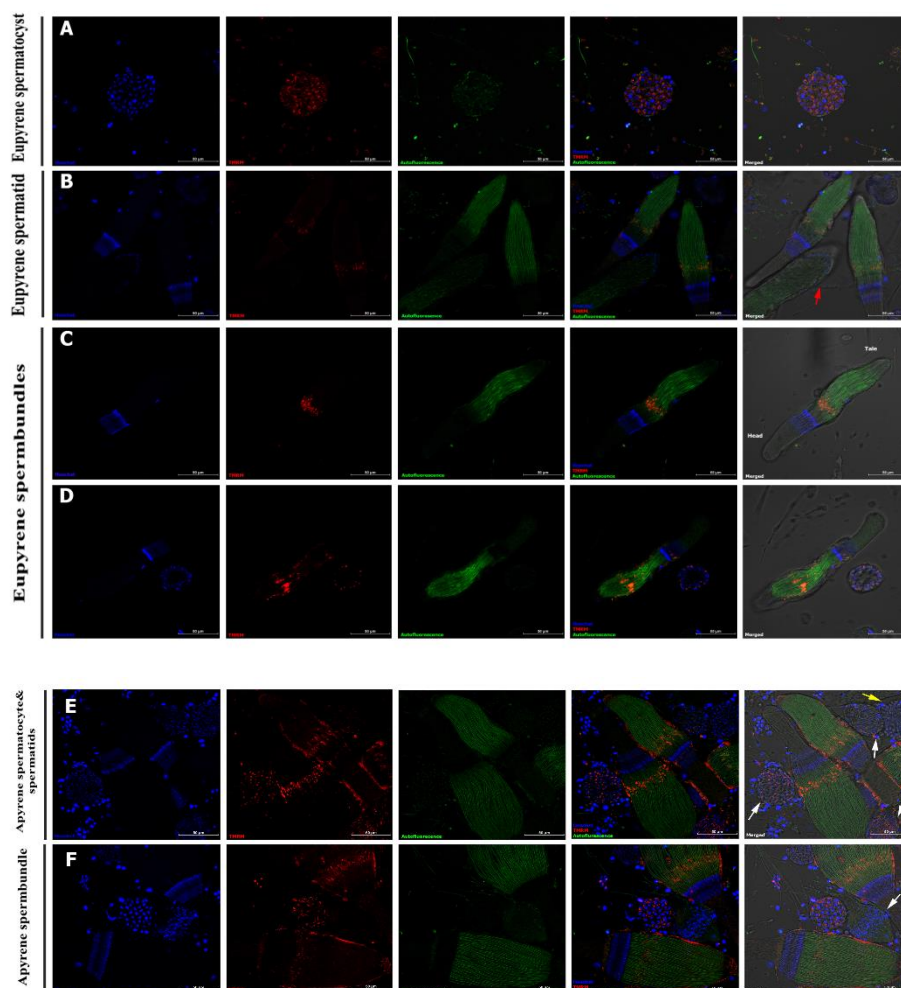


Plate-VI: Fig A- F. Laser scanning confocal microscopy of spermatocyst and spermatozoa of *S. mauritia* (Control) showing double labelling with Hoechst-33342 (blue), TMRM (red) and FITC (green) A: Eupyrene spermatocysts; B: elongating eupyrene spermatid (Red arrow); C: elongating eupyrene spermatozoa; D: eupyrene sperm bundle and spermatogonia cell; E: Apyrene spermatocyst (Yellow arrow), elongating apyrene spermatozoa (white arrow); F: Apyrene sperm bundle (White arrow)

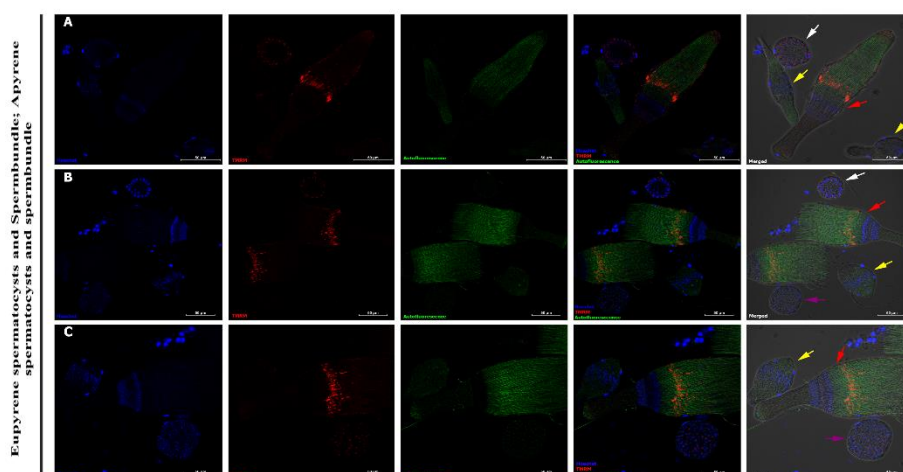


Plate VII: Fig A- C. Laser scanning confocal microscopy of spermatocyst and spermatozoa of *S. mauritia* after treatment with flufenoxuron (ED_{25}) showing double labelling with Hoechst-33342 (blue), TMRM (red) and FITC (green). A: Eupyrene spermatozoa (Red arrow), Apyrene spermatozoa (Yellow arrow), eupyrene spermatocyst (White arrow); B: eupyrene spermatocyte (White arrow), apyrene spermatocyst (Pink arrow) apyrene spermatozoa (Yellow arrow), eupyrene spermatozoa (Red arrow); C: apyrene spermatocyst (Pink arrow), apyrene spermatozoa (Yellow arrow), eupyrene sperm bundle (Red arrow)

The present study examined the impact of flufenoxuron on the dichotomous spermiogenesis of *S. mauritia*. In order to investigate the effects of two different concentrations (ED_{25} and ED_{50}) of flufenoxuron on spermiogenesis in sixth instar larvae of *Spodoptera mauritia*, testes were dissected from day one pupa and analyzed for abnormalities in dichotomic sperm. Fluorescent staining utilizing confocal microscopy was employed to assess the changes in sperm structure and development.

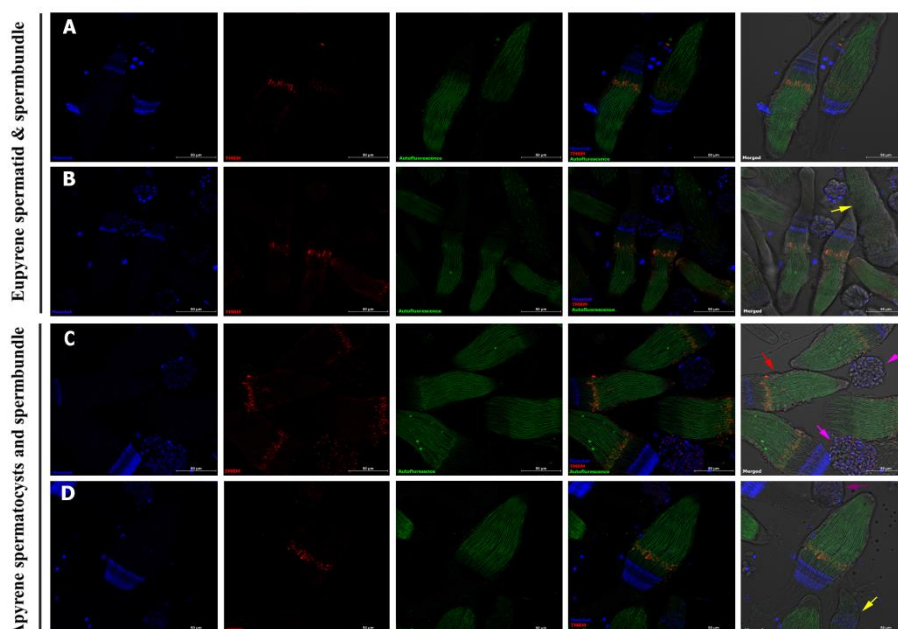


Plate VIII: Fig A- D. Laser scanning confocal microscopy of spermatocyst and spermatozoa of *S. mauritia* after treatment with flufenoxuron (ED_{50}) showing double labelling with Hoechst-33342 (blue), TMRM (red) and FITC (green). A: eupyrene sperm bundles; B: eupyrene sperm bundles and spermatid (Yellow arrow); C: apyrene spermatocyte (Pink arrow), eupyrene sperm bundle (Red arrow); D: Apyrene spermatocyst (Pink arrow), eupyrene sperm bundle (Red arrow), and apyrene spermatozoan (Yellow arrow).

Examination of the normal (control) pupal testis of *S. mauritia* revealed that the pupal instar is a crucial period marked by eupyrene and apyrene spermatozoa formation. During the spermiogenesis of *S. mauritia* two forms of spermatids (Eupyrene and apyrene) are identified. Eupyrene spermatids exhibit a densely packed nuclear head, while apyrene spermatids display a nucleus arranged randomly (Plate VI: Figs B & E). Further, developing eupyrene and apyrene spermatozoa also show distinct morphologies and size differences (Plate VI: Figs D & F). Distinct developmental trajectories are observed for each spermatozoon starting from the spermatocyst stage.

Eupyrene spermatocytes are characterized by closely packed cells with large, round nuclei, whereas apyrene spermatocytes exhibit randomly arranged, small, rounded nuclei (Plate VI: Fig A & E). Subsequently, eupyrene spermatocytes undergo elongation and transform into spermatids, readily recognizable by their uniform arrangement within the follicles, with head regions oriented in the same direction (Plate VI: Fig B). Conversely, during elongation, the nuclei in apyrene spermatocytes initially display a random arrangement, gradually shifting towards the tail (Plate VI: Fig E). Further elongation results in the formation of structurally distinct eupyrene and apyrene spermatozoa (Plate VI: Fig D & F).

After 144 hours of treatment, the larva undergoes pupation, enabling clear identification of the developing stages of eupyrene and apyrene sperm. The study examined that the administration of flufenoxuron during the late larval stage could induce changes in spermatocyte differentiation, including chromatin condensation and mitochondrial membrane potential. The effects of flufenoxuron on spermiogenesis were analyzed, explicitly focusing on eupyrene and apyrene spermatozoa. The assessment involved determining the fluorescence ratio of mitochondrial membrane potential in each sperm bundle. TMRM, a cell-permeant dye, was used, which accumulates in active mitochondria with intact membrane potentials. The red fluorescence of Texas Red indicates the presence of mitochondrial derivatives, while chromatin material is marked by blue fluorescence from the DAPI filter. The stain Hoechst-33342 was utilized to assess chromatin condensation during spermiogenesis following flufenoxuron application. Plate VI illustrates various stages of spermiogenesis, each

displaying distinct levels of nuclear and mitochondrial development in both apyrene and eupyrene spermiogenesis. These stages exhibit different degrees of Hoechst and TMRM activity. In the control group, eupyrene and apyrene spermatocytes demonstrate high mitochondrial activity (Plate VI: Fig A & E), with eupyrene spermatocytes exhibiting greater mitochondrial activity than apyrene spermatocysts. The fluorescence of both spermatocyte types indicates the presence of actively proliferating mitochondrial derivatives. Due to their small cytoplasmic content, the initial stages of development display faint to bright Texas Red signals. As the cells progress towards becoming eupyrene, those with strong signals start appearing in the cytoplasm as the nucleus continues to grow, accompanied by an increase in cytoplasmic volume. Apyrene spermatocytes show a slight increase in cytoplasmic volume.

The elongation commence of from early spermatocyst, and mitochondria from the eupyrene spermatocyst stage also began to undergo modification, forming mitochondrial derivatives, which exhibited high TMRM activity (Plate VI: Fig B). Apyrene spermatids are clearly distinguishable (Plate VI: Fig 6E), characterized by scattered nuclei and pronounced TMRM activity. As elongation progresses, eupyrene and apyrene sperm bundles are distinctly identifiable by their mitochondrial activity in the elongating region of each bundle (Plate VI: Fig C, D & F). Plate VI: Figure A shows strong Texas Red signals from eupyrene spermatocytes in the control group. After elongation into eupyrene sperm bundles, each mitochondrion elongated into mitochondrial derivatives without diminishing the strong signal from the Texas Red channel.

Examination of ED₂₅ treated testes exhibited that the mitochondrial activity labelled by TMRM on cyst cells notably decreased, with a more pronounced decrease observed in spermatocysts, indicating a reduced appearance of TMRM activity (Plate VII: Fig A, B & C). The TMRM activity of eupyrene spermatocysts (Plate VII: Fig A & B) and apyrene spermatocysts (Plate VII: Fig C) was lower than the control. After 144 hours of ED₂₅ flufenoxuron treatment, the fluorescence signals from spermatocytes were faint, indicating reduced mitochondrial potential. This observation was also noted in the sperm bundles. However, in the case of eupyrene sperm bundles, the Texas Red signal was not significantly reduced compared to the control. It is evident that the TMRM signals from eupyrene sperm bundles indicate that the lower dose of flufenoxuron did not significantly affect the formation of eupyrene spermatozoa (Plate VII: Fig A, B, C, & D). However, the formation of apyrene sperm bundles was certainly affected by the lower dose of flufenoxuron treatment (Plate VII: Fig A & C), with a considerable decrease in TMRM activity compared to the control apyrene sperm bundle.

The confocal micrograph depicted in Plate VIII, reveals that treatment with a high dose of flufenoxuron (ED₅₀) significantly affects the elongation and formation of mitochondrial derivatives in spermatocytes as well as sperm bundles, resulting in very low Texas Red signal in the treatment group, indicating reduced TMRM activity in all treated testicular organelles. This abnormality in elongating spermatozoa leads to defects in the total elongation process of both eupyrene and apyrene spermatozoa. Elongating eupyrene spermatid cysts and sperm bundles are observed with reduced TMRM activity compared to normal eupyrene spermatozoan development, indicating

that the formation of mitochondrial derivatives is affected by the high dose of flufenoxuron treatment (Plate VIII: Fig A & B). Observations from Plate VIII: Fig C reveal a visible reduction in the TMRM activity of apyrene spermatocytes. Under normal conditions, elongating apyrene spermatids always exhibit lower TMRM activity compared to eupyrene spermatids, but there is a clear indication of a decrease in mitochondrial activity in apyrene spermatids after the application of a high dose of flufenoxuron concentration (Plate VIII: Fig D).

In the present study, it was observed that there was no evidence of chromatin condensation or any other damage in genetic material, as the signals from Hoechst were similar in both the control and treated groups (Plate VI, VII, VIII), suggesting no impact on the nuclear material of sperm. However, a cytotoxic effect was observed with TMRM loss in spermatocytes and spermatozoa after treatment with flufenoxuron, indicating developmental arrest at the stage of spermatocyte and sperm bundles, followed by apoptosis. The confocal micrograph of dye-loaded cells in the figures illustrates a clear inverse relationship between TMRM and flufenoxuron-treated individuals. Each spermatocyte's mitochondria emit their TMRM fluorescence (Plate VI, VII, and VIII). Confocal microscopy examination revealed that, after 144 hours of treatment, flufenoxuron-treated samples (ED₅₀ & ED₂₅) exhibited a dose-dependent decrease in mitochondrial membrane potential compared to the control group. However, results indicate that the damage in mitochondria of spermatogonia, spermatocyst, and spermatozoa may result in the improper development of the sperm bundle and further may lead to male sterility.

2.5 DISCUSSION

2.5.1 Chitin synthesis inhibitor flufenoxuron affects reproductive impairment in *Spodoptera mauritia*

It is well documented that chitin synthesis inhibitors (CSIs) can inhibit chitin synthesis in insects and act as a viable alternative against several insect species. Earlier reports have shown that CSIs can disrupt chitin synthesis in insects, leading to various detrimental effects, including reproductive dysfunction. Moreover, understanding the relationship between CSIs and reproductive dysfunction is crucial for validating these compounds as potential insecticides (Acheuk et al., 2012; Costa et al., 2017; Fansiri et al., 2022). Studies have highlighted the impact of CSIs on the reproductive behaviour and fertility of several insect pests, revealing their potential as an effective solution for pest management. The present study examined the effects of a chitin synthesis inhibitor (CSI), flufenoxuron, on sixth instar larvae of *Spodoptera mauritia*, focusing on its impact on testicular development, spermiogenesis and male reproductive dysfunction. The study revealed that topical application of flufenoxuron in *S. mauritia* reduced biochemical components and morphological aberrations in the testis of *Spodoptera mauritia*.

2.5.2 Flufenoxuron influence on biochemical alteration in *Spodoptera mauritia*

The current investigation clearly demonstrated that the biochemical constituent's carbohydrates, lipids, and proteins in the haemolymph and testes of flufenoxuron treated sixth instar larvae of *Spodoptera*

mauritica were significantly reduced compared to the control groups. Carbohydrates are essential for providing energy and play a critical role in various processes, including defence against harmful substances like insecticides (Arrese et al., 2010; Rosas-Mejía et al., 2015). The carbohydrate reserves in insects can vary depending on their developmental stage, and few studies have shown that chitin synthesis inhibitors (CSIs) typically affect carbohydrate synthesis (Shen et al., 2018; Wang et al., 2018). The biochemical effects of flufenoxuron on insects have been previously documented in the Lepidopteran species *Spodoptera littoralis* (Bakr et al., 2013).

Many reports have highlighted the effects of chitin synthesis inhibitors (CSIs) on the total carbohydrate content of insect bodies. Zhu et al. (2012) reported a decrease in total carbohydrate content in the haemolymph of *Spodoptera litura* after treatment with hexaflumuron. Similarly, hexaflumuron reduced the trehalose and glucose content in *Apolygus lucorum* (Tan et al., 2014). Further, Hamadah, (2014) observed that flufenoxuron inhibited the carbohydrate content in the haemolymph of *Schistocerca gregaria*. A similar reduction in carbohydrate content was affected with lufenuron treatment in *Glyphodes pyloalis* (Aliabadi et al., 2016) and *Schistocerca gregaria* (Tanani et al., 2012). In the penultimate larval instar of *Musca domestica*, CSIs such as lufenuron, flufenoxuron, and hexaflumuron were shown to block carbohydrate synthesis (Tanani et al., 2022).

Perveen (2012a) found that the total carbohydrate content in both the testes and ovaries of *Spodoptera litura* decreased following treatment with chlorfluazuron. Analysis of the main metabolic components of

Agrotis ipsilon females treated with chlorfluazuron and flufenoxuron showed a significant decrease in the carbohydrate content of the ovaries (Shaurub et al., 2018). Costa et al. (2017) observed that treatment with lufenuron led to significant changes in the histochemical contents of the midgut, ovaries, and testes, with a reduction in neutral carbohydrates in testicular spermatozooids. Similarly, a reduction in testicular carbohydrate content was noted in the present study in the last instar larvae of *S. mauritia*. However, studies using various CSIs have consistently demonstrated decreased levels of ovarian carbohydrate content in *Cydia pomonella*, *Spodoptera litura*, and *Spodoptera littoralis* (Soltani & Mazouni, 1992; Perveen & Miyata, 2000; Abdel-Aal, 2012).

Proteins are biological macromolecules composed of one or more long chains of amino acids, and they are essential for growth and reproduction. Protein synthesis is crucial for cell division and regulating chemical processes during cell metabolism (Tanani et al., 2022). The current study observed fluctuations in the total protein content of haemolymph and testes with flufenoxuron treatment. Studies have shown that total protein content changes significantly after treatment with different chitin synthesis inhibitors (Bakr & Ghoneim, 2008). In *Agrotis ipsilon*, total protein levels exhibited severe fluctuations after treatment with chlorfluazuron and triflumuron (Fahmy, 2014).

Abdel-Aal (2003) observed that the total protein content in the haemolymph of *Spodoptera littoralis* larvae decreased after treatment with chlorfluazuron and flufenoxuron. Similarly, chitin synthesis

inhibitors (CSIs) such as lufenuron, flufenoxuron, and hexaflumuron were found to block total protein synthesis in the penultimate larval instar of *Musca domestica* (Tanani et al., 2022). Chlorfluazuron also caused a significant reduction in the total protein content of *Rhynchophorus ferrugineus* (El-Sobki and Ali, 2020). In *Glyphodes pyloalis*, larval protein content decreased significantly following lufenuron treatment (Aliabadi et al., 2016).

Likewise, the protein content in adult female *Spodoptera litura* significantly decreased following treatment with chlorfluazuron (Perveen & Miyata, 2000). Similarly, female *Agrotis ipsilon* treated with chlorfluazuron and flufenoxuron exhibited significantly lower total protein content in the ovaries (Shaurub et al., 2018). These findings align with our results of reduction of protein content in the tissue after flufenoxuron treatment. Several studies have reported that the administration of various chitin synthesis inhibitors against *Cydia pomonella*, *Spodoptera litura*, and *Spodoptera littoralis* has consistently shown decreased ovarian protein content (Soltani & Mazouni, 1992; Perveen & Miyata, 2000; Abdel-Aal, 2012).

Further, the protein content per ovary in diflubenzuron-treated *Cydia pomonella* was also significantly reduced (Soltani & Soltani-Mazouni, 1992). Bebas et al. (2008) noted that one of the yolk protein precursors, YP2, has a dual function in insects. In females, YP2 accumulates in the oocytes to support embryo development, while in males, it interacts with sperm in the seminal fluid after being produced in the testes. These findings suggest an unexpected shared biochemical component in developing insect sperm and eggs.

Lipids supply the metabolic energy required for egg formation and are the primary energy source during embryonic development. A complex network of hormonal signals tightly regulates the availability and utilization of lipids. The central nervous system and associated endocrine glands primarily orchestrate this regulation. Various hormones and neuropeptides are produced through this endocrine axis, significantly influencing the tissues involved in reproduction, thereby ensuring successful reproductive outcomes (Leyria et al., 2024). Lipids are synthesized and stored in the fat body and then transported through the haemolymph to the ovaries, where they are essential for the process of vitellogenesis (Shaurub et al., 2018). Research by Hamadah (2014) demonstrated that flufenoxuron reduced the haemolymph lipid content of *Schistocerca gregaria*. Similarly, Reda et al. (2007) reported that lufenuron significantly decreased haemolymph lipid content in *Schistocerca gregaria*.

2.5.3 Flufenoxuron interaction on protein metabolism

The electrophoretic protein patterns observed in this study reveal differences between control and flufenoxuron-treated haemolymph and testis samples. Similar findings were reported by Bakr et al. (2013) in *Spodoptera littoralis* after treatment with flufenoxuron, where variations in protein expression were linked to potential deformities. In our study, differential expression of protein bands was observed depending on the dose and duration of flufenoxuron treatment. This suggests the possible induction of new proteins in activating the insect's immune system in response to foreign substances (Bakr et al., 2013).

The role of binding proteins or chitin recognition in the immunological response of insects has been relatively understudied, with limited data available (Chae et al., 1999). In insects, proteins present in the haemolymph are crucial for carrying out humoral immune responses (Royet, 2004). Several haemolymph proteins that contribute directly or indirectly to immunological processes have been identified and reported (Chung & Ourth, 2002; Park et al., 2005). Chae et al. (1999) identified three types of chitin-binding proteins (CBPs) with molecular weights of 9 kDa, 15 kDa, and 66 kDa in the haemolymph of a sphingid moth, *Agrius convolvuli*.

In the present study, flufenoxuron was observed to negatively affect proteins with molecular weights of 71 kDa, 67 kDa, 65 kDa, 55 kDa, and 12 kDa in *S. mauritia* haemolymph. This suggests a possible defect in chitin-binding proteins following flufenoxuron treatment. Similarly, a chitin-binding protein with a molecular weight ranging from 75-80 kDa was reported by Terwilliger (1999), supporting the idea that these proteins may play a significant role in insect physiology and could be impacted by flufenoxuron.

Guedes et al. (2003) identified several stress-related and heat-shock proteins in *Drosophila melanogaster* haemolymph, each with distinct roles. A 72 kDa protein (Heat Shock 70 kDa Protein Cognate Precursor) was reported to have molecular chaperone activity, while a 53 kDa protein (glutathione reductase or thioredoxin reductase) was involved in detoxification processes. Additionally, a 60 kDa heat shock protein (Hsp 60, mitochondrial precursor) exhibited molecular chaperone activity, and 22 kDa (Glutathione S-transferase, GST class-

theta) and 23 kDa (Glutathione Transferase D) proteins were also linked to detoxification activities. These proteins play key roles in maintaining cellular integrity by assisting in protein transport, folding newly synthesized proteins, degrading unstable or misfolded proteins, and preventing protein aggregation (Guedes et al., 2003).

In the current study, similar molecular weight protein bands were detected in the haemolymph and testis of *S. mauritia*, suggesting that flufenoxuron may affect proteins involved in cellular maintenance, particularly those critical for testicular development. The disruption of these proteins could lead to impaired cellular processes, including detoxification and protein folding, further highlighting the broader impacts of flufenoxuron on insect physiology.

These findings are consistent with the report of Bakr et al. (2005), on the effects of chlorfluazuron and pyriproxyfen on the protein fractionation pattern in *Spodoptera littoralis*. The study identified new and distinct proteins in samples treated with chlorfluazuron and pyriproxyfen. An earlier study by Ghoneim et al. (2012) also investigated the impact of pyriproxyfen (an insect growth regulator) on the protein fractionation pattern of *Schistocerca gregaria* with six protein bands present in control samples were absent in treated samples. Additionally, research by Anan et al. (1993) on the biochemical effects of pyriproxyfen on *Pectinophora gossypiella* larvae demonstrated a significant reduction in protein bands following treatment with the insect growth regulator, indicating that the genes responsible for producing the mRNA for these proteins might have been inactivated.

2.5.4 Potential effects of flufenoxuron on spermatogenesis

Previous studies have primarily focused on chitin synthesis inhibitors (CSIs) and their effects on female reproduction in insects. Lufenuron, a CSI, was shown to impact vitellogenesis in *Aedes aegypti* (Moreira et al., 2007) and *Rhodnius prolixus* (Mansur et al., 2010). Chitin, a crucial component of eggs in these species, was affected by lufenuron treatment, which prevented N-acetylglucosamine's incorporation into egg chitin. This resulted in smaller and decreased chorionated eggs, suggesting that chitin inhibition can lead to insect reproductive arrest.

Recent studies have demonstrated that CSIs targeting CHS1 (Chitin Synthase 1) groups are particularly effective in inhibiting adult emergence and disrupting the reproductive systems of male and female insects (Fansiri et al., 2022). Despite these findings, there have been limited investigations into the morphological and ultrastructural changes that the reproductive organs of insect species undergo anomalies following exposure to CSIs (Acheuk et al., 2012; Costa et al., 2017). Notably, studies on the reproductive toxicity of Lepidopteran species are scarce, and only a few reports are available (Chen et al., 2019; Santorum et al., 2021).

Therefore, there remains a significant gap in our understanding of the complex effects of CSIs on the physiological makeup of these organisms. Intensive investigations into the histo-morphological, ultrastructural, and biochemical alterations affected by CSIs in the reproductive structures of Lepidopteran species are crucial. Such

studies are essential to unravel the intricate relationship between CSI exposure and the reproductive efficiency and survival of the organisms.

Different derivatives of chitin synthesis inhibitors (CSIs) have been shown to significantly influence insect reproduction, fertility, emergence, and development (Yasir et al., 2012; Gad et al., 2022). Previous research by Cloyd et al. (2004), and Joseph (2020) has documented the impact of novaluron, a CSI, on the reproductive functions of crop pests. Additionally, the study conducted by Acheuk et al. (2012) demonstrated the deleterious effects of teflubenzuron on the reproductive system of *Locusta migratoria*. Further expanding on these findings, Costa et al. (2017) investigated the consequences of lufenuron exposure on reproductive development, revealing its impact on the midgut, which disrupts the insects' ability to reproduce by interacting with gametogenesis. Moreover, Yasir et al. (2012) highlighted flufenoxuron exposure through diet interferes with crucial reproductive processes, reducing fecundity and fertility rates and hampering population growth.

More recently, Amein and Mohammad (2022) explored the impacts of flufenoxuron on the histopathological aspects of insects, highlighting the intricate interplay between this chemical compound and their reproductive mechanisms. This comprehensive analysis provided valuable insights into the potential ecological implications of using flufenoxuron and other CSIs in pest control strategies, emphasizing the need for further research in this domain. CSIs significantly hinder the growth and development process of the gonads, ultimately leading to the production of sterile eggs (Soltani-Mazouni, 1994). These

disturbances caused by CSIs on the reproductive cycle and embryogenesis have been thoroughly documented and studied (Mian & Mulla, 1982; Fansiri et al., 2022). A previous study demonstrated that chitin is present in the ovaries of *Rhodnius prolixus*, and it was hypothesized that chitin plays a significant role in the oogenesis process (Mansur et al., 2010). Furthermore, studies have shown that female insects treated with diflubenzuron and teflubenzuron exhibit unfertilized eggs, as these CSIs primarily act through ingestion and impact the fertility of female insects (Kodandaram et al., 2010). Moreira et al. (2007) previously reported the presence of a chitin-like component in the eggshells, eggs, and ovaries of *Aedes aegypti*, which suggests that chitin synthesis plays a crucial role in ovarian development. This finding highlights the potential impact of chitin synthesis inhibitors on impairing ovarian development in insects, as these inhibitors could disrupt the formation of essential chitin structures within reproductive tissues.

Several studies have demonstrated the harmful effects of chitin synthesis inhibitors (CSIs) on different stages of Lepidopteran species, such as *Spodoptera littoralis* (Reda et al., 2010) and *Agrotis ipsilon* (Shaurub et al., 2018; Amein & Mohammad, 2022). CSIs have proven effective in producing significant changes in the population control of Lepidopteran pests, impacting females and males. After exposure to diflubenzuron treatment, newly emerged adults of *Cydia pomonella* exhibited a significant decrease in basal oocyte size and the number of oocytes per ovary (Soltani & Soltani-Mazouni, 1992). In *Anthonomus grandis*, lufenuron consumption led to the structural disorganization of

follicular cells in ovarioles and reduced yolk formation (Costa et al., 2017).

The occurrence of a single set of kidney-shaped testes in the larvae and distinct spherical testes in the adult stage has been previously documented in several species, including *Atrophaneura alcinous* (Kubo-Irie et al., 1998), *Galleria mellonella* (Polanska et al., 2005), *Grapholita molesta* (de Moraes et al., 2009), and *Diatraea saccharalis* (Bilha et al., 2014). The anatomical structures seen in *S. mauritia* are similar to those documented in other Lepidopteran insects. During the pupal stage, most Lepidopteran species undergo a fusion of testes (Mari et al., 2018); likewise, a similar condition was noted in the pupa of *S. mauritia*.

Moreover, all Lepidopteran pests exhibit unique attributes in their male reproductive biology, particularly in their possession of two distinct types of sperm; one type of sperm, called eupyrene sperm or sperm, contains a nucleus, while the other type, known as apyrene sperm or perisperm, is without a nucleus (Friedlander et al., 2005). Eupyrene sperm fertilizes eggs with paternal genes. Apyrene sperm helps male fitness in other ways. Both types are essential for Lepidopteran fertilization (Chen et al., 2020). It is possible to interfere specifically with male reproduction in order to control Lepidopteran pests, and dichotomous spermatogenesis provides an extensive range of opportunities for such interference (Seth et al., 2023), and males were found to be more sensitive to pesticides than females (Abro et al., 1997).

A remarkably wide variety of organisms exhibit sperm heteromorphism, and numerous studies have demonstrated the significance of parasperm for male fertility (Whittington et al., 2015). Chitin synthesis inhibitors (CSIs) have been reported to prevent chitin formation and cause reproductive disruption in Lepidopteran pest species (Perveen, 2000). Perveen (2000) also observed the impact of chlorfluazuron on the development of testes and the process of spermatogenesis. Similarly, lufenuron-treated larvae reduced the number of spermatozooids in the testicles of *Anthonomus grandis* (Costa et al., 2017).

Histopathological observations of the present study revealed that spermiogenesis was directly affected by two doses of flufenoxuron applied during the sixth instar stage. The present study is on par with the findings of Abdel Aal and Abdel Wahab (2007) on the histological changes in the cotton leafworm after lufenuron exposure. Histological analysis of *S. maurita* testes after flufenoxuron treatment showed a dose-dependent reduction in testicular tissue compared to the control, suggesting that spermiogenesis was affected. Additionally, defects in follicular formation, vacuolation, and cyst degeneration were observed, indicating potential damage to connective tissue and supporting cells. Studies on morphometric alterations in the testicular area after treatment with lufenuron and buprofezin in *Euschistus heros* suggested the effects of chemical application on testicular development (Cremonez et al., 2018). In *Rhynchophorus ferrugineus* treated with lower concentrations of flufenoxuron, narrower follicles and a reduction in cellular content were observed. Higher concentrations of

flufenoxuron in the testicular follicles led to pronounced disorganization, characterized by decreased cellular content and a significant reduction in the cysts of spermatocytes and spermatogonia (El-Bokl et al., 2010).

Agüero et al. (2014) noted that diflubenzuron impacts both females and males' development and reproductive systems. According to Abd-Alla et al. (2003), lufenuron is genotoxic to the germ-line cells of male *Drosophila melanogaster*, showing varying degrees of genotoxic potential across the three separate phases: spermatozoa, spermatids, and spermatocytes. Furthermore, chlorfluazuron application on *Spodoptera litura* has been shown to disrupt spermatogenesis, causing a decrease in both the quantity and size of eupyrene and apyrene sperm bundles in the testes (Perveen, 2000). Histopathological examination conducted by Ghazawy (2012) on *S. gregaria* males revealed disorganized and damaged sperm tubes, vacuolated small cells, disorganized spermatids, abnormal cell organelles, thickened and vacuolated testicular epithelia, detachment from the peritoneal sheath, degenerated sperm bundles, and incomplete spermatid development. These findings are consistent with our histological results.

Perveen & Miyata (2000) reported significant damage to the ovarian cells of *Spodoptera litura* females of fourth-instar larvae treated with chlorfluazuron. Similarly, Amein and Mohammad (2022) investigated the effects of flufenoxuron on the histopathology of *A. ipsilon* ovaries, observing notable changes such as a thin and vacuolated follicular epithelium, oocyte shrinkage and the absence of nurse cells. Ghazawy (2012) found that topical application of lufenuron to newly moulted

fifth-instar desert locusts (*Schistocerca gregaria*) resulted in a range of ultrastructural alterations in the ovarioles. Similarly, in male *Schistocerca gregaria*, vacuolated testicular epithelia and loosely fragmented testicular tissues were observed. An earlier study by Perez-Farinos et al. (1998) investigated the penetration, distribution, and excretion of labelled hexaflumuron in young and old adults of *Aubeonymus mariaefranciscae*. Their study revealed that hexaflumuron penetration was slightly higher in females than males. However, males exhibited lower elimination rates, leading to more significant accumulation of the insecticide. Additionally, young adult weevils showed higher levels of hexaflumuron in their reproductive systems than older weevils.

2.5.5 Flufenoxuron-induced dysfunction on dimorphic spermiogenesis of *S. mauritia*

In Lepidoptera, testicular formation and spermiogenesis occur simultaneously (Liang et al., 2019). Friedlander et al. (2005) state that eupyrene spermiogenesis occurs before apyrene spermiogenesis. In *Spodoptera litura*, the primary phase of eupyrene spermiogenesis spans from the sixth instar larvae to the pupal stage. However, meiosis for eupyrene sperm begins as early as the fifth instar larvae (Wen et al., 2022). Apyrene sperm meiotic divisions, on the other hand, occur during the pupal stage in *S. litura* (Liang et al., 2019), *Bombyx mori* (Yamashiki & Kawamura, 1997), and *Manduca sexta* (Elliott & Zeidler, 2008; Friedlander, 1997).

Mitochondria are intracellular organelles in eukaryotic cells that show considerable morphological variability and flexibility. Extensive biochemical research has demonstrated that mitochondria are crucial in synthesizing the energy required for cellular functions (Kawamura et al., 1998). The evaluation of mitochondrial membrane potential is often used to indicate sperm effectiveness (Li et al., 2012).

The present study explored significant changes in spermiogenesis, and corresponding mitochondrial membrane potential signals were observed using confocal electron microscopy, particularly in control and flufenoxuron-treated individuals. Although the effects of chitin synthesis inhibitors (CSIs) on spermiogenesis have not been extensively documented in previous studies, their impact on testicular development remains poorly understood. Earlier studies have documented that in silkworms, spermatocytes that initiate meiotic divisions after the spinning stage are destined to produce apyrene sperm. In contrast, those undergoing meiosis during the larval stages differentiate into eupyrene sperm. It is well known that the silkworm undergoes substantial internal changes as it transitions from larva to pupa (Kawamura et al., 1998). Mitochondria are essential for the functional maturation of eupyrene sperm. The behavioural characteristics of mitochondrial derivatives in the two sperm types differ significantly, suggesting a crucial, possibly selective, role in the spermatogenesis of dimorphic sperm (Friedlander, 1997; Kawamura et al., 1998).

The findings demonstrated that spermatozoa with both high and low mitochondrial membrane potential could be identified using TMRM

(Uribe et al., 2017; Barbarosie et al., 2021). Under our experimental conditions, TMRM fluorescence effectively detected sperm mitochondrial membrane potential, showing a visible difference between the control and treated groups. TMRM reliably identifies variations in mitochondrial membrane potential due to different sperm qualities (Uribe et al., 2016; Barbarosie et al., 2021), which aligns with our results, confirming similar outcomes.

Mitochondria are integral for developing spermatids, with the axoneme and mitochondrial derivatives constituting most of the mature sperm tail (Laurinyecz et al., 2019). In eupyrene sperm, mitochondria are essential not only for providing the energy needed for sperm tail movement but also for other functions beyond energy supply (Kawamura et al., 1998). Indirect immunofluorescence studies have shown that eupyrene spermatocytes have significantly more microtubules than apyrene spermatocytes. This is due to tubulin, a component of the flagellum's spindle and axoneme (Yamashiki & Kawamura, 1997). These observations suggest that mitochondria may play a role in tubulin synthesis and microtubule formation (Kawamura et al., 1998).

Studies have reported that two mitochondrial derivatives are present in eupyrene sperm but cannot move independently through the female reproductive system. The functions of these eupyrene sperm mitochondrial derivatives during fertilization remain unclear (Chen et al., 2020). Insect sperm typically feature two elongated mitochondrial derivatives along the flagellum (Werner et al., 2008; Moreira et al., 2010; Rego et al., 2016). Laurinyecz et al. (2019) observed that defects

in the more extensive mitochondrial derivatives can lead to insect male sterility. Our results suggest that low mitochondrial membrane potential may be due to defects in mitochondrial derivatives during spermiogenesis. Overall, the biochemical, histological, and live imaging using confocal microscopy in *S. mauritia* testis suggests that the observed effects may be associated with flufenoxuron-induced disorders. These disarrays could be related to the inhibition, delay, or disruption of nutrient absorption (Costa et al., 2017). Furthermore, nutritional deficiencies and feeding challenges have been previously correlated with declines in reproductive capabilities (Engelmann, 1997).

2.6 Conclusion

In conclusion, the findings of this study demonstrated that sublethal concentrations of flufenoxuron significantly impacted reproduction in male *S. mauritia*. The exposure notably impaired testicular development disrupted spermiogenesis, and altered key biochemical parameters. These results highlight the potential reproductive risks associated with flufenoxuron exposure.

Flufenoxuron-treated groups exhibited high sensitivity in various biochemical indices, reflecting the pesticide's impact. The effects of flufenoxuron were associated with a significant reduction in proteins, lipids, and carbohydrates in the haemolymph and testes of treated individuals, which are critical for the normal development of the testes. This depletion likely contributed to the failure to produce healthy spermatozoa. The observed depletion may be due to damage in the larval midgut epithelium, impairing nutrient absorption and metabolism, ultimately affecting testicular development and

reproductive success. The results clearly demonstrated that flufenoxuron exposure on sixth instar larvae of *S. mauritia* induced testicular reproductive toxicity, significantly impairing spermiogenesis. This is evidenced by a reduction in the viability of spermatogonia, spermatocytes, and sperm bundles, ultimately compromising the survival of future generations. Histological analysis of the testes following flufenoxuron treatment revealed visible damage to the developing gonads, including vacuolation and degeneration of the testicular epithelium and follicular septa. These effects led to a reduction in the number of follicular septa and further damage to the epithelium. Additionally, flufenoxuron exposure affected different stages of spermatogenesis within each follicle. Vacuolation of the spermatogonial cysts, along with varying degrees of degeneration in the spermatocysts and sperm bundles, were observed. The extent of damage varied depending on the concentration and duration of flufenoxuron exposure. Confocal microscopy using Hoechst and TMRM validated these findings by indicating reduced mitochondrial membrane potential in developing spermatogonia, spermatocytes, and dichotomous sperm bundles.

The available literature shows that, this study provides the first direct evidence of the impact of flufenoxuron, a chitin synthesis inhibitor, on the spermiogenesis of *S. mauritia*. Future research building on these findings could offer valuable insights into the specific mechanisms by which chitin synthesis inhibitors cause reproductive toxicity in pest species, highlighting their potential as a targeted approach for insect control.

Impact of flufenoxuron on metabolomics profiling and chitin synthesis pathway of *Spodoptera mauritia*

3.1 INTRODUCTION

In recent years, rapid technological advancements in biological sciences have catalysed research breakthroughs in systems biology. "Omics" technologies such as genomics, transcriptomics, proteomics, and metabolomics offer comprehensive insights into the components of organisms, tissues, or cells (Horgan & Kenny, 2011). Among these, metabolomics specifically investigates disrupted metabolic processes and aids in identifying potential targets for hazardous compounds. This field enhances our understanding of how substances function and provides critical information on affected target organs.

Metabolomics, a rapidly evolving discipline, integrates statistical and multivariate techniques for data interpretation with advanced analytical methods to detect and measure biological metabolites (Roessner & Bowne, 2009). Through this approach, numerous metabolites involved in biochemical and physiochemical processes within biological systems can be identified (Patti et al., 2012; Wang et al., 2015). Typically, metabolomics employs a variety of analytical platforms, including NMR, GC-MS, LC-MS, and CE-MS (Kim et al., 2016;

Panda et al., 2018). Mass spectrometry, in particular, is widely regarded as the most popular tool for analyzing and identifying analytes in both proteomics and metabolomics (Horgan & Kenny, 2011).

Among the available techniques, GC-MS (gas chromatography-mass spectrometry) is a versatile analytical method (Tsugawa et al., 2011). It is particularly well-suited for metabolomics research, enabling the identification and quantification of small molecular weight metabolites such as sugars, sterols, alcohols, amino acids, fatty acids, and organic acids. The high sensitivity and repeatability of quantitative analyses in metabolomics make it a preferred technique in many studies (Feizi et al., 2021). GC-MS-based metabolomics has been widely adopted in toxicity studies and biomarker identification due to its superior chromatographic resolution and analyte specificity compared to other analytical techniques (Mudiam et al., 2013).

The metabolome is defined as a metabolite's quantitative and qualitative compositions that vary across an organism's different compartments or organs within "normal" ranges, and each organism functions as a complex entity. Environmental factors, diet, developmental stage, and genetic composition influence these metabolomic variations. When an organism encounters stress, it undergoes physiological and behavioural changes, leading to a new metabolic equilibrium (Aliferis et al., 2012). Monitoring these metabolic regulatory networks can provide crucial insights into the factors that disrupt them and help to determine whether an organism is in a "normal" or healthy state (Fiehn, 2002). Metabolomics has the

potential to offer a more accurate assessment of environmental stress than other omics technologies (Van Aggelen et al., 2010). Its use is increasingly expanding in various applications, including monitoring pathophysiological processes, toxicity assessments and target identification, and identifying potential biomarkers for toxicant exposure (Ch et al., 2015). Metabolomics is also a widely employed technique in studying the multivariate dynamic responses of both target and non-target organisms to pesticide exposure at the metabolite level (Chen et al., 2021).

The vital signals in vertebrates, such as blood and urine, contain metabolic traces from various biochemical pathways, providing valuable insights into their physiological state. These metabolic signs are crucial for understanding the overall physiology of organisms. Based on this concept, metabolomics techniques have been successfully developed for early disease detection, tracking the progression of human diseases, and identifying disease biomarkers in blood and urine (Madsen et al., 2011). Moreover, exposure to stress disrupts metabolic pathways, leading to decreased energy availability for growth, reproduction, and survival; changes in metabolite profiles can predict an organism's fitness (Hines et al., 2010). Many studies have demonstrated that insect haemolymph is a stress marker for toxicity analysis, reflecting the systemic physiological and biochemical changes induced by pesticide exposure (Ali & Felton, 1990; Lindh & Lehane, 2011). However, despite its potential, metabolomics has not been as widely applied to the study of haemolymph as it has to mammalian serum (Aliferis et al., 2012). The available reports suggest

that, to date, a few studies have focused on toxicity investigations using insect haemolymph metabolomes (Wu et al., 2024).

Chitin plays a vital structural role in insects' trachea, peritrophic matrix (PM), and epidermis. The administration of insect growth regulators, particularly chitin synthesis inhibitors, disrupt chitin synthesis and metabolic pathways, making it a promising target for novel pest management strategies (Liu et al., 2023). Despite extensive research over many years on the mode of action of chitin synthesis inhibitors (CSIs), the precise inhibition of a specific target enzyme has yet to be conclusively demonstrated. Several research has focused on investigating the effects of chitin synthesis inhibitors on the developmental, biochemical, and reproductive processes of several insect species (Kellouche & Soltani, 2006; Reda et al., 2010; Fahmy, 2014). These studies suggest that chitin synthesis inhibitors may indirectly influence other developmental pathways. Chitin metabolism is regulated by a dynamic equilibrium between the chitin hydrolase and synthase systems, governed by multiple signalling pathways and hormones (Zhang et al., 2021). The pathway to insect chitin production begins with trehalose, which is hydrolyzed into glucose by trehalases (Chen et al., 2010). The balance between dietary intake and the energy demands of maintenance, development, and reproduction dictates the availability of lipids. Disruptions to this balance caused by toxic substances can lead to developmental dysfunctions (Tanani et al., 2022). However, the mechanisms by which metabolites influence biochemical pathways and regulate chitin metabolism remain poorly understood (Yu et al., 2021; Liu et al., 2023).

The ultimate result of biological activity and metabolic patterns are the endpoints of the actions of enzymes, and they can be used to immediately identify abnormal physiological conditions (Huang et al., 2016). In order to minimize metabolic volatility and provide increased contrast capacity, exposure study designs are essential to focus on the reproductive aspects (Jeong & Simpson, 2019). Moreover, several reports have revealed the harmful effects of flufenoxuron exposure on the male reproductive system of Lepidopteran insects (Shaurub et al., 2018; El-Sabrou et al., 2019). Male infertility may arise from flufenoxuron's known effects on biochemical constituents of the testis, inhibition of spermatogenesis, and impairment of reproductive organ development. Earlier research by Loeb (1986) demonstrated that steroids present in the testicular sheath of insects are not synthesized within the testes themselves but are instead sequestered from the haemolymph. This process suggests that the haemolymph acts as a medium for circulating steroid hormones crucial for reproductive functions, particularly in the development and maturation of the testes. So, in this context, it is crucial to understand the relationship between biochemical pathways and reproductive development on chitin synthesis inhibitor exposure, particularly in insect haemolymph. Understanding the alterations in haemolymph composition throughout the reproductive maturation process would also facilitate the development of tactics intended to control the insect's capacity for reproduction (Lewis et al., 2001).

The field of metabolomics holds great promise for identifying indicator metabolites that enable the rapid detection of various disease states

(Aliferis et al., 2012). Insect species have already been the subject of successful studies locating such metabolic indicators (Qian et al., 2020; Gao et al., 2022). Based on these findings, it is assumed that metabolite biomarkers may respond to physiological stress induced by chitin synthesis inhibition in *Spodoptera mauritia*. Therefore, to test this hypothesis, the present study employs gas chromatography-mass spectrometry (GC-MS) on metabolomics profile to detect alterations in the haemolymph of *S. mauritia* following exposure to flufenoxuron, a known chitin synthesis inhibitor. Based on the available information, this study represents the first metabolomics investigation using haemolymph analysis to explore the physiological effects of flufenoxuron in *Spodoptera mauritia*. To validate the mechanism by which this chitin synthesis inhibitor regulates growth, development and reproduction, we employed untargeted gas chromatography-mass spectrometry (GC-MS) metabolomics. This approach allowed us to evaluate differences in haemolymph metabolite profiles between flufenoxuron-treated and untreated individuals. By identifying the metabolic pathways influenced by flufenoxuron, this study aims to provide a deeper understanding of its physiological impact on male *S. mauritia*, offering new insights into the metabolic responses to chitin synthesis inhibition.

3.2 REVIEW OF LITERATURE

3.2.1 Metabolomics: An Overview

The growing public concern for food safety and environmental health has prompted the agrochemical industry to seek innovative crop protection agents. In this context, the current efforts focus on harnessing natural sources of bioactivity, leading to the successful development of natural products and their analogues for crop protection (Copping & Duke, 2007; Dayan et al., 2009; Huter, 2011). Advances in synthetic and natural product chemistry have provided scientists access to a wide range of molecules with potential bioactivity. These molecules are expected to exhibit low toxicity, efficacy at low doses, minimal environmental persistence, and the ability to address pest resistance through novel modes of action (MoA). A molecule's MoA determines its bioactivity through specific biochemical interactions (Casida, 2009; Aliferis & Jabaji, 2011). Early understanding of the MoA of bioactive molecules can significantly accelerate the development of new crop protection agents, as it helps researchers eliminate compounds with undesirable properties with existing pesticides (Aliferis & Jabaji, 2011).

Metabolomics, the study of small molecule compounds, encompasses both endogenous and exogenous molecules that are products and substrates of chemical reactions within biological systems. It examines cellular and organismal metabolism on a global or network scale, providing insight into metabolism's physiological and pathophysiological endpoints. A metabolomics experiment directly

reflects the activity of the metabolic network, offering crucial information about the biological status of the system under study (Liu & Locasale, 2017). The extensive qualitative and quantitative characterization of numerous metabolites within a biological system is commonly referred to as metabolomics (Trethewey et al., 1999; Fiehn et al., 2000). By allowing for a detailed investigation of global metabolic networks, metabolomics adds a new dimension to multidimensional biology (Spratlin et al., 2009; Vinayavekhin et al., 2010). The increasing use of techniques like LC-MS/MS, GC-MS/MS, and NMR in metabolomics research is due to their ability to detect a wide range of compounds and their relative ease of sample pretreatment (Gika et al., 2014; Zarate et al., 2016).

The standardization of metabolomic analysis has been advanced by recent developments in analytical tools, specialized software for handling large datasets, and the creation of online databases (Aliferis & Chrysai-Tokousbalides, 2011). As with other "omics" technologies, bioinformatics plays a critical role in facilitating the storage, sharing, and interpretation of metabolomic data (Xia & Wishart, 2011). Several software tools have recently been developed for metabolomics analysis, allowing for the progression from simple compound identification to functional interpretation and pathway analysis, often called systems biology (Xia & Wishart, 2011). These software tools are widely used for untargeted metabolomic analysis.

In metabolomics, there are two primary approaches: chemometric and quantitative methods. Chemometric methods, or nontargeted or untargeted approaches, utilize raw, unannotated peak lists, binned

spectral data, or aligned spectral profiles combined with multivariate statistical techniques to identify spectral features that differ significantly between two or more sample populations. These significant features (peaks, retention times, masses, and chemical shifts) may or may not be identified in subsequent analysis steps (Xia & Wishart, 2011).

Metabolomics enables the simultaneous monitoring of multiple metabolites, providing a comprehensive overview of a chemical's impact on living organisms. As a complement to traditional methods, metabolomics has shown significant promise in pharmacology and toxicology (Bouhifd et al., 2013; Wang & Wu, 2015). A key application of metabolomics in toxicology is identifying patterns of metabolite changes and biomarkers that serve as indicators of toxicity, helping to predict potential hazards (Wang & Wu, 2015). By systematically detecting the dynamic physiological changes caused by exposure to toxicants, metabolomics can identify early signs of exposure to harmful substances, as minor metabolic shifts often precede the biochemical changes induced by xenobiotics. In toxicological research, metabolomics is commonly applied in safety screening, biomarker discovery, and investigating mechanisms of toxicity (Robertson et al., 2011).

3.2.2 Stress biomarker using metabolomic profiling

Metabolomics is rapidly gaining momentum as a valuable omics technology for analyzing insect haemolymph and tissues. It enables identifying a wide range of metabolites from even small sample

extracts, offering insights into metabolites specific to cells and tissues and their roles within cellular processes (Kumar et al., 2017). In ecotoxicology, the emerging field of environmental metabolomics holds promise for understanding the impact of pesticide exposure on stressed organisms. Non-targeted metabolomics allows for profiling the global state of metabolites in biological fluids and tissues (Zhang et al., 2016, 2019) while also providing real-time detection of organisms adaptive responses to pathophysiological stimuli, environmental changes, and genetic modifications (Bordag et al., 2016). This approach can comprehensively assess biological activities in individuals following molecular-level interactions with environmental stressors (Lankadurai et al., 2013).

Several physical (e.g., radiation, temperature, noise, vibration), chemical (e.g., drugs, metals, smog, abnormal oxygen levels), and physiological (e.g., diseases, injury, ageing, inflammation) stressors can disrupt functional homeostasis, leading to stress (Kodrík et al., 2015). In recent years, metabolomics has been increasingly applied to study stress-induced changes in insect species under various conditions (Aliferis et al., 2012; Wang et al., 2019; Jousse et al., 2020). For instance, metabolite profiling of haemolymph in worker honey bees (*Apis mellifera* L.) infected by the intracellular parasite *Nosema ceranae* revealed clear signs of stress in the haemolymph after infection (Aliferis et al., 2012). Likewise, a study of *Bombyx mori* haemolymph metabolome infected with nucleopolyhedrovirus (BmNPV) demonstrated stress-related metabolic changes (Wang et al., 2019). Additionally, Zhang et al. (2022) identified the tryptophan

metabolic pathway as a key mechanism in the pathogenicity of the entomopathogenic nematode (EPN) *Steinernema feltiae*.

Agrochemicals, including fungicides, insecticides, and herbicides, can significantly alter enzyme activity, motility, orientation, development, learning, and immune function in insects (Boncristiani et al., 2012; Eiri & Nieh, 2012; Gregorc et al., 2012). In a study investigating the effects of pesticides on the metabolic profile of *Apis mellifera*, UPLC-MS metabolomics analysis was employed to examine the impact of carbendazim on bee brain metabolism (Chen et al., 2021). Similarly, the metabolic pathway analysis of *Aphis gossypii* exposed to imidacloprid revealed high energy consumption, excitotoxicity, and oxidative stress in the treated group (Lv et al., 2021). Moreover, 3-hydroxy anthranilic acid (3-HAA) was identified as a potential biomarker mediating the effect of entomopathogenic nematodes (EPN) in killing host insects.

Lv et al. (2018) found that juglone altered the haemolymph metabolomic processes in *Aphis gossypii*, leading to physiological changes that ultimately caused death. In another study, UHPLC-MS untargeted metabolomics conducted by Zarate et al. (2016) revealed changes in metabolite levels in the epidermis and viscera of *Helicoverpa armigera* larvae treated with ZQ-8. This study reported that decreased glycolysis, increased TCA cycle activity, and reduced amino acid synthesis were the leading causes of growth inhibition and death in *H. armigera* larvae (Liu et al., 2023). Several reports indicate that pesticides can significantly disrupt metabolic pathways in insects, affecting their ability to maintain normal physiological functions. The

three major metabolic pathways that are predominantly affected in insects are carbohydrate, fatty acid, and amino acid metabolic pathways.

3.2.3 Carbohydrate metabolic pathway

Glycolysis is the primary metabolic pathway responsible for producing adenosine triphosphate (ATP) and generating intermediate metabolites necessary for biosynthesis (Gunn & Gatehouse, 1988; Tian et al., 2010; Vander Heiden et al., 2009). This pathway plays a crucial role in regulating insect blood sugar and glycogen levels. Disruptions in glycolytic flux can lead to various metabolic disorders (Bar-Even et al., 2012; Sanman et al., 2016). The chemical agents, such as pyrethroids and nicotinoids, on insect energy metabolism and glycolysis have been extensively investigated (Ali et al., 2013; Derecka et al., 2013; Dewar et al., 2016). Recent studies revealed that pesticide stress or detoxification metabolism adversely affects the pathways involved in glycolysis and the activities of associated enzymes (Lv et al., 2021; Thornton et al., 2010; Xu et al., 2022).

ATP is initially used to provide energy for detoxification enzymes and ATP-binding cassette (ABC) transporters that eliminate harmful compounds during insect detoxification metabolism (Wu et al., 2019; Xu et al., 2021). This detoxification process is energy-intensive and often necessitates an increase in glycolysis to compensate for ATP depletion (Konus et al., 2013; Rand et al., 2015). Additionally, certain pesticides influence trehalose metabolism by affecting insect glycolysis (Ge et al., 2011; Reyes-DelaTorre et al., 2012; Thompson, 2003). For instance, sublethal concentrations of specific pesticides have been

shown to significantly decrease trehalose content while simultaneously increasing the activity of soluble trehalase, which promotes trehalose hydrolysis in the haemolymph (Ge et al., 2011; Tang et al., 2017).

Elevated lactate levels in insecticide-exposed *Bombyx mori* indicate a reduction in pyruvate mobilization into the Krebs cycle, which is essential for producing NADH used in various metabolic processes and providing metabolites for biosynthesis (Nath, 2000). Lactic acid is a building block for gluconeogenesis, the pathway through which insects synthesize carbohydrates. Consequently, its increased concentration may be attributed to reduced gluconeogenic rates, which can also result from depleted amino acid pools (Aliferis et al., 2012). Several studies on *Helicoverpa armigera* (Liu et al., 2023) and *Apis mellifera* (Shi et al., 2018) have reported high energy demands following pesticide exposure. Energy metabolism primarily comprises anaerobic glycolysis and aerobic respiration (TCA), and it also involves the breakdown of cholesterol, amino acids, and other compounds to create substrates that enter the TCA cycle (Liu et al., 2023). Similarly, significant changes in the concentration of pyruvate, a glycolysis byproduct and gluconeogenesis precursor, indicate that treatments with insecticides such as DDT, malathion, and deltamethrin increase the energy required for *Aedes aegypti* to cope with exposure-induced stress. Further, it is evidenced that pyruvate metabolism through glycolysis and gluconeogenesis is a critical aspect of oxidative stress management (Singh et al., 2022).

Zhou et al. (2020) reported that azadirachtin affects carbohydrate metabolism in *Bactocera dorsalis* larvae, leading to the differential enrichment of two metabolites, succinic and malic acid, within the TCA cycle. The TCA cycle serves two essential functions: it provides

intermediary compounds necessary for the synthesis of fatty acids and amino acids, and it generates substantial amounts of ATP, which is vital for various metabolic processes (Pallardy, 2008). Additionally, Liu et al. (2020) reported that treatment with anaxadiol saponins inhibited glucose metabolism and synthesis in the third instar larvae of *Ostrinia furnacalis*.

3.2.4 Fatty acid metabolic pathway

A recent study has shown that the biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, and linoleic acid metabolism in *Bactrocera dorsalis* larvae are notably influenced by exposure to insecticides such as azadirachtin, pyriproxyfen, and tebufenozide (Zhou et al., 2024). Liu et al. (2020) identified a metabolic-based mechanism linked to ginsenoside-mediated defense against *Ostrinia furnacalis*, showing that sixteen free fatty acids decreased after treatment, while the elevated levels of palmitoleic acid, palmitic acid, and 9-octadecenoic acid indicated that palmitic acid triggered lipotoxicity and endoplasmic reticulum stress. Recently, using spatial metabolomics and lipidomics, Li et al. (2022) investigated the spatial distribution and metabolic expression in *Plutella xylostella* treated with rotenone, and findings revealed that rotenone damaged the cell membrane by inhibiting purine and amino acid metabolism.

In addition, metabolomics analysis of *Bactrocera dorsalis* larvae exposed to azadirachtin revealed a significant reduction in fatty acid quantity and relative composition (Zhou et al., 2020). Similarly, Balabanidou et al. (2016) reported that lipid biosynthesis was significantly higher in insecticide-resistant mosquitoes than in susceptible ones, likely due to the essential role of lipids in stress

reduction strategies (Hochachka, 2002). Singh et al. (2022) also demonstrated that exposure to temperature followed by malathion could alter mosquito cellular behaviour, particularly through changes in phospholipid composition.

3.2.5 Amino acid metabolic pathway

The fat body in insects plays a crucial role in synthesizing several proteins in the haemolymph and managing the movement of metabolites and intermediaries throughout the body. Among its key functions, the fat body produces lipophorin, a lipid transport protein that facilitates lipid movement in the haemolymph, vitellogenins, which are essential for egg maturation, and storage proteins that supply amino acids for insect morphogenesis (Arrese & Soulages, 2010). Additionally, the fat body synthesizes frost-protection proteins at low temperatures and detoxification enzymes that aid in removing harmful substances, such as pesticides (Li et al., 2019).

In a study by Liu et al. (2020), fifteen free amino acids reduced following treatment indicated a metabolic mechanism potentially associated with ginsenoside-mediated defence against *Ostrinia furnacalis* infestation. Similarly, azadirachtin influenced the amino acid metabolism of *Bactrocera dorsalis* larvae, leading to increased levels of glutamic acid in treated larvae. This elevation could result from the metabolism of histidine, d-glutamine, d-glutamate, alanine, aspartate, and glutamate, which may facilitate the conversion of nitrogenous metabolites into intermediates of the TCA cycle to compensate for energy deficits and the need for essential compounds (Zhou et al., 2020).

3.2.6 Action of BPUs on insect metabolism

In recent years, molecular design and structure optimization based on natural products have become crucial for developing novel pesticides. Managing pest populations is closely tied to the energy dynamics of insects (Shaurub et al., 2018). The AMP-activated protein kinase (AMPK) signalling pathway, which regulates development and nutrient utilization, is particularly sensitive to fluctuations in energy status (Carling & Viollet, 2015; Hardie et al., 2012). Under pesticide stress, insects require more energy to metabolize pesticides and their by-products, which disrupts the body's energy balance (Ge et al., 2011; Rand et al., 2015; Xu et al., 2022). This increased energy demand triggers shifts in metabolic processes, particularly affecting the activity of digestive enzymes that are critical for nutrient assimilation (Timmins & Reynolds, 1992; Vyjayanthi & Subramanyam, 2002). Simultaneously, detoxification enzymes, which play a pivotal role in neutralizing and processing pesticides, draw energy from biomass reserves to facilitate these processes (Nathan & Kalaivani, 2005).

Certain insect growth regulators (IGRs) and conventional pesticides can reduce larval feeding and digestion efficiency, even causing larvae to refuse food after treatment (Mondal & Parween, 2000). However, the physiological mechanisms by which benzoylphenyl ureas (BPUs) affect insect growth, food consumption, food conversion, and energy metabolism remain largely unknown (He et al., 2022; Shaurub et al., 2020). Previous studies have shown that BPUs disrupt larval growth and physiological regulation across varied metabolic pathways, particularly by inhibiting chitin biosynthesis (Merzendorfer, 2013; Mommaerts et al., 2006). In particular, inhibitors that impair the development of the peritrophic matrix and cuticle cause severe

physiological dysfunctions (Merzendorfer, 2013). The exact mechanism of action of acyl ureas is unknown, although their interference with chitin formation is well established (Van Eck, 1979). As demonstrated in cell-free chitin-producing insect systems, several experimental lines contend that acyl urea chemicals fail to block catalysis (Cohen & Casida, 1980; Mayer et al., 1980; Cohen, 1985; Zimoch et al., 2005). Instead of directly blocking chitin synthase, acyl ureas changes vesicle transport or fusion, prevents chitin polymers from moving across membranes, or disrupts the hormonal control of chitin synthesis by affecting the production of ecdysteroid hormones (Nakagawa & Matsumura, 1994; Cohen, 2001; Fournet et al., 1995).

Glycogen phosphorylase acts on fat body glycogen, the primary source of sugar residues for chitin production. This enzymatic process generates glucose-1-phosphate, which is converted into trehalose and released into the haemolymph (Muthukrishnan et al., 2012). Chitin synthase (UDP-N-acetyl-D-glucosamine: chitin 4- β -N-acetylglucosaminyltransferase) then catalyzes the synthesis of chitin (Merzendorfer, 2006). The rate of chitin synthesis during moulting is influenced by the amount of trehalose in insects' haemolymph (Candy & Kilby, 1962). Two cytosolic glycolytic enzymes convert glucose to fructose-6-phosphate, which is required for chitin production. Hexokinase and glucose-6-phosphate isomerase are the enzymes that convert glucose into fructose-6-phosphate. As the initial committed step in the biosynthesis of amino sugars, glutamine fructose-6-phosphate amidotransferase is the first enzyme to catalyze the branch of the chitin biosynthesis pathway that arises from the latter (Muthukrishnan et al., 2012). Pan et al., 2019 demonstrated that the defects in G6Pase and HK genes in glycolysis and gluconeogenesis

pathways will lead to the downregulation of the genes involved in chitin metabolism in the brown planthopper. Figure (1) provides an overview of the chitin biosynthesis pathway (Muthukrishnan et al., 2012).

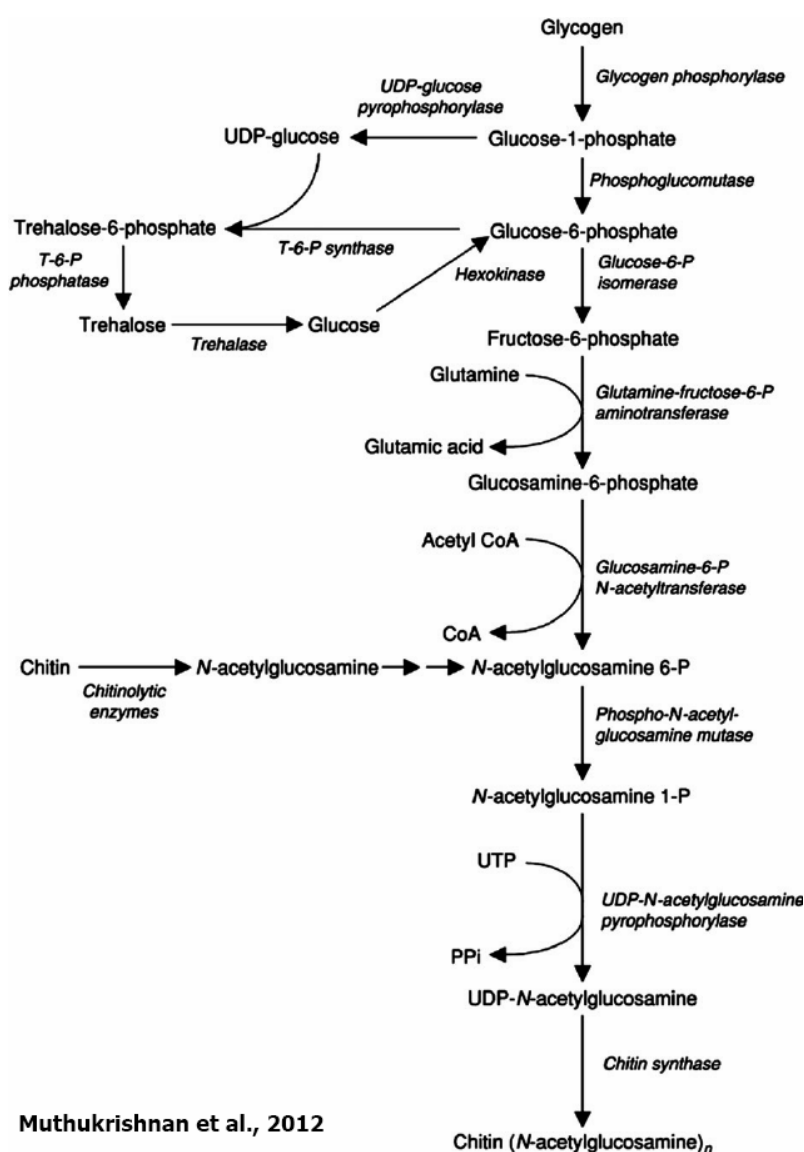


Figure 1 Chitin biosynthesis pathway (Muthukrishnan et al., 2012)

In addition to chitin synthase (CHS), the final enzyme in the pathway and a member of the β -glycosyltransferases (GT2) family, which is closely related to cellulose synthases (CES) and hyaluronan synthases (HS) (Merzendorfer, 2006), other enzymes that supply precursors and those that commit precursors to the formation of amino sugars, such as trehalase (TRE), glutamine-fructose-6-phosphate aminotransferase, and UDP-N-acetylglucosamine pyrophosphorylase (UAP), also regulate the flow of carbon into the chitin biosynthetic pathway (Zhu et al., 2016).

Three sub-reactions can be considered part of the chitin biosynthesis pathway. The amino sugar N-acetylglucosamine (GlcNAc) is formed in the first set, followed by its activated form UDP-GlcNAc in the second, and the polymeric chitin from the amino sugar in the last set (Merzendorfer, 2011; Muthukrishnan et al., 2012). The first stage of the glycogen synthesis, glycolysis, or pentose phosphate pathways is ATP-dependent glucose phosphorylation, which is irreversibly catalyzed by hexokinase and results in the production of ADP and glucose-6-phosphate (G-6-P) (Morris et al., 2006; Calmettes et al., 2013; Al-aryahi et al., 2014). Crucially, it is the second step leading to chitin production.

The chitin biosynthesis pathway is lacking in plants and humans (Nagpure et al., 2014). Hence, the proteins involved in chitin metabolism have long been seen as desirable targets for creating ecologically friendly and effective pesticides. Benzoylphenylurea (BPU) is the oldest and largest class of chitin synthesis inhibitors used as insecticides (Douris et al., 2016; Zhu et al., 2016). Studies have

shown that in pharmacological research conducted on two insect species (*D. melanogaster* and *Blattella germanica*), the primary mechanism by which acyl ureas (diflubenzuron) prevent the synthesis of chitin is their binding to a sulfonyleurea-sensitive transporter (Abo-Elghar et al., 2004). Sulfonyleureas (SUs) have been reported to alter the carbohydrate levels in insect haemolymph by acting on sulfonyleurea receptor (SUR) analogues in adipokinetic hormone (AKH) secreting cells (Bharucha, 2009). Evidence suggests that BPU can inhibit K⁺ channels in insects by binding to SUR, which implies that BPUs may regulate blood sugar and glycometabolism (Meyer et al., 2013; Gangishetti et al., 2009). Additionally, BPUs and SUs may compete to regulate insect SUR (Abo-Elghar et al., 2004). Therefore, a deeper understanding of how BPUs regulate nutritional indicators, energy metabolism, and carbohydrate metabolism is needed (Zhao et al., 2023).

Zhao et al. (2023) demonstrated that in *Hyphantria cunea* larvae, chlorbenzuron treatment significantly reduced levels of pyruvic acid (PA), adenosine triphosphate (ATP), and carbohydrates. Further studies revealed that chlorbenzuron treatment increased the mRNA expression and enzyme activity of key glycolytic enzymes, including pyruvate kinase (PK), phosphofructokinase (PFK), and hexokinase (HK), leading to elevated glycolytic flow. Genes related to the AMPK signalling pathway also showed increased expression, which suggests higher energy consumption. Zhao et al. (2023) attributed the higher ATP consumption observed with chlorbenzuron treatment to the energy demand of detoxification metabolism in insects.

Previous studies have shown that chlorbenzuron enhances the activity of insect detoxification enzymes, such as glutathione S-transferases (GSTs) and acetylcholinesterase (AChE) (He et al., 2022). Activating these enzymes requires energy allocation from the insect's reserves to counteract the toxic effects of insecticides (Guedes et al., 2006; Rand et al., 2015). Zhao et al. (2023) also highlighted chlorbenzuron's impact on alanine, aspartate, glutamate metabolism, glycerophospholipid metabolism, and starch and sucrose metabolism in *Hyphantria cunea* larvae. Additionally, chlorbenzuron increased levels of pantothenate and coenzyme A (CoA), which are involved in fatty acid metabolism and pyruvic acid (PA) production. These metabolites were suggested to indirectly regulate the rate of the TCA cycle by influencing the synthesis of acetyl-CoA, a key substrate (Xia et al., 2019; Xu et al., 2020). Though polymerisation may be linked to translocation and fibrillogenesis in the complicated process of chitin biosynthesis, many classes of chitin synthesis inhibitors may target distinct processes, all of which lead to a loss of chitin creation and extrusion (Merzendorfer, 2013)

3.3 MATERIALS AND METHODS

3.3.1 Treatment of Experimental animal

This chapter explores the impact of the chitin synthesis inhibitor, flufenoxuron, on the sixth instar day-zero male larvae of *Spodoptera mauritia*. Using a metabolomics approach, the study aims to elucidate the effects of flufenoxuron on various metabolites and the associated metabolic pathways. As outlined in previous chapters, the newly

moulted sixth instar male larvae were isolated from the laboratory stock culture and treated with an ED₅₀ concentration of flufenoxuron (0.00068µg). The treated and control larvae were kept separately in plastic tubs and monitored daily for survival. After 144 hours of flufenoxuron exposure, both groups were sacrificed, and haemolymph was extracted by incision on the pupal integument by applying gentle pressure on the abdomen (Loughton & West, 1965). The haemolymph was collected into an Eppendorf tube containing phenylthiourea crystals to prevent melanization. The pooled samples were centrifuged, and the supernatant was then stored at -80°C until further analysis.

3.3.2 Sample preparation and derivation for GC MS analysis

The haemolymph sample derivation was subjected to the protocol Lu et al. (2014) with slight modification. Each sample (100µL haemolymph) was mixed with 800µL of methanol (Himedia) and 100µL of distilled water containing 0.1 mg/mL sucrose (used as an internal standard) for metabolomic analysis. The mixture was then chilled on ice for an hour, briefly vortexed for 10 seconds, and centrifuged at 10,000 g for 15 minutes. The metabolites extracted in the supernatant were collected and labelled separately. Subsequently, 600µL of supernatant was transferred to a GC vial and vacuum-dried for approximately six hours. The resultant extract was taken as it is for derivation before GC-MS analysis. The derivation reaction was carried out in two steps: methoxyamination and trimethylsilylation. To carry out the methoxyamination reaction, the GC vial was supplemented with 90µL of freshly prepared methoxyl amine hydrochloride (15 mg/mL in pyridine; Sigma), and the sample was allowed to incubate overnight at room temperature. Following this, 90µL of BSTFA

containing 1% TMCS (Sigma) was added to the mixture for one hour of trimethylsilylation. Finally, 120 μ L of hexane (Himedia) was added to terminate the derivatization reaction. The derivatized sample vortexed collected the hexane part and transferred it to a new GC vial. The sample was then diluted to make 500 μ L final volume before GC-MS analysis.

3.3.3 Metabolites analysis using GC-MS

The derivatized sample (1 μ L) was loaded to a Shimadzu Nexis GC-2030 GC–MS system equipped with an autosampler AOC-30/20i and analysed in splitless mode utilizing an SH-I-5Sil MS capillary column (30 meters in length, 0.25 mm internal diameter, and 0.25 μ m thickness). The GC–MS analysis parameters were set according to a previous study (Zhou et al., 2020). Helium was served as the carrier gas at a constant flow rate of 1.20 mL/min. The instrument was initially held at 50°C for 1 minute, then ramped to 100°C at a rate of 10°C/min for 1 minute, further ramped to 200°C at a rate of 10°C/min for 1 minute, and finally to 280°C at a rate of 10°C/min for 1 minute. The injector, transfer line, and ion source temperatures were maintained at 250°C, 280°C, and 230°C, respectively. Mass spectra were acquired using electron ionization mode (70 eV) in full scan mode (m/z 50–650), with a solvent delay set at 5 minutes.

3.3.4 Data pre-processing and statistical analysis

The original GC-MS data, obtained through the GCMS Solutions Software, were automatically processed using the GCMS Postrun analysis software. Metabolite identification was then performed by comparing the results with the WILEY 8 and NIST20 libraries based

on mass similarity matches and retention times (RT). Peaks originating from the BSTFA derivatization process and column bleeding were excluded from the analysis. Additionally, exogenous substances such as drugs and compounds derived from plants were filtered out based on their likelihood of being found in biological samples, aiming to refine the metabolite identification process. The blank sample was run between each test sample during the analysis to reduce contamination from sample processing, instrument contamination, and reagent impurities. The collected data were then subjected to multivariate statistical analysis.

3.3.5 Multivariate pattern recognition analysis

Multivariate analysis of haemolymph samples of *S. mauritia* was performed using MetaboAnalyst 6.0 software (Xia & Wishart, 2011). The total area of each sample was first adjusted using the data matrix's mode (Rt/(m/z) and intensities. Total data normalization was conducted to mitigate systematic biases within the dataset. Normalization involved row-wise normalization by the sample median. Subsequently, the data were log-transformed (base 10) to adjust the importance of high and low-abundance metabolites to an equal level. Fold changes in metabolite abundance were calculated by comparing mean concentrations between control and treated groups, with significance determined using Student's t-test to identify differentially abundant metabolites.

Three types of multidimensional statistical analyses—orthogonal partial least squares discriminant analysis (OPLS-DA), partial least squares discriminant analysis (PLS-DA), and principal component analysis (PCA)—were employed to visualize the original data

distribution and variable classification. PCA elucidated the original data distribution, utilizing the PRcomp package for analysis based on singular value decomposition. The differential metabolites contributing to sample group separation were identified using PCA and supervised partial least square discrimination analysis PLS-DA.

The features of the metabolite expression patterns were assessed to ascertain differences in metabolite abundance between flufenoxuron-treated and control samples through Variable Importance for the Projection (VIP) analysis of OPLS-DA model, which will be used to identify the differential metabolites with VIP score greater than 1.5. The OPLS-DA method was implemented using the R ropls package, following the approach described by Thevenot et al. (2016). The OPLS-DA model was also validated using 2000 times permutation tests and a 5-fold internal cross-validation approach, with R^2 and Q^2 values used to assess its quality.

Metabolite correlation analysis utilized Pearson's correlation coefficient to examine the linear association between metabolite richness in haemolymph samples of control and treated groups. Furthermore, the Debiased Sparse Partial Correlation (DSPC) network was employed to assess the degree of betweenness among selected metabolites, following the methodology outlined by Basu et al. (2017).

3.3.6 Pathway analysis

With the assistance of MetabAnalyst 6.0 software, the metabolic pathways affected by flufenoxuron treatment were analysed. For the enrichment analysis, the Quantitative enrichment analysis (QEA) was employed with the aid of package global test, using a generalized

linear model to estimate the *Q-statistic* for each metabolic set. For the pathway topology analysis, the relative between centrality method was utilized. Pathway impact values from pathway topology analysis or P values from pathway enrichment analysis were used to choose the significantly affected pathways. The study's criteria were set at effect levels greater than 0.1 and negative log(P) values greater than 2.0.

3.3.7 Model pathway construction

To examine the effects of flufenoxuron on *S. mauritia* physiology and the construction of metabolic networks, data were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.kegg.jp/>) of *Bombyx mori*.

3.4 RESULTS

3.4.1 Impact of flufenoxuron on haemolymph metabolome of *Spodoptera mauritia*

The current investigation comprehensively explores metabolic changes in the haemolymph of male *Spodoptera mauritia* following exposure to the chitin synthesis inhibitor flufenoxuron, administered at a specific dose of 0.00068 µg. The findings of this study are significant as they shed light on the impact of flufenoxuron on the haemolymph metabolome of *S. mauritia*. To monitor alterations in metabolic levels post-treatment, haemolymph was collected 144 hours after flufenoxuron administration, pooled, and grouped into two categories: the flufenoxuron-treated and the control groups. A significant detrimental effect was observed in the male larvae of *S. mauritia* treated with flufenoxuron compared to the control.

3.4.2 Haemolymph metabolome composition of male *S. mauritia*

Following GC-MS analysis of the haemolymph samples, many metabolites were identified. Specifically, 14 fatty acids and lipid derivatives, six carbohydrates, nine organic acids, six alcohols, one amino acid, and thirty-one other compounds were detected. These metabolites are associated with several metabolic pathways, including starch and sucrose metabolism, galactose metabolism, fructose and mannose metabolism, steroid biosynthesis, nitrogen metabolism, phenylalanine metabolism, fatty acid biosynthesis, ascorbate and aldarate metabolism, glycerolipid metabolism, insect hormone biosynthesis, pentose phosphate pathway, glycolysis/gluconeogenesis, inositol phosphate metabolism, amino sugar and nucleotide sugar metabolism, fatty acid elongation, fatty acid degradation (Table 1).

Table 1. Metabolites identified from the haemolymph of *S. mauritia*

Fatty acids and lipids Hexadecanoic acid Hexanedioic acid Octadecanoic acid Tetradecanoic acid Tridecanoic acid beta-Sitosterol beta-Tocopherol Cholesterol gamma-Sitosterol Oleic Acid Stigmasterol	Sugars D-Ribose Alpha-D-Glucose Sucrose D-Allose D-Trehalose D-Maltose	Organic acids 1,2-Benzenedicarboxylic acid 2,5-Dihydroxybenzoic acid Docosanoic acid 1,3-Benzenedicarboxylic acid Carbonic acid Benzeneacetic acid Benzoic acid phthalic acid l-(+)-Ascorbic acid	Others Eugenol 1,2,4-Trimethylbenzene 2,4-Dimethyldodecane 3,3-Dimethylhexane 4-Allylphenol Bisphenol Ethoxyquin Allyl isothiocyanate Benzothiazole Dodecane Dotriacontane Ectoine Eicosane Floxaduridine 2-Mercaptobenzothiazole Propylene glycol	Heneicosane Hentriacontane Heptadecane Hexadecane Lutidine n-Octacosane n-Tetradecane Octadecane Octane Pentadecane Squalene Tetracosane Tridecane Undecane
	Amino acids L-Norleucine			
	Alcohols Myo-Inositol alpha-Amyrin D-Sorbitol Glycerol Dodecyl acetate Glycerol monostearate			

3.4.3 Statistical analyses overview

The data obtained from the Total Ion Chromatograms (TICs) of *Spodoptera mauritia* haemolymph from the control and flufenoxuron-exposed groups was subjected to multivariate analysis. Unsupervised Principal Component Analysis (PCA) was performed to assess the quality of the GC-MS data. This analysis revealed significant clustering of all treated and control samples, as depicted in Figure 1A. Notably, no outliers were detected among the samples.

The PCA plots illustrate a clear distinction between the metabolic profiles of the control group and those exposed to the chitin synthesis inhibitor (CSI). The metabolic profiles of the haemolymph samples exposed to CSI showed substantial changes compared to the control group. The first principal component (PC1), second principal component (PC2), third principal component analysis (PC3), fourth principal component (PC4), and fifth principal component (PC5) accounted for 73.4%, 17.6%, 5.9%, 1.4%, and 1.1% of the total variance, respectively, in the GC-MS analysis.

Furthermore, the loading plots for Component 1 (PC1) and Component 2 (PC2) highlight the key metabolites in distinguishing the groups. These plots show that the variance was significantly more pronounced in the CSI-exposed group (Fig. 1B), indicating that the CSI treatment had a notable impact on the metabolic profiles.

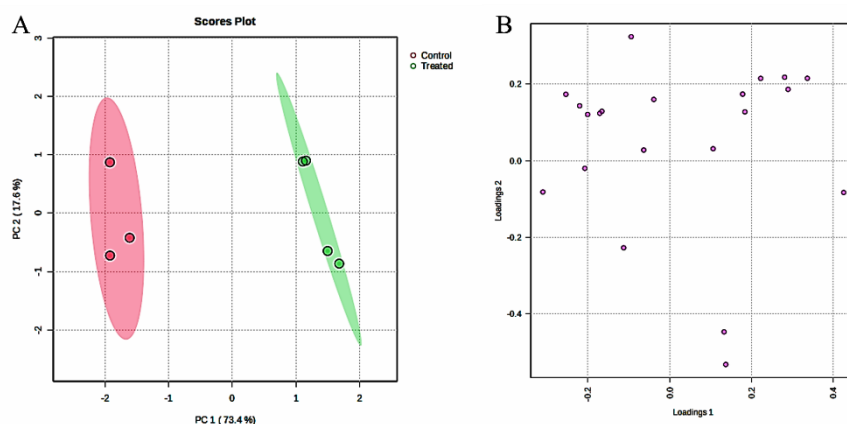


Figure 1: (A) PCA Score plot for separating haemolymph control sample from flufenoxuron treated haemolymph sample and **(B)** Loading plot of the flufenoxuron treated group and the control group representing the key metabolites contributing to the separation of groups observed in the PCA score plot.

Using supervised orthogonal partial least squares discrimination analysis (OPLS-DA), the metabolites accountable for the clear separation between the flufenoxuron-exposed and control groups were found. In these OPLS-DA models, the control and treated groups demonstrated distinct separation and were inside the score plot. The OPLS-DA score plot (Fig. 2) showed significant differences in the metabolic characteristics between the haemolymph control and treated flufenoxuron groups. A cross-validation method was used to test the model quality. The 5-fold internal cross-validation and 2000 times permutation test were further conducted to assess these models' predictive accuracy and statistical significance, and the resulting model's predictive accuracy were $R^2X=0.652$, $R^2Y=0.978$, and $Q^2=0.954$ (Fig 3 A); with its corresponding statistical significance were R^2Y and Q^2 values were 0.994 and 0.987 (Fig. 2 B). The cumulative values of R^2 and Q^2 show a good fit for the model. 2000 times

permutation numbers were used to validate the supervised models, and the p-value was to be less than 0.031. These results indicate that the model was reliable and reproducible, suggesting that the metabolites in the haemolymph of the *S. mauritia* treated with chitin synthesis inhibitor were significantly different from those in the haemolymph of the control groups.

The OPLS-DA analysis has identified 20 differential variables/metabolites based on the score of VIP, which include Tridecanoic acid, Myo-Inositol, Glycerol monostearate, Octadecanoic acid, Cholesterol, l-(+)-Ascorbic acid, Alpha-D-glucopyranose, 3-Picoline, Carbonic acid, Propylene glycol, Beta-Sitosterol, Tetradecanoic acid, 2-Mercaptobenzothiazole, Trehalose, Eugenol, Hexadecanoic acid, Tridecanoic acid, Palmitic acid, Phthalic acid, Glycerol (Fig. 3 B).

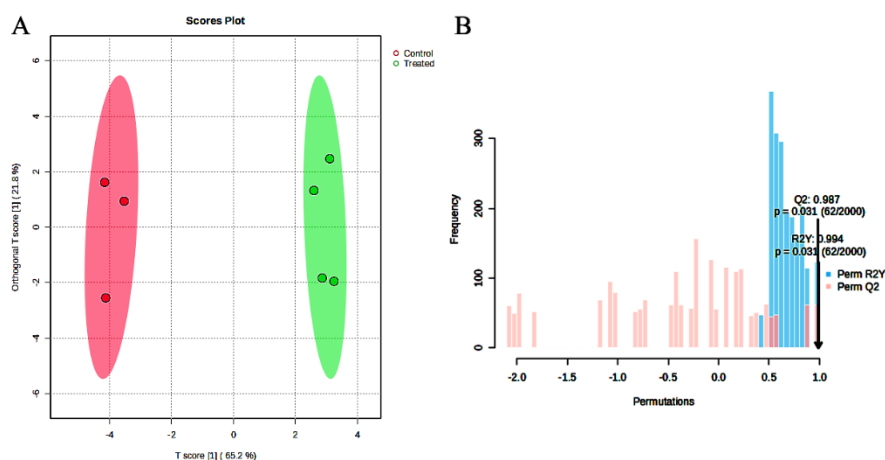


Figure 2: (A) OPLS-DA score plots for separating control haemolymph sample from flufenoxuron treated haemolymph sample **(B)** Permutation test plot (permutation numbers-2000) showing the statistical significance and a p-value.

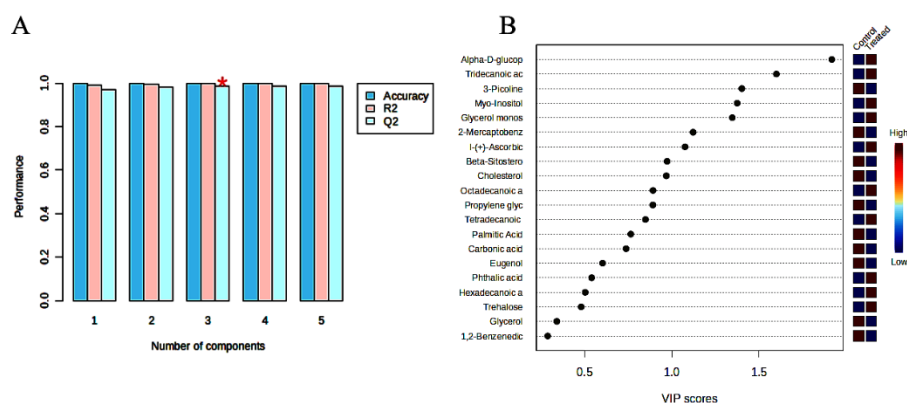


Figure 3. (A): Bar graphs with varying numbers of components displaying the three performance measures (prediction accuracy, R^2 , and Q^2). The best values of the currently chosen measures (Q^2) in cross-validation are indicated by the red "*". **(B)** The top 20 compounds ranked by VIP scores for control versus flufenoxuron-treated groups.

3.4.4 Altered haemolymph metabolites profile of *S. mauritia* on flufenoxuron treatment

The representative GC-MS Total Ion Chromatograms (TICs) of *Spodoptera mauritia* haemolymph samples are depicted in Figure 4. Significant fluctuations were observed in the shape and quantity of peaks between the control and flufenoxuron-treated groups. The overall metabolite profiles of *S. mauritia* haemolymph from the control and flufenoxuron-exposed larvae were further analyzed using Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) and univariate analysis. Metabolites with differential abundance were identified based on the p-value from Student's t-test ($p < 0.05$) and the Variable Importance in Projection (VIP) score from the OPLS-DA model ($VIP \geq 1.5$), as shown in Figure 3b.

Figure 5 displays the volcano plots illustrating the differentially expressed metabolites in the haemolymph of *Spodoptera mauritia* following treatment with flufenoxuron on newly ecdysed sixth-instar larvae. As listed in Table 2, sixteen metabolites were found to have altered abundance, with nine metabolites downregulated and eight upregulated during GC-MS analysis. The primary metabolites affected by flufenoxuron treatment include carbohydrates, lipids, organic acids, and cofactors. Specifically, the saturated fatty acids tridecanoic acid, octadecanoic acid, and tetradecanoic acid, the key metabolites involved in lipid biosynthesis, were downregulated. Additionally, myoinositol, a sugar alcohol involved in energy metabolism, and L-(+)-ascorbic acid, a potent antioxidant, were down-regulated. Alpha-D-glucopyranose, a crucial component in glucose metabolism, was found to be downregulated, potentially disrupting carbohydrate metabolism. Glycerol monostearate, a product of fat breakdown, also showed reduced levels, indicating a decrease in fat biosynthesis. Trehalose, the major haemolymph sugar involved in chitin biosynthesis, was similarly downregulated after flufenoxuron treatment.

In contrast, specific metabolites were upregulated with post-treatment of flufenoxuron. These include cholesterol (a precursor for insect hormone synthesis), 3-picoline, carbonic acid, propylene glycol, beta-sitosterol, and 2-mercaptobenzothiazole. These findings indicate that on day six, after flufenoxuron treatment, significant changes occurred in the metabolic profile of the haemolymph of *S. mauritia*. Heat map analysis further confirmed that the metabolite abundances between the control and flufenoxuron-treated groups differed markedly, as depicted in Figure 6.

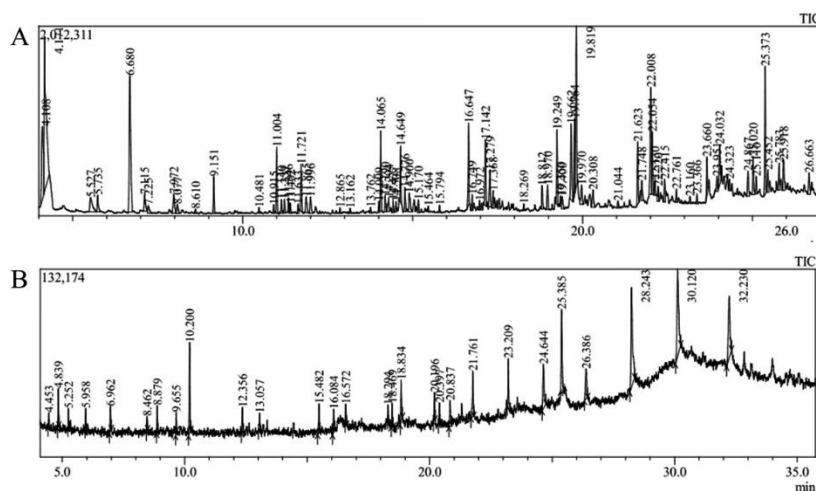


Figure 4: Representative total ion current (TIC) chromatograms of *S. mauritia* haemolymph obtained from GC-MS. A- the haemolymph control sample, and B- the haemolymph of the treated group.

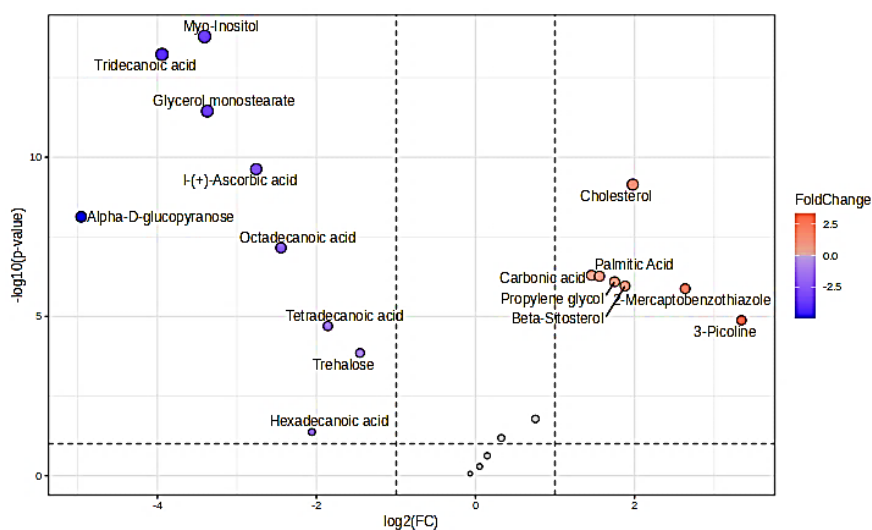


Figure 5: Volcano plot of metabolites in the haemolymph of flufenoxuron treated and control *S. mauritia*. Each point represents a metabolite, and the greater the scattered point, the greater the value of fold change.

Table 2. List of differentially abundant metabolites after flufenoxuron treatment.

Metabolites	Regulation	P-value	Fold Change	VIP
Tridecanoic acid	Down	0.00013003	0.079153	1.5997
Myo-Inositol	Down	0.00016687	0.11468	1.3746
Glycerol monostearate	Down	0.00041249	0.11748	1.347
Octadecanoic acid	Down	0.0011708	0.2307	0.89195
Cholesterol	Up	0.0014832	4.7911	0.9683
l-(+)-Ascorbic acid	Down	0.0015318	0.17983	1.0755
Alpha-D-glucopyranose	Down	0.0017741	0.039111	1.9177
3-Picoline	Up	0.0050648	11.799	1.4014
Carbonic acid	Up	0.0070262	3.2532	0.73744
Propylene glycol	Up	0.0072488	4.0908	0.89137
Beta-Sitosterol	Up	0.0076372	5.1375	0.97322
Tetradecanoic acid	Down	0.0083783	0.26668	0.84843
2-Mercaptobenzothiazole	Up	0.012132	7.3555	1.1218
Trehalose	Down	0.088902	0.44458	0.48046
Eugenol	Up	0.15076	2.0502	0.60249
Hexadecanoic acid	Down	0.41794	0.31442	0.50329

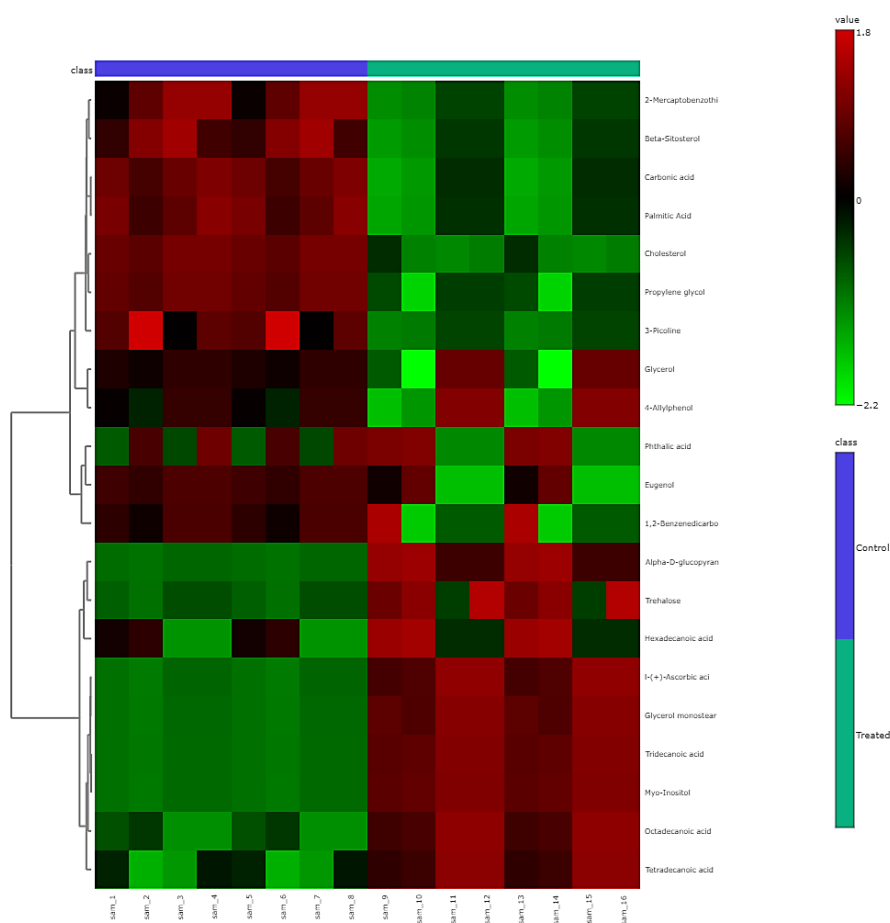


Figure 6: Heat map of differentially abundant metabolites in haemolymph following flufenoxuron treatment. One sample is represented by each column, and one DE metabolite is represented by each row. The relative level of the up-regulated metabolites is shown by the colour red, and the down-regulated metabolites are shown by the colour green.

3.4.5 Pathway analysis

To identify the key functional metabolic pathways affected by flufenoxuron treatment in haemolymph, a metabolic pathway analysis was conducted using the metabolic concentration data obtained from GC-MS analysis. Figure 7 presents the pathway enrichment results.

The enrichment ratio is calculated by dividing the number of hits in a specific metabolic pathway by the predicted number of hits, with a higher enrichment ratio indicating more significant pathway enrichment. Another metric, the p -value, ranges from 0 to 1, with values closer to 0 indicating more significant enrichment. Figure 7 highlights the top 25 pathways activated by flufenoxuron treatment. Notably, metabolic pathways such as ascorbate and aldarate metabolism, inositol phosphate metabolism, pentose phosphate pathway, starch and sucrose metabolism, tyrosine metabolism, phenylalanine metabolism, galactose metabolism, steroid biosynthesis, steroid hormone biosynthesis, fructose and mannose metabolism, glycolysis/gluconeogenesis, and amino sugar and nucleotide sugar metabolism are strongly associated with the effects of the chitin synthesis inhibitor. As shown in Figure 7, ascorbate and aldarate metabolism, along with inositol phosphate metabolism, are significantly enriched, with a relatively higher number of metabolites involved.

Based on negative $\log(P)$ and impact values obtained from the pathway enrichment analysis and pathway topological analysis, four differentially enriched metabolic pathways had pathway impact values more than 0.1, indicating their significance. In accordance with the above results, inositol phosphate metabolism, steroid biosynthesis, starch sucrose metabolism and glycerolipid metabolism in *S. mauritia* are the significantly relevant pathways affected by chitin synthesis inhibitor, and their impact values were 0.11, 0.14, 0.21 and 0.23 respectively (Fig. 8).

The normalized concentrations of metabolite intensities were further analyzed to investigate differences in the metabolites of flufenoxuron-exposed *S. mauritia* haemolymph compared to controls. Significant differences in metabolite abundances were observed between the flufenoxuron-treated and control groups, particularly in carbohydrate-related metabolites (Fig. 10). Metabolomics analysis revealed that the haemolymph of treated individuals showed significantly higher relative levels of alpha-D-glucose, trehalose, D-ribose ($p < 0.05$), and the sugar alcohol myo-inositol ($p < 0.05$) compared to non-treated controls.



Figure 7: Metabolome map of top 25 KEGG-enriched pathways based on metabolites in the haemolymph of *S. mauritia* after treatment with flufenoxuron.

Myristic acid (tetradecanoic acid) and stearic acid (octadecanoic acid) are saturated fatty acids found in *S. mauritia* haemolymph; their relative abundance found increased a little after the flufenoxuron treatment when compared to control. Cholesterol and Beta-sitosterol are relatively less abundant after treatment with flufenoxuron (Fig. 11). Other metabolites like phenylacetic acid, gentisic acid and hydrogen carbonate were also found to have relatively less abundance in flufenoxuron treated individuals when compared to the control (Fig. 12).

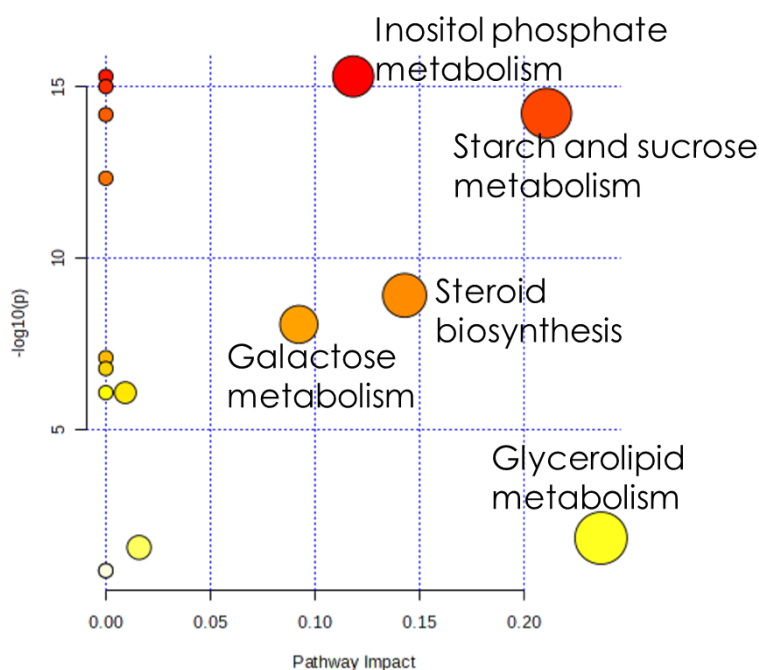


Figure 8: Dot plot representation of pathway topology analysis representing significant metabolic pathways characterized in *S. mauritia* of flufenoxuron treated and control groups. Significantly changed pathways based on enrichment and topology analysis are shown. The x-axis represents pathway enrichment, whereas the y-axis represents pathway impact. Large sizes and dark colours represent major pathway enrichment and high pathway impact values, respectively.

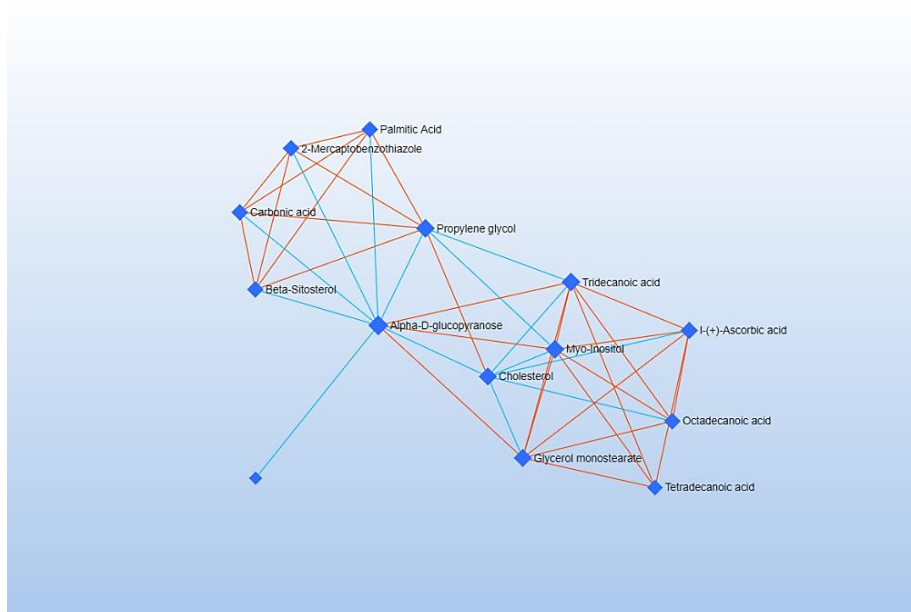


Figure 9: Correlation network of differential metabolites in the flufenoxuron treated *S. mauritia*.

A correlation network analysis of differential metabolites was conducted using Pearson's correlation coefficient. Several edges were formed among the 16 differentially abundant metabolites, creating a complex topological network (Fig. 9). The number of linkages indicates the significance of each metabolite, with glucose emerging as a key metabolite. According to the pathway map, flufenoxuron treatment notably impacts carbohydrate metabolism.

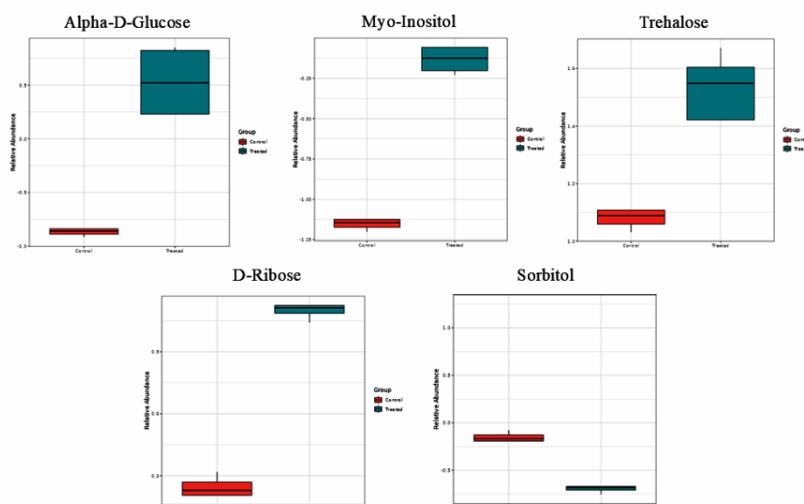


Figure 10: Relative Abundance plot of key metabolites involved in carbohydrate metabolism in the haemolymph of *S. mauritia* after treatment with flufenoxuron. Data were derived from metabolomic enrichment analysis.

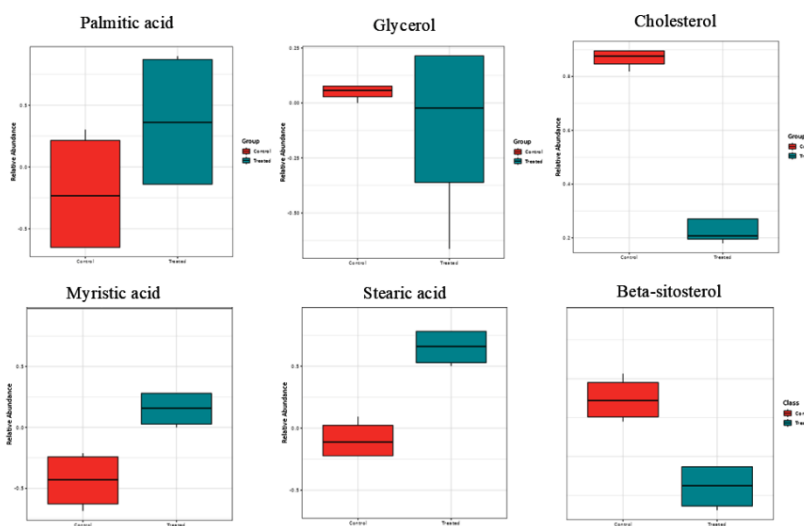


Figure 11: Relative Abundance plot of key metabolites involved in lipid metabolism in the haemolymph of *S. mauritia* after treatment with flufenoxuron. Data were derived from metabolomic enrichment analysis.

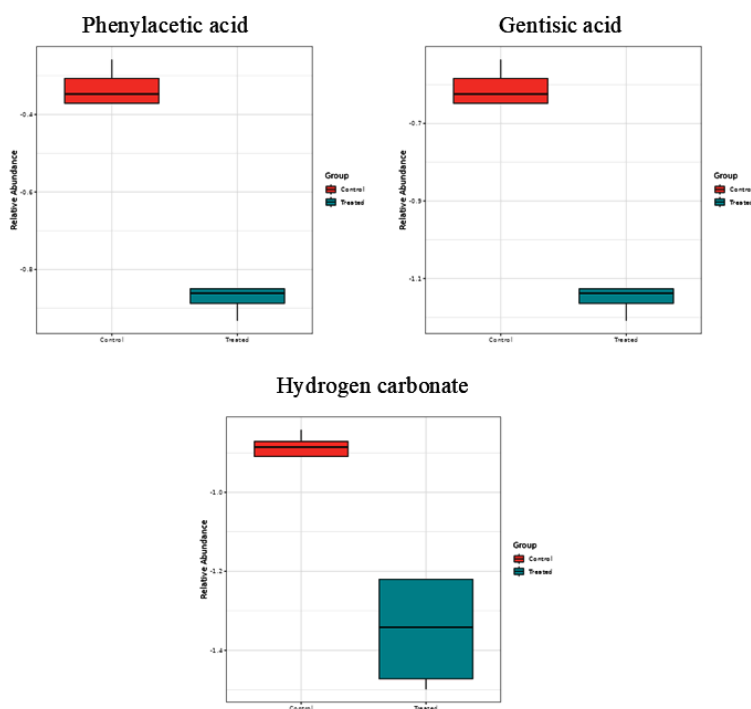


Figure 12: Relative Abundance plot of key metabolites involved in other metabolisms in the haemolymph of *S. mauritia* after treatment with flufenoxuron. Data were derived from metabolomic enrichment analysis.

To identify the metabolic pathways directly responsible for chitin synthesis inhibition and reduced reproductive capacity in *S. mauritia*, the changes in metabolic pathways were analyzed using KEGG pathway analysis of differentially abundant metabolites. A functional model was constructed using the reference map from the KEGG database (Fig. 13). A group of metabolites was associated with carbohydrate and fatty acid metabolism pathways. Seven of these metabolites were downregulated, while one was upregulated. Notably, trehalose and alpha-D-glucose were downregulated, impacting key

pathways such as gluconeogenesis/glycolysis, sugar and starch metabolism, galactose metabolism, and chitin biosynthesis. This disruption negatively affected various other pathways related to carbohydrate metabolism. Myo-inositol and L-ascorbic acid, two critical metabolites in ascorbic acid and aldarate metabolism, were also downregulated. This pathway is closely linked to carbohydrate metabolism. Additionally, tetradecanoic acid, hexadecanoic acid, and octadecanoic acid—initial metabolites involved in fatty acid biosynthesis—were downregulated following flufenoxuron treatment. These findings suggest that flufenoxuron exposure in *S. mauritia* larvae may impair the ability to produce subsequent metabolites in the pathway network, leading to an insufficient supply of essential metabolites for development and reproduction.

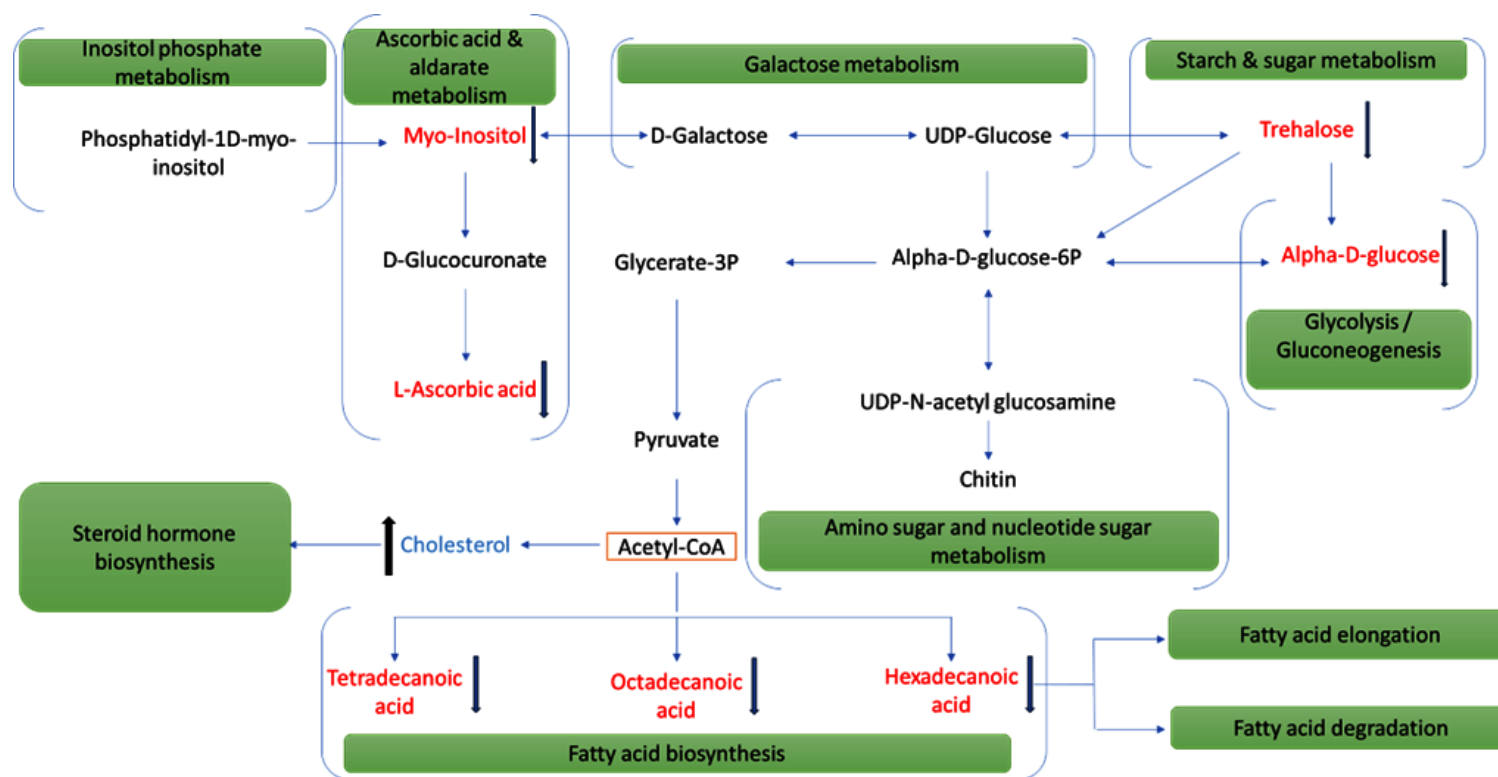


Figure 13: Map of the primarily affected metabolic pathways in the haemolymph of *S. mauritia* treated with flufenoxuron. The paths were simplified and organized based on KEGG pathways. The blue arrows indicate downregulated metabolites, and the Black arrow indicates upregulated metabolites.

3.5 DISCUSSION

3.5.1 Impact of flufenoxuron on metabolomics

Several studies have shown that various chemicals have been developed to target chitin biosynthesis, breakdown, and regulation in insects, inducing moulting deformities and mortality (Liu et al., 2019). These include benzoylphenyl ureas, such as etoxazole, which inhibits chitin synthesis (Nauen & Smagghe, 2006), as well as compounds like allosamidin (Blattner et al., 1997) and psammaplin (Tabudravu et al., 2002) that disrupt chitin breakdown, and benzoyl hydrazines that regulate chitin formation (Liu et al., 2019). Flufenoxuron is a halogenated benzoylurea and effective stomach and contact insecticide. The benzoylurea insecticide, flufenoxuron, inhibits chitin synthesis, a crucial component of the arthropod exoskeleton, making it effective against pests such as phytophagous insects and mites on fruits, vegetables, and ornamentals (Hsiao et al., 2013; Shaurub et al., 2018; Amein et al., 2022). Studies have shown that flufenoxuron application can lead to hatching defects and premature moulting by reducing chitin biosynthesis (Salokhe et al., 2006). Ultrastructural analysis also revealed abnormal endocuticle deposition of lamellate structure dissolution in *Tribolium castaneum* (Merzendorfer et al., 2012).

Chitin production, an intricate physiological and biochemical process absent in vertebrates and plants, is critical in insect moulting (Andersen, 1979; Zhu et al., 2016). Candy and Kilby (1962) were the first to describe the insect chitin biosynthesis pathway, which commences with trehalose and culminates with chitin formation. The

present study attempts to explore the GC-MS-based metabolomics analysis to characterize the haemolymph metabolite profiles of the chitin synthesis inhibitor, flufenoxuron, in *Spodoptera mauritia*. The analysis revealed differential metabolites, including fatty acids, lipid derivatives, carbohydrates, organic acids, alcohols, amino acids, and other compounds. Among these, some key metabolites were up-regulated while others were down-regulated in treated *S. mauritia* compared to the control. Metabolic pathway analysis further highlighted that the affected metabolites were primarily involved in carbohydrate metabolism, lipid metabolism, and steroid biosynthesis.

Metabolomics, complementing transcriptomics and proteomics, bridges the gap between genotype and phenotype, making it a valuable tool for elucidating disease-resistant mechanisms. In insect toxicological studies, transcriptomics and proteomics have been widely employed to investigate molecular responses to toxicants (Xu et al., 2016; Li et al., 2016; Zhang et al., 2018). However, metabolomics has gained increasing attention for its ability to provide a more direct understanding of metabolic alterations. Recently, Li et al. (2022) utilized spatial metabolomics and lipidomics to analyze the metabolic expression levels and spatial distribution in *Plutella xylostella* treated with rotenone. Their findings revealed that rotenone inhibited purine and amino acid metabolism, ultimately damaging the cell membrane of *P. xylostella*.

Several studies have shown that insect haemolymph is a critical fluid that reflects changes in physiological and pathological states, as it plays a vital role in immune defence, physiological regulation, and

nutrient transport (Feng et al., 2014; Wu et al., 2022). Therefore, to validate the metabolomics model, GC/MS analysis was performed on haemolymph samples to determine that the flufenoxuron exposure affected the metabolic profile of male *S. mauritia*. The results demonstrated that flufenoxuron exposure significantly altered metabolic pathways related to energy metabolism. These pathways included ascorbate and aldarate metabolism, inositol phosphate metabolism, the pentose phosphate pathway, starch and sucrose metabolism, tyrosine metabolism, phenylalanine metabolism, galactose metabolism, steroid biosynthesis, steroid hormone synthesis, fructose and mannose metabolism, glycolysis/gluconeogenesis, and amino sugar and nucleotide sugar metabolism. These findings emphasize the potential implications of understanding the broad metabolic disruptions caused by flufenoxuron, providing valuable insights into its impact on insect physiology at the molecular level.

Chitin synthesis inhibitors are a broad class of chemical substances with varying effects on the growth and reproduction of chitin-producing organisms (Merzendorfer, 2013). However, the mechanisms of action for chitin synthesis inhibitors are not well understood, but indirect effects of benzoylureas on the activities of enzymes involved in chitin breakdown and cuticular sclerotization, the transport of uridinediphosphate N- acetylglucosamine across membranes, and hormonal regulation of transcriptional activities were investigated (Aravindarajan et al., 2017). Pyrimidine-nucleoside peptides, for example, interfere with the catalytic site of chitin synthase by affecting nucleotide binding. At the same time, compounds like benzoylureas,

oxazolines, and thiazolidinones are considered to block a post-catalytic stage of chitin production, potentially by a similar mechanism (Asahara et al., 2008; Van Leeuwen et al., 2012). Moreover, these inhibitors may induce physiological stress, triggering compensatory mechanisms to mitigate the stress effects. Sex-specific variations in metabolism, essential for the production of eggs and sperm, are linked to physiological differences in organs responsible for nutrient acquisition (Shingleton & Veal, 2023).

The results obtained from the previous chapters demonstrated that the last instar larvae of *Spodoptera mauritia* treated with flufenoxuron altered complete metamorphosis. Despite their ability to withstand sublethal doses of the insecticide and obtain essential nutrients, the treatment negatively impacted their overall metabolism and reproduction. The impact of flufenoxuron on the biochemical parameters of male *Spodoptera mauritia* was evident from the changes observed in the haemolymph and testis. Specifically, exposure to sublethal doses of flufenoxuron in the sixth instar larvae caused a significant decrease in carbohydrate and lipid levels at 48, 96, and 144 hours. Conversely, the total protein content in both haemolymph and tissue fluctuated significantly in response to the sublethal doses. In the present study, a sublethal dose ($ED_{50} = 0.00068\mu\text{g}$) of flufenoxuron in male *S. mauritia* notably influenced the regulation of metabolite reserves in the haemolymph, particularly those involved in energy metabolism. From the observations, it is evident that exposure to flufenoxuron in the last instar male larvae of *Spodoptera mauritia* led to significant alterations in the haemolymph metabolomic profile, with

over 20 metabolic pathways affected. These changes disrupted key biochemical processes, including carbohydrate metabolism, lipid metabolism, vitamin and cofactor metabolism, and amino acid metabolism. These findings suggest that flufenoxuron disrupts multiple metabolic pathways, which could explain the developmental failures observed in *S. mauritia* after treatment.

Studies have shown that in cell-free systems, chitin synthesis inhibitors (CSIs) do not appear to directly impact the enzymes responsible for chitin synthesis (Liu et al., 2019). However, the study revealed that chitin biosynthesis inhibitors have been reported to target the CHS1 gene (IRAC, 2018). Genomic studies on *Tribolium castaneum* have shown that diflubenzuron significantly alters global gene expression, particularly impacting genes encoding cuticular proteins (Merzendorfer et al., 2012). Moreover, mass spectrometry analysis revealed the upregulation of UDP-N-acetylglucosamine pyrophosphorylase (UAP) and glutathione synthase, adding complexity to the findings and prompting researchers to explore alternative mechanisms of action (Merzendorfer et al., 2012).

3.5.2 Altered carbohydrate metabolic pathway in response to flufenoxuron toxicity

The physiological balance of living organisms relies on the metabolism of carbohydrates. In Lepidopteran insects, sugars obtained from the diet are converted into trehalose, predominantly stored in the haemolymph (Deng et al., 2015). Trehalose is the primary energy reserve in the insect's haemolymph (Tang et al., 2008). This sugar

plays a crucial role in meeting energy demands during stressful environmental conditions such as heat, freezing, desiccation, dehydration, and oxidative stress while also acting as a protective barrier against protein and membrane damage (Crowe et al., 1984; Elbein et al., 2003).

Trehalase is the only known enzyme capable of irreversibly breaking down trehalose, although other organisms may employ different pathways for trehalose metabolism (Avonce et al., 2006). Trehalase converts trehalose into glucose, which meets the energy needs required for flight, development, and defence against severe environmental conditions (Gu et al., 2009; Shukla et al., 2015). At certain developmental stages, trehalose can comprise approximately 20% of the total carbohydrates, leading to its designation as the "blood sugar" of insects (Saltiel & Kahn, 2001; Shukla et al., 2015).

The regulation of trehalose concentration is controlled by a process known as enantiostasis, which ensures metabolic balance in response to environmental changes and is critical for normal physiological functioning (Wharton, 2009; Thompson, 2003). Consequently, trehalose is essential in insect development and various physiological processes (Pan et al., 2019). The concentration of trehalose in the haemolymph has been shown to influence several key functions, including chitin synthesis during moulting (Candy & Kilby, 1962), cold resistance (Wyatt, 1967), food selection and feeding behaviour (Thompson & Dahlman, 1999), and oogenesis (Santos et al., 2012). Once converted into glucose, trehalose is involved in the synthesis of various macromolecular organic substances, such as through carbon

metabolism (Avonce et al., 2006), lipid biosynthesis (Shi et al., 2016), and chitin biosynthesis (Shen et al., 2017). These processes underline the importance of trehalose conversion in supporting vital physiological functions.

The interactions between chitin synthesis inhibitors and carbohydrate content have been the subject of several investigations in recent years (Zhu et al., 2012; Hamadah, 2014). The balance of cellular energy and the production of new cellular building blocks depend on the metabolism of carbohydrates (Mattila & Heitakangas, 2017). Hamadah, (2014) reported that trehalose content after treatment with flufenoxuron decreased considerably in *Schistocerca gregaria*. The present study also revealed that the different carbohydrate metabolites in the haemolymph were down-regulated after flufenoxuron exposure, indicating a suppression of the glycolytic pathway. This suggests that flufenoxuron interferes with normal carbohydrate metabolism.

In the current study, trehalose levels in the haemolymph were consistently downregulated following flufenoxuron treatment. Trehalose plays a central role in starch and sugar metabolism, glycolysis/gluconeogenesis, and amino and nucleotide sugar metabolism. It is also a key precursor in insect chitin synthesis, which begins with glucose and trehalose, ultimately forming the chitin polymer (Merzendorfer & Zimoch, 2003). The reduction in trehalose and glucose levels aligns with the observation that flufenoxuron inhibits chitin formation by disrupting these essential metabolites. Physiological and nutritional factors significantly influence blood trehalose levels, which are not regulated in a strictly homeostatic

manner (Thompson et al., 2003). Several studies indicate that insects' Trehalase (TRE) and Trehalose-6-phosphate synthase (TPS) genes regulate trehalose metabolism, which controls chitin synthesis (Chen et al., 2018; Chen et al., 2010a, b). Xu et al. (2009) demonstrated that trehalose is critical for insect growth, development, and survival, and its downregulation results in various developmental abnormalities.

Simpson and Raubenheimer (1993) suggested that trehalose levels in insect haemolymph reflect the nutritional status and may influence feeding behaviour and nutrient intake. This idea correlates well with the reduced nutrient uptake observed in *S. mauritia* larvae treated with flufenoxuron. Similarly, Xu et al. (2017) reported decreased nutrient intake in *Leptinotarsa decemlineata* after treatment with a chitin synthesis inhibitor. Thompson and Redak (2000) reported a link between trehalose levels and the effects of dietary nutrients on gluconeogenesis in *Manduca sexta*.

In the present study, the downregulation of both glucose and trehalose suggests that other metabolites in downstream pathways, such as glycolysis and the TCA cycle, may also be suppressed. Carbohydrate intake, which influences pyruvate cycling, regulates the balance between glycolysis and gluconeogenesis by controlling pyruvate metabolism (Thompson et al., 2003). Furthermore, flufenoxuron also impacted vitamin and cofactor metabolism, specifically disrupting ascorbic acid and aldarate metabolism, as evidenced by the downregulation of metabolites such as myo-inositol and L-ascorbic acid. Moriwaki et al. (2003) found that *Nilaparvata lugens* contain higher levels of trehalose and myo-inositol than other insects,

highlighting the interconnection between metabolites involved in carbohydrate metabolism. Earlier studies indicate that L-ascorbic acid serves as a cofactor for various enzymes (Smirnoff, 2018) and plays key roles in several metabolic processes, including tyrosine metabolism, collagen production, steroid synthesis, detoxification responses, moulting, phagostimulation, and neuromodulation (Kramer & Seib, 1982). The downregulation of L-ascorbic acid could lead to a shortage of critical metabolomic intermediates, thereby impairing the growth and development of *S. mauritia*.

Myo-inositol is a six-carbon sugar alcohol found in both eukaryotic and many prokaryotic cells, recognized as an essential nutrient (Combs & McClung, 2017). It plays significant roles in signal transduction, endoplasmic reticulum stress, and osmoregulation. Additionally, it serves as a precursor to phosphatidylinositol (PI), a key component of phospholipid membranes (Henry et al., 2014; Chow et al., 2015; Raghu et al., 2019). Alterations in myo-inositol metabolism have been associated with reproductive defects, affecting fertilization and the early stages of embryonic development in human (Croze & Soulage, 2013; Bevilacqua et al., 2015) and critical for balanced metabolism and development in *Drosophila melanogaster* (Rivera et al., 2021). The Inos gene, responsible for encoding myo-inositol-3-phosphate synthase (MIPS) in *D. melanogaster*, is highly expressed in the head and testes in high-throughput expression analyses (Chen et al., 2014). Similarly, Chintapalli et al. (2007) reported that MIPS synthesizes large quantities of inositol in the male reproductive organs of *D.*

melanogaster. Further, Jackson et al. (2018) demonstrated that the absence of myo-inositol is linked to male sterility in *D. melanogaster*.

Myo-inositol also participates in the TCA cycle through galactose metabolism (Zhou et al., 2020), helping to compensate for energy deficits and producing intermediate compounds. The TCA cycle generates significant amounts of ATP, which fuels various synthetic processes and provides intermediate molecules essential for the production of amino acids and fatty acids (Pallardy, 2008). Therefore, the downregulation of myo-inositol in *S. mauritia* following flufenoxuron treatment is expected to indicate a shortage of energy and intermediate molecules, as the TCA cycle is a central metabolic pathway for lipids, carbohydrates, and amino acids (Dong et al., 2017).

The data obtained from previous chapters noted that flufenoxuron induces aberrant reproductive variations in the testes of *S. mauritia* at both cellular and biochemical analysis. Moreover, these impairments can be correlated with the altered haemolymph metabolite profile on flufenoxuron treatment in *S. mauritia*. In an earlier study by Loeb (1986), it was evidenced that the steroids observed in the testicular sheath were sequestered from the haemolymph, suggesting that haemolymph acts as a barrier for transporting materials. Therefore, the present study suggests that the sex-specific haemolymph metabolites in flufenoxuron treated *S. mauritia* might induce a variation in spermatogenesis and reproduction.

3.5.3 Influence of flufenoxuron toxicity on lipid and cholesterol metabolic pathway

In insects, sterols are essential nutrients that must be continuously acquired from their diet for proper growth and development (Dong et al., 2017). Few reports have shown that a lack of sterols can impair ecdysone synthesis and oviposition while having adequate sterols but insufficient fatty acids can restrict insect growth (Niwa et al., 2010; Salini et al., 2015). Phosphatidylethanolamine, phosphatidylcholine, and ether lipids are particularly abundant in the lipid profile of male and female reproductive organs, highlighting the crucial role of lipids as components of the sperm membrane (Chintapalli et al., 2013). The non-protein fraction of seminal fluid comprises lipids, nucleic acids, amino acids, fatty acids, hormones (such as 20E), and other bioactive substances (Scolari et al., 2021). Metabolomic analysis of the ovary revealed high metabolic activity, with prominent pathways related to energy reserves, lipid and terpenoid biosynthesis, stem cell development, and antioxidant defence (Chintapalli et al., 2013). An imbalance in fatty acid and lipid content can lead to physiological dysfunction in male reproductive organs.

In the present study, the levels of free fatty acids, including tetradecanoic acid, hexadecenoic acid, and octadecanoic acid, were downregulated following treatment with flufenoxuron, whereas cholesterol levels increased. These findings indicate significant alterations in the lipid metabolic pathways of male *S. mauritia*. Pesticide-induced oxidative stress can disrupt various metabolic homeostasis, including amino acids, nucleic acids, and fatty acids

(Broadrup et al., 2019). Huang et al. (2012) noted that azadirachtin has the potential to reduce both the quantity and content of fatty acids significantly. The balance of nutritional intake, maintenance, development, and reproductive needs influences the availability of lipids. Toxic substances can disrupt this balance (Tanani et al., 2022). Consequently, fatty acids are considered crucial chemical stress indicators (Filimonova et al., 2016).

Lipids are a vital insect energy source, regulated by neuroendocrine feedback loops (Downer, 1985). They are essential components of insect cell membranes and act as precursors for various pheromones (Arrese & Soulagès, 2010). In holometabolous insects, the accumulation and mobilization of lipids are crucial for the significant remodelling of body structure and biochemistry. The primary function of lipid accumulation during the larval stage is to support metamorphosis during the pupal stage and, often, to fuel the energy-intensive activities of non-feeding adult stages, such as flying and reproduction (Huang et al., 2012). A finely tuned balance exists during feeding phases between the lipid and fatty acid content derived from food and the *de novo* synthesis of fatty acids from dietary carbohydrates.

Ziegler et al. (2006) previously demonstrated the roles of fatty acids in insect reproduction. For instance, linoleic acid, an octadecanoic acid, is likely vital for insect reproduction due to its substantial presence in the dry mass of oocytes and its various functions, including providing energy to the developing embryo (Huang et al., 2012). Zhang et al. (2022) reported that the consumption of azadirachtin may decrease

linolenic acid metabolism in *Chrysopa sinica* larvae, potentially affecting pupation and eclosion. Lipidomics studies indicate that the composition of triglycerides and other membrane lipids is altered by impaired lipolysis in females, negatively impacting female fecundity (Yang et al., 2024). Additionally, phosphatidylinositol lipids are crucial for spermatocyte cytokinesis, cyst polarity, and axoneme development (Ben-David et al., 2015).

The current study demonstrated that metabolomic analysis is an efficient method to examine the changes in the haemolymph metabolome induced by the chitin synthesis inhibitor flufenoxuron. Prolonged exposure to flufenoxuron is anticipated to reduce fitness and survival due to lipid and carbohydrate reserve depletion. Our model provides insights into the physiological effects of flufenoxuron by elucidating how alterations in metabolite regulation contribute to the stress response observed in the haemolymph.

3.6 CONCLUSION

The current study comprehensively assessed the impact of the chitin synthesis inhibitor, flufenoxuron, on the haemolymph metabolome of *S. mauritia*. The findings revealed profound metabolic disruptions, with treated individuals displaying markedly altered profiles compared to controls. Notably, carbohydrate and fatty acid levels were significantly reduced, indicating severe nutritional and energetic stress. These changes were accompanied by elevated levels of certain metabolites, suggesting inefficient metabolic utilization under pesticide stress.

The pathway analysis further demonstrated that flufenoxuron disrupted essential processes, including glycolysis/gluconeogenesis, starch and sucrose metabolism, the pentose phosphate pathway, steroid biosynthesis, and amino sugar and nucleotide sugar metabolism. These pathways are vital for energy homeostasis, developmental regulation, and hormonal synthesis, highlighting the systemic impact of flufenoxuron exposure. The study represents a significant advancement in using GC-MS for lepidopteran haemolymph metabolomics, advancing our understanding of insect biochemistry under pesticide stress.

The findings of the study deepen our understanding of the physiological disruptions caused by flufenoxuron, highlighting its potential hazards in agricultural applications. By elucidating the intricate relationship between flufenoxuron and *S. mauritia* physiology, particularly its impact on chitin biosynthesis and reproductive development, this study underscores the need to evaluate CSIs to mitigate ecological risks carefully. The investigation also provides a baseline for future studies focusing on the broader implications of CSIs on non-target organisms and agricultural ecosystems.

General Conclusion

The current study examined the effect of the chitin synthesis inhibitor, flufenoxuron, on the changes in the male reproductive development and spermiogenesis of sixth instar larvae of *Spodoptera mauritia*. Based on the results obtained from the current study, it can be concluded the following aspects.

- From the toxicity study, it was observed that flufenoxuron treatment severely affected the pupal metamorphosis. Moreover, flufenoxuron exhibited multiple effects on the morphology, metamorphosis, and development of *S. mauritia*.
- The study demonstrates that flufenoxuron adversely affects key life parameters of *Spodoptera mauritia*, including egg hatchability, larval duration, larval-pupal moulting, and adult emergence, by inhibiting chitin synthesis.
- Flufenoxuron inhibited the growth and development of the male reproductive organs in *S. mauritia*. The study envisages that the chemical treatment may reduce the insect species' population growth through acute toxicity or by diminishing its reproductive capacity.
- Flufenoxuron-treated groups exhibited high sensitivity in various biochemical indices, reflecting the pesticide's impact.

The effects of flufenoxuron were associated with a significant reduction in proteins, lipids, and carbohydrates in the haemolymph and testes of treated individuals. This depletion likely contributed to the normal development of the testes and the failure to produce healthy spermatozoa.

- The observed depletion may be due to damage in the larval midgut epithelium, impairing nutrient absorption and metabolism, ultimately affecting testicular development and reproductive success.
- The results demonstrated that flufenoxuron exposure on sixth instar larvae of *S. mauritia* induced testicular reproductive toxicity, significantly impairing spermiogenesis. This is evidenced by a reduction in the viability of spermatogonia, spermatocytes, and sperm bundles, ultimately compromising the survival of future generations. Confocal microscopy using Hoechst and TMRM validated these findings by indicating reduced mitochondrial membrane potential in developing spermatogonia, spermatocytes, and dichotomous sperm bundles.
- The results confirmed the hypothesis that GC/MS metabolic profiles of haemolymph of *S. mauritia* exposed to flufenoxuron reflect the disturbance of the insect's metabolism and are substantially different compared to those of the untreated group. The metabolites with the highest contribution to the observed discrimination were carbohydrates and fatty acids,

which provide insights into the effects on the physiology of *S. mauritia*.

- The chemical exerts nutritional and energetic stress on *S. mauritia* in its attempt to divert the metabolism, mainly in decline in the levels of the majority of carbohydrates and lipids. Corresponding to this, elevated levels of some metabolites in the exposed group may indicate a general disruption in *S. mauritia* metabolism or the incapacity to use the metabolites for its nutritional requirements.
- Exposure to flufenoxuron induced significant alterations in pathways associated with energy metabolism in *S. mauritia* haemolymph, including ascorbate and aldarate metabolism, inositol phosphate metabolism, pentose phosphate pathway, starch sucrose metabolism, tyrosine metabolism, phenylalanine metabolism, galactose metabolism, steroid biosynthesis, steroid hormone synthesis, fructose and mannose metabolism, glycolysis/gluconeogenesis and amino sugar and nucleotide sugar metabolism.
- Glycolysis/ gluconeogenesis, ascorbate and aldarate metabolism and starch sucrose metabolism are interconnected with the chitin biosynthetic pathway and reduction in the metabolites of these pathways subsequently restricts chitin biosynthesis in treated individuals. Precursors for chitin biosynthesis (glucose and trehalose) are generated from these

pathways and transported to initiate the chitin biosynthetic pathway.

Future research building on these findings could offer valuable insights into the specific mechanisms by which chitin synthesis inhibitors cause reproductive toxicity in pest species, highlighting their potential as a targeted approach for insect control. This study suggests that the examined CSI, flufenoxuron, considered an environmentally safer alternative to synthetic chemical insecticides, is a potential insecticide that can be a suitable candidate to be integrated into insect pest management strategies.

Future Perspectives

1. **Complete haemolymph metabolomic profiling:** Hemolymph metabolites have recently been used in research to identify biomarkers that indicate a viral infection, toxic exposure, or other biological response. Metabolomics is the first option for investigating the metabolic phenotypes and phenotypic abnormalities impacted by dynamic environmental changes. In our future studies, complete metabolomic profiling of *S. mauritia* will be done to identify the total metabolome of the insect, which can be used to study how an insect's metabolism responds to environmental stressors. Documentation of complete metabolomics of an organism that will make easy access to the metabolic database to observe and analyze metabolic changes during its life cycle.
2. **Testicular metabolomic profile:** Testicular metabolic profiling needs to be conducted in future to understand the exact mechanism undergoing in the testicular development after the treatment with flufenoxuron on *S. mauritia*. A complete metabolome is essential to study the further reproductive level evaluation of *S. mauritia* development.
3. **Proteomic studies on haemolymph and testis:** proteomics studies may unravel the molecular mechanisms behind insect feeding, diapause, metamorphosis, gametogenesis, embryogenesis, etc. therefore correlation of metabolomic profile with proteomic analysis will provide insight into complete pathway behind the regulatory action of pesticide in insect development.

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Appendix

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Effects of flufenoxuron on the male reproductive development of *Spodoptera mauritia* Boisduval (Lepidoptera: Noctuidae)

V. Reshma · E. M. Manogem

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Abstract The insecticide flufenoxuron, one of the insect growth regulators used in pest control, has been presented as a viable alternative against several insects and mites by inhibiting chitin synthesis and leading to changes in the midgut epithelium. The current study aimed to examine whether flufenoxuron interferes with the structural organisation, weight, volume, fecundity and fertility of the male reproductive system of *Spodoptera mauritia*, a sporadic pest that occasionally inflicts significant losses to rice crops. Median effective dose (ED_{50}) indicates that flufenoxuron is effective against *S. mauritia*, and the moulting inhibition percentage, when calculated with probit analysis, indicates that ED_{50} were $0.00068 \mu\text{g}$ for 144 h. Topical application of two selected doses (ED_{25} and ED_{50}) was applied to find out the effects of flufenoxuron exposure on male gonadal development. The flufenoxuron treatment significantly impaired the organization and development of the testis follicles and other parameters like weight, volume and gonadosomatic index. Moreover, reductions in fecundity and fertility rate, 27.33 ± 13.86 and 85.36 ± 12 for treated male $\times$ untreated female, 47 ± 23.33 and 97.16 ± 22.92 for untreated male $\times$ treated female,

and 4 ± 4 and zero for both treated pairs, were found in the emerged *S. mauritia* adult following the larval treatment, suggesting the occurrence of defects in reproductive organs in both male and female moths. These findings highlight the reproductive toxicity of flufenoxuron on the rice swarming caterpillar, offering insights into the male reproductive toxicity associated with chitin synthesis inhibition. The impairment of reproductive organ development suggests the potential use of flufenoxuron for controlling pest populations effectively.

Keywords Chitin synthesis inhibitor · Army worm · Reproductive toxicity · Testis morphology · Gonadosomatic index · Fecundity

Introduction

The intensive use of synthetic pesticides has led to insecticide resistance, environmental pollution, and health hazards to humans and non-target organisms. As a result, eco-friendly safe compounds, like Insect Growth Regulators, were developed as alternative pest control agents (Li & Fantke, 2023). Over the past few decades, investigation has concentrated on identifying chemicals that impede insect development and reproduction that target the endocrine regulating systems. Since then, several substances known as "insect growth regulators" (IGRs) have been in use that negatively affect the development and reproduction

V. Reshma (✉) · E. M. Manogem (✉)
Division of Insect Endocrinology, Department of Zoology,
University of Calicut, 673635 Malappuram, Kerala, India
e-mail: reshma_do@uoc.ac.in

E. M. Manogem
e-mail: dr.manogem_em@uoc.ac.in

of insects. Their mode of action is highly selective and species specific (Tunaz & Uygun, 2004). IGRs are classified as juvenile hormone analogues (methoprene, pyriproxifen, and fenoxycarb), ecdysone agonists (methoxyfenozide, tebufenozide), and chitin synthesis inhibitors (diflubenzuron, lufenuron) (Oberlander & Silhacek, 2000; Poppenga & Oehme, 2010).

The armyworm, or rice-swarming caterpillar, *Spodoptera mauritia* Boisduval (Lepidoptera: Noctuidae), is a sporadic paddy pest in India that causes severe loss in rice yield production. This pest is highly prevalent in Asia and Australia, with infestations reported in Indian states such as Kerala, Tamil Nadu, Orissa, Bihar, Jharkhand, and Chhattisgarh (Ramaiah & Maheswari, 2018). Moreover, in recent years, the armyworm has become a significant pest in southern and eastern India, severely impacting wet-season rice production (Jena et al., 2017; Tanwar et al., 2010). Reports indicate that swarming caterpillars ravaged 1,304 hectares of land in Kerala, in Alappuzha district causing severe damage to paddy crops (Jena et al., 2017). In India, rice swarming caterpillars are typically controlled using insecticides such as chlorpyrifos, quinalphos, triazophos, or dichlorvos. During *S. mauritia* infestation in Odisha in 2015, various insecticides, including chlorpyrifos, imidacloprid, thiamethoxam, triazophos, and acephate, were employed for rapid larval control. Studies have shown that exposure to these pesticides, particularly organophosphates and neonicotinoids, is associated with endocrine disruption and reproductive abnormalities, primarily in mammals (Suwannarin et al., 2021).

Due to the perceived safety, IGR insecticides have been used to reduce adverse effects on the environment and beneficial species that are not their intended targets. Among the benzophenylureas, several insect growth regulators work effectively against *Spodoptera* species larvae through contact and ingestion inhibition of the biochemical processes that lead to the formation of chitin synthetase, resulting in abnormal endocuticle deposition and interrupting moulting (Clarke & Jewess, 1990; Mead & Khedr, 2018; Tabozada et al., 2014). Flufenoxuron, belonging to the benzophenylurea class insecticide, has been employed for the control of insect pests belonging to the orders Diptera (Matsuda, 2022), Coleoptera (Ali et al., 2022), and Lepidoptera (Amein & Mohammad, 2022; Bakr et al., 2013; Clarke & Jewess, 1990; Tabozada et al., 2014). Flufenoxuron is an effective

stomach and contact insecticide (Hsiao et al., 2013), inhibiting chitin synthesis during insect development. Additionally, flufenoxuron has been used as an insecticide for public hygiene, primarily to control fleas and cockroaches (Clarke & Jewess, 1990). Nevertheless, earlier research has indicated that flufenoxuron may also have harmful impacts on insects' ability to reproduce (Salokhe et al., 2003; Shaurub et al., 2018; Yasir et al., 2012). To date, only a few studies are available on the reproductive toxicity of flufenoxuron; therefore, the underlying processes are still not fully understood. For a better understanding and more focused application of these chemicals, the effects of compounds on insect nutrition and reproduction are also crucial. In light of the above context, the present study aimed to examine the male reproductive level toxicity of flufenoxuron in the serious paddy pest *S. mauritia* (<https://www.cabi.org/isc/datasheet/51071>).

Materials and methods

Experimental organism

The fertilized *S. mauritia* moths were collected from paddy fields using fluorescent light traps. The adults were maintained in glass beakers with muslin cloth coverings and fed with diluted honey solution (1:1). The females deposited their eggs in the container provided, and within two to three days, the eggs developed into larvae. The stock colony was maintained as per the earlier standardized laboratory protocol (Banu et al., 2022). In the present study, newly moulted sixth instar day zero larvae of *S. mauritia* males (yellow-coloured paired testis visible through the dorsal skin of larvae) were selected for the experimental analysis.

Tested compound

The insecticide Flufenoxuron (Technical grade; 98% purity), was purchased from Sigma-Aldrich for the studies.

Toxicity bioassay

Acute toxicity studies were carried out under laboratory conditions at room temperature $RH 90 \pm 3\%$ and 10:14 light: dark photoperiod. The freshly ecdysed sixth instar day zero larvae of *S. mauritia*

were taken from the laboratory stock culture. The acetone-dissolved flufenoxuron was applied topically at doses of 0.025 μM to 5 μM . Five microliters of the chemical were topically administered to the abdomen of newly ecdysed larvae using a Hamilton microsyringe. Equal amounts of acetone were utilized to maintain the vehicle control group and larval group without treatment considered as control. Four replicates of each concentration were maintained (10 larvae/replicate). The experimental and control larvae were observed daily for morphological abnormalities until 144 h (pupal stage). Abbott's formula (1925) was used to determine and corrected the percentage moulting. Probit analysis was employed to estimate the median effective dose (ED_{50}) (Finney, 1971).

$$\text{Corrected Moulting inhibition} = \frac{P - P_0}{100 - P_0} \times 100$$

P Moulting inhibition percentage of treated group.

P_0 Moulting inhibition percentage of control group.

The resulting ED values were determined and applied in the toxicological analysis to compute the developmental aspects. Two concentrations of the flufenoxuron, ED_{25} and ED_{50} were employed for all the experiments. Three replicates of each concentration were maintained (10 larvae/replicate). After 48, 96, and 144 h of flufenoxuron exposure, samples were taken separately for further analysis.

Surgical techniques

The treated larvae and the controls were anaesthetized with ethyl acetate after 48, 96, and 144 h of treatment. The dorsal side of the larvae was pinned in a wax-lined Petri dish. The abdomen was cut longitudinally, and the testis was removed with fine forceps under a stereoscopic microscope (Manogem & Nair, 2013). After being cleaned with insect ringer solution (NaCl—0.65 gm, KCl—0.025 gm, CaCl—0.03 gm, NaHCO_3 —0.025 gm dissolved in 100 ml distilled water), the isolated testis were transferred to 50 mM PBS and examined further for reproductive developmental analysis (Banu et al., 2022).

Effect of flufenoxuron on the development of *S. mauritia*

Newly moulted sixth instar day zero larvae of *S. mauritia* were treated with the ED_{50} and ED_{25} values of flufenoxuron as described above. The treated individuals were examined after 48 h, 96 h, and 144 h to determine developmental parameters such as instar body weight and adult emergence. Each treatment was replicated thrice (10 larvae/replicate) using different insect batches. A parallel control and vehicle control was also run.

Effect of flufenoxuron on the male reproductive development of *S. mauritia*

The dissected-out testes of the flufenoxuron exposed larvae at different intervals were observed for morphological modifications with a Zeiss stereomicroscope (Zeiss Discovery V20 Stereomicroscope attached with a Zeiss 506 camera) and photographed. The morphometric measurement was performed using the ZEN application. The testis volume was determined after 48 h and 96 h (larval stage) according to Hiroyoshi et al., (2021), assuming that the testis is an ellipsoid. The paired testis was examined and tabulated for statistical analysis. The fused pupal testis forms into a single spherical organ, and the radius was determined using the mean diameter from 2 measurements across the organ's central axis divided by two and calculated the testis volume (size) using a formula according to Liu et al. (2021), where r is the radius of the testis.

$$\text{Volume(Larval testis)} = \pi/6 \times \text{Length} \times \text{Width}^2$$

$$\text{Volume(Pupal testis)} = 4/3\pi r^3$$

The weight of the dissected testis from the experimental and control sets was determined using a digital weighing balance (Shimadzu). The Gonadosomatic index (GSI) was computed using the equation according to (Sturm, 1978).

$$\text{GSI} = \text{Weight of } \frac{\text{Gonad}}{\text{Insect}} \times 100$$

To determine fecundity and fertility, the adults that survived treatment of freshly moulted sixth

instar larvae of *S. mauritia* with the ED₂₅ values of flufenoxuron were allowed to mate with untreated individuals. One treated male and one untreated female, one untreated male and one treated female, and one treated male and one treated female were the three mating combinations used for the study. One untreated male and one untreated female were used as a parallel control. Three replicates were carried out for each combination using different batches at various periods. Fecundity (total number of eggs laid by a female) and fertility (% of eggs hatch successfully) were calculated.

Statistical analysis

All data were provided as Mean $\pm$ Standard Error (SE) for each group. SPSS 24.0 were used for the analysis. The differences between and among the control and treatment groups were examined using one-way ANOVA, followed by Duncan's Multiple Range as a post-hoc test. The significance level was chosen at $P > 0.05$. Statistical analysis for developmental parameters was performed using R version 4.2.3 (Eye Holes). Differences were evaluated using a one-way ANOVA after confirming equal variance (Levene test, $p = 0.5$) and normality (Shapiro test, $p > 0.5$ for all groups).

Results

Acute toxicity

Our findings indicate that the impact of flufenoxuron on the moulting process of sixth-instar larvae varies with dosage, demonstrating an apparent dose-dependent effect. Data represented in Table 1 demonstrated that flufenoxuron has a significant effect against newly moulted sixth instar larvae of *S. mauritia*. Larvae exposed to a higher dose (5 μM) exhibited complete moulting inhibition and metamorphosis. A relatively lesser rate of moult inhibition was observed when a lower dose of flufenoxuron (0.025 μM) was administered, and significant moulting inhibition was exhibited by the dose 0.1 μM to 5 μM . The ED₅₀ value for the flufenoxuron treatment on the sixth instar larvae was 0.00068 μg (Table 1). Regression analysis showed a dose-dependent correlation with a value $R^2 = 0.964$ and a slope value of 0.81. The calculated ED₂₅ and ED₅₀ values are 0.00015 μg and 0.00068 μg , respectively.

In the toxicity analysis, after 144 h of observation, severe morphological anomalies were noticed compared to control pupae (Fig. 1A), such as the formation of larval pupal intermediates and abnormal pupae, followed by further developmental arrest (Fig. 1B, C, D, E and F). Malformations in the larval stage result in the formation of larval pupal intermediates with the larval body and pupal body part (Fig. 1B

Table 1 Effect of flufenoxuron on the median effective dose for 144 h (ED₅₀-144 h) in *Spodoptera mauritia* by Probit analysis

Dose(μM)	Moult inhibition (%) ($\bar{x} \pm \text{SE}$)	%CM	ED ₅₀ (μM)	ED ₅₀ (μg)	χ^2 (df = 7)	Variance (F)	Slope value	R ²	P value
Control	2 $\pm$ 0.25	0	0.266	0.00068	16.72	7.61	0.81	0.964	0.019
V control	2 $\pm$ 0.25	0							
0.025	16 $\pm$ 0.4	14.28							
0.05	32 $\pm$ 0.4	30.61							
0.1	40 $\pm$ 0.7 ^a	38.77							
0.25	48 $\pm$ 1.0 ^b	46.93							
0.5	52 $\pm$ 0.86 ^c	51.02							
1.0	64 $\pm$ 1.7 ^d	63.26							
2.5	80 $\pm$ 2.3 ^d	79.59							
5.0	100 $\pm$ 2.5 ^d	100							

Control–Nil mortality, CM– Corrected moult inhibition, $\bar{x} \pm \text{SE}$ – Mean $\pm$ Standard error, χ^2 –Chi-square value, df–degrees of freedom. The mean in the same column followed by the same superscript does not differ significantly at $P < 0.05$ (Duncan test). Four replicates (10 larvae in each set) were treated at each concentration



Fig. 1 Morphological malformations of pupal instar of *Spodoptera mauritia* on post-treatment with 0.025 μM – 5.00 μM concentrations of flufenoxuron on the sixth instar larvae. **A**-Normal (Control) pupa; **B** and **C**- larval pupal intermediates

containing larval body part and sclerotized body; **D**- Abnormal pupae with malformed thoracic region; **E**- Abnormal pupae with highly sclerotized thorax and failed to moult larval integument; **F**- Abnormal pupae with malformed head region

and D). Moreover, the diverse degree of pupal malformations was evidenced as a abnormal pupae with malformed thoracic region (Fig. 1C), highly sclerotized thoracic region that failed to shed larval integument, inverted shape and a malformed head region, and pupae with reduced size and shrunken cuticle (Fig. 1E and F). Figure 2 indicates a clear correlation between higher concentrations of flufenoxuron and increased pupal mortality and malformations, a decrease in pupation rates, followed by an increase in the percentage of larval-pupal intermediates.

Toxicity effect on adult emergence

The data of *S. mauritia* adults emerged after treatment of sixth instar larvae indicated a significant difference

in adult emergence treated with flufenoxuron (Table 2). There was a significant reduction in the number of adults emerged after the treatment with the ED₂₅ dose when compared to control (96.66 ± 0.33%); it allowed only 23.33 ± 0.33 per cent of adults to emerge. Conversely, adult emergence was blocked entirely with the treatment of ED₅₀ concentration, and the survival rate of pupae was highly reduced. Moreover, the corresponding rate of formation of larval pupal intermediate with ED₂₅ and ED₅₀ significantly increased to 66.67 ± 0.88% and 96.7 ± 0.33%, respectively.

Toxicity effect on instar body weight

The Body weight was significantly impaired by flufenoxuron treatment on *S. mauritia*. The instar

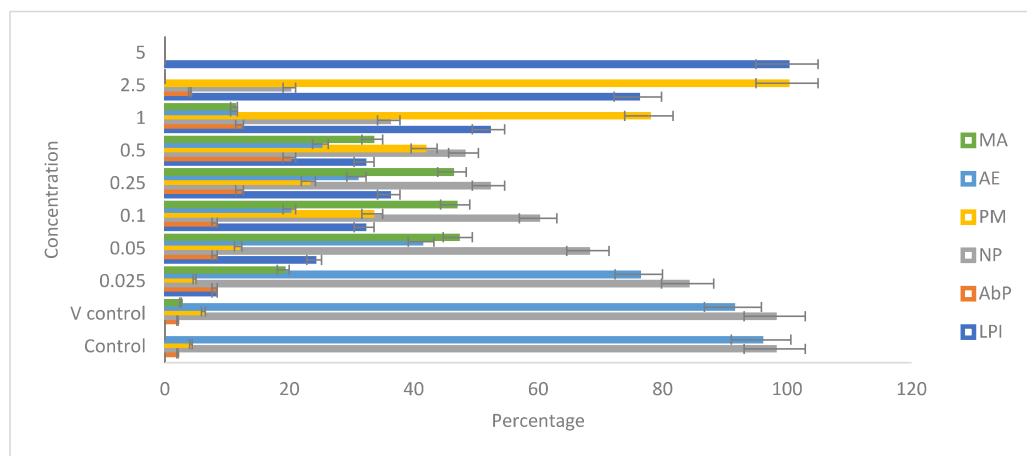


Fig. 2 Effect of flufenoxuron on developmental stages of *Spodoptera mauritia*. The data expressed in the percentage mean values of four independent experiments ($N=10$). LPI- larval

pupal intermediate, AbP- Abnormal pupal formation, NP- Normal pupal formation, PM- Pupal mortality, AE-Adult emergence, MA- Malformed adult

Table 2 Effect of flufenoxuron on adult emergence of *S. mauritia*

Experimental group	Larval-pupal intermediates (Mean% ± SE)	No. of Normal Pupae (Mean% ± SE)	Dead pupae (Mean% ± SE)	Adult emerged (Mean% ± SE)
Control	0	100 ± 0	3.33 ± 0.33	96.66 ± 0.33
V Control	0	100 ± 0	6.67 ± 0.33	93.33 ± 0.33
ED ₂₅	66.67 ± 0.88*	33.33 ± 0.88*	10 ± 0.57	23.33 ± 0.33*
ED ₅₀	96.7 ± 0.33**	3.34 ± 0.33**	3.33 ± 0.33	0**

The data expressed in the mean% ± SE values of three independent experiments ($N=10$). Statistical differences were considered significantly by one-way ANOVA (the same number of asterisks do not differ significant at $P < 0.05$)

body weight decreased with increasing flufenoxuron concentrations (ED_{25} and ED_{50}) and at different hours of exposure (Fig. 3). For the treatment groups ED_{50} and ED_{25} , significant reductions in body weight were noted with 0.377 ± 0.009 g and 0.349 ± 0.011 g after 48 h compared to control and vehicle control, 0.430 ± 0.010 g and 0.436 ± 0.010 g, respectively. After 96 h, the weight of instar in control and vehicle control was 0.241 ± 0.005 g and 0.240 ± 0.005 g; after the treatment, the weight of larvae reduced considerably, at a rate of 0.184 ± 0.005 g and 0.169 ± 0.012 g for ED_{25} and ED_{50} , respectively. At the final interval (144 h) weight of pupal instar were 0.136 ± 0.003 g and 0.136 ± 0.004 g in control and vehicle control groups, the Larval—pupal intermediate weight for the ED_{50} treated group was 0.102 ± 0.005 g, far less than the ED_{25} treated group, 0.12 ± 0.004 g.

Effect of flufenoxuron on the male reproductive development of *S. mauritia*

Testis morphology

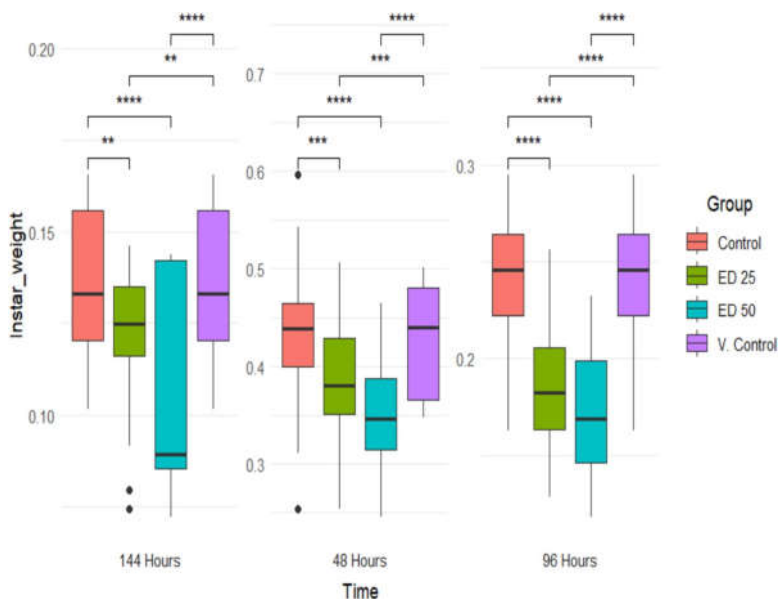
Flufenoxuron impacted the growth and development of the testis and induced morphological aberrations (Fig. 4). Microscopic examination of the *S. mauritia*

larval testis after the post-treatment of flufenoxuron ED_{25} and ED_{50} doses at 48 and 96 h exhibited relatively slight changes in the morphological features. In contrast with the controls (Fig. 4A and B), flufenoxuron's application resulted in changes in the physical arrangements of four follicles (irregularly arranged follicles), which led to more structural changes in individual testis (Fig. 4D, E, G and H). However, marked structural deformities in the testis were observed at 144 h after flufenoxuron (ED_{25} , ED_{50}) treatment on the sixth instar larvae of *S. mauritia*. The treatment resulted in the lack of a testis spherical shape, and irregularity was noticed (Fig. 4F and I), which was entirely different from the completely fused spherical testis from the control (Fig. 4C). After 144 h of flufenoxuron treatment, abnormal fusion was more prominent in the pupal testis. The size of the testis is enlarged due to irregular edges and a defective fusion process (Fig. 4I).

Testis weight

After flufenoxuron administration, the size of the larval testes was considerably reduced in a dose-dependent manner (Fig. 5). A decrease in testis weight was observed in response to the lower dose

Fig. 3 Instar weight of flufenoxuron treated sixth instar larvae of *Spodoptera mauritia* at different intervals. Boxes represent the interquartile range, bars indicate the median, and whiskers span 1.5 times the interquartile range. Statistical differences were considered significant by one-way ANOVA (***) $p < 0.0001$, **** $p < 0.00001$)



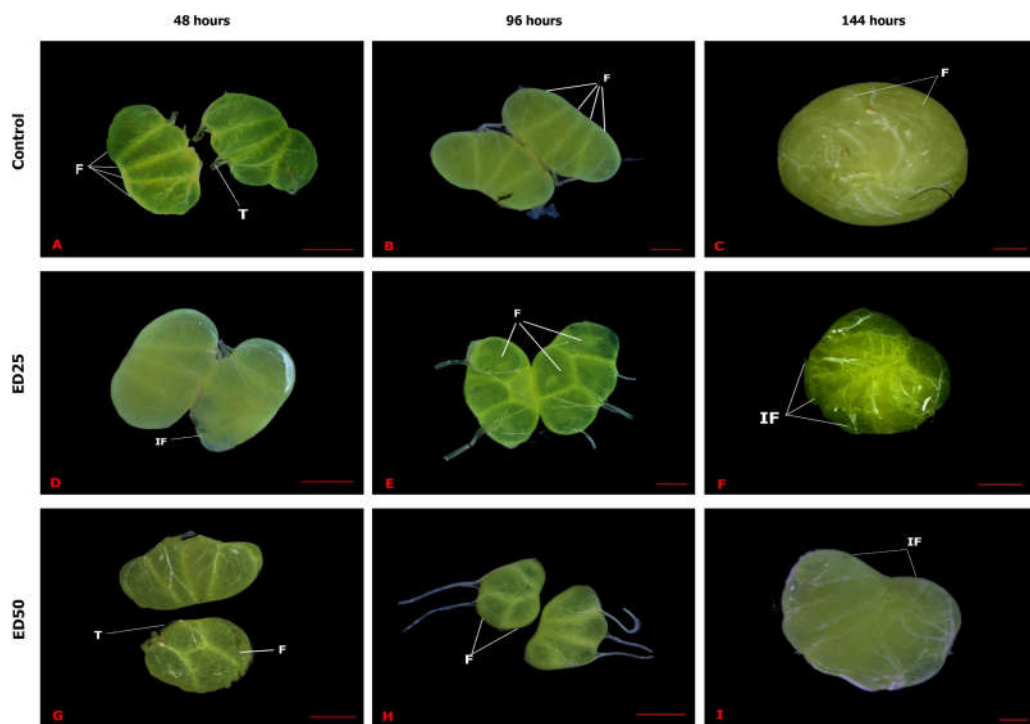


Fig. 4 Testis morphology of sixth instar larvae of *Spodoptera mauritia* with post-treatment of flufenoxuron (after 48, 96, and 144 h of treatment). **A & B:** Controls set after 48 h and 96 h: paired kidney-shaped testis of *S. mauritia* with four follicles (F). **C:** Control: fused rounded testis of *S. mauritia*. **D:** Flufenoxuron treated (ED_{25}) 48 h after treatment- Paired kidney-shaped testis with slightly irregularly arranged follicles (IF). **E:** Flufenoxuron treated (ED_{25}) 96 h after treatment- paired testis with complete lack of kidney shape and dif-

ferently oriented follicle (F). **F:** Flufenoxuron treated (ED_{25}) 144 h after treatment- Abnormally fused testis with irregular edges. **G:** Flufenoxuron treated (ED_{50}) 48 h after treatment- Testis Follicle (F) orientation alteration from normal kidney shape. **H:** Flufenoxuron treated (ED_{50}) after 96 h of treatment- reduced paired testis with differently oriented follicles. **I:** Flufenoxuron treated (ED_{50}) after 144 h of treatment- abnormal fusion more prominent. Size enlarged with irregular edges and defective fusion process (Scale Bar: 500 μ m)

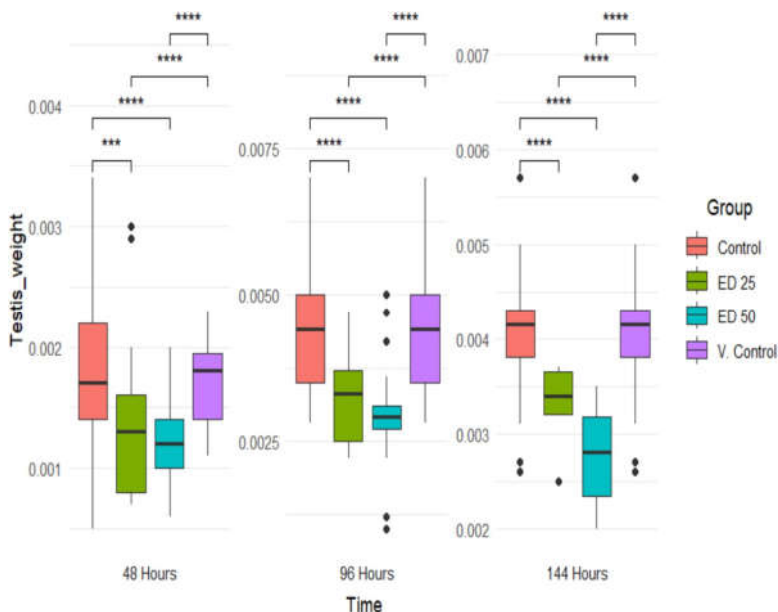
(ED_{25}) of flufenoxuron. The testis weight was significantly lower than the control groups at 48 h, 96 h, and 144 h post-treatment with flufenoxuron. However, in ED_{50} treatment groups, the testis mass drastically decreased after 48, 96, and 144 h of administration. The weight of testis at 48 h was 0.0017 ± 0.0001 g and 0.0016 ± 0.00008 g in control and vehicle control samples, but in flufenoxuron, treated samples have a weight of 0.0013 ± 0.00008 g and 0.0011 ± 0.00004 g respectively in ED_{25} and ED_{50} doses. After 96 h, testis weights were 0.0044 ± 0.0002 g and 0.0043 ± 0.0002 g in control and vehicle control; after treatment, weights were recorded as 0.0038 ± 0.0002 g and 0.0033 ± 0.0001 g

in ED_{25} and ED_{50} respectively. The 144-h pupal testis weighed about 0.0040 ± 0.0001 g and 0.0040 ± 0.0001 g in control and vehicle control, weight estimated after ED_{25} and ED_{50} dose treatment were 0.0034 ± 0.00005 g and 0.0032 ± 0.00004 g respectively.

Testis volume

The treatment of *S. mauritia* six instar larvae with flufenoxuron showed a substantial decrease in the total volume of the testis. The volume of the testes in the larval stage (after 48 h of treatment) was 1.44 ± 0.11 mm³ and 1.38 ± 0.06 mm³ in control

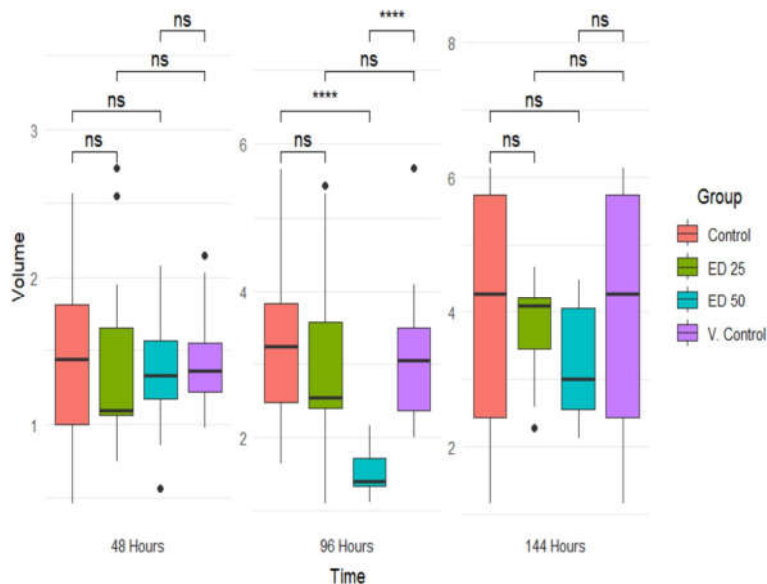
Fig. 5 Testes weight of flufenoxuron treated sixth instar larvae of *Spodoptera mauritia*. Boxes represent the interquartile range, bars indicate the median, and whiskers span 1.5 times the interquartile range. Statistical differences were considered significant by one-way ANOVA (*** $p < 0.0001$, **** $p < 0.00001$)



and vehicle control and in ED₅₀ doses of flufenoxuron administered exhibited a decrease of $1.35 \pm 0.087 \text{ mm}^3$ (Fig. 6), there was not much change occurred at dose ED₂₅, $1.38 \pm 0.10 \text{ mm}^3$. At 96 h post-treatment,

control and vehicle control samples had testicular volumes of $3.21 \pm 0.21 \text{ mm}^3$ and $3.10 \pm 0.21 \text{ mm}^3$, then the testicular volume decreased in the ED₂₅ group to $3.02 \pm 0.28 \text{ mm}^3$ but experienced a more substantial

Fig. 6 Testis volume of flufenoxuron treated sixth instar larvae of *Spodoptera mauritia*. Boxes represent the interquartile range, bars indicate the median, and whiskers span 1.5 times the interquartile range. Statistical differences were considered significant by one-way ANOVA (**** $p < 0.00001$, ns- not significant)



reduction of $1.51 \pm 0.051 \text{ mm}^3$ after the application of ED_{50} . During the pupal stage (144 h post-treatment), testicular volume was $3.95 \pm 0.61 \text{ mm}^3$ and $3.93 \pm 0.62 \text{ mm}^3$ in control and vehicle control, respectively; there was a slight decrease in the volume of the fused testes in the ED_{25} treated group, $3.76 \pm 0.25 \text{ mm}^3$. In contrast, the higher dose treated group showed a reduction of $3.26 \pm 0.27 \text{ mm}^3$. These effects were statistically significant for all doses.

Gonadosomatic index

After 48 h of treatments, the gonadosomatic index (GSI) values indicate that the ED_{50} dose was more effective. There was a significant reduction in the value of the GSI of the ED_{25} and ED_{50} dose at 48 h, 0.35 ± 0.021 and 0.346 ± 0.012 compared to control and vehicle control, 0.40 ± 0.019 and 0.38 ± 0.023 respectively. The GSI values show a gradual decline after 96 h of treatment and at the pupal period (144 h) (Fig. 7). For control and vehicle control, the GSI values at 96 h were 1.87 ± 0.11 and 1.9 ± 0.12 . In treated samples, GSI values for ED_{25} and ED_{50} treated larvae after the 96 h were 1.83 ± 0.08 and 1.79 ± 0.10 , respectively. The GSI value at 144 h recorded as 3.01 ± 0.09 and 3.1 ± 0.17 in control and vehicle control, GSI estimated after treatment with ED_{25} and

ED_{50} , GSI reduced to 2.95 ± 0.13 and 2.88 ± 0.16 respectively in a dose dependent manner. Data show a notable decrease in groups treated with high-dose flufenoxuron.

Fecundity and fertility

The findings revealed that the treatments disrupted the adult fecundity and fertility of *S. mauritia*. Flufenoxuron treatment significantly decreased the number of eggs laid and the percentage of egg hatchability compared to the control. The most significant reduction occurred in the treated group of both mated sexes, followed by a treated male mated with an untreated female. The adult female moths exposed to flufenoxuron with untreated males laid an average of 47 eggs per female (Table 3). The observations show that the treatment significantly reduced from the control mean value of 854.33 ± 55.54 eggs per female. However, when treated males were paired with non-treated females, the mean fecundity decreased to 27.33 ± 13.86 eggs per female. This mean value was also significantly different from the number of eggs laid from the treated female/nontreated male pairing and control group. An effective dose of flufenoxuron can negatively affect the total number of eggs laid by a nontreated female that mates with a treated

Fig. 7 Gonado-somatic index (GSI) of flufenoxuron-treated sixth instar larvae of *Spodoptera mauritia*. Boxes represent the interquartile range, bars indicate the median, and whiskers span 1.5 times the interquartile range. Statistical differences were considered significant by one-way ANOVA (* $p < 0.005$, ** $p < 0.001$, ns-not significant)

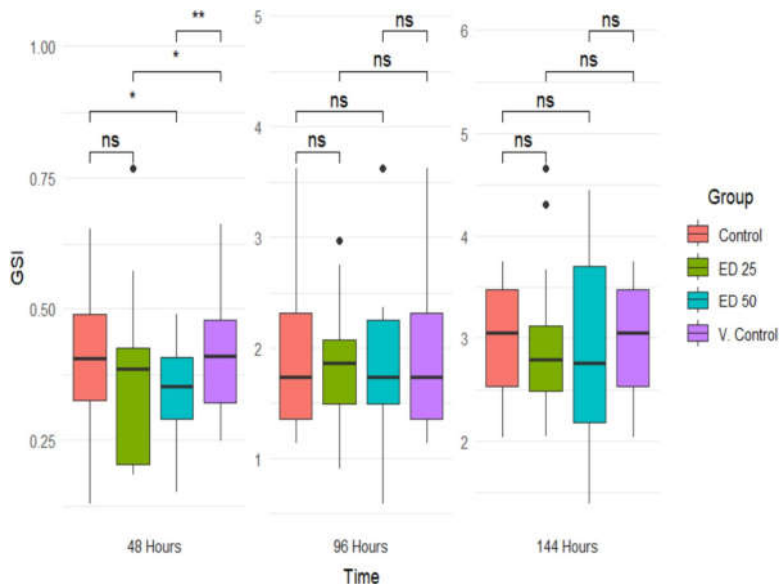


Table 3 Effect of flufenoxuron on fecundity and egg hatchability of *S. mauritia*

Fecundity (Total no. of eggs/♀) and fertility (% Egg hatchability)							
Untreated male × Untreated female		Treated male × Untreated female		Untreated male × Treated female		Treated male × Treated female	
No. of eggs/♀	% Egg hatchability	No. of eggs/♀	% Egg hatchability	No. of eggs/♀	% Egg hatchability	No. of eggs/♀	% Egg hatchability
854.33 ± 55.54	99.10 ± 56.96	27.33 ± 13.86*	85.36 ± 12**	47 ± 23.33*	97.16 ± 22.92**	4 ± 4*	0**

Means ± SE. Values followed by the number of stars in a column within each regime are significantly different ($P < 0.05$) (ANOVA followed by LSD post-test). The data expressed in the mean ± SE values of three independent experiments

(exposed) male and the eggs laid by a treated female that mates with a nontreated male.

In the larva exposed to flufenoxuron treatments, the per cent egg hatchability found in the treated female with nontreated male pairing was 97.16% (Table 3). This was not significantly different from the 85.36% egg fertility found in the nontreated female with treated male pairing. However, both values were significantly less than the control hatchability. The percentage of egg hatchability in treated females paired with treated males was nil. Larval exposure to flufenoxuron by topical treatment significantly reduced the rate of eggs that hatched, regardless of whether only the females or males were exposed. This suggests that flufenoxuron induces more effect on the males than females.

The data suggested that the compound may have ovicidal activity, and administration of flufenoxuron at ED₂₅ dose decreased egg lay by females in untreated male and treated female pairs (Table 3). Furthermore, the eggs laid by treated and untreated mating pairs of *S. mauritia* with ED₂₅ dose of flufenoxuron were represented in Fig. 8. It is also clearly evident that, the egg laid by treatment pairs did not hatch, and the dead or unhatched eggs had turned brownish-black in colour (Fig. 8). It is also noted that the unfertilized eggs were found to be yellow.

Discussion

The current investigation revealed that flufenoxuron had highly detrimental effects on the larvae of *S. mauritia*. Several studies have highlighted the considerable impact of chitin synthesis inhibitors (CSIs) on lepidopteran pests (Aliabadi et al., 2016; Santorum et al., 2021), making them increasingly

favourable for integration into agricultural practices. Assessments of CSI compounds, including flufenoxuron, lufenuron, and chlorfluazuron, have revealed both lethal and sublethal effects across diverse insect species.

The application of flufenoxuron on the sixth instar larvae of *S. mauritia* resulted in severe morphological defects in subsequent developmental stages. Similar findings were documented in various insect species. Reda et al. (2010) noted mortality in the second and fourth instars and observed morphological abnormalities in larvae, pupae, and adults of *S. littoralis* treated with flufenoxuron. Additionally, Khajepour et al. (2012) found that lufenuron, a chitin synthesis inhibitor, induced mortality with the formation of larval pupal intermediates, defective pupal and adults of *Ephestia figulilella*. Several studies reported similar outcomes for other lepidopteran species treated with IGRs, such as chlorfluazuron-treated in *S. littoralis* (Abdel-Aal, 2003, 2012), methoprene-treated *Corcyra cephalonica* (Tripathi & Tiwari, 2014), and hexaflumuron-treated in *Heliothis armigera* (Taleh et al., 2015).

In the present study, sub lethal dose slowed down the emergence of adults in *S. mauritia*. At high concentrations, flufenoxuron completely inhibited the emergence of adults. These findings align with the report of Reda et al. (2010) on the reduction of adult emergence in *S. littoralis* with flufenoxuron treatment. Similar findings of a decline in adult emergence of *H. armigera* treated with lufenuron were reported by Butter et al. (2003). Similarly, comparable results were observed in *Tenebrio molitor* when treated with diflubenzuron, a chitin synthesis inhibitor that disrupted pupal and adult development (Soltani et al., 1984). Likewise, the treatment of *Agrotis ipsilon* larvae with chlorfluazuron and flufenoxuron

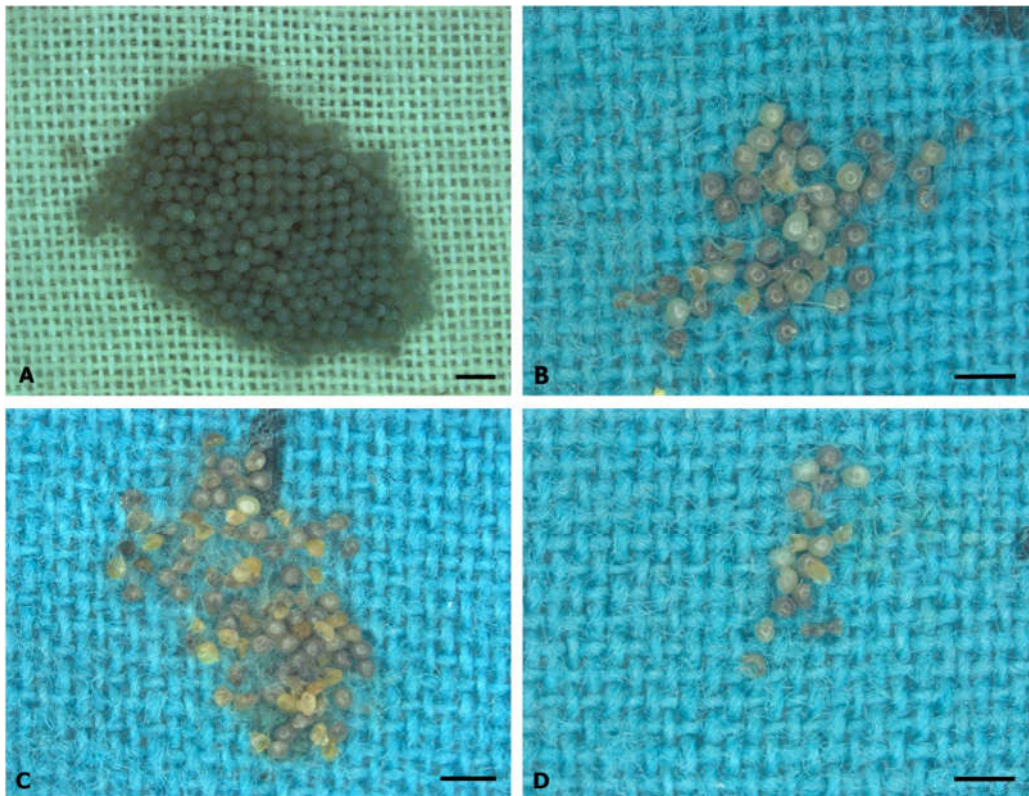


Fig. 8 **A:** Egg mass of *Spodoptera mauritia* (control), **B:** egg laid by treated male $\times$ untreated female pair, **C:** the egg laid by treated female $\times$ untreated male pairs **D:** egg laid by treated male and treated female pair (treated with ED₂₅ dose).

Yellow-coloured shrunk eggs are unfertilized. Brownish-Black colour eggs represent the dead or unhatched eggs (Scale Bar: 1000 μ m)

significantly decreased the adult emergence of both sexes (Shaurub et al., 2018).

In the current investigation, it was observed that the effective dose of flufenoxuron led to a reduction in larval weight; this decrease in weight is attributed to the physiological and metabolic disruptions induced by flufenoxuron (Amein & Mohammad, 2022) in *S. mauritia*. Similar observations have been reported in other studies where applying Chitin Synthesis Inhibitors (CHIs) through direct or topical treatment decreased total body mass. Mervat et al. (2012) noted a considerable reduction in larval weight and a significant prolongation of the larval and pupal stages in *P. gossypiella* larvae at the LC₅₀ concentration of lufenuron. Zhu et al. (2012) found that sublethal doses of

hexaflumuron on the 3rd, 4th, and 5th instars of *S. litura* prolonged developmental time and decreased larval body weight. The observed reduction in weight across different instars may be attributed to decreased food consumption or reduced food utilization efficiency following flufenoxuron treatment. Abdel-Aal (2003) made similar observations, noting that flufenoxuron and chlorfluazuron significantly reduced the efficiency of converting consumed and digested food into biomass in the last instars of *S. littoralis* pre-treated on fourth instars.

These findings highlight that flufenoxuron causes insect mortality and induces various detrimental outcomes. The larvae treated with the compound experienced reduced fecundity and abnormalities in their

testes, likely slowing from diminished nutrition. In effect, the disorders induced by flufenoxuron, altering chitin formation across multiple body areas, constrain and delay the absorption of nutrients, potentially linking to toxicological consequences and morphological modifications observed in the testis.

Studies on the effects of chitin synthesis inhibitors on the reproductive aspects of insects are very sparse (Costa et al., 2017; Santorum et al., 2021). Only a limited number of studies have explicitly delved into the morphological and ultrastructural alterations induced by chitin synthesis inhibitors in the reproductive organs of Lepidoptera (Santorum et al., 2021). The current study observed that effective doses of flufenoxuron administered to sixth-instar larvae induced various abnormalities in the development and arrangement of follicles. Notably, alterations in the overall shape and arrangement of the testis were evident after treatment. Similar effects were noted with the insecticide novaluron, a benzoylphenylurea class insect growth regulator, in *Bombyx mori* and demonstrated that novaluron induced ruptures in the cellular tunica and septa surrounding the testicular follicles (Santorum et al., 2021). Additionally, similar reports highlighted the action of azadirachtin, indicating a reduction in the size of testicular structures with internal morphological changes in *Spodoptera eridania* (Scudeler et al., 2023). The morphometric changes observed in the testicular region in our study suggest that flufenoxuron has a discernible impact on reproductive development during the latter stages of *S. mauritia* growth.

From earlier studies (Cutler, 2006; Mommaerts et al., 2006; Doucet & Retnakaran, 2012), it is established that the benzoylphenylurea class of insecticides exerts control over insect development by inhibiting chitin synthesis and concurrently reducing reproductive capabilities. Perez-Farinos et al. (1998) detected a higher concentration of radioactive hexaflumuron in the gonads of younger males compared to older ones. This raised level of hexaflumuron was suggested to be sufficient to impact spermatogenesis, potentially leading to unsuccessful fertilization. As highlighted by Aliabadi et al. (2016), lufenuron has been shown to have detrimental effects on the reproductive processes of *Glyphodes pyloalis*.

The impact of chitin synthesis inhibitors on the testicular measurements of insects has been previously documented. According to Seth et al. (2004),

treating *S. litura* last instar larvae with IGRS has detrimental effects on the development of the male reproductive system. Sublethal dosages significantly reduce testicular size, volume, and length and width measures. Moreover, there is a decrease in the number of spermatozooids in the testicles following chitin synthesis inhibitor treatment, indicating an impact on spermiogenesis (Costa et al., 2017). Perveen (2000) also reported similar results, demonstrating that topical application of a sublethal dose of the chitin synthesis inhibitor chlorfluazuron negatively affects the development of testes by reducing their weight, volume, and sheath thickness.

The present study documented a reduction in fecundity in female and male moths treated with flufenoxuron in the sixth larval instar of *S. mauritia*. These observations are on par with the findings of other studies (Khebbeh et al., 2008; Reda et al., 2010; Wang & Tian, 2009). The results suggest that flufenoxuron may have a more pronounced effect on males than females. The current study is in agreement with previous reports on black cutworm (*Agrotis ipsilon*) (Shaurub et al., 2018), codling moth (*Cydia pomonella*) (Sun & Barrett, 1999), and *S. litura* treated with chlorfluazuron (Perveen & Miyata, 2000).

The negative impact of flufenoxuron on egg hatchability suggests potential developmental effects on both male and female reproductive processes. Similar observations reported a significant reduction in the number of eggs deposited by *P. gossypiella* larvae treated with LC₅₀ concentrations of lufenuron (Mervat et al., 2012). Shaurub et al. (2018) observed that the pre-oviposition period of *A. ipsilon* females, surviving treatment with chlorfluazuron and flufenoxuron, did not significantly decrease. However, oviposition time was markedly reduced, particularly with chlorfluazuron treatment. Similarly, Reda et al. (2010) demonstrated a dose-dependent reduction in fertility in *S. littoralis* following flufenoxuron treatment.

A recent study observed that novaluron decreased egg production and oviposition in *B. mori* (Santorum et al., 2021). Yao et al. (2021) noticed aberrant development in the egg-hatching of litchi fruit borer, reduced chitin content, and modified chitin distribution in freshly hatched larvae. Joseph (2020) found that Novaluron reduced the populations of *Stephanitis pyrioides* (Hemiptera: Tingidae) by influencing egg hatching and disrupting nymphal development

through abnormal ecdysis. These findings on the developmental processes of embryos, particularly concerning egg hatching and nymphal development, serve as crucial insights that can contribute to a theoretical understanding of the reproductive toxicity of flufenoxuron in the target species.

Conclusions

The CSIs had multiple effects on the morphology, metamorphosis, and development of lepidopterans. The results of this study indicate that flufenoxuron adversely affected most *S. mauritia* life parameters, including egg hatchability, larval duration, larval pupal moulting, and adult emergence. Importantly, flufenoxuron inhibited the growth and development of the male reproductive organ of *S. mauritia*. Furthermore, they may directly inhibit this insect species population growth through their acute toxic effects or, more specifically, by lowering its capacity for reproduction. Nevertheless, we suggest that the examined Chitin Synthesis Inhibitors (CSIs), deemed environmentally safe alternatives to synthetic chemical insecticides, can be endorsed within an integrated insect pest management strategy.

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Author contributions V. Reshma wrote the main manuscript text and E. M. Manogem reviewed the manuscript as supervisor of the research work.

Declarations

Conflicts of interest The authors declare no competing interests.

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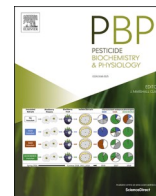
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Sublethal effects of flufenoxuron on *Spodoptera mauritia* Bosid. (Lepidoptera: Noctuidae) testicular biochemical profiling and Spermatogenic alterations

V. Reshma, E.M. Manogem*

Division of Insect Endocrinology, Department of Zoology, University of Calicut, 673635, Kerala, India

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ABSTRACT

Flufenoxuron, a widely used chitin synthesis inhibitor, is effective against several insect and mite pests. This study evaluated the reproductive toxicity in male sixth-instar larvae of *Spodoptera mauritia*, a sporadic lepidopteran pest of rice crops. Larvae were topically treated with sublethal concentrations of flufenoxuron (ED_{25} –0.00068 μ g and ED_{50} –0.00015 μ g), and biochemical responses were assessed at 48, 96, and 144 h post-treatment. Significant reductions in lipid, protein, and carbohydrate levels were observed in the haemolymph and testes of treated larvae, indicating a potential impairment of reproductive physiology. The electrophoretic profiling showed significant alterations in testicular and haemolymph proteins, particularly in the range of 65 kDa to 79 kDa and 61 kDa to 77 kDa, respectively. Histopathological analysis of the testes revealed spermatogenesis disruptions, including germ and somatic cell alterations. Flufenoxuron affected the organization, distribution, and development of male germ cell cysts, particularly during spermiogenesis. These findings highlight flufenoxuron's toxicity in *S. mauritia* male reproductive development and provide a basis for further studies on its reproductive effects in other insect species.

1. Introduction

Chitin, a key structural component of the exoskeleton, supports the lining of the digestive tract, respiratory system, reproductive ducts, and certain glandular ducts in insects. The insect cuticle serves as a critical interface between the organism and its environment (Tunaz and Uygun, 2004). Consequently, alterations in chitin production following the administration of chitin synthesis inhibitors (CSIs) can impact insects, affecting the histophysiology of multiple systems, including the reproductive system (Costa et al., 2017). Several CSIs, such as diflubenzuron, chlorflazuron, lufenuron, and novaluron, inhibit chitin synthase, the enzyme responsible for chitin biosynthesis. This inhibition indirectly disrupts spermatogenesis by affecting testicular development, spermatocyte differentiation, and sperm maturation (Perveen, 2000; Cremonese et al., 2018). Insect reproduction and hormone modulation are possible targets for developing biorational pesticides. In this context, identifying chemicals that interfere with insect reproduction and development, and targeting the corresponding endocrine-regulating systems, has been the core objective of successful research over the last few decades (Dhadialla et al., 2005).

The larvae of *S. mauritia*, an agricultural pest that feeds on paddy, are responsible for significant economic losses in many countries (Grant, 1982). These swarming caterpillars can severely impact paddy, damaging immature seedlings, leaves and the base of rice panicles (Sarma and Salam, 2018). The older larvae feed voraciously, with the fifth instar lasting about three days and the sixth instar lasting about six days, almost entirely defoliating the plants. While younger larvae, each instar lasts about 2–3 days, cause the plants to appear sickly, with wilted tips and clipped leaves. The rapid movement from field to field leads to crop loss within a week and causes severe losses in rice production. Due to this large-scale damage, it is important to control its population over time.

In Lepidopteran insects, sperm development and behaviour are interesting targets for target-specific, environmentally safer biorational control techniques. Sperm dimorphism offers a broad range of opportunities to modify sperm dynamics and functionality, reducing the reproductive fitness of pest species (Seth et al., 2023). Morphological analysis of the insect testes throughout their development may reveal important alterations in the germ and somatic cells involved in spermatogenesis. Researching the male reproductive biology of pests can aid

* Corresponding author.

E-mail addresses: reshma_do@uoc.ac.in (V. Reshma), dr.manogem_em@uoc.ac.in (E.M. Manogem).

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in developing male sterility techniques and provide a theoretical framework for creating novel biological controls for insect pests (Ahmadi et al., 2018).

This study highlights key aspects of male reproductive biology in lepidopteran pests, particularly the presence of two distinct forms of spermatozoa, which play a crucial role in fertility regulation. Understanding dichotomous spermiogenesis in Lepidoptera and identifying potential targets for reproductive disruption could offer novel strategies for pest management. Disrupting male reproductive processes, particularly in migratory lepidopteran pests, may be an effective means of population control. Recent studies have revealed that flufenoxuron impairs testicular development in *S. mauritia* (Reshma and Manogem, 2024). However, the effects of flufenoxuron on spermiogenesis in this species remain unexplored. Therefore, this study investigates the impact of flufenoxuron on key biochemical indices, testicular development, and spermiogenesis in *S. mauritia*, providing new insights into its reproductive toxicity and potential applications in insect population management.

2. Materials and methods

2.1. Collection and rearing of test organisms

Fertilized *S. mauritia* moths were collected from rice fields of Kunnungal-Kavancheri, Purathur, Kerala, India (10°49'16"N 75°56'00"E) using fluorescent light traps. *S. mauritia* exhibit sexual dimorphism; the following characteristics are used to identify moths: the adult male has a dark grey colour with white markings on the forewings and a tuft of hair on the forelegs. The adult female had a lightly grey coloured forewing without white markings and a lack of a tuft of hair on the foreleg. Both males and females had a prominent black spot on each forewing. Then, we selectively collected *S. mauritia* females with large abdomens from the light trap. A markedly enlarged and firm abdomen is considered a sign of mating and the onset of egg development (Wunderlich et al., 2025), indicating that the moth is ready to lay eggs. The adults were maintained in glass beakers covered with muslin cloth and provided with a diluted honey solution (1:10) as a food source. Females laid eggs in designated containers, which hatched into larvae within two to three days. The stock colony was maintained according to standardized laboratory protocols (Banu et al., 2022). The alternative host plant, *Ischaemum aristatum*, was used to feed the larvae; the entire colony was maintained in plastic tubs covered with muslin cloth under laboratory conditions at room temperature (RH 90 ± 3 % and a 10:14 light: dark photoperiod). For the experimental investigation, newly moulted sixth-instar male larvae (day zero) were selected based on yellow-coloured paired testes visible through the dorsal skin (Reshma and Manogem, 2024).

2.2. Tested compound

Flufenoxuron (C₂₁H₁₁ClF₆N₂O₃, CAS No. 101463–69-8) (Analytical standard, 98 % purity) was obtained from Sigma-Aldrich and used for the investigations.

2.3. Treatment of flufenoxuron on sixth instar larvae

The newly ecdysed *S. mauritia* sixth instar larvae were obtained from a laboratory stock culture. Flufenoxuron dissolved in acetone was applied topically at the ED₅₀ (0.00068 µg) and ED₂₅ (0.00015 µg) concentrations. Based on our previous study on the toxicity bioassay, the effective doses, ED₅₀ (0.00068 µg) and ED₂₅ (0.00015 µg), were used for the study (Reshma and Manogem, 2024). Five microliters of the chemical were topically applied to the abdomen of newly ecdysed larvae using a Hamilton microsyringe. Equal amounts of acetone were used for the vehicle control group, while untreated larvae served as an additional control. Four replicates were maintained for each concentration, with 10

larvae per replica. The experimental and control larvae were kept in separate plastic tubs covered with muslin cloth. The larvae were collected at different intervals (48, 96, and 144 h) for all examinations.

2.4. Sample preparation for biochemical analysis

To extract the haemolymph and tissue, the ED₅₀ and ED₂₅ (0.00068 µg and 0.00015 µg respectively) flufenoxuron-treated larvae and control groups at specific intervals of 48 h, 96 h and 144 h were anaesthetized with diethyl ether. Then, thoracic legs are surface-sterilized by ethyl alcohol, one of its thoracic legs was amputated with sharp scissors, and the haemolymph oozed out immediately and was drawn into a calibrated capillary tube. The haemolymph is transferred into a centrifuge tube containing phenylthiourea crystals. Centrifuge the haemolymph for 10 min in cold conditions (4 °C) at 10,000 rpm and pipetted out the cell-free suspension and kept it at –80 °C for further analysis (Banu et al., 2022).

For collecting tissue samples, the treated and control sixth instar larvae were dissected to remove the testes at specific intervals (48 h, 96 h, and 144 h). The testes of treated and control samples (100 µg/100 µl) were homogenized in prechilled 10 mM phosphate buffer (pH 7, with 1 mM PMSF as a protease inhibitor) using a polytron homogenizer (Remi, India) under cold conditions (–20 °C) for 3–5 min. The homogenate was centrifuged for 10 min at 4 °C and 20,000 rpm, and the supernatant was collected and stored at –80 °C until use (Banu et al., 2022). Collected and stored haemolymph and tissue samples from each treatment and control group were diluted according to the demand of each biochemical experiment.

2.5. Biochemical assays

The haemolymph and tissue glucose levels were determined using the phenol-sulphuric acid method (Van Handel, 1985), with D-glucose as the standard. The absorbance was measured at 490 nm in a microplate reader (Synergy™ HT, Bio-Tek Instruments).

The total amount of lipids in each treatment and control group was determined using the vanillin assay procedure (Van Handel, 1985), with cholesterol as the standard. The absorbance was measured at 525 nm using a microplate reader (Synergy™ HT, Bio-Tek Instruments).

The protein assays of haemolymph and tissues were performed using the Bradford method (Bradford, 1976), with bovine serum albumin as a standard. The absorbance was measured at 595 nm using a Microplate reader (Synergy™ HT, Bio-Tek Instruments). Protein profiling of haemolymph and tissue was conducted on a maxi vertical system (Genie). The samples were subjected to 10 % SDS-PAGE analysis (Laemmli, 1970). The Coomassie Brilliant Blue R-250-stained gel was photographed and analysed using GelAnalyser 19.1 software for band detection. Band intensity was calculated using ImageJ 1.54f software (USA). A hierarchical cluster based on treatment group and band intensity was examined using Origin software (2022 version, USA).

2.6. Effect of flufenoxuron on the histology of testes

The flufenoxuron-exposed ED₅₀ and ED₂₅ (0.00068 µg and 0.00015 µg, respectively) sixth instar larval testes were reclaimed at 48 h, 96 h, and 114 h, and the testes were fixed overnight in Bouin's fixative. The tissues were washed in distilled water, dehydrated in a graded ethanol series, cleared in methyl benzoate, infiltrated, embedded in paraffin wax, and sectioned on a Reichert precision rotary microtome to a thickness of 5 µm. The sections were stained using Harris haematoxylin-eosin and mounted with DPX. The control and treated tissue sections were observed and photographed under a Zeiss Axio-Scope A.1 microscope. Morphometric analysis of histopathological alterations in all experimental groups was assessed using ImageJ 1.54f software (USA) (de Souza et al., 2022). For morphometric analysis, ten photographs of each group were used, and the thickness of the epithelium, the average

area of cyst cells, and the average length of cyst cells were measured. Twenty-five measurements were performed for each parameter.

2.7. Statistical analysis

Statistical analysis for biochemical analysis was performed using R version 4.2.3 (Eye Holes). Differences were evaluated using a one-way ANOVA after confirming equal variance (Levene test, $p = 0.05$) and normality (Shapiro test, $p > 0.05$) for all groups. The data are presented as the mean $\pm$ Standard Error (SE) of separate experiments. Statistical differences were considered significant by one-way ANOVA, followed by Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). Morphometric data were analysed using IBM SPSS (version 24.0). Differences were evaluated using one-way ANOVA, followed by Duncan's Multiple Range as a post-hoc test ($*p < 0.05$).

3. Results

3.1. Effect of flufenoxuron on haemolymph and testicular carbohydrate concentration of *Spodoptera mauritia*

Treatment of final instar larvae with two different effective doses of flufenoxuron adversely affected the total carbohydrate concentration of haemolymph. There was a significant reduction in carbohydrate concentration in a dose-dependent way (Fig. 1). After 48 h of treatment with ED₂₅ (0.00015 μ g) and ED₅₀ (0.00068 μ g) doses, the concentrations dropped significantly to 8.75 ± 0.21 μ g/ μ l and 2.67 ± 0.39 μ g/ μ l, respectively, compared to the control and vehicle control groups at 23.57 ± 0.92 μ g/ μ l and 21.13 ± 0.26 μ g/ μ l, respectively ($F = 352.3$; $df = 3$; $p < 0.0001$). Similar reductions were observed after 96 h of treatment. The lower dose of flufenoxuron (0.00015 μ g) led to a concentration of 13.57 ± 0.82 μ g/ μ l. In comparison, the higher ED₅₀ dose resulted in a more drastic decrease of 4.26 ± 0.43 μ g/ μ l, which is highly significant compared to the control and vehicle control at 17.27 ± 0.16 μ g/ μ l and 30.91 ± 2.15 respectively ($F = 116.2$; $df = 3$; $p < 0.0001$) (Fig. 1). Further, at 144 h, the total carbohydrate concentration increased as

compared to both 48 h and 96 h. However, significant reductions were also noted in the ED₂₅ and ED₅₀ concentrations of carbohydrate, at 31.93 ± 0.19 μ g/ μ l and 26.42 ± 0.25 μ g/ μ l, respectively, which were far lower than those of the control and vehicle control groups, 48.80 ± 1.8 μ g/ μ l and 45.51 ± 2.4 μ g/ μ l, respectively ($F = 50.139$; $df = 3$; $p < 0.0001$).

The data depicted in Fig. 2 reveal that after 48 h of treatment, the carbohydrate concentration in larval testes following administration of ED₂₅ and ED₅₀ doses of flufenoxuron decreased by 22.27 ± 1.25 μ g/ μ l and 17.84 ± 3.87 μ g/ μ l, respectively, compared to control and vehicle control, 31.36 ± 0.34 μ g/ μ l and 30.79 ± 0.23 μ g/ μ l, respectively ($F = 31.572$; $df = 3$; $p < 0.0001$). After 96 h of exposure, two concentrations (ED₂₅ and ED₅₀) of flufenoxuron induced a significant decrease in testicular carbohydrate levels of 24.2 ± 0.68 μ g/ μ l and 17.61 ± 0.68 μ g/ μ l, respectively, relative to controls ($F = 111.275$; $df = 3$; $p < 0.0001$). At 144 h of treatment, a rapid and considerable reduction in carbohydrate concentration was observed, with ED₂₅ and ED₅₀ concentrations of 8.97 ± 0.9 μ g/ μ l and 3.4 ± 1.2 μ g/ μ l, respectively ($F = 709.87$; $df = 3$; $p < 0.0001$).

3.2. Effect of flufenoxuron on haemolymph and testicular lipid concentration of *Spodoptera mauritia*

Post-treatment with ED₂₅ and ED₅₀ concentrations of flufenoxuron showed a significant reduction in haemolymph lipid concentration of 125.80 ± 1.44 μ g/ μ l and 93.58 ± 5.1 μ g/ μ l, respectively after 48 h compared to the control and vehicle control groups (295.17 ± 2.26 μ g/ μ l and 292 ± 1.45 μ g/ μ l, respectively) ($F = 2580.8$; $df = 3$; $p < 0.0001$) (Fig. 3). Similarly, a significant decrease after 96 h of treatment, was noted in the ED₂₅ and ED₅₀ compared to the control and vehicle control groups, of 189.3 ± 0.75 μ g/ μ l and 197.23 ± 0.41 μ g/ μ l with a decrease of 164.61 ± 0.36 μ g/ μ l and 126.12 ± 2.1 μ g/ μ l, respectively ($F = 1541.8$; $df = 3$; $p < 0.0001$). The lipid levels significantly declined to 141.68 ± 0.91 μ g/ μ l and 110.96 ± 0.52 μ g/ μ l, respectively, compared to 247.23 ± 0.76 μ g/ μ l and 267.63 ± 1.37 μ g/ μ l in the control and vehicle control groups after 144 h, respectively ($F = 6604.2$; $df = 3$; $p < 0.0001$).

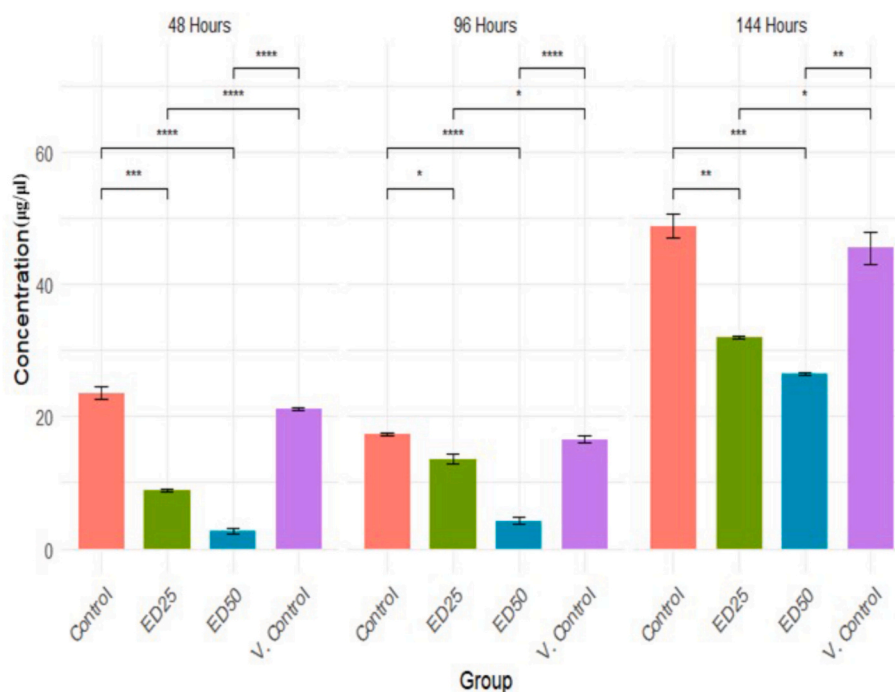


Fig. 1. Haemolymph carbohydrate concentration of flufenoxuron-treated sixth instar larvae of *S. mauritia*. Bars represent the mean $\pm$ SE of three independent experiments. Statistical differences were considered significant by one-way ANOVA followed by Tukey post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

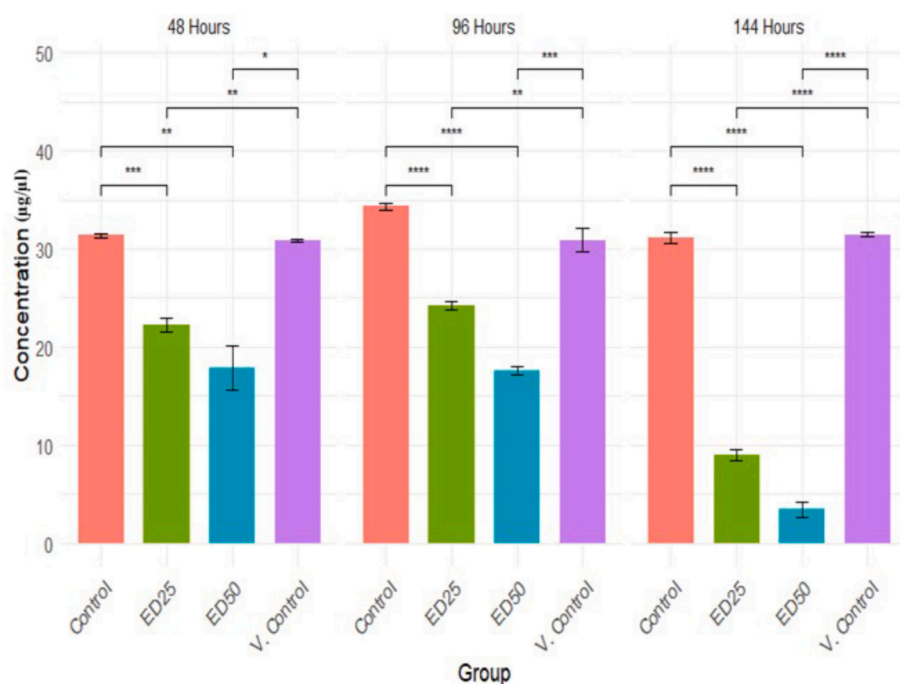


Fig. 2. Testis carbohydrate concentration of flufenoxuron-treated sixth instar larvae of *S. mauritia*. Bars represent the mean $\pm$ SE of three independent experiments. Statistical differences were considered significant by one-way ANOVA followed by Tukey post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

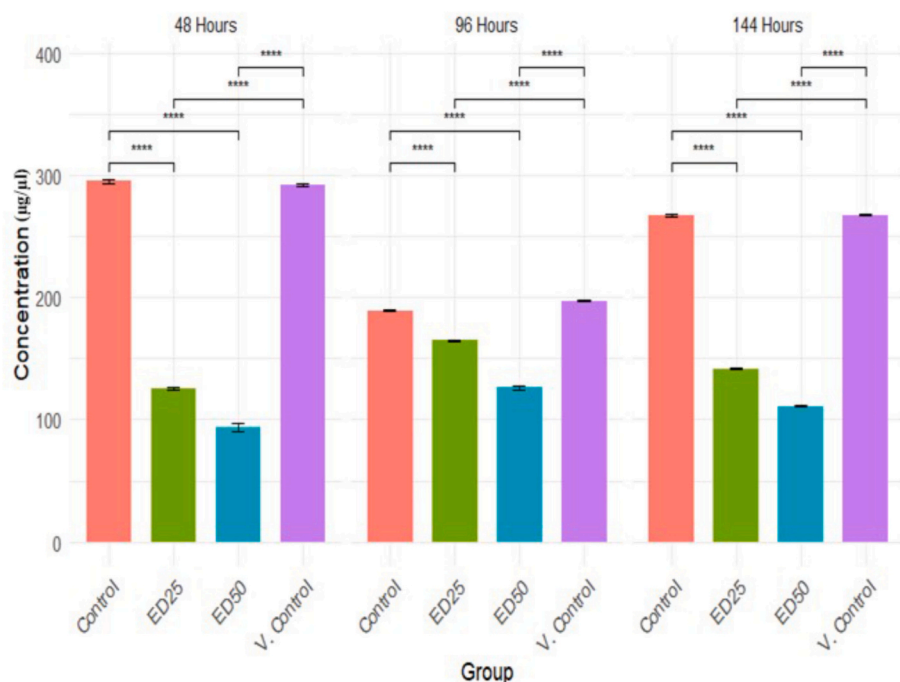


Fig. 3. Haemolymph lipid concentration of flufenoxuron-treated sixth instar larvae of *S. mauritia*. Bars represent the mean $\pm$ SE of three independent experiments. Statistical differences were considered significant by one-way ANOVA followed by Tukey post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

The effective doses of flufenoxuron led to a significant decrease in lipid concentration in the testes of sixth instar larvae of *S. mauritia* after 48, 96, and 144 h of treatment (Fig. 4). In the control and vehicle control groups, the total lipid concentration after 48 h of treatment was 212.71 ± 0.25 $\mu\text{g}/\mu\text{l}$ and 218.54 ± 0.57 $\mu\text{g}/\mu\text{l}$, respectively but significantly reduced following treatment with ED₂₅ and ED₅₀ to 173.84 ± 0.6 $\mu\text{g}/\mu\text{l}$ and 168.01 ± 0.56 $\mu\text{g}/\mu\text{l}$, respectively ($F = 2488.5$; $\text{df} = 3$; $p < 0.0001$). Similarly, after 96 h of treatment, testicular lipid concentration showed

a notable decrease to 169.67 ± 1.13 $\mu\text{g}/\mu\text{l}$ and 138.13 ± 0.66 $\mu\text{g}/\mu\text{l}$, respectively, compared to the control and vehicle control (244.5 ± 1.57 $\mu\text{g}/\mu\text{l}$ and 244.5 ± 2.32 $\mu\text{g}/\mu\text{l}$, respectively) ($F = 1204.1$; $\text{df} = 3$; $p < 0.0001$). A similar pattern was observed after 144 h, where the lipid amount significantly decreased with ED₂₅ concentration, with a more pronounced reduction at ED₅₀ compared to the controls ($F = 2246.2$; $\text{df} = 3$; $p < 0.0001$) (Fig. 4).

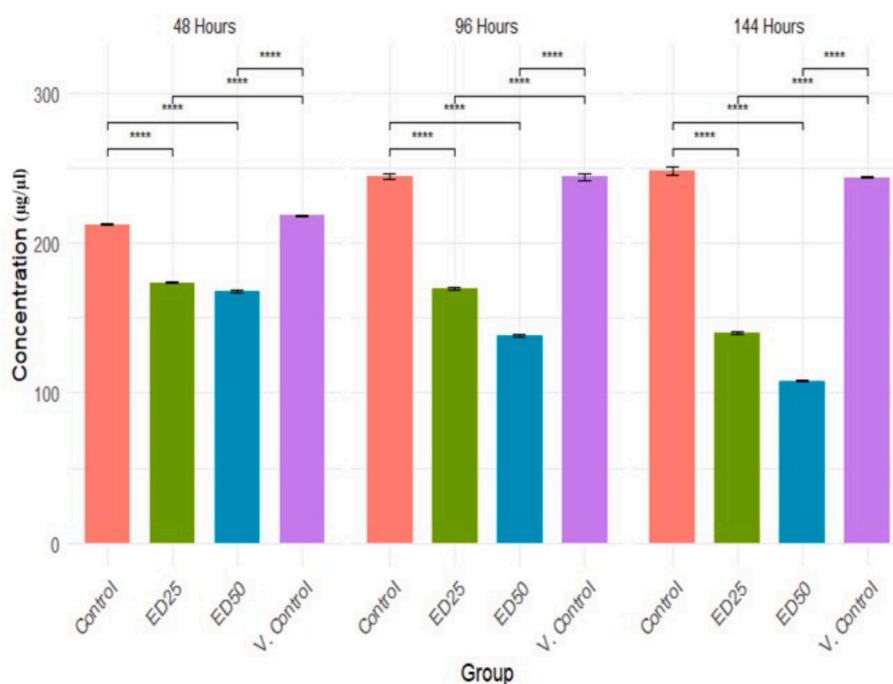


Fig. 4. Testis lipid concentration of flufenoxuron-treated sixth instar larvae of *S. mauritia*. Bars represent the mean $\pm$ SE of three independent experiments. Statistical differences were considered significant by one-way ANOVA followed by Tukey post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3.3. Effect of flufenoxuron on haemolymph and testicular total protein concentration of *Spodoptera mauritia*

The total soluble protein levels in the haemolymph after 48 h were $18.51 \pm 0.18 \mu\text{g}/\mu\text{l}$ and $19.45 \pm 0.22 \mu\text{g}/\mu\text{l}$ in the control and vehicle control groups, respectively ($F = 1.3$; $df = 3$; $p = 0.31$). Following treatment with the CSI, no significant difference in total protein levels was observed compared to controls. However, after 96 h, a substantial

reduction in protein levels was evident in larvae treated with ED₂₅ and ED₅₀ doses of flufenoxuron, measuring $15.43 \pm 0.07 \mu\text{g}/\mu\text{L}$ and $18.02 \pm 0.26 \mu\text{g}/\mu\text{L}$, respectively ($F = 141$; $df = 3$; $p < 0.0001$).

The observations at 144 h post-treatment showed a reverse trend, with a significant increase in haemolymph protein concentration of $21.42 \pm 0.06 \mu\text{g}/\mu\text{l}$ and $20.78 \pm 0.19 \mu\text{g}/\mu\text{l}$ for ED₂₅ and ED₅₀ doses, respectively, compared to controls ($F = 40.7$; $df = 3$; $p < 0.0001$) (Fig. 5). Thus, flufenoxuron demonstrated a noticeable but slight impact

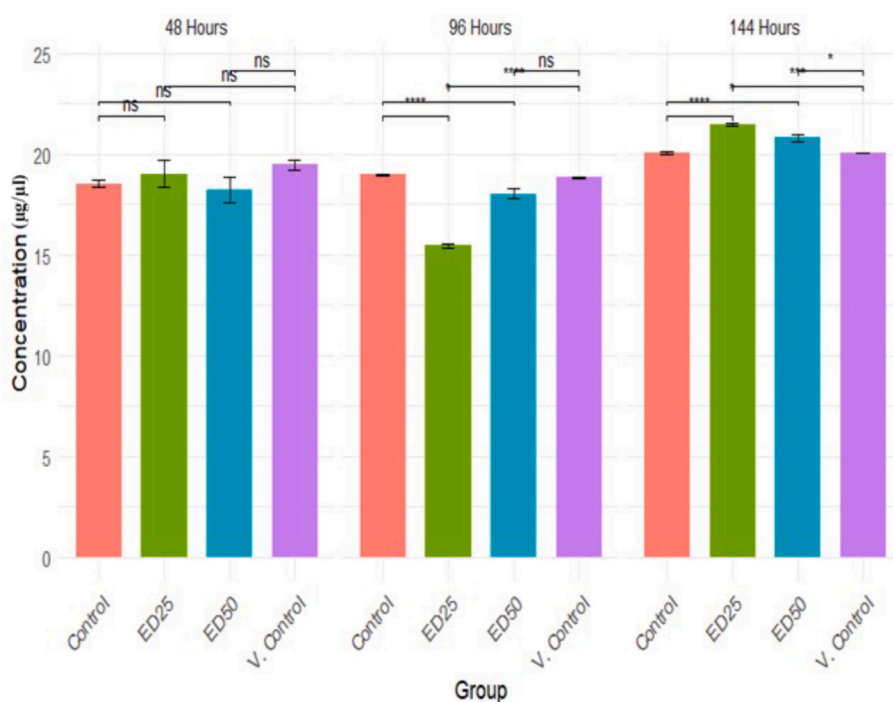


Fig. 5. Haemolymph protein concentration of flufenoxuron-treated sixth instar larvae of *S. mauritia*. Bars represent the mean $\pm$ SE of three independent experiments. Statistical differences were considered significant by one-way ANOVA followed by Tukey post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

on haemolymph protein concentration in *S. mauritia* larvae.

After 48 h of treatment, the testicular protein concentration in the ED₂₅ group decreased significantly ($F = 82.7$; $df = 3$; $p < 0.0001$). In contrast, it led to a notable increase in the ED₅₀-treated group. At 96 h post-treatment, a significant increase was observed in the ED₅₀ group, with a concentration of $16.69 \pm 0.24 \mu\text{g}/\mu\text{L}$ compared to the control and vehicle control, 14.89 ± 0.1 and 14.42 ± 0.26 respectively ($F = 20.1$; $df = 3$; $p < 0.0001$). Subsequently, after 144 h of post-treatment, a significant increase in testicular protein levels was observed at $16.20 \pm 0.11 \mu\text{g}/\mu\text{L}$ and $15.39 \pm 0.06 \mu\text{g}/\mu\text{L}$ for the ED₂₅ and ED₅₀ groups, respectively, as compared to the control and vehicle control groups, $12.20 \pm 0.05 \mu\text{g}/\mu\text{L}$ and $11.83 \pm 0.017 \mu\text{g}/\mu\text{L}$, respectively ($F = 969.4$; $df = 3$; $p < 0.0001$) (Fig. 6).

3.4. Electrophoretic SDS haemolymph protein profile of *Spodoptera mauritia*

The SDS-PAGE analysis of haemolymph proteins of ED₂₅ (0.00015 μg) and ED₅₀ (0.00068 μg) flufenoxuron-treated sixth instar larvae of *S. mauritia* after 48, 96 and 144 h and control samples revealed distinct variations in the expression of different proteins (Fig. 7A). The electrophoretogram reveals significant differences in the percentage of protein concentration and the bands within the middle-range proteins (61 kDa to 77 kDa) after 48 h of flufenoxuron treatment. Notably, the expressions of the bands at 71 kDa and 77 kDa were inhibited, while the expressions at 61 kDa showed a higher rate. The band at 48 kDa was also not expressed (Fig. 7 B). At 96 h, relatively less expressed haemolymph protein bands were observed at 182 kDa and 250 kDa and in the middle range (61 kDa to 77 kDa) in individuals treated with ED₂₅. Conversely, haemolymph from individuals treated with ED₅₀ showed a slight increase in protein band intensity compared to the control (Fig. 7C). After 144 h of treatment, bands corresponding to high-range proteins (182 kDa, 250 kDa) and middle-range proteins (55 kDa, 61 kDa, and 71 kDa) displayed an increase in band intensity in Lanes 11 and 12. Additionally, noticeable changes were observed in the other two bands (22 kDa, 35 kDa) compared to the control (Fig. 7D).

The protein profiling analysis was validated using a dendrogram. The

dendrogram represented the distance based on the band intensity of each band of haemolymph proteins (Fig. 8). From the dendrogram, it is clear that the variations in band intensity with respect to the molecular weight of haemolymph proteins were found to vary highly with the concentration of flufenoxuron applied. Clear-cut variations exist in the haemolymph protein samples from the flufenoxuron-treated and control/vehicle control groups.

3.5. Electrophoretic SDS testicular protein profile of *Spodoptera mauritia*

The SDS-PAGE analysis of testicular protein profiles following treatment with ED₂₅ and ED₅₀ flufenoxuron on sixth instar day zero larvae after exposure at 48 h, 96 h, and 144 h displayed notable differences compared to control groups (Fig. 9A). Specifically, significant alterations were observed in testicular proteins, particularly in the range of 65 kDa to 79 kDa.

After 48 h of treatment with flufenoxuron, a notable decrease in intensity was observed in protein bands with molecular weights of 65 kDa and 79 kDa. Other bands with faint intensity (34 kDa, 33 kDa, 32 kDa, and 28 kDa) were also detected compared to the controls (Fig. 9 B). At 96 h post-treatment, proteins in the middle range (65 kDa and 79 kDa) were observed. Their band intensity levels increased gradually from the ED₂₅ concentration to the ED₅₀ concentration of flufenoxuron (Fig. 9C). By 144 h of treatment, these protein bands exhibited increased intensity, particularly at the ED₂₅ concentration, followed by a decrease at the ED₅₀ concentration. Additionally, in Lane 12, proteins with a molecular weight range of 28 kDa to 34 kDa were expressed, compared to other control and treated lanes, with reduced intensity observed in Lane 11 (Fig. 9D). The 34 kDa, 32 kDa, 28 kDa, and 26 kDa protein bands exhibited a similar pattern of significant increase in both ED₂₅ and ED₅₀ concentrations at 96 h and 144 h of treatment.

The dendrogram represented the distance between each band based on the molecular weight and band intensity of the testicular protein (Fig. 10). The colour indicates the closely related groups based on their molecular weight and band intensity. *S. mauritia* testis protein samples from treated and control/vehicle control groups differ significantly.

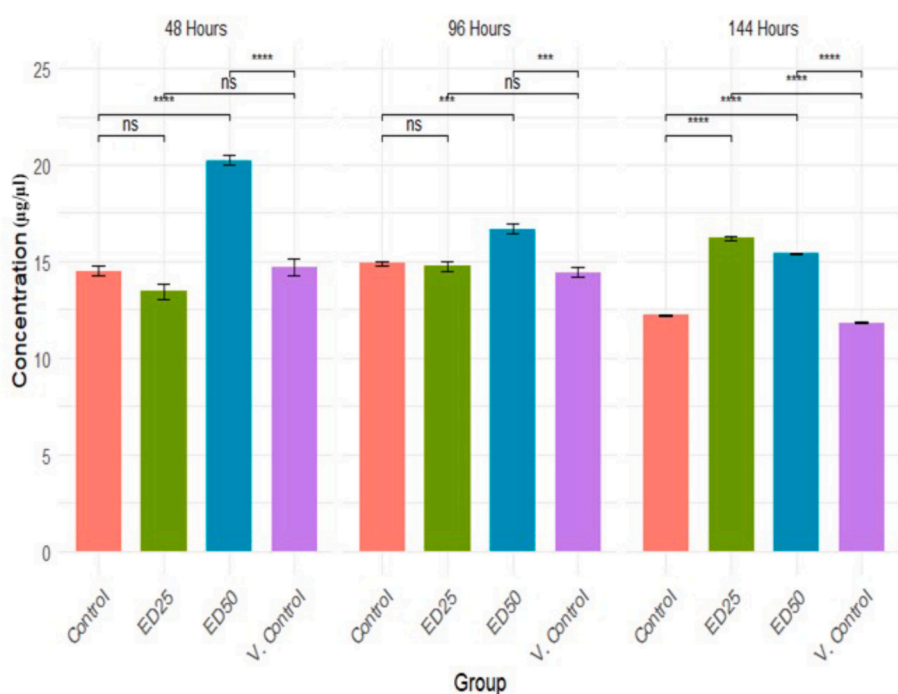


Fig. 6. Testis protein concentration of flufenoxuron-treated sixth instar larvae of *S. mauritia*. Bars represent the mean $\pm$ SE of three independent experiments. Statistical differences were considered significant by one-way ANOVA followed by Tukey post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

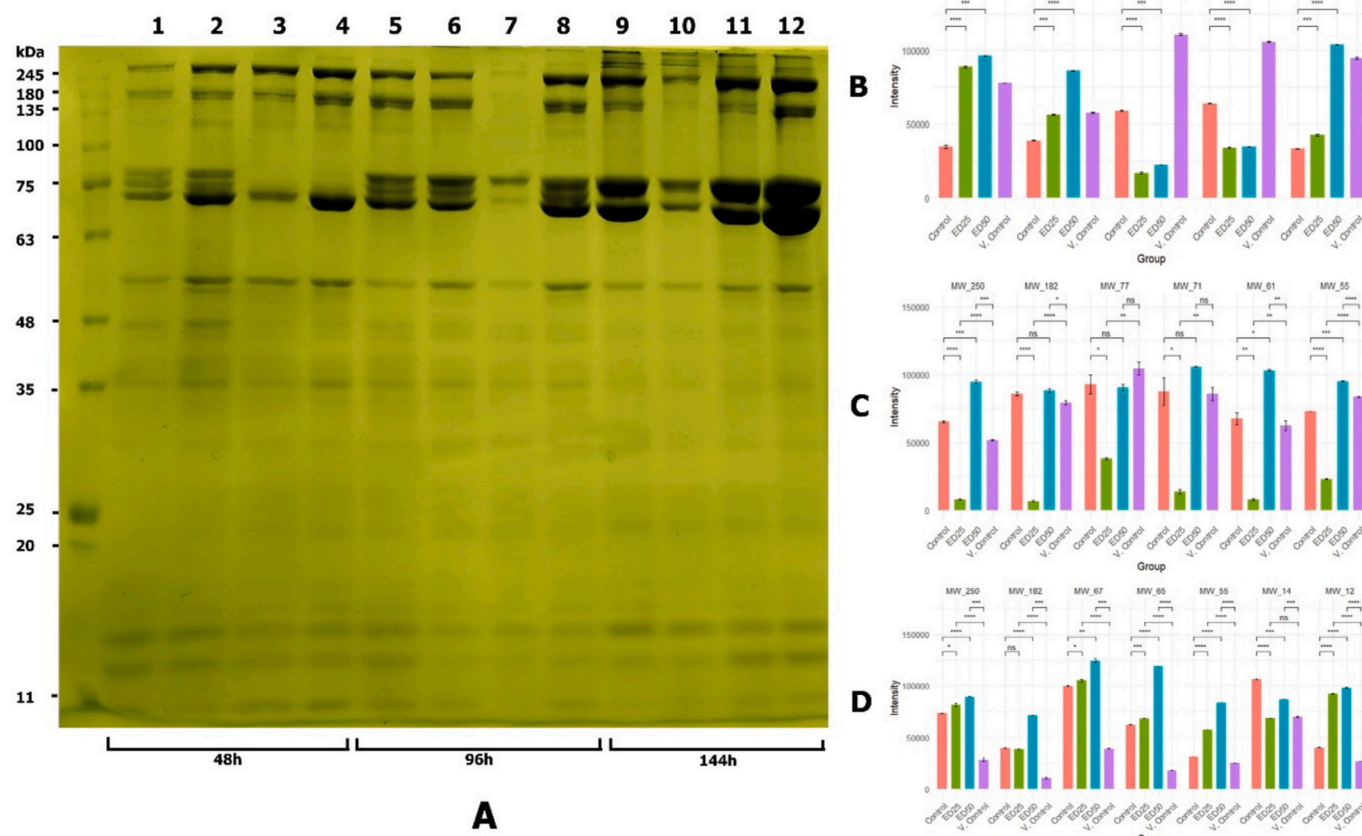


Fig. 7. A: Electrophoretogram of SDS protein profile of *S. mauritia* haemolymph control and flufenoxuron-treated haemolymph samples. Pre-stained protein ladder (Lane 0), Haemolymph sample after 48 h of treatment [Control (Lane 1), Vehicle control (Lane 2), Flufenoxuron treated ED₂₅ (Lane 3) and ED₅₀ (Lane 4)]; Haemolymph sample after 96 h of treatment [Control (Lane 5), Vehicle control (Lane 6), Flufenoxuron treated ED₂₅ (Lane 7) and ED₅₀ (Lane 8)]; Haemolymph sample after 144 h of treatment [Control (Lane 9), Vehicle control (Lane 10), Flufenoxuron treated ED₂₅ (Lane 11) and ED₅₀ (Lane 12)]. Protein intensity with a specific molecular weight in the control and treated haemolymph protein samples of *S. mauritia* after 48 h (B), 96 h (C), and 144 h (D) of ED₂₅ and ED₅₀ flufenoxuron treatment. The data are expressed in mean $\pm$ SE values of three independent experiments. Statistical differences were considered significant by one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.6. Effect of flufenoxuron on testicular histopathology of *Spodoptera mauritia*

The treatment of *S. mauritia* with flufenoxuron showed severe histopathological changes in the testicular development. The exposure to flufenoxuron resulted in pronounced alterations in the testicular cysts, characterized by disorganization of the cell arrangement. After 48 h of treatment (Fig. 11), highly vacuolated germarium is evident in the treated groups. Further, a large number of disorganized spermatocysts and spermatids were also present. Slight malformation was found in the eupyrene spermatocyst (Fig. 11C, E, and F). Moreover, a noticeable void was observed within the spermatocyst cells, and the germ cells were unevenly arranged. Elongating spermatocyst also had a highly vacuolated area due to degeneration in the tail portion of developing cells. The follicular septum and outer epithelium were also observed to exhibit vacuolation, resulting from the irregular arrangement and damage within the epithelial cells.

The damages were more pronounced with higher concentrations of flufenoxuron. Within 96 h of flufenoxuron treatment, these damages were more pronounced, and disruption or degeneration inside the spermatid cells was more evident (Fig. 12). The typical size of both eupyrene and apyrene spermatids significantly decreased, creating space between the cells within the spermatid and the surrounding cyst cells.

Exposure to flufenoxuron resulted in striking alterations in the spermatocyst and spermatozoa, characterized by extreme necrosis and disorganization of the cyst cells. Spermatid cyst cells showed a larger space between the developing cystic cells and the septa. Significant tissue reduction was also visible inside the spermatids. A reduction in connective tissue also represents the area between each spermatid cyst. Cells in the spermatocyst lost their normal shape due to damage in the cytoplasm. The results showed a significant decrease in the number of developing spermatids in the proximal region, characterized by a large amount of cyst-free cellular region, resulting in an uneven distribution of cysts compared to the controls. The follicular septum contains many vacuoles, which induce structural disorganization of cells within the septa (Fig. 12D). The application of a higher dose (ED₅₀) revealed extensive degeneration and necrosis of spermatids (Fig. 12E), accompanied by malformed eupyrene sperm bundles (EUS).

The examination of the testis revealed that the number of degenerated spermatozoa and a highly vacuolated follicular septum was evident after 144 h of flufenoxuron treatment (Fig. 13). ED₂₅-treated groups exhibited more pronounced necrosis of spermatozoa and spermatocytes (Fig. 13C and D). Highly degenerated spermatids (eupyrene) with nuclear heads are noticed, which blocks the further development of spermatozoa. Flufenoxuron post-treatment with ED₅₀ resulted in eupyrene and apyrene spermatozoa exhibiting severe degeneration and necrosis; therefore, they cannot be easily distinguished (Fig. 13E).

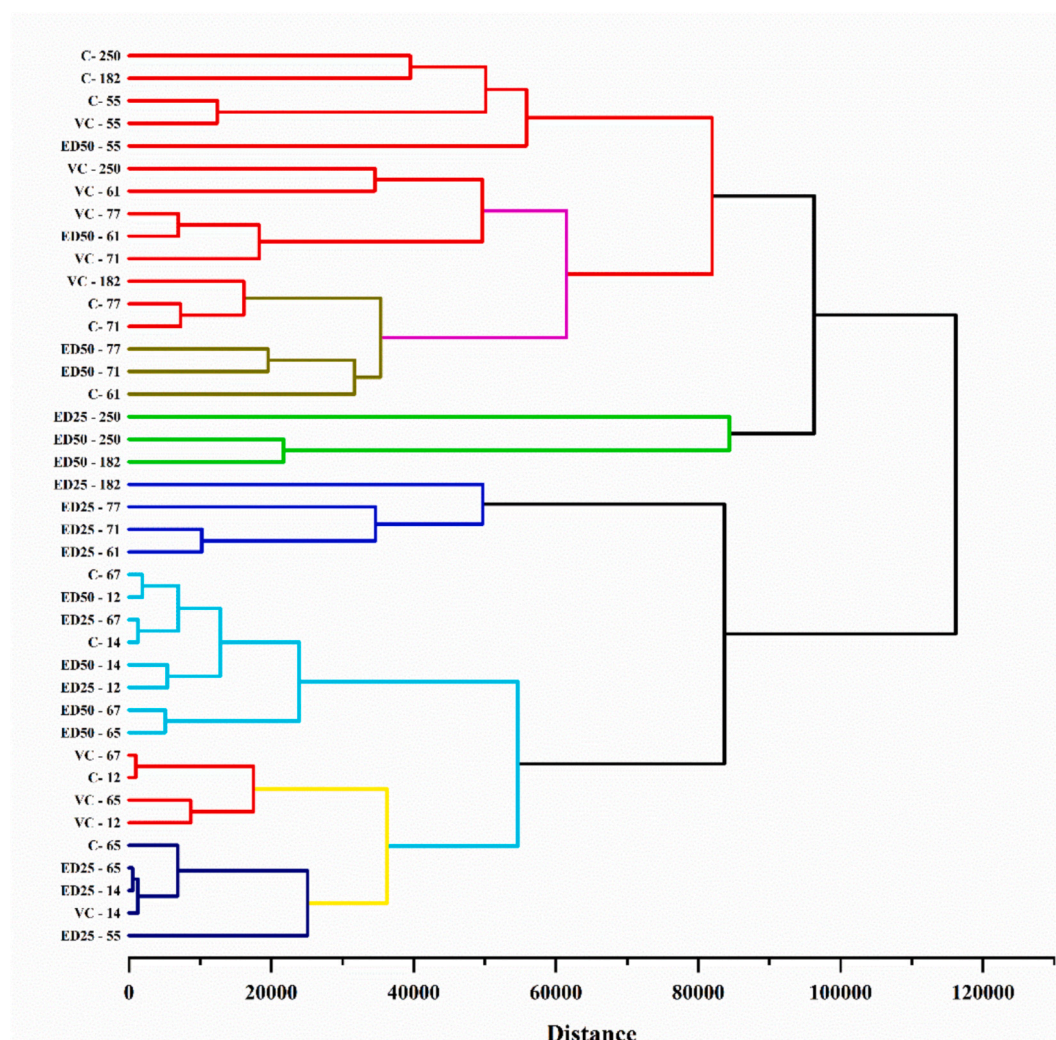


Fig. 8. Dendrogram representation of haemolymph protein bands in the control and treated haemolymph protein samples of *S. mauritia* after 48 h, 96 h, and 144 h of ED₂₅ and ED₅₀ flufenoxuron treatment.

Moreover, it was evident that a few immature spermatogonia lie between the follicular septum and epithelium, which forms a sizeable epithelial area. The epithelium, characterized by numerous vacuoles, was evident in this stage (Fig. 13D).

3.7. Morphometric measurements of histopathological examinations of *Spodoptera mauritia*

The histopathological data revealed no notable changes in the control and vehicle control groups of the sixth instar larval testes of *S. mauritia*. In contrast, tissue degeneration in the *S. mauritia* testis was observed after post-treatment with two different doses (ED₂₅, ED₅₀) of flufenoxuron. Table 1 depicts the morphometric analysis of histopathological examinations from different groups (controls and treated) of testicular tissue associated with flufenoxuron application in the sixth instar stage of *S. mauritia*. Significant differences were observed between the measured parameters for ED₂₅ and ED₅₀ treated insects and controls. The thickness of the outer epithelium and the follicular epithelium significantly reduced after the treatment. In a way, the average length of the elongating spermatocyst also reduced considerably after the flufenoxuron treatment. The thickness of the outer epithelium after 144 h of treatment significantly reduced in the ED₂₅-treated group ($11.69 \pm 0.65 \mu\text{m}$), and enlarged considerably in the ED₅₀ ($69.9 \pm 9.49 \mu\text{m}$) due to high vacuole formation, compared to the control and vehicle control

groups ($28.06 \pm 2.33 \mu\text{m}$ and $28.82 \pm 2.44 \mu\text{m}$, respectively). Most importantly, the average length of early spermatid cyst, the initial stage of sperm development, reduced considerably in all treatment groups and time periods. At 144 h (just after metamorphosis), the average length reduced to $48.49 \pm 4.54 \mu\text{m}$ and $40.33 \pm 3.58 \mu\text{m}$ in ED₂₅ and ED₅₀, respectively, compared to control and vehicle control ($93.24 \pm 4.76 \mu\text{m}$ and $102.51 \pm 5.07 \mu\text{m}$, respectively).

4. Discussion

4.1. Chitin synthesis inhibitor flufenoxuron affects reproductive impairment in *Spodoptera mauritia*

It is well-documented that CSIs can inhibit chitin synthesis in insects and serve as a viable alternative against several insect species. Earlier reports have shown that CSIs can disrupt chitin synthesis in insects, leading to various detrimental effects, including reproductive dysfunction. Moreover, understanding the relationship between CSIs and reproductive dysfunction is crucial for validating these compounds as potential insecticides (Acheuk et al., 2012; Costa et al., 2017; Fansiri et al., 2022). Studies have highlighted the impact of CSIs on the reproductive behaviour and fertility of several insect pests, revealing their potential as an effective solution for pest management.

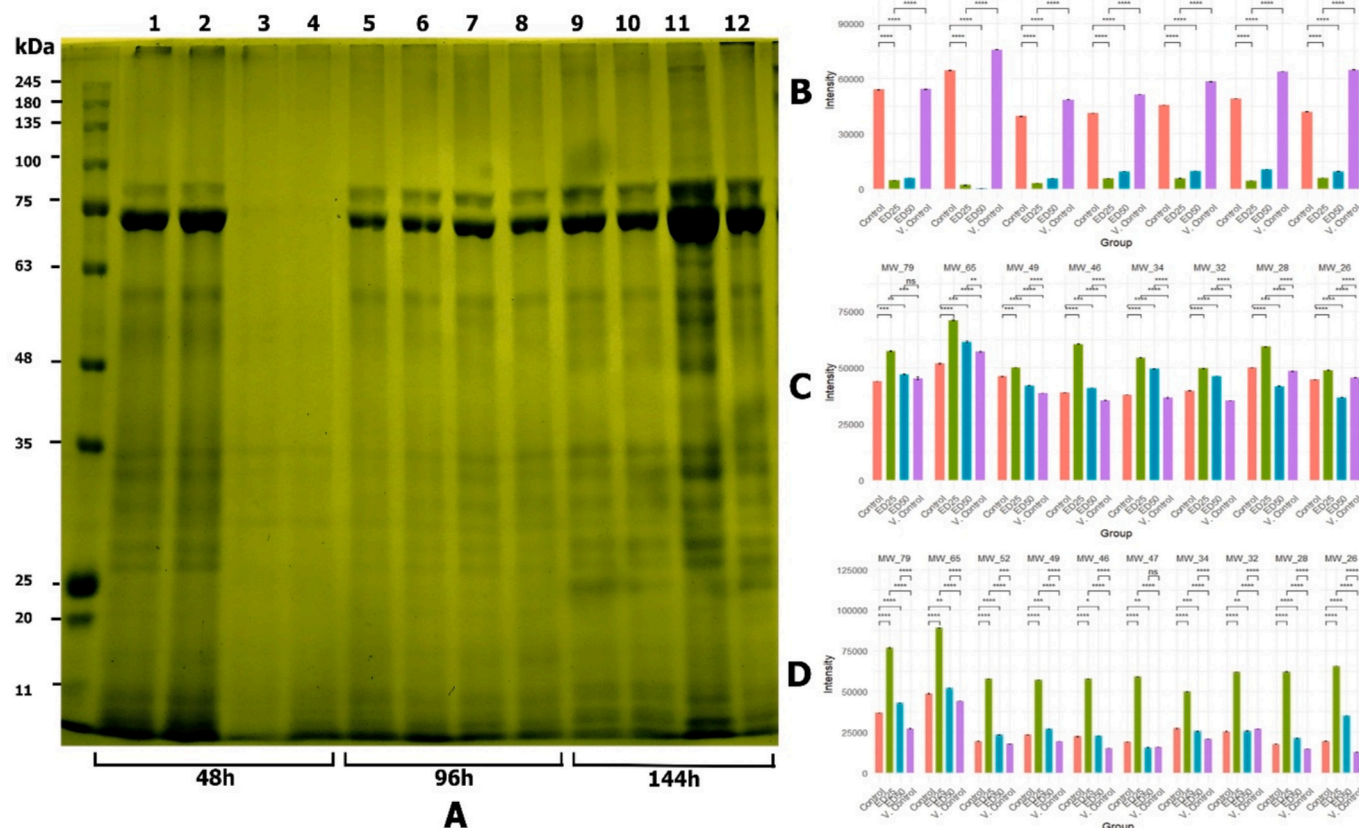


Fig. 9. A: Electrophoretogram of SDS protein profile of *S. mauritia* control and flufenoxuron-treated testis samples. Pre-stained protein ladder (0), Haemolymph sample after 48 h of treatment [Control (1), Vehicle control (2), Flufenoxuron treated ED₂₅(3) and ED₅₀ (4)]; Haemolymph sample after 96 h of treatment [Control (5), Vehicle control (6), Flufenoxuron treated ED₂₅ (7) and ED₅₀ (8)]; Haemolymph sample after 144 h of treatment [Control (9), Vehicle control (10), Flufenoxuron treated ED₂₅ (11) and ED₅₀ (12)].

Protein intensity with a specific molecular weight in control and treated testicular protein samples of *S. mauritia* after 48 h (B), 96 h (C) and 144 h (D) of treatment. The data are expressed in mean $\pm$ SE values of three independent experiments. Statistical differences were considered significant by one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.2. Flufenoxuron influence on biochemical alteration in *Spodoptera mauritia*

Carbohydrates are essential for providing energy and play a critical role in various processes, including defence against harmful substances like insecticides (Arrese et al., 2010; Rosas-Mejía et al., 2015). The carbohydrate reserves in insects can vary depending on their developmental stage, and few studies have shown that CSIs typically affect carbohydrate synthesis (Shen et al., 2018; Wang et al., 2018). The biochemical effects of flufenoxuron on insects have been previously documented in the Lepidopteran species *Spodoptera littoralis* (Bakr et al., 2013).

Many reports have highlighted the effects of CSIs on the total carbohydrate content of insect bodies. Zhu et al. (2012) reported a decrease in total carbohydrate content in the haemolymph of *Spodoptera litura* after treatment with hexaflumuron. Similarly, hexaflumuron reduced the trehalose and glucose content in *Apolygus lucorum* (Tan et al., 2014). Further, Hamadah (2014) observed that flufenoxuron inhibited the carbohydrate content in the haemolymph of *Schistocerca gregaria*. A similar reduction in carbohydrate content was observed with lufenuron treatment in *Glyphodes pyloalis* (Aliabadi et al., 2016) and *S. gregaria* (Tanani et al., 2012). In the penultimate larval instar of *Musca domestica*, CSIs such as lufenuron, flufenoxuron, and hexaflumuron were shown to block carbohydrate synthesis (Tanani et al., 2022).

Perveen (2012a) found that the total carbohydrate content in both the testes and ovaries of *S. litura* decreased following treatment with

chlorfluazuron. Analysis of the main metabolic components of *Agrotis ipsilon* females treated with chlorfluazuron and flufenoxuron showed a significant decrease in the carbohydrate content of the ovaries (Shaurub et al., 2018). Costa et al. (2017) observed that treatment with lufenuron led to significant changes in the histochemical contents of the midgut, ovaries, and testes, with a reduction in neutral carbohydrates in testicular spermatozooids. Similarly, a reduction in testicular carbohydrate content was noted in the present study in the last instar larvae of *S. mauritia*. However, studies using various CSIs have consistently demonstrated decreased levels of ovarian carbohydrate content in *Cydia pomonella*, *S. litura*, and *S. littoralis* (Soltani-Mazouni, 1994; Perveen and Miyata, 2000; Abdel-Aal, 2012).

Lipids supply the metabolic energy required for egg formation and are the primary energy source during embryonic development. A complex network of hormonal signals tightly regulates the availability and utilization of lipids. The central nervous system and associated endocrine glands primarily orchestrate this regulation. Various hormones and neuropeptides are produced through this endocrine axis, significantly influencing the tissues involved in reproduction, thereby ensuring successful reproductive outcomes (Leyria et al., 2024). Lipids are synthesized and stored in the fat body and then transported through the haemolymph to the ovaries, where they are essential for the process of vitellogenesis (Shaurub et al., 2018). Research by Hamadah (2014) demonstrated that flufenoxuron reduced the haemolymph lipid content of *S. gregaria*. Similarly, Reda et al. (2007) reported that lufenuron significantly decreased haemolymph lipid content in *S. gregaria*.

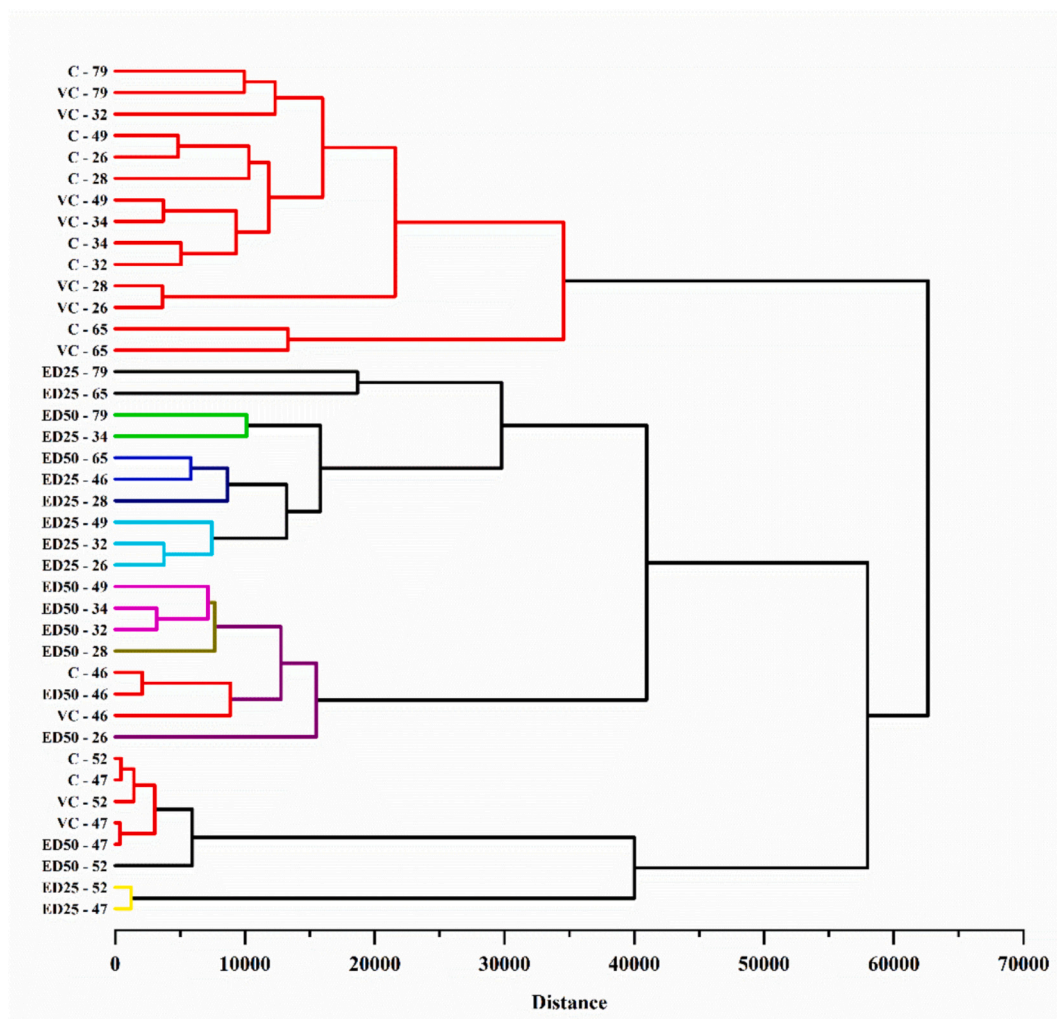


Fig. 10. Dendrogram representation of testis protein bands in control and treated testicular protein samples of *S. mauritia* after 48 h, 96 h, and 144 h of ED₂₅ and ED₅₀ flufenoxuron treatment.

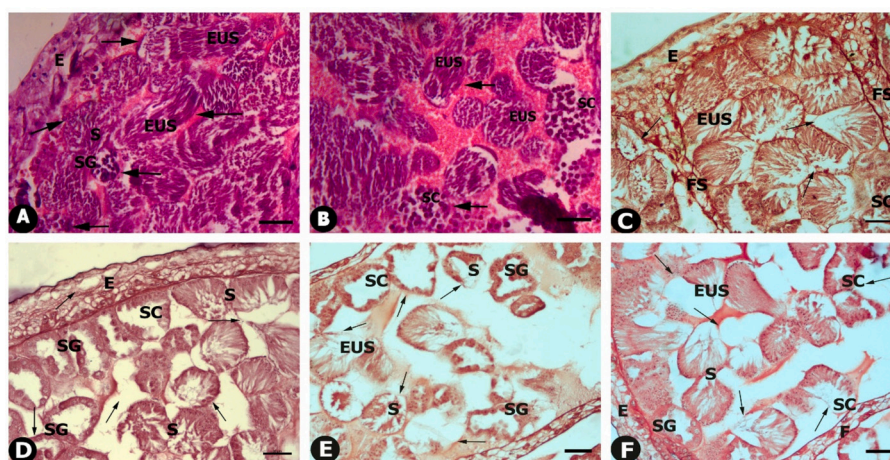


Fig. 11. Histological sections of *S. mauritia* testis after 48 h of flufenoxuron treatment. Arrow indicates the damage in epithelium, follicular septum, and spermatocyst (Scale Bar:20 μ m).

A & B: Control & Vehicle control (Sections of *S. mauritia* testis after 48 h). C & D: Flufenoxuron treated (ED₂₅): note the rupture in tunica cell and septa (FS), with marked irregularity; necrotic eupyrene spermatid (EUS) and spermatocyst (SC) with irregularly arranged cellular components and large cavity. E & F: Flufenoxuron treated (ED₅₀): necrotic spermatocytes (SC) with a large cavity inside; visible damage in spermatocyte cyst cells; large vacuolated area developed within the elongating spermatid cyst (S) due to degeneration of tail portion; Epithelium (E) and follicular septa (FS) found with vacuolated cells and degenerated spermatogonia (SG).

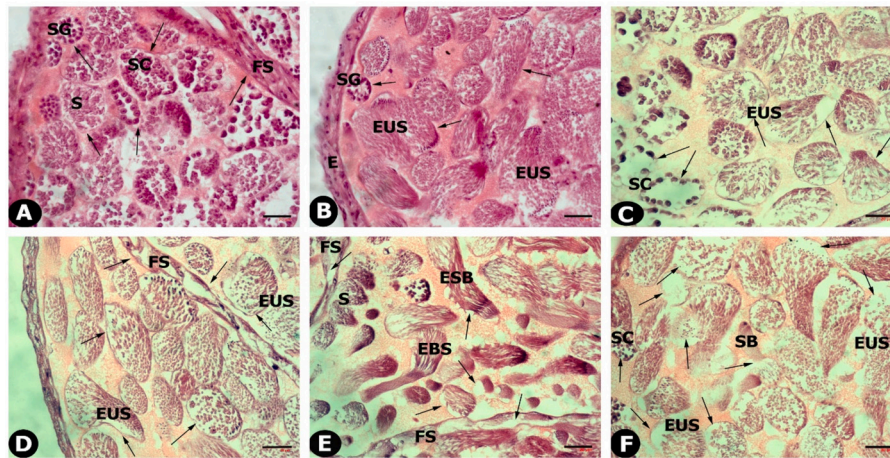


Fig. 12. Histological sections of *S. mauritia* testis after 96 h of flufenoxuron treatment (Scale Bar:20 μ m).

A & B: Control & Vehicle control (*S. mauritia* testis section at 96 h). C & D: Flufenoxuron treated (ED_{25}): Malformed arrangement within developing spermatids (S); visible gaps found with different intensity; eupyrene spermatid (EUS) with vacuolated tail end; number and arrangement of cells within spermatocyte cyst cells were found defective; follicular septum (FS) with vacuolated cells. E & F Flufenoxuron treated (ED_{50}): Highly vacuolated spermatids (S), spermatocyte (SC) and eupyrene spermatids and degenerated cellular components are visible; vacuolated area with degeneration of cells within the cyst; thin and vacuolated follicular septum (FS) formed.

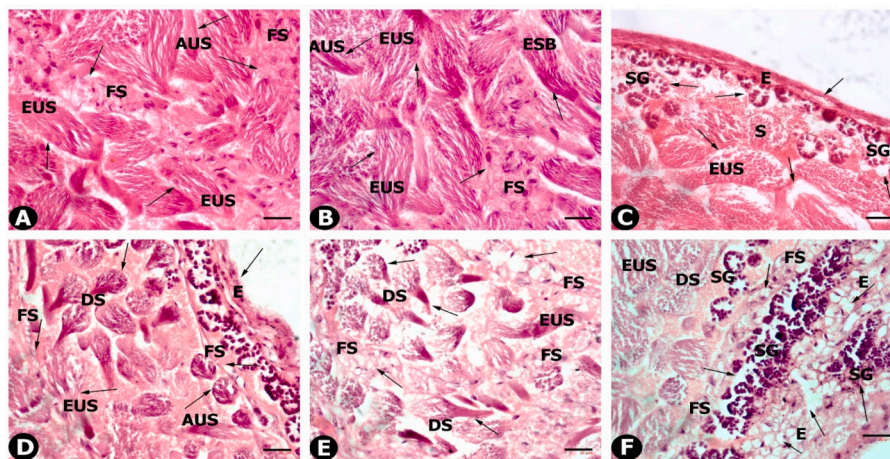


Fig. 13. Histological sections of *S. mauritia* testis after 144 h of flufenoxuron treatment (Scale Bar:20 μ m).

A & B: Control & Vehicle control. C & D: Flufenoxuron treated (ED_{25}): Degenerated spermatids (DS) with large vacuolated area; necrotic eupyrene and apyrene spermatids (EUS & AUS) are evident; narrowing of follicular septa (FS) and that run nearby the epithelium, spermatogonial cysts trapped in between. E & F: Flufenoxuron treated (ED_{50}): Highly degenerated spermatids (DS) and spermatozoa (EUS) with many empty spaces within the cyst. Vacuolated Epithelium and necrotic spermatogonia (SG) present between the vacuolated epithelium; follicular septum (FS) highly vacuolated.

Proteins are biological macromolecules composed of one or more long chains of amino acids, and they are essential for growth and reproduction. Protein synthesis is crucial for cell division and the regulation of chemical processes during cellular metabolism (Tanani et al., 2022). The current study observed fluctuations in the total protein content of haemolymph and testes with flufenoxuron treatment. Studies have shown that total protein content changes significantly after treatment with different CSIs (Bakr and Ghoneim, 2008). In *A. ipsilon*, total protein levels exhibited severe fluctuations after treatment with chlorfluazuron and triflurumuron (Fahmy, 2014).

Abdel-Aal (2003) observed that the total protein content in the haemolymph of *S. littoralis* larvae decreased after treatment with chlorfluazuron and flufenoxuron. Similarly, CSIs such as lufenuron, flufenoxuron, and hexaflumuron were found to block total protein synthesis in the penultimate larval instar of *M. domestica* (Tanani et al., 2022). Chlorfluazuron also caused a significant reduction in the total protein content of *Rhynchophorus ferrugineus* (El-Sobki and Ali, 2020). In *G. pyralis*, larval protein content decreased significantly following

lufenuron treatment (Aliabadi et al., 2016).

Likewise, the protein content in adult female *S. litura* significantly decreased following treatment with chlorfluazuron (Perveen and Miyata, 2000). Similarly, female *A. ipsilon* treated with chlorfluazuron and flufenoxuron exhibited significantly lower total protein content in the ovaries (Shaurub et al., 2018). These findings align with our results, which show a reduction in protein content in the tissue after flufenoxuron treatment. Several studies have reported that the administration of various CSIs against *C. pomonella*, *S. litura*, and *S. littoralis* has consistently shown decreased ovarian protein content (Soltani & Mazouni, 1992; Perveen and Miyata, 2000; Abdel-Aal, 2012).

Further, the protein content per ovary in diflubenzuron-treated *C. pomonella* was also significantly reduced (Soltani and Soltani-Mazouni, 1992). Bebas et al. (2008) noted that one of the yolk protein precursors, YP2, has a dual function in insects. In females, YP2 accumulates in the oocytes to support embryo development, while in males, it interacts with sperm in the seminal fluid after being produced in the testes. These findings suggest an unexpected shared biochemical

Table 1Morphometric measurements of histopathological examinations of *Spodoptera mauritia*.

Histological alterations (μm)	(Hours)	Treatment groups			
		Control	Vehicle Control	ED ₂₅	ED ₅₀
Thickness of outer epithelium(μm)	48	52.68 ± 0.88	40.87 ± 3.12	28.85 ± 1.13*	25.64 ± 0.4*
		19.80 ± 1.18	15.16 ± 0.55	9.13 ± 0.50*	8.09 ± 0.65*
	96	28.06 ± 2.33	28.82 ± 2.44	11.69 ± 0.65*	69.9 ± 9.49*
		15.98 ± 0.40	15.39 ± 0.15	9.98 ± 0.25*	8.48 ± 0.48*
	144	19.81 ± 0.80	18.72 ± 0.55	10.19 ± 0.87*	10.71 ± 0.99*
		21.44 ± 1.58	27.93 ± 1.53	14.45 ± 1.28*	21.78 ± 1.27*
Thickness of follicular epithelium(μm)	48	675.84 ± 80.33	510.09 ± 41.34	945.88 ± 46.50*	842.88 ± 82.19*
		576.32 ± 63.84	556.65 ± 35.47	996.91 ± 156.23*	506.78 ± 101.7
	96	259.96 ± 27.49	248.25 ± 22.95	154.19 ± 25.38*	153.60 ± 23.02*
		5550.51 ± 912.39	6040.65 ± 530.94	988.29 ± 41.18*	851.25 ± 90.71*
	144	1030.0 ± 57.93	1173.02 ± 132.24	772.53 ± 78.01*	852.8 ± 115.4
		953.32 ± 98.21	953.31 ± 98.21	189.73 ± 1.65*	316.39 ± 32.79*
Average area of spermatogonia (μm ²)	48	37.60 ± 3.32	36.31 ± 4.49	10.19 ± 1.32974*	8.32 ± 0.44*
		27.38 ± 2.13	36.9 ± 2.13	21.2 ± 2.10*	22.48 ± 1.50*
	96	42.67 ± 3.51	45.14 ± 1.26	24.18 ± 1.15*	20.26 ± 1.47*
		306.24 ± 52.45	229.20 ± 48.69	22.84 ± 1.71*	19.23 ± 3.07*
	144	76.02 ± 12.24	76.02 ± 12.24	59.62 ± 7.60	71.62 ± 5.44
		93.24 ± 4.76	102.51 ± 5.07	48.49 ± 4.54*	40.33 ± 3.58*
Average length of elongating spermatocyst (μm)	48	37.60 ± 3.32	36.31 ± 4.49	10.19 ± 1.32974*	8.32 ± 0.44*
		27.38 ± 2.13	36.9 ± 2.13	21.2 ± 2.10*	22.48 ± 1.50*
	96	42.67 ± 3.51	45.14 ± 1.26	24.18 ± 1.15*	20.26 ± 1.47*
		306.24 ± 52.45	229.20 ± 48.69	22.84 ± 1.71*	19.23 ± 3.07*
	144	76.02 ± 12.24	76.02 ± 12.24	59.62 ± 7.60	71.62 ± 5.44
		93.24 ± 4.76	102.51 ± 5.07	48.49 ± 4.54*	40.33 ± 3.58*
Average length of early spermatid cyst(μm)	48	37.60 ± 3.32	36.31 ± 4.49	10.19 ± 1.32974*	8.32 ± 0.44*
		27.38 ± 2.13	36.9 ± 2.13	21.2 ± 2.10*	22.48 ± 1.50*
	96	42.67 ± 3.51	45.14 ± 1.26	24.18 ± 1.15*	20.26 ± 1.47*
		306.24 ± 52.45	229.20 ± 48.69	22.84 ± 1.71*	19.23 ± 3.07*
	144	76.02 ± 12.24	76.02 ± 12.24	59.62 ± 7.60	71.62 ± 5.44
		93.24 ± 4.76	102.51 ± 5.07	48.49 ± 4.54*	40.33 ± 3.58*

The data expressed in the Mean ± SE. Statistical differences were considered significantly by one-way ANOVA, followed by Duncan post-hoc test (the same number of asterisks do not differ significant at $P < 0.05$).

component in developing insect sperm and eggs.

4.3. Flufenoxuron interaction on protein metabolism

The electrophoretic protein patterns observed in this study reveal differences between control and flufenoxuron-treated haemolymph and testis samples. Bakr et al. (2013) reported similar findings in *S. littoralis* after treatment with flufenoxuron, where variations in protein expression were linked to potential deformities. In our study, differential expression of protein bands was observed depending on the dose and duration of flufenoxuron treatment. This suggests the possible induction of new proteins in activating the insect's immune system in response to foreign substances (Bakr et al., 2013).

The role of binding proteins or chitin recognition in the immunological response of insects has been relatively understudied, with limited data available (Chae et al., 1999). In insects, proteins present in the haemolymph are crucial for carrying out humoral immune responses (Royet, 2004). Several haemolymph proteins that contribute directly or indirectly to immunological processes have been identified and reported (Chung and Ourth, 2002; Park et al., 2005). Chae et al. (1999) identified three types of chitin-binding proteins (CBPs) with molecular weights of 9 kDa, 15 kDa, and 66 kDa in the haemolymph of a sphingid moth, *Agrius*

convolvuli.

In the present study, flufenoxuron was observed to negatively affect proteins with molecular weights of 71 kDa, 67 kDa, 65 kDa, 55 kDa, and 12 kDa in the haemolymph of *S. mauritia*. This suggests a possible defect in chitin-binding proteins following flufenoxuron treatment.

4.4. Potential effects of flufenoxuron on spermatogenesis

Different derivatives of CSIs have been shown to significantly influence insect reproduction, fertility, emergence, and development (Yasir et al., 2012; Gad et al., 2022). Previous research by Cloyd et al. (2004) and Joseph (2020) has documented the impact of novaluron, a CSI, on the reproductive functions of crop pests. Additionally, the study conducted by Acheuk et al. (2012) demonstrated the deleterious effects of teflubenzuron on the reproductive system of *Locusta migratoria*. Further expanding on these findings, Costa et al. (2017) investigated the consequences of lufenuron exposure on reproductive development, revealing its impact on the midgut, which disrupts the insects' ability to reproduce by interacting with gametogenesis. Moreover, Yasir et al. (2012) highlighted that exposure to flufenoxuron through the diet interferes with crucial reproductive processes, reducing fecundity and fertility rates and hindering population growth.

All Lepidopteran pests exhibit unique attributes in their male reproductive biology, particularly in their possession of two distinct types of sperm; one type of sperm, called eupyrene sperm or sperm, contains a nucleus, while the other type, known as apyrene sperm or perisperm, is without a nucleus (Friedländer et al., 2005). Eupyrene sperm fertilizes eggs with paternal genes. Apyrene sperm also contribute to male fitness in other ways. Both types are essential for Lepidopteran fertilization (Chen et al., 2020). It can interfere with male reproduction to control Lepidopteran pests, and dichotomous spermatogenesis offers a precise and extensive range of opportunities for such interference (Seth et al., 2023).

A remarkably wide variety of organisms exhibit sperm heteromorphism, and numerous studies have demonstrated the significance of parasperm for male fertility (Whittington et al., 2015). CSIs have been reported to prevent chitin formation and cause reproductive disruption in Lepidopteran pest species (Perveen, 2000). Perveen (2000) also observed the impact of chlorfluazuron on testis development and the process of spermatogenesis. Similarly, lufenuron-treated larvae reduced the number of spermatozooids in the testicles of *Anthonomus grandis* (Costa et al., 2017).

Histopathological observations of the present study revealed that spermiogenesis was directly affected by two doses of flufenoxuron applied during the sixth instar stage. The present study aligns with the findings of Abdel-Aal and Abdel-wahab (2007) regarding the histological changes in the cotton leafworm following exposure to lufenuron. Histological analysis of *S. mauritia* testes after flufenoxuron treatment showed a dose-dependent reduction in testicular tissue compared to the control, suggesting that spermiogenesis was severely affected. Additionally, defects in follicular formation, vacuolation, and cyst degeneration were observed, indicating potential damage to connective tissue and supporting cells. Studies on morphometric alterations in the testicular area after treatment with lufenuron and buprofezin in *Euschistus heros* suggested the effects of chemical application on testicular development (Cremonez et al., 2018). In *R. ferrugineus* treated with lower concentrations of flufenoxuron, narrower follicles and a reduction in cellular content were observed. Higher concentrations of flufenoxuron in the testicular follicles led to pronounced disorganization, characterized by decreased cellular content and a significant reduction in the cysts of spermatocytes and spermatogonia (El-Bokl et al., 2010).

Agüero et al. (2014) noted that diflubenzuron impacts both females' and males' development and reproductive systems. According to Abd-Alla et al. (2003), lufenuron is genotoxic to the germ-line cells of male *D. melanogaster*, showing varying degrees of genotoxic potential across the three separate phases: spermatozoa, spermatids, and spermatocytes.

Furthermore, the application of chlorfluazuron on *S. litura* has been shown to disrupt spermatogenesis, resulting in a decrease in both the quantity and size of eupyrene and apyrene sperm bundles in the testes (Perveen, 2000). Histopathological examination conducted by Ghazawy (2012) on *S. gregaria* males treated with the CSI lufenuron revealed disorganized and damaged sperm tubes, vacuolated small cells, disorganized spermatids, abnormal cell organelles, thickened and vacuolated testicular epithelia, detachment from the peritoneal sheath, degenerated sperm bundles, and incomplete spermatid development. These findings are consistent with our histological results.

Similarly, in male *S. gregaria*, vacuolated testicular epithelia and loosely fragmented testicular tissues were observed. An earlier study by Perez-Farinos et al. (1998) investigated the penetration, distribution, and excretion of labelled hexaflumuron in young and old adults of *Aubeonymus mariaefranciscas*. Their study revealed that hexaflumuron penetration was slightly higher in females than in males. However, males exhibited lower elimination rates, resulting in a more significant accumulation of the insecticide. Additionally, young adult weevils showed higher levels of hexaflumuron in their reproductive systems than older weevils.

5. Conclusion

The ED₂₅ and ED₅₀ were used to understand the consequences of flufenoxuron on the biochemical and spermatogenic alterations in *S. mauritia*. Our results demonstrated that exposure of male *S. mauritia* larvae to flufenoxuron can alter key metabolites, including carbohydrates, lipids, and proteins, as well as testicular development. We have shown that the flufenoxuron exposure induced testicular reproductive toxicity, significantly impairing spermiogenesis. However, the results from a small-scale experiment may not apply to a larger population in field conditions. Therefore, an additional field experiment is necessary to understand the potential of flufenoxuron before integrating it as a tool in the management of lepidopteran pests. Further studies based on this type of male-targeted pest control model should provide new insight into population control over many serious pest species.

CRediT authorship contribution statement

V. Reshma: Writing – original draft, Methodology, Investigation, Conceptualization. **E.M. Manogem:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare no potential conflicts of interest.

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Data availability

Data will be made available on request.

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RESEARCH ARTICLE

GC-MS Based Study of Haemolymph Metabolomics in Male *Spodoptera mauritia* Boisduval, on Post-Treatment of Flufenoxuron

V. Reshma  | E. M. Manogem 

Department of Zoology, Division of Insect Endocrinology, University of Calicut, Malappuram, Kerala, India

Correspondence: E. M. Manogem (dr.manogem_em@uoc.ac.in)**Received:** 25 February 2026 | **Revised:** 31 March 2026 | **Accepted:** 3 April 2026**Keywords:** chitin biosynthesis pathway | energy metabolism | flufenoxuron | GC-MS | insect haemolymph | insect metabolomics | nutritional regulation

ABSTRACT

Flufenoxuron exhibits excellent chitin-inhibiting activity against several insect species, including Lepidoptera. The disruption of the chitin synthesis process can have a significantly negative impact on several developmental systems of insects. However, current knowledge of the metabolomic effect on *Spodoptera mauritia* larvae remains underdeveloped. This study employed Gas Chromatography–Mass Spectrometry (GC-MS)-based untargeted metabolomics to investigate the changes in metabolic activity and biochemical effects of flufenoxuron in *S. mauritia* haemolymph, thereby illustrating the possible mode of action. Metabolomic analysis of haemolymph was conducted on exposure to two different sublethal concentrations of flufenoxuron (0.00068 and 0.00015 μg) in the sixth instar stage of *S. mauritia*. The effect of flufenoxuron was investigated on selected differently abundant metabolites in GC-MS-based metabolomics results. Notably, carbohydrate and fatty acid levels were significantly reduced, indicating severe nutritional and energetic stress. Pathway analysis indicated that carbohydrate, lipid and amino acid metabolism are strongly associated with the effects of the chitin synthesis inhibitor. The findings substantiate the flufenoxuron-mediated disturbance in metabolites and metabolic pathways. The findings of the study deepen our understanding of the physiological disruptions caused by flufenoxuron, highlighting its potential hazards in agricultural applications against *S. mauritia*.

1 | Introduction

The administration of insect growth regulators, particularly chitin synthesis inhibitors (CSIs), disrupts chitin synthesis and related metabolic pathways, making them promising targets for novel pest management strategies (Liu et al. 2023). Despite extensive research over many years on the mode of action of CSIs, the precise inhibition of a specific target enzyme has yet to be conclusively demonstrated. Several studies have investigated the effects of CSIs on the developmental, biochemical, and reproductive processes of several insect species (Kellouche and Soltani 2006; Reda et al. 2010; Fahmy 2014). These studies suggest that CSIs may indirectly influence other developmental pathways.

Chitin metabolism is regulated by a dynamic equilibrium between the chitin hydrolase and synthase systems, governed by

multiple signalling pathways and hormones (Zhang et al. 2021). The pathway to insect chitin production begins with trehalose, which is hydrolyzed into glucose by trehalase (Chen et al. 2010a). The balance between dietary intake and the energy demands of maintenance, development, and reproduction dictates the availability of lipids. Disruptions to this balance caused by toxic substances can lead to developmental dysfunctions (Tanani et al. 2022). However, the mechanisms by which metabolites influence biochemical pathways and the regulation of chitin metabolism remain poorly understood (Yu et al. 2021; Liu et al. 2023).

Rice-swarming caterpillar, *Spodoptera mauritia* Boisduval (Lepidoptera: Noctuidae), is a sporadic paddy pest in India that causes substantial yield and economic losses (Grant 1982). Flufenoxuron, a benzophenylurea derivative belonging to the

Summary

- Flufenoxuron affected the differently abundant metabolites of *Spodoptera mauritia*.
- Exposure to flufenoxuron resulted in changes in the total metabolic profile.
- Flufenoxuron had a significant impact on the chitin synthetic pathway.

chitin synthesis inhibitor (CSI) class, have been used in several insects (Fisk et al. 1993; El-Bendary and El-Helaly 2015). The biological and metabolic responses to such compounds reflect underlying enzymatic activity, which serves as a sensitive indicator of physiological disturbance (Huang et al. 2016). To minimize metabolic variability and enhance toxicological interpretation, exposure assessments should also emphasize reproductive endpoints (Jeong and Simpson 2019). Moreover, CSIs such as diflubenzuron, chlorfluazuron, lufenuron, and novaluron inhibit chitin synthase, thereby disrupting chitin formation and indirectly affecting spermatogenesis by altering testicular development, spermatocyte differentiation, and sperm maturation in insects (Perveen 2000; Cremonese et al. 2018). Previous studies have shown that exposure to flufenoxuron adversely affects the male reproductive system in lepidopteran insects (Shaurub et al. 2018; El-Sabroun et al. 2019; Reshma and Manogem 2024). Recent evidence further suggests that flufenoxuron disrupts major biochemical pathways, suppresses spermatogenesis, and impairs reproductive organ development, leading to male infertility in *S. mauritia* (Reshma and Manogem 2026).

To date, few investigations have applied haemolymph metabolomics to examine physiological responses, with only a limited number of studies addressing toxicity (Poynton et al. 2011), disease, oxidative stress (Schock et al. 2010), and infections (Aliferis et al. 2012). The haemolymph acts as a medium for circulating steroid hormones, which are crucial for reproductive functions (Merzendorfer 2025), particularly in the development and maturation of the testes (Shinbo and Happ 1989). Earlier research by Loeb (1986) demonstrated that steroids present in the testicular sheath of insects are not synthesized within the testes themselves but are instead sequestered from the haemolymph. Consequently, tracking metabolic regulatory networks may yield substantial insights into the elements driving the disruption. Understanding alterations in haemolymph composition during reproductive maturation would also facilitate the development of strategies to control the insect's reproductive capacity (Lewis et al. 2001).

Despite the complexity of metabolomes, metabolomics has emerged as a powerful bioanalytical tool for monitoring and diagnosing metabolic networks of organisms and their fluctuations (Aliferis et al. 2012; Aliferis and Jabaji 2010; Simpson et al. 2011). Insect species have already been the subject of successful studies that have identified such metabolic indicators (Qian et al. 2020; Gao et al. 2022). Therefore, it is assumed that metabolite biomarkers may respond to physiological stress induced by the inhibition of chitin synthesis in *S. mauritia*. Therefore, to test this hypothesis, the present study employs gas chromatography-mass spectrometry (GC-MS) on a metabolomics

profile to detect alterations in the haemolymph of male *S. mauritia* following exposure to flufenoxuron. Based on the available information, this study represents the first metabolomics investigation using haemolymph analysis to explore the physiological effects of flufenoxuron in male *S. mauritia*, offering new insights into the metabolic responses to chitin synthesis inhibition.

2 | Materials and Methods

2.1 | Chemicals and Reagents

The chemicals and reagents used in this study were analytical grade. Flufenoxuron ($C_{21}H_{11}ClF_6N_2O_3$) (purity > 98%, Product Number: 31594, CAS No. 101463-69-8) was procured from Sigma Aldrich. The chemical solvents n-hexane, methanol, and Phenylthiourea were purchased from Himedia Chemicals. O-methoxyamine hydrochloride, N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% Trimethylchlorosilane (TMCS), and pyridine were procured from Sigma-Aldrich.

2.2 | Treatment of Experimental Animals

The toxicity study conducted on the male gonadal development of *S. mauritia* (Reshma and Manogem 2024) was extended to analyze the effect on haemolymph metabolomics of *S. mauritia* males. The population of *S. mauritia* was maintained in a laboratory at room temperature, with RH of $90\% \pm 3\%$ and a 10:14 light: dark photoperiod, as per the earlier standardized laboratory protocol (Banu et al. 2022; Reshma and Manogem 2024).

The newly moulted sixth instar male larvae were isolated from the laboratory stock culture and treated with flufenoxuron at Effective dose 50% (ED_{50} -0.00068 μ g) and Effective dose 25% (ED_{25} -0.00015 μ g) concentrations. Five microliters of flufenoxuron were topically applied to the abdomen of larvae using a Hamilton microsyringe. Untreated larvae served as the control, and acetone-treated individuals served as the vehicle control. After 144 h of flufenoxuron exposure, all groups were sacrificed, and haemolymph was extracted by incision on the pupal integument by applying gentle pressure on the abdomen (Loughton and West 1965). The haemolymph was collected into an Eppendorf tube containing phenylthiourea crystals to prevent melanization. Haemolymph from 10 individuals in the same experimental group was pooled into a single sample. The pooled samples were centrifuged, and the supernatant was then stored at -80°C until further analysis.

2.3 | Sample Preparation and Derivation for GC-MS Analysis

The haemolymph sample derivation was subjected to the protocol of Lu et al. (2014) with slight modification. Each sample (100 μ L haemolymph) was mixed with 800 μ L of methanol and 100 μ L of distilled water containing 0.1 mg/mL sucrose (used as an internal standard) for metabolomic analysis. The mixture was then chilled on ice for an hour, briefly vortexed for 10 s, and centrifuged at 10,000 g for 15 min. Subsequently, 600 μ L of supernatant was transferred to a GC vial and vacuum-dried for approximately 6 h.

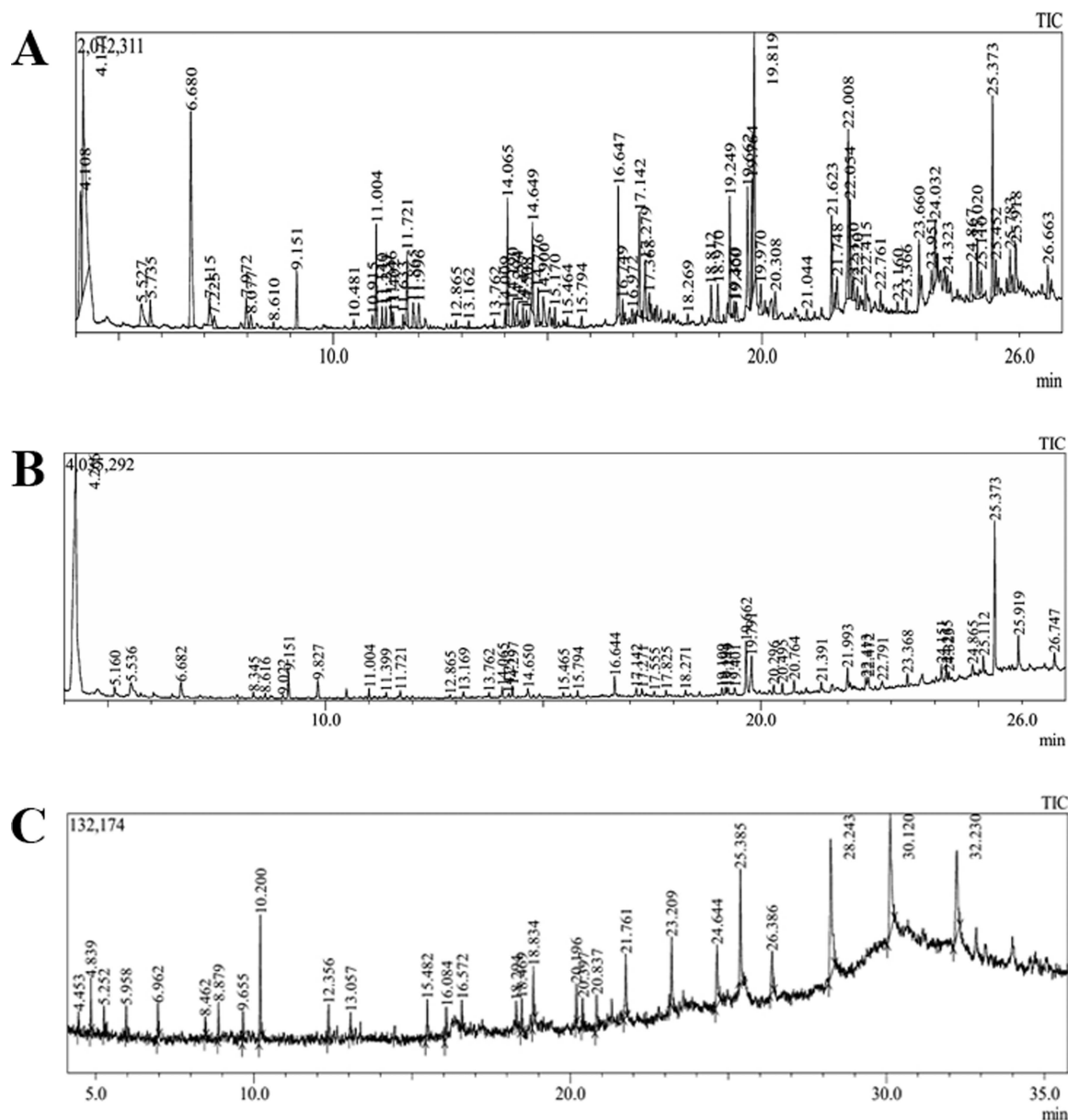


FIGURE 1 | Representative total ion current (TIC) chromatograms of *Spodoptera mauritia* haemolymph obtained from GC-MS. A- the haemolymph control sample, B- the haemolymph of the Vehicle control, and C- the haemolymph of the treated group.

The resultant extract was used for derivatization prior to GC-MS analysis. The GC vial was supplemented with 90 μ L of freshly prepared methoxylamine hydrochloride (15 mg/mL in pyridine), and the sample was incubated overnight at room temperature. Following this, 90 μ L of BSTFA containing 1% TMCS was added to the mixture for 1 h of trimethylsilylation. Finally, 120 μ L of hexane was added to terminate the derivatization reaction. The derivatised sample was vortexed, and the hexane phase was collected and transferred to a new GC vial. The sample was then diluted to a final volume of 500 μ L before GC-MS analysis.

The derivatized sample (1 μ L) was loaded onto a Shimadzu Nexis GC-2030 GC-MS system equipped with an autosampler AOC-30/20i and analysed in splitless mode utilizing an SH-I-5Sil MS capillary column (30 m in length, 0.25 mm internal diameter, and 0.25 μ m thickness). The GC-MS analysis parameters were set according to a previous study (Zhou et al. 2020). Helium served as the carrier gas at a constant flow

rate of 1.20 mL/min. The instrument was initially held at 50°C for 1 min, then ramped to 100°C at a rate of 10°C/min for 1 min, further ramped to 200°C at a rate of 10°C/min for 1 min, and finally to 280°C at a rate of 10°C/min for 1 min. The injector, transfer line, and ion source temperatures were maintained at 250°C, 280°C, and 230°C, respectively. Mass spectra were acquired using electron ionization mode (70 eV) in full scan mode (m/z 50–650), with a solvent delay set at 5 min.

2.4 | Data Pre-Processing and Multivariate Pattern Recognition Analysis

The original GC-MS data, obtained with GCMS Solutions Software, were automatically processed with GCMS Postrun analysis software. Metabolite identification was then performed by comparing results with the WILEY 8 and NIST20 libraries using mass-similarity matches and retention times (RT).

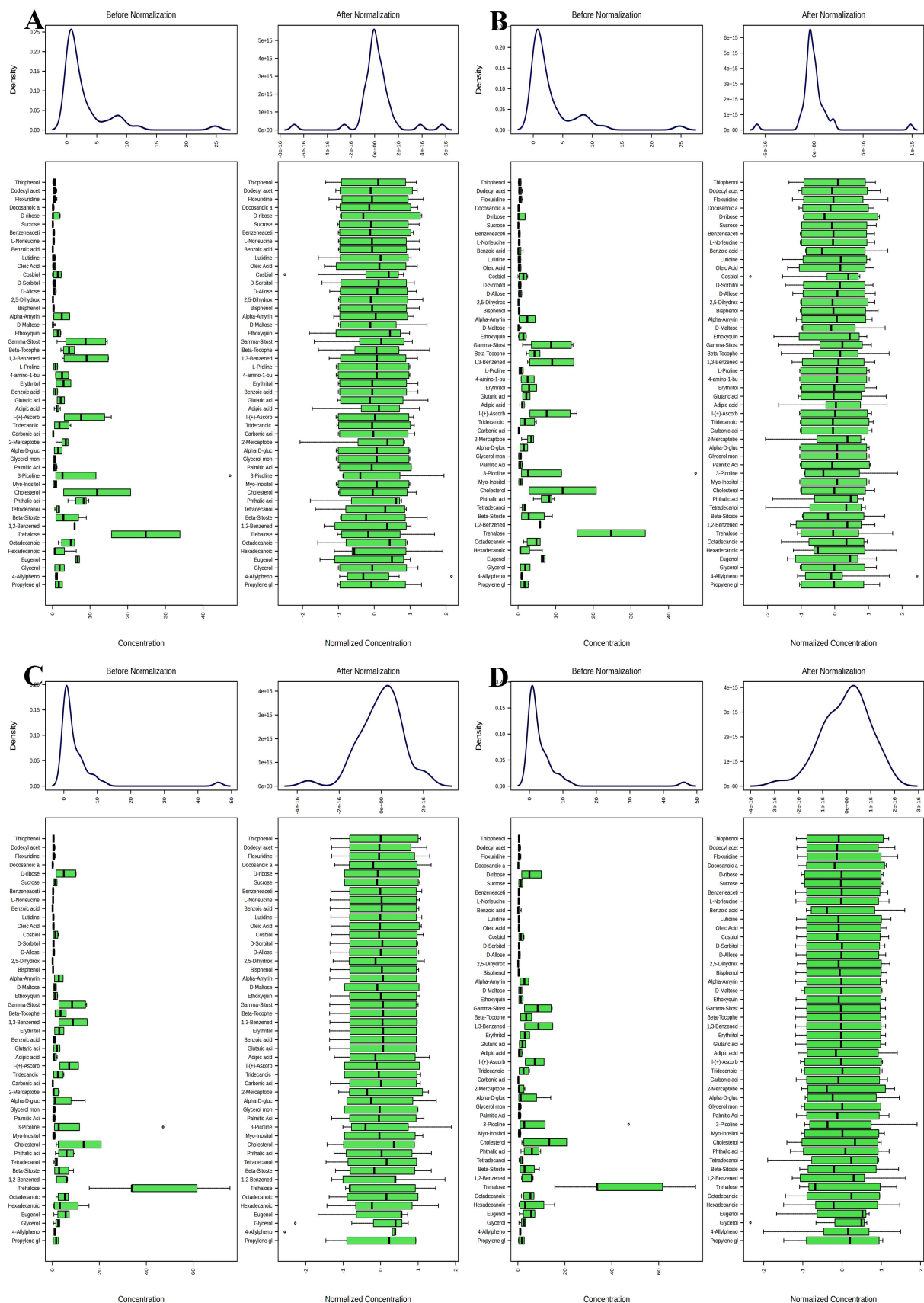


FIGURE 2 | (A) Normalised data of control and ED₂₅ group; (B) Normalised data of V. control and ED₂₅ group; Normalised data of control and ED₅₀ group; Normalised data of V. control and ED₅₀ group.

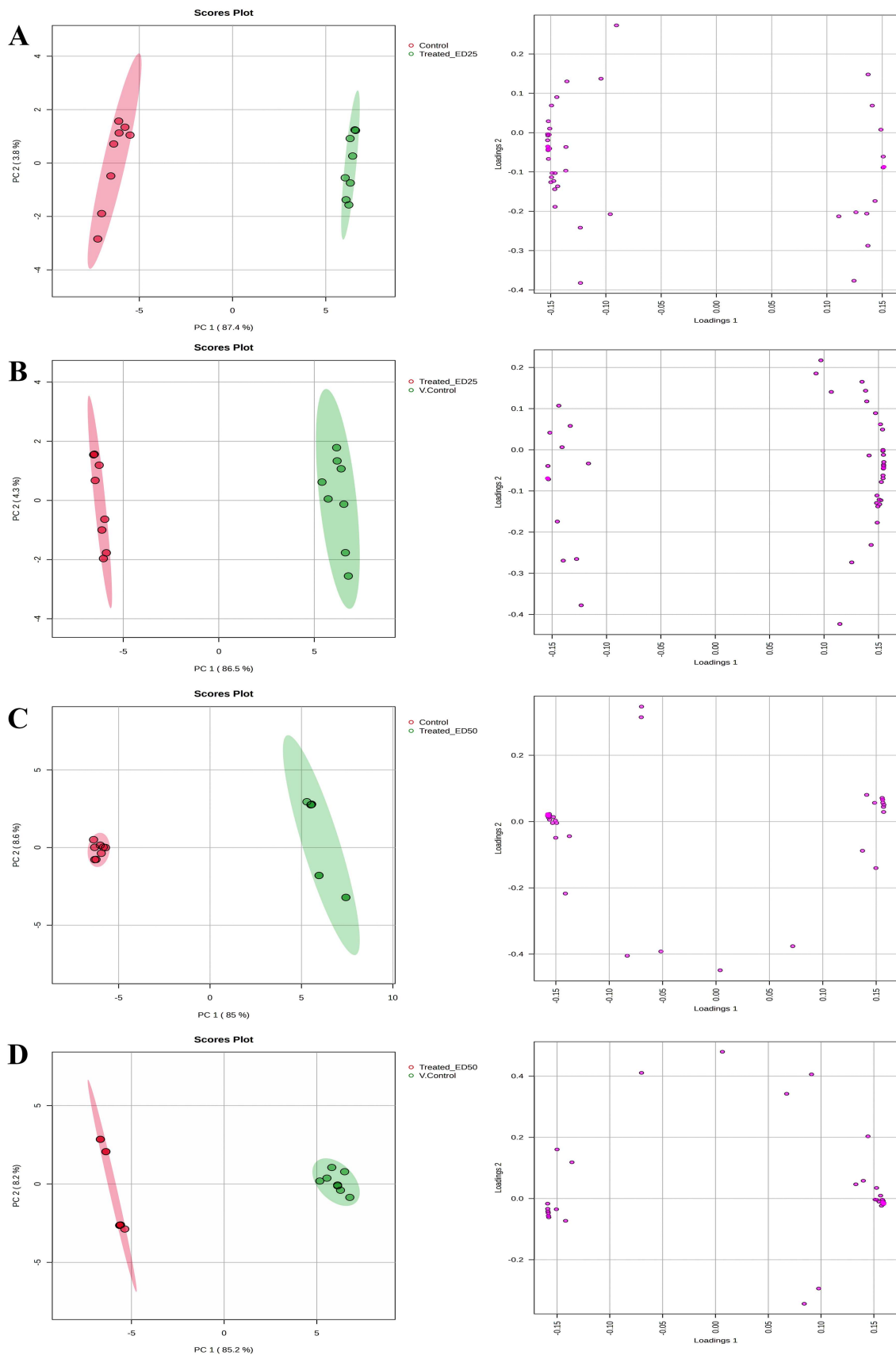


FIGURE 3 | PCA Score plot for separating haemolymph control sample from flufenoxuron treated haemolymph sample and corresponding Loading plot of the flufenoxuron treated group and the control group representing the key metabolites contributing to the separation of groups observed in the PCA score plot. (A) control/ED₂₅; (B) V. control/ED₂₅; (C) Control/ED₅₀; (D) V. control/ED₅₀.

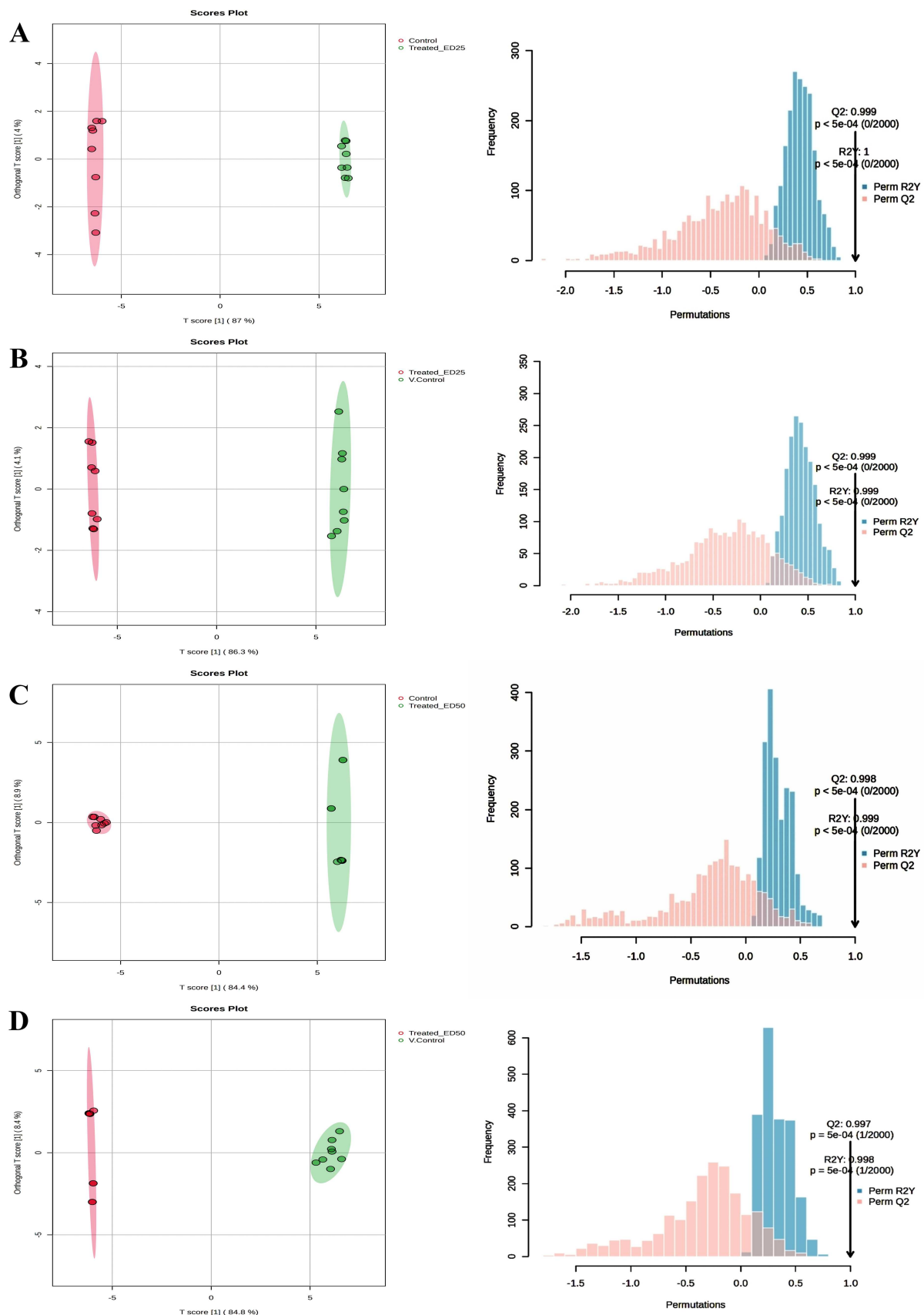


FIGURE 4 | OPLS-DA score plots of control haemolymph sample from flufenoxuron treated haemolymph sample and corresponding Permutation test plot (permutation numbers-2000) showing the statistical significance and a p-value. (A) control/ED₂₅; (B) V. control/ED₂₅; (C) Control/ED₅₀; (D) V. control/ED₅₀.

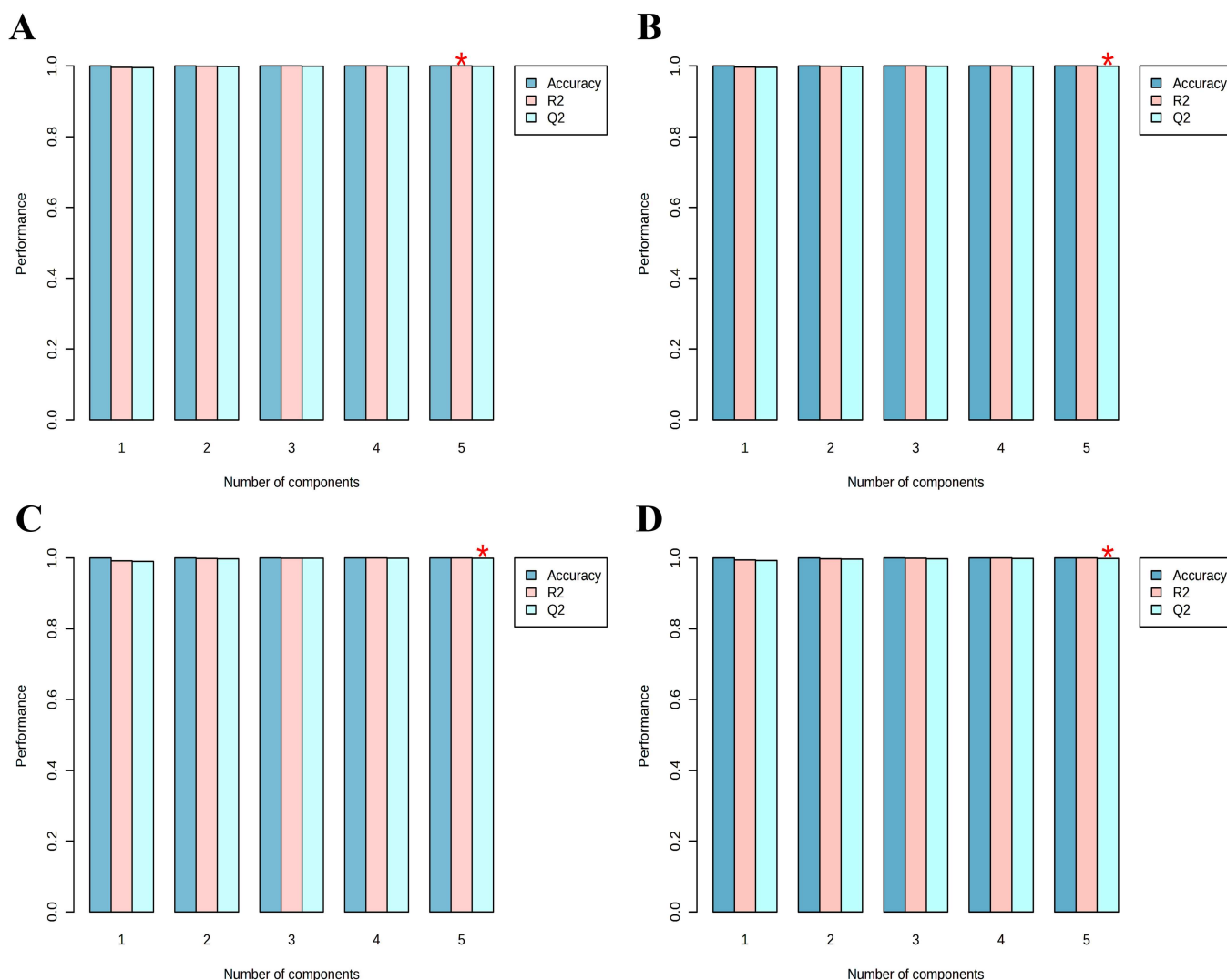


FIGURE 5 | Bar graphs with varying numbers of components displaying the three performance measures (prediction accuracy, R^2 , and Q^2). The best values of the currently chosen measures (Q^2) in cross-validation are indicated by the red “*”. (A) control/ED₂₅; (B) V. control/ED₂₅; (C) Control/ED₅₀; (D) V. control/ED₅₀.

Multivariate analysis of haemolymph samples of *S. mauritia* was performed using MetaboAnalyst 6.0 software (Xia and Wishart 2011). The total area of each sample was first adjusted using the data matrix's mode (Rt/(m/z) and intensities. Total data normalization was conducted to mitigate systematic biases within the dataset. Fold changes in metabolite abundance were calculated by comparing mean concentrations between the control and treated groups, and significance was determined using Student's *t*-test to identify differentially abundant metabolites.

The differential metabolites contributing to sample-group separation were identified using PCA and supervised partial least squares discriminant analysis (PLS-DA). The features of the metabolite expression patterns were assessed to determine differences in metabolite abundance between flufenoxuron-treated and control samples. This was done using Variable Importance for the Projection (VIP) analysis of the OPLS-DA model, which identifies differential metabolites with VIP scores greater than 1.5. The OPLS-DA method was implemented using the R ropls package, following the approach described by Thevenot (2016). The OPLS-DA model was also validated using 2000 permutations and a fivefold internal cross-validation, with R^2 and Q^2

values to assess its quality. Metabolite correlation analysis utilized Pearson's correlation coefficient to examine the linear association between metabolite richness in haemolymph samples of control and treated groups.

2.5 | Pathway Analysis

Using MetaboAnalyst 6.0, the metabolic pathways affected by flufenoxuron treatment were analysed. For the enrichment analysis, the Quantitative Enrichment Analysis (QEA) was employed with the aid of the global test package, using a generalized linear model to estimate the *Q*-statistic for each metabolic set (Goeman et al. 2004). For the pathway topology analysis, the relative centrality method was utilized. Pathway impact values from pathway topology analysis or P-values from pathway enrichment analysis were used to identify the significantly affected pathways. The study's criteria were set at impact values greater than 0.1, and negative log(P) values greater than 2.0 (Wang et al. 2012, 2015; Zhou et al. 2020), to select significant pathways affected by flufenoxuron. To examine the effects

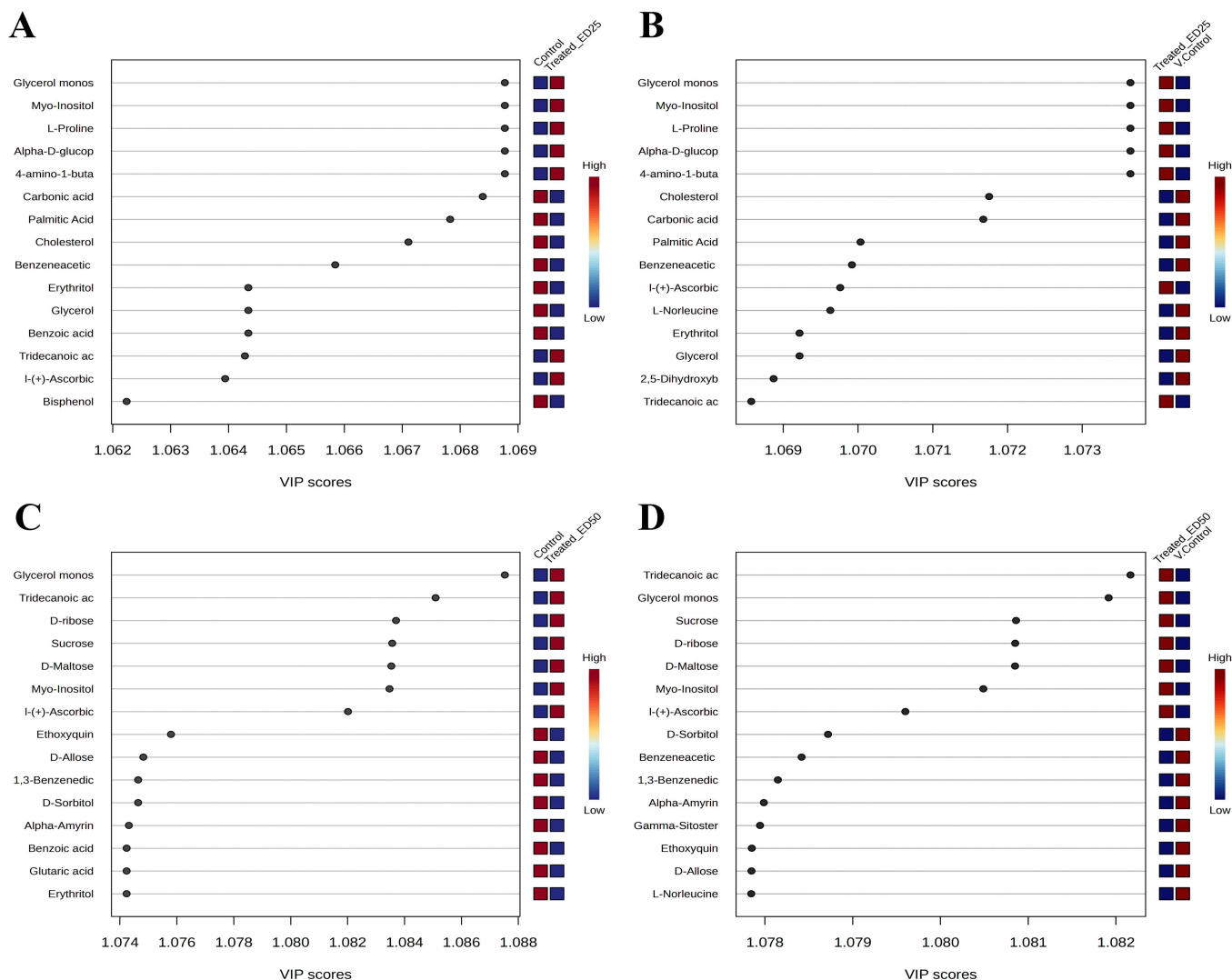


FIGURE 6 | The top 20 compounds ranked by VIP scores for control versus flufenoxuron-treated groups. (A) control/ED₂₅; (B) V. control/ED₂₅; (C) Control/ED₅₀; (D) V. control/ED₅₀.

of flufenoxuron on *S. mauritia* physiology and metabolic network construction, data were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.kegg.jp/>) for *Bombyx mori*.

3 | Results

3.1 | Metabolic Profile of *S. mauritia* After Exposure to the ED₅₀ and ED₂₅ Doses of Flufenoxuron

The current investigation comprehensively examines metabolic changes in the haemolymph of male *S. mauritia* following exposure to the chitin synthesis inhibitor flufenoxuron at doses of 0.00068 and 0.00015 μg . The findings of this study are significant as they shed light on the impact of flufenoxuron on the haemolymph metabolome of *S. mauritia*. To monitor post-treatment alterations in metabolic levels, haemolymph was collected 144 h after flufenoxuron administration, pooled, and divided into two groups: flufenoxuron-treated and control. GC-MS was used to individually analyse 32 samples into two groups (8 per control and vehicle groups, and 8 per flufenoxuron-

treated groups). A significant detrimental effect was observed in male *S. mauritia* larvae treated with flufenoxuron compared to controls.

The representative GC-MS Total Ion Chromatograms (TICs) of *S. mauritia* haemolymph samples are depicted in Figure 1. Significant fluctuations were observed in the shape and quantity of peaks between the control and flufenoxuron-treated groups. The data obtained from the TICs, the control, and the flufenoxuron-exposed groups were normalized before multivariate analysis (Figure 2). Unsupervised Principal Component Analysis (PCA) was performed to assess the quality of the GC-MS data. This analysis revealed significant clustering of all treated and control samples. Notably, no outliers were detected among the samples. The PCA plots illustrate a clear distinction between the metabolic profiles of the control group and those of the group exposed to the flufenoxuron. The metabolic profiles of the haemolymph samples exposed to flufenoxuron showed substantial changes compared to the control group (Figures 3A–D). The PCA models obtained from Control-ED₂₅, V. control-ED₂₅, V. control-ED₅₀, and control-ED₅₀ revealed perfect separation between groups and indicated significant differences (Figure 3A,C). There were also differences in

metabolomics among the four groups. Furthermore, the corresponding loading plots for each group highlighted the key metabolites that distinguished them. These plots show that the variance was significantly more pronounced in the flufenoxuron-exposed group (Figure 3), indicating that the flufenoxuron treatment had a notable impact on the metabolic profiles.

Orthogonal partial least squares discriminant analysis (OPLS-DA) was employed to enhance cluster visibility, and using the model, the metabolites responsible for the clear separation between the flufenoxuron-exposed and control groups were identified (Figure 4A–D). In these OPLS-DA models, the control and treated groups showed distinct separation on the score plot. The OPLS-DA score plot revealed significant differences in metabolic characteristics between the haemolymph control and flufenoxuron-treated groups. The 2000-permutation test and 5-fold internal cross-validation were further conducted to assess the statistical and predictive significance of these models. The results from the permutation test confirmed that there was no overfitting in the OPLS-DA model (Figure 4E–H). The resulting model's predictive accuracy was for control/ED₂₅-R²X = 0.87, R²Y = 0.996, and Q² = 0.996, V. control/ED₂₅-R²X = 0.863, R²Y = 0.997, and Q² = 0.996, control/ED₅₀-R²X = 0.844,

R²Y = 0.992, and Q² = 0.991, and V. control/ED₅₀-R²X = 0.848, R²Y = 0.994, and Q² = 0.993 (Figure 5). These results indicate that the model was reliable and reproducible.

3.2 | Altered Haemolymph Metabolites Profile of *S. mauritia* on Flufenoxuron Treatment

Metabolites with differential abundance were identified using PCA and OPLS-DA model analyses (Figure 6, Table S1); a total of 45 metabolites were identified. Volcano plots illustrated the differentially expressed metabolites in Control-ED₂₅, V. control-ED₂₅, Control-ED₅₀, and V. control-ED₅₀ (Figure 7). Sixteen metabolites were significantly down-regulated and 26 metabolites were significantly up-regulated in ED₂₅ compared to the control groups (Figure 7A,B). However, compared with the control groups, 31 metabolites were significantly up-regulated and 11 were markedly down-regulated in the ED₅₀ group (Figure 7C,D). The details of differentially abundant metabolites from treated and control groups are listed in Table 1. The hierarchical clustering analysis revealed significant differences in heatmap distributions among the control-ED₂₅, V. control-ED₂₅, control-ED₅₀, and V. control-ED₅₀ groups, indicating that

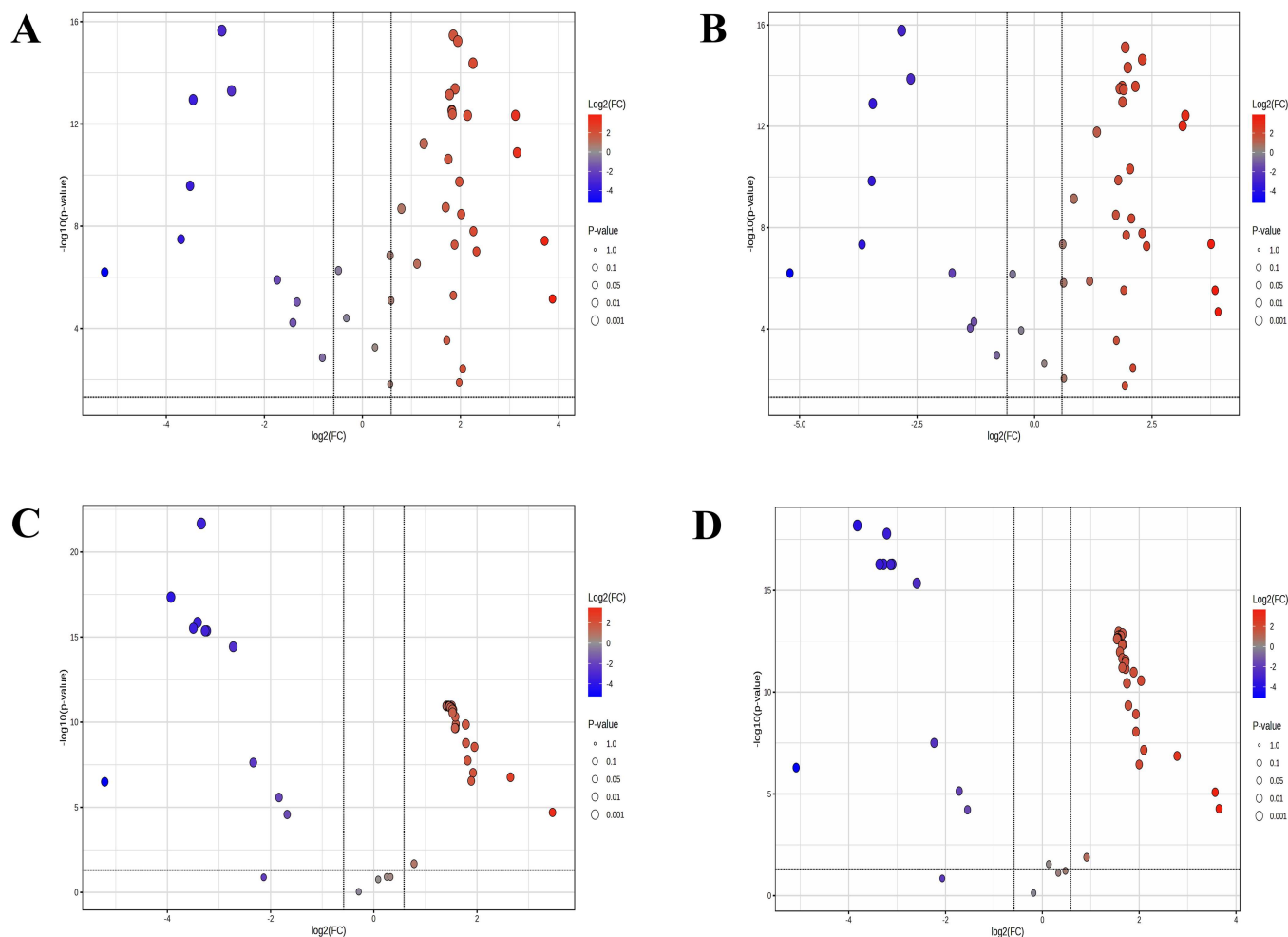


FIGURE 7 | Volcano plot of metabolites in the haemolymph of flufenoxuron treated and control *Spodoptera mauritia*. Each point represents a metabolite, and the greater the scattered point, the greater the value of fold change. (A) control/ED₂₅; (B) V. control/ED₂₅; (C) Control/ED₅₀; (D) V. control/ED₅₀.

TABLE 1 | List of differentially abundant metabolites after flufenoxuron treatment.

	Fold change (vs Control)				Fold change (vs V. Control)			
	ED ₂₅		ED ₅₀		ED ₂₅		ED ₅₀	
1,3-Benzenedicarboxylic acid	1.699(<i>p</i> < 0.0001)	↑	1.4303(<i>p</i> < 0.0001)	↑	1.7305(<i>p</i> < 0.0001)	↑	1.5652(<i>p</i> < 0.0001)	↑
2,5-Dihydroxybenzoic acid	2.144(<i>p</i> < 0.0001)	↑	1.7817(<i>p</i> < 0.0001)	↑	2.147(<i>p</i> < 0.0001)	↑	1.8869(<i>p</i> < 0.0001)	↑
2-Mercaptobenzothiazole	−1.4173 (<i>p</i> < 0.0001)	↓	2.6483(<i>p</i> < 0.0001)	↑	−1.3681 (<i>p</i> < 0.0001)	↓	2.7839(<i>p</i> < 0.0001)	↑
3-Picoline	3.8759(<i>p</i> < 0.0001)	↑	3.4622(<i>p</i> < 0.0001)	↑	3.8421(<i>p</i> < 0.0001)	↑	3.57(<i>p</i> < 0.0001)	↑
4-amino-1-butanol	−2.8668 (<i>p</i> < 0.0001)		<i>Nil</i>		−2.8298 (<i>p</i> < 0.0001)	↓	<i>nil</i>	
Adipic acid	<i>Nil</i>		1.8177(<i>p</i> < 0.0001)	↑	0.6237(0.009)	↑	1.934(<i>p</i> < 0.0001)	↑
Alpha-Amyrin	3.1201(<i>p</i> < 0.0001)	↑	1.4112(<i>p</i> < 0.0001)	↑	3.1525(<i>p</i> < 0.0001)	↑	1.5506(<i>p</i> < 0.0001)	↑
Alpha-D-glucopyranose	−2.8668 (<i>p</i> < 0.0001)	↓	−5.2139 (<i>p</i> < 0.0001)	↓	−2.8298 (<i>p</i> < 0.0001)	↓	−5.0849 (<i>p</i> < 0.0001)	↓
Benzeneacetic acid	1.8897(<i>p</i> < 0.0001)	↑	1.525(<i>p</i> < 0.0001)	↑	1.8637(<i>p</i> < 0.0001)	↑	1.593(<i>p</i> < 0.0001)	↑
Benzoic acid	1.8269(<i>p</i> < 0.0001)	↑	1.4076(<i>p</i> < 0.0001)	↑	3.9056(<i>p</i> < 0.0001)	↑	3.6502(<i>p</i> < 0.0001)	↑
Beta-Sitosterol	2.3265(<i>p</i> < 0.0001)	↑	1.9233(<i>p</i> < 0.0001)	↑	2.385(<i>p</i> < 0.0001)	↑	2.0964(<i>p</i> < 0.0001)	↑
Beta-Tocopherol	<i>Nil</i>		1.4076(<i>p</i> < 0.0001)	↑	0.61757(<i>p</i> < 0.0001)	↑	1.5437(<i>p</i> < 0.0001)	↑
Bisphenol	1.8233(<i>p</i> < 0.0001)	↑	1.4521(<i>p</i> < 0.0001)	↑	1.8728(<i>p</i> < 0.0001)	↑	1.6028(<i>p</i> < 0.0001)	↑
Carbonic acid	1.853(<i>p</i> < 0.0001)	↑	1.4899(<i>p</i> < 0.0001)	↑	1.9315(<i>p</i> < 0.0001)	↑	1.6661(<i>p</i> < 0.0001)	↑
Cholesterol	2.2555(<i>p</i> < 0.0001)	↑	1.8885(<i>p</i> < 0.0001)	↑	2.2939(<i>p</i> < 0.0001)	↑	2(<i>p</i> < 0.0001)	↑
Cosbiol	2.0457(<i>p</i> < 0.0001)	↑	1.5793(<i>p</i> < 0.0001)	↑	2.0929(0.003)	↑	1.7216(<i>p</i> < 0.0001)	↑
D-Allose	1.9761(<i>p</i> < 0.0001)	↑	1.5104(<i>p</i> < 0.0001)	↑	2.033(<i>p</i> < 0.0001)	↑	1.657(<i>p</i> < 0.0001)	↑
D-Maltose	−3.6995 (<i>p</i> < 0.0001)	↓	−3.2362 (<i>p</i> < 0.0001)	↓	−3.6735 (<i>p</i> < 0.0001)	↓	−3.1002 (<i>p</i> < 0.0001)	↓
Docosanoic acid	3.1545(<i>p</i> < 0.0001)	↑	1.9515(<i>p</i> < 0.0001)	↑	3.209(<i>p</i> < 0.0001)	↑	2.0398(<i>p</i> < 0.0001)	↑
Dodecyl acetate	1.7512(<i>p</i> < 0.0001)	↑	1.5719(<i>p</i> < 0.0001)	↑	1.7813(<i>p</i> < 0.0001)	↑	1.7491(<i>p</i> < 0.0001)	↑
D-ribose	−5.2546 (<i>p</i> < 0.0001)	↓	−3.4968 (<i>p</i> < 0.0001)	↓	−5.2046 (<i>p</i> < 0.0001)	↓	−3.3609 (<i>p</i> < 0.0001)	↓
D-Sorbitol	2.0148(<i>p</i> < 0.0001)	↑	1.4441(<i>p</i> < 0.0001)	↑	2.0609(<i>p</i> < 0.0001)	↑	1.5739(<i>p</i> < 0.0001)	↑
Erythritol	1.777(<i>p</i> < 0.0001)	↑	1.4076(<i>p</i> < 0.0001)	↑	1.8141(<i>p</i> < 0.0001)	↑	1.5437(<i>p</i> < 0.0001)	↑
Ethoxyquin	1.7201(0.0003)	↑	1.4952(<i>p</i> < 0.0001)	↑	1.7418(0.0003)	↑	1.6477(<i>p</i> < 0.0001)	↑
Eugenol	<i>Nil</i>		0.78124(0.02)	↑	<i>Nil</i>		0.91441(0.013)	↑
Floxuridine	1.1145(<i>p</i> < 0.0001)	↑	1.5832(<i>p</i> < 0.0001)	↑	1.1719(<i>p</i> < 0.0001)	↑	1.7774(<i>p</i> < 0.0001)	↑
Gamma-Sitosterol	1.8546(<i>p</i> < 0.0001)	↑	1.4233(<i>p</i> < 0.0001)	↑	1.9007(<i>p</i> < 0.0001)	↑	1.5614(<i>p</i> < 0.0001)	↑
Glutaric acid	0.79524(<i>p</i> < 0.0001)	↑	1.4076(<i>p</i> < 0.0001)	↑	0.83677(<i>p</i> < 0.0001)	↑	1.5437(<i>p</i> < 0.0001)	↑
Glycerol	1.777(<i>p</i> < 0.0001)	↑	<i>Nil</i>		1.8141(<i>p</i> < 0.0001)		<i>nil</i>	
Glycerol monostearate	−2.8668 (<i>p</i> < 0.0001)	↓	−3.343 (<i>p</i> < 0.0001)	↓	−2.8298 (<i>p</i> < 0.0001)	↓	−3.213 (<i>p</i> < 0.0001)	↓
Hexadecanoic acid	1.974(0.013)	↑	<i>Nil</i>		1.9231(0.017)		<i>nil</i>	
l-(+)-Ascorbic acid	−2.6703 (<i>p</i> < 0.0001)	↓	−2.7249 (<i>p</i> < 0.0001)	↓	−2.635 (<i>p</i> < 0.0001)	↓	−2.5934 (<i>p</i> < 0.0001)	↓
L-Norleucine	1.8349(<i>p</i> < 0.0001)	↑	1.4632(<i>p</i> < 0.0001)	↑	1.8935(<i>p</i> < 0.0001)	↑	1.6226(<i>p</i> < 0.0001)	↑
L-Proline	−2.8668 (<i>p</i> < 0.0001)	↓	<i>Nil</i>		−2.8298 (<i>p</i> < 0.0001)	↓	<i>nil</i>	
Lutidine	3.7156(<i>p</i> < 0.0001)	↑	1.5222(<i>p</i> < 0.0001)	↑	3.7569(<i>p</i> < 0.0001)	↑	1.7193(<i>p</i> < 0.0001)	↑
Myo-Inositol	−2.8668 (<i>p</i> < 0.0001)	↓	−3.4154 (<i>p</i> < 0.0001)	↓	−2.8298 (<i>p</i> < 0.0001)	↓	−3.2804 (<i>p</i> < 0.0001)	↓
Octadecanoic acid	−1.7374 (<i>p</i> < 0.0001)	↓	−2.3363 (<i>p</i> < 0.0001)	↓	−1.7538 (<i>p</i> < 0.0001)	↓	−2.2364 (<i>p</i> < 0.0001)	↓
Oleic acid	2.2636(<i>p</i> < 0.0001)	↑	1.5376(<i>p</i> < 0.0001)	↑	2.2892(<i>p</i> < 0.0001)	↑	1.6524(<i>p</i> < 0.0001)	↑
Palmitic acid	1.9446(<i>p</i> < 0.0001)	↑	1.5888(<i>p</i> < 0.0001)	↑	1.9841(<i>p</i> < 0.0001)	↑	1.7208(<i>p</i> < 0.0001)	↑
Phthalic acid	−0.81614(0.0014)	↓	<i>Nil</i>		−0.80149(0.001)	↓	<i>nil</i>	
Propylene glycol	1.2516(<i>p</i> < 0.0001)	↑	1.7853(<i>p</i> < 0.0001)	↑	1.3263(<i>p</i> < 0.0001)	↑	1.9344(<i>p</i> < 0.0001)	↑
Tetradecanoic acid	−1.3313 (<i>p</i> < 0.0001)	↓	−1.8371 (<i>p</i> < 0.0001)	↓	−1.2878 (<i>p</i> < 0.0001)	↓	−1.7185 (<i>p</i> < 0.0001)	↓
Thiophenol	1.8799(<i>p</i> < 0.0001)	↑	1.5018(<i>p</i> < 0.0001)	↑	1.9507(<i>p</i> < 0.0001)	↑	1.6527(<i>p</i> < 0.0001)	↑

(Continues)

TABLE 1 | (Continued)

	Fold change (vs Control)		Fold change (vs V. Control)	
	ED ₂₅	ED ₅₀	ED ₂₅	ED ₅₀
Trehalose	<i>Nil</i>	<i>-1.6789</i> ($p < 0.0001$) ↓	0.59838($p < 0.0001$) ↑	<i>-1.5454</i> ($p < 0.0001$) ↓
Tridecanoic acid	<i>-3.452</i> ($p < 0.0001$) ↓	<i>-3.9317</i> ($p < 0.0001$) ↓	<i>-3.4423</i> ($p < 0.0001$) ↑	<i>-3.8223</i> ($p < 0.0001$) ↓

Note: List of significantly regulated metabolites with concentration changes after flufenoxuron treatment. (Numbers in bold and italic mean the significantly down-regulated fold change with p -value; others are up-regulated).

the two effective doses of flufenoxuron exerted a significant influence on metabolism in *S. mauritia*. Heat map analysis further confirmed differential clustering among treated and control groups, as depicted in Figure 8.

3.3 | Metabolic Pathway Analysis

To identify the key functional metabolic pathways affected by flufenoxuron treatment in haemolymph, a metabolic pathway analysis was conducted using the metabolic concentration data obtained from GC-MS analysis. Figure 9 presents the pathway enrichment results. The enrichment ratio is calculated as the number of hits in a specific metabolic pathway divided by the predicted number of hits. A higher enrichment ratio indicates more significant pathway enrichment. Another metric, the p -value, ranges from 0 to 1, with values closer to 0 indicating greater enrichment significance. Figure 9 highlights the top 25 pathways activated by flufenoxuron treatment. Notably, metabolic pathways such as ascorbate and aldarate metabolism, inositol phosphate metabolism, pentose phosphate pathway, starch and sucrose metabolism, tyrosine metabolism, phenylalanine metabolism, galactose metabolism, steroid biosynthesis, steroid hormone biosynthesis, fructose and mannose metabolism, glycolysis/gluconeogenesis, and amino sugar and nucleotide sugar metabolism are strongly associated with the effects of the chitin synthesis inhibitor. As shown in Figure 9, ascorbate and aldarate metabolism, along with inositol phosphate metabolism, are significantly enriched, with a relatively higher number of metabolites involved.

Based on negative $\log(P)$ and impact values obtained from the pathway enrichment and topological analyses, four differentially enriched metabolic pathways had impact values greater than 0.1, indicating their significance. In accordance with the above results, the most intensively modified pathways between control and ED₂₅ were Inositol phosphate metabolism, Arginine and proline metabolism, Glycolysis or Gluconeogenesis, Amino sugar and nucleotide sugar metabolism, and Insect hormone biosynthesis. Furthermore, the most significant changes were observed in Inositol phosphate metabolism, Ascorbate and aldarate metabolism, and the Pentose phosphate pathway in the control-ED₅₀ group (Figure 10A–D).

To identify the metabolic pathways directly responsible for chitin synthesis inhibition and reduced reproductive capacity in *S. mauritia*, the changes in metabolic pathways were analyzed using KEGG pathway analysis of differentially abundant metabolites. A functional model was constructed using the reference map from the KEGG database (Figure 11), and summarized significant metabolic pathways to reveal the effect of flufenoxuron.

4 | Discussion

4.1 | Impact of Flufenoxuron on Metabolomics

Several studies have shown that insect haemolymph is a critical fluid that reflects physiological and pathological changes, as it plays a vital role in immune defence, physiological regulation, and nutrient transport (Feng et al. 2014; Wu et al. 2022). Therefore, to validate the metabolomics model, GC/MS analysis was performed on haemolymph samples to determine that the flufenoxuron exposure affected the metabolic profile of male *S. mauritia*. The results demonstrated that flufenoxuron exposure significantly altered metabolic pathways related to energy metabolism. These findings emphasize the potential implications of understanding the broad metabolic disruptions caused by flufenoxuron, providing valuable insights into its impact on insect physiology at the molecular level.

CSIs are a broad class of chemical substances with varying effects on the growth and reproduction of chitin-producing organisms (Merzendorfer 2013). However, the mechanisms of action for CSIs are not well understood, but the indirect effects of benzoylureas on the activities of enzymes involved in chitin breakdown and cuticular sclerotization, the transport of uridine diphosphate N- acetylglucosamine across membranes, and hormonal regulation of transcriptional activities were investigated (Aravindarajan et al. 2017). Pyrimidine-nucleoside peptides, for example, interfere with the catalytic site of chitin synthase by affecting nucleotide binding. At the same time, compounds such as benzoylureas, oxazolines, and thiazolidinones are considered to block a post-catalytic stage of chitin production, potentially via a similar mechanism (Asahara et al. 2008; Van Leeuwen et al. 2012). Moreover, these inhibitors may induce physiological stress, triggering compensatory mechanisms to mitigate the stress effects. Sex-specific variations in metabolism, essential for egg and sperm production, are linked to physiological differences in organs responsible for nutrient acquisition (Shingleton and Veal 2023).

Genomic studies on *Tribolium castaneum* have shown that diflubenzuron significantly alters global gene expression, particularly impacting genes encoding cuticular proteins (Merzendorfer et al. 2012). Moreover, mass spectrometry analysis revealed the upregulation of UDP-N-acetylglucosamine pyrophosphorylase (UAP) and glutathione synthase, adding complexity to the findings and prompting researchers to explore alternative mechanisms of action (Merzendorfer et al. 2012).

4.2 | Altered Carbohydrate Metabolic Pathway in Response to Flufenoxuron Toxicity

The physiological balance of living organisms depends on carbohydrate metabolism. In lepidopteran insects, dietary

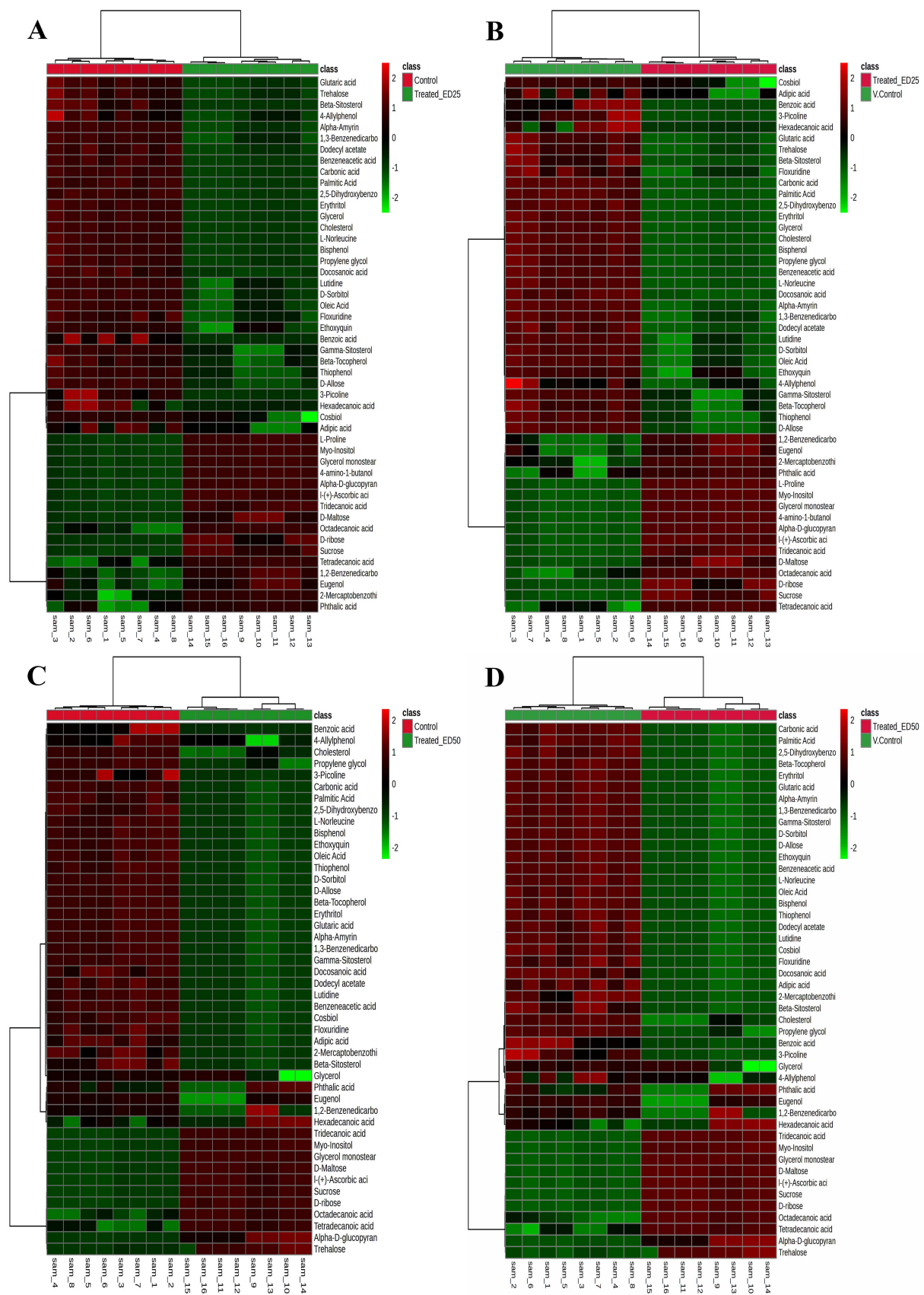


FIGURE 8 | Heat map of differentially abundant metabolites in haemolymph following flufenoxuron treatment. Each column represents one sample, and each row represents one DE metabolite. The colour red shows the relative level of the up-regulated metabolites, and the down-regulated metabolites are in green. (A) control/ED₂₅; (B) V. control/ED₂₅; (C) Control/ED₅₀; (D) V. control/ED₅₀.

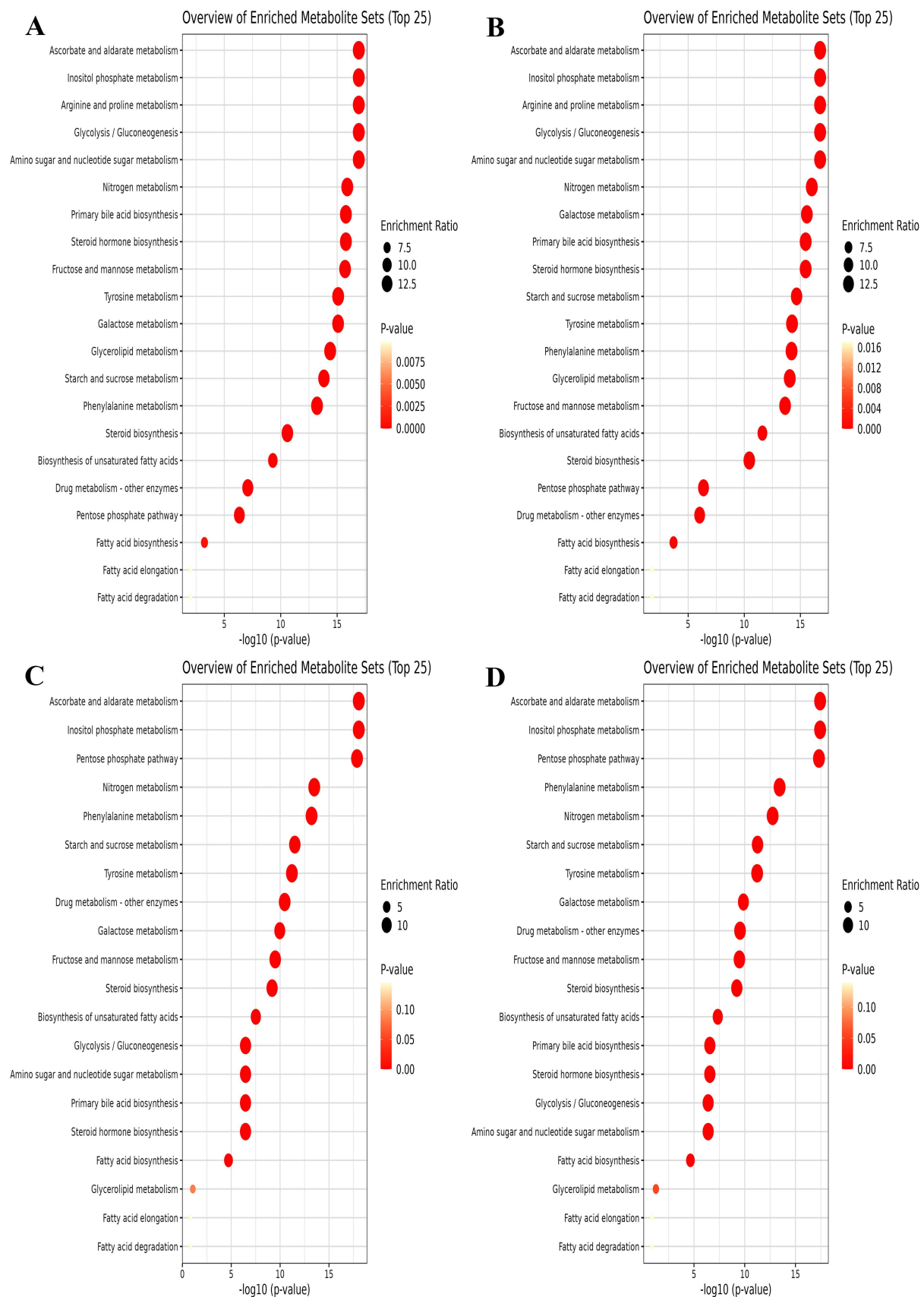


FIGURE 9 | Metabolome map of top 25 KEGG-enriched pathways based on metabolites in the haemolymph of *Spodoptera mauritia* after treatment with flufenoxuron. (A) control/ED₂₅; (B) V. control/ED₂₅; (C) Control/ED₅₀; (D) V. control/ED₅₀.

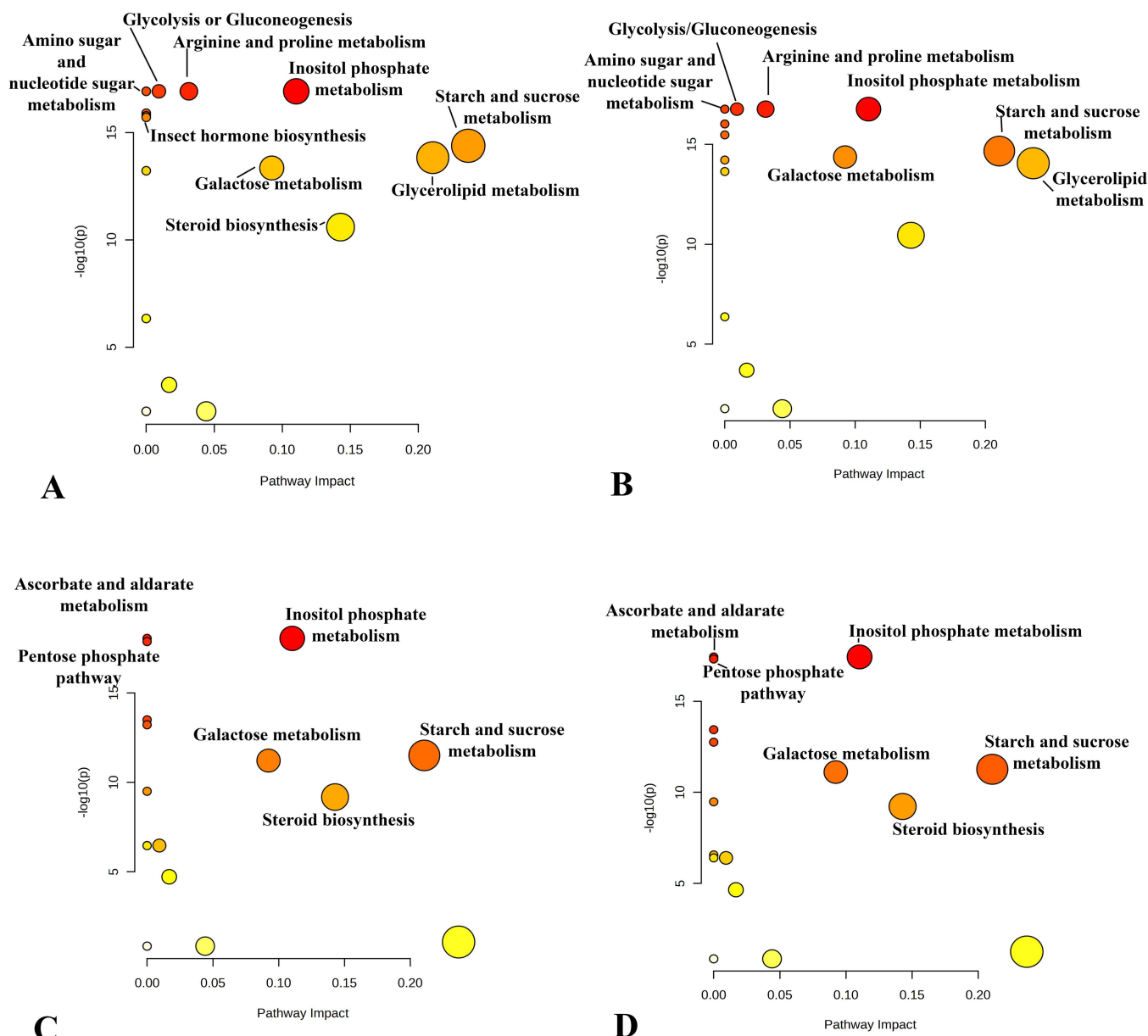


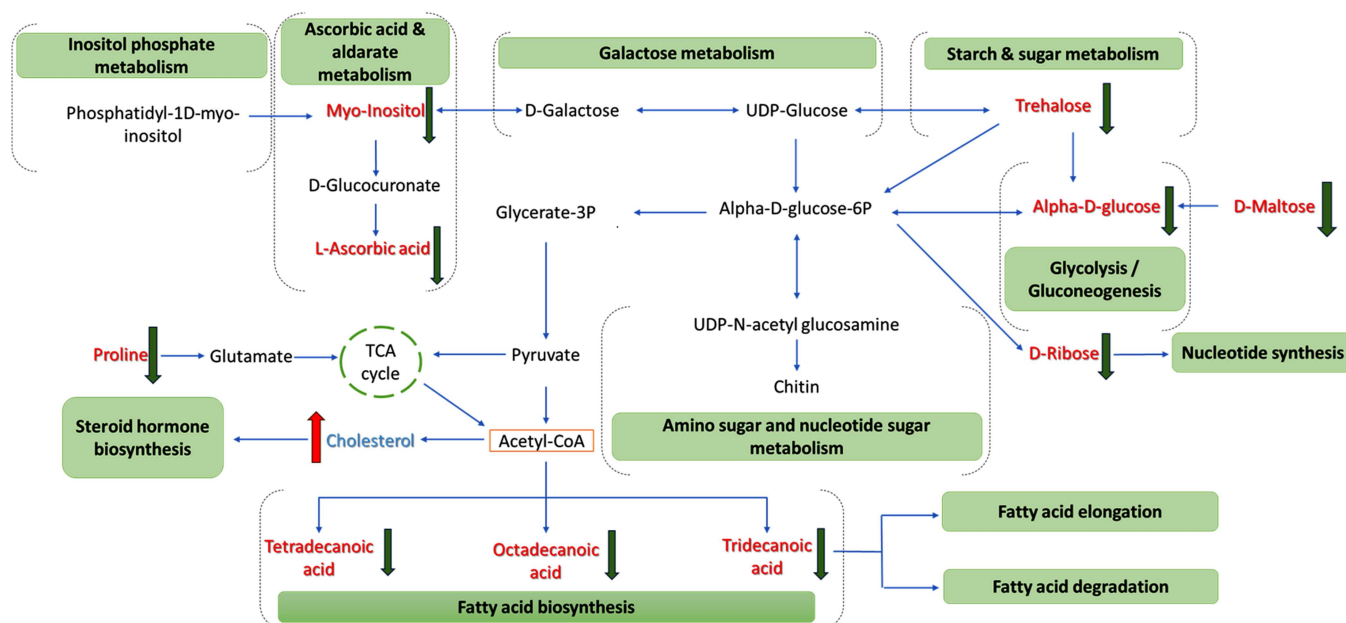
FIGURE 10 | Dot plot representation of pathway topology analysis representing significant metabolic pathways characterized in *Spodoptera mauritia* of flufenoxuron treated and control groups. Significantly changed pathways based on enrichment and topology analysis are shown. The x-axis represents pathway enrichment, whereas the y-axis represents pathway impact. Large sizes and dark colours represent major pathway enrichment and high pathway impact values, respectively. (A) control/ED₂₅; (B) V. control/ED₂₅; (C) Control/ED₅₀; (D) V. control/ED₅₀.

sugars are converted to trehalose, which is predominantly stored in the haemolymph (Deng et al. 2015). Trehalose is the primary energy reserve in the insect's haemolymph (Tang et al. 2008). This sugar plays a crucial role in meeting energy demands under stressful environmental conditions, such as heat, freezing, desiccation, dehydration, and oxidative stress, while also acting as a protective barrier against protein and membrane damage (Crowe et al. 1984; Elbein 2003).

Trehalase is the only known enzyme that irreversibly breaks down trehalose in insects, though alternative pathways exist in other organisms (Avonce et al. 2006). Trehalase converts trehalose into glucose, which meets the energy needs required for flight, development, and defence against severe environmental conditions (Gu et al. 2009; Shukla et al. 2015). At certain

developmental stages, trehalose can comprise approximately 20% of the total carbohydrates, leading to its designation as the “blood sugar” of insects (Saltiel and Kahn 2001; Shukla et al. 2015).

Consequently, trehalose is essential in insect development and various physiological processes (Pan et al. 2019). The concentration of trehalose in the haemolymph has been shown to influence several key functions, including chitin synthesis during moulting (Candy and Kilby 1962), cold resistance (Wyatt 1967), food selection and feeding behaviour (Thompson and Dahlman 1999), and oogenesis (Santos et al. 2012). Once converted into glucose, trehalose is involved in the synthesis of various macromolecular organic substances, including carbon metabolism (Avonce et al. 2006), lipid biosynthesis (Shi et al. 2016), and chitin biosynthesis (Shen et al. 2017). These



processes underline the importance of trehalose conversion in supporting vital physiological functions.

Trehalose plays a central role in starch and sugar metabolism, glycolysis/gluconeogenesis, and amino and nucleotide-sugar metabolism. It is also a key precursor in insect chitin synthesis, which begins with glucose and trehalose and ultimately forms the chitin polymer (Merzendorfer and Zimoch [2003](#)). Recently, Yang, Liang, et al. ([2024](#)) reported that trehalose metabolism

Simpson and Raubenheimer (1993) suggested that trehalose levels in insect haemolymph reflect the nutritional status and may influence feeding behaviour and nutrient intake. This idea correlates well with the reduced nutrient uptake observed in *S. mauritia* larvae treated with flufenoxuron (Reshma and Manogem 2026). Similarly, Xu et al. (2017) reported decreased nutrient intake in *Leptinotarsa decemlineata* after treatment with a chitin synthesis inhibitor. Thompson and Redak (2000) reported a link between trehalose levels and the effects of dietary nutrients on gluconeogenesis in *Manduca sexta*.

metabolites involved in carbohydrate metabolism. Earlier studies indicate that L-ascorbic acid serves as a cofactor for various enzymes (Smirnoff 2018) and plays key roles in several metabolic processes, including tyrosine metabolism, collagen production, steroid synthesis, detoxification responses, moulting, phagostimulation, and neuromodulation (Kramer and Seib 1982). Kramer and Seib (1982) reported that many insects require dietary ascorbic acid for normal growth and completion of their life cycles. Similarly, Vanderzant et al. (1962) reported that ascorbic acid is an essential nutrient for insect growth and development; in their studies, ascorbic acid deficiency reduced fertility. Ascorbic acid is also involved in the tanning process in insects and controls hydroxylations that convert cholesterol into ecdysone (Chippendale 1975). In *S. littoralis* ascorbic acid deficiency resulted in moulting cycle failure, and also had control over cuticle melanisation and sclerotization (Navon 1978). The downregulation of L-ascorbic acid could lead to a shortage of critical metabolomic intermediates, thereby impairing the growth and development of *S. mauritia*.

Myo-inositol is a six-carbon sugar alcohol found in both eukaryotic and many prokaryotic cells and is recognized as an essential nutrient (Combs and McClung 2017). It plays significant roles in signal transduction, endoplasmic reticulum stress, and osmoregulation. Additionally, it serves as a precursor to phosphatidylinositol (PI), a key component of phospholipid membranes (Henry et al. 2014; Chow et al. 2015; Raghu et al. 2019). Alterations in myo-inositol metabolism have been associated with reproductive defects, affecting fertilization and the early stages of embryonic development in humans (Croze and Soulage 2013; Bevilacqua et al. 2015) and are critical for balanced metabolism and development in *Drosophila melanogaster* (Rivera et al. 2021). The Inos gene, responsible for encoding myo-inositol-3-phosphate synthase (MIPS) in *D. melanogaster*, is highly expressed in the head and testes in high-throughput expression analyses (Chen et al. 2014). Similarly, Chintapalli et al. (2007) reported that MIPS synthesizes large quantities of inositol in the male reproductive organs of *D. melanogaster*. Further, Jackson et al. (2018) demonstrated that the absence of myo-inositol is linked to male sterility in *D. melanogaster*.

Myo-inositol also participates in the TCA cycle via galactose metabolism (Zhou et al. 2020), helping to compensate for energy deficits and producing intermediates. The TCA cycle generates significant amounts of ATP, which fuels various synthetic processes and provides intermediate molecules essential for the production of amino acids and fatty acids (Pallardy 2008). Therefore, the downregulation of myo-inositol in *S. mauritia* following flufenoxuron treatment is expected to indicate a shortage of energy and intermediate molecules, as the TCA cycle is a central metabolic pathway for lipids, carbohydrates, and amino acids (Dong et al. 2017).

4.3 | Influence of Flufenoxuron Toxicity on Lipid and Cholesterol Metabolic Pathways

In insects, sterols are essential nutrients that must be continuously acquired from their diet for proper growth and development (Dong et al. 2017). Few reports have shown that a lack of sterols can impair ecdysone synthesis and oviposition, while

having adequate sterols, but insufficient fatty acids can restrict insect growth (Niwa et al. 2010; Salini et al. 2015). Phosphatidylethanolamine, phosphatidylcholine, and ether lipids are particularly abundant in the lipid profile of male and female reproductive organs, highlighting the crucial role of lipids as components of the sperm membrane (Chintapalli et al. 2013). The non-protein fraction of seminal fluid comprises lipids, nucleic acids, amino acids, fatty acids, hormones (such as 20E), and other bioactive substances (Scolari et al. 2021). Metabolomic analysis of the ovary revealed high metabolic activity, with prominent pathways related to energy reserves, lipid and terpenoid biosynthesis, stem cell development, and antioxidant defence (Chintapalli et al. 2013). An imbalance in fatty acid and lipid content can lead to physiological dysfunction in male reproductive organs. The examination of the haemolymph lipid constituents of male *S. mauritia* subjected to flufenoxuron treatment revealed a notable reduction in the lipid levels (Reshma and Manogem 2026). Hamadah (2014) also reported that flufenoxuron treatment decreased the haemolymph lipid content of *S. gregaria*.

In the present study, the levels of free fatty acids, including tetradecanoic acid, tridecanoic acid, and octadecanoic acid, were downregulated following flufenoxuron treatment. In contrast, the levels of hexadecenoic acid, cholesterol, and docosanoic acid increased. These findings indicate significant alterations in the lipid metabolic pathways of male *S. mauritia*. Pesticide-induced oxidative stress can disrupt the homeostasis of various metabolites, including amino acids, nucleic acids, and fatty acids (Broadrup et al. 2019). The balance of nutritional intake, maintenance, development, and reproductive needs influences lipid availability. Toxic substances can disrupt this balance (Tanani et al. 2022). Consequently, fatty acids are considered crucial chemical indicators of stress (Filimonova et al. 2016). Saturated fatty acids maintain membrane stability and stiffness, increasing resilience to diverse stresses (Huber et al. 2025). Tetradecanoic acid (myristic acid) and octadecanoic acid (stearic acid) are saturated fatty acids that are frequently found in Lepidoptera, Hemiptera, Orthoptera, Diptera, Hymenoptera and Dictyoptera, but are especially prevalent in some species (Thompson 1973). Myristic acid acts as a pheromone component, as well as a chemoattractant to female insects to lay eggs (Klüber et al. 2024). Stearic acid has been recognized as a significant component of phospholipids (Aboshi et al. 2013). Tridecanoic acid is less common in insects and acts as a precursor for pheromones (Pinilla et al. 1999; Abad et al. 2001).

As mentioned above, insect survival is greatly influenced by lipids, which are essential parts of cells (Kaczmarek et al. 2020) and serve as a vital energy source for insects, regulated by neuroendocrine feedback loops (Downer 1985). Internal free fatty acids are also significant energy sources and building blocks for secondary metabolites, waxes, pheromones, and defence secretions (Soulages and Wells 1994). They are essential components of insect cell membranes and act as precursors for various pheromones (Arrese and Soulages 2010). In holometabolous insects, the accumulation and mobilization of lipids are crucial for the significant remodelling of body structure and biochemistry. The primary function of lipid accumulation during the larval stage is to support metamorphosis during the pupal stage and, often, to fuel the energy-intensive activities of non-feeding adult stages, such as flying and

reproduction (Huang et al. 2012). A finely tuned balance exists during feeding phases between the lipid and fatty acid content derived from food and the de novo synthesis of fatty acids from dietary carbohydrates.

Ziegler and van Antwerpen (2006) previously demonstrated the roles of fatty acids in insect reproduction. For instance, linoleic acid, an octadecanoic acid, is likely vital for insect reproduction due to its substantial presence in oocyte dry mass and its various functions, including providing energy to the developing embryo (Huang et al. 2012). Lipidomics studies indicate that the composition of triglycerides and other membrane lipids is altered by impaired lipolysis in females, negatively impacting female fecundity (Yang, Xu, et al. 2024). Additionally, phosphatidylinositol lipids are crucial for spermatocyte cytokinesis, cyst polarity, and axoneme development (Ben-David et al. 2015).

4.4 | Influence of Flufenoxuron Toxicity on Amino Acid Biosynthesis

Amino acids are indispensable for insect development, functioning as the fundamental components of proteins and as signalling molecules (Liu et al. 2020). A previous report noted that the chitin synthesis inhibitor interferes with vitellogenesis, particularly protein synthesis in the ovaries (Soltani-Mazouni and Soltani 1995). In response to diflubenzuron, a CSI, a family of proteins known to function as transporters was up-regulated, binding hydrophobic substances such as odorants and pheromones (Vogt and Riddiford 1981; Merzendorfer et al. 2012).

The amino acid proline serves as a major energy substrate for many insect species (Gäde and Auerswald 2002) and as an alternative metabolic fuel for flight (Teulier et al. 2016). However, many insects can oxidize the amino acid proline at a fast rate, which is a unique trait of this group (Teulier et al. 2016). Proline oxidation may be linked to the release of fuel from fat bodies, which can be transported as proline instead of being oxidized directly as fatty acids (Bursell 1977; Arrese and Soulages 2010). Furthermore, proline ingestion improves the thermotolerance of insects (Li et al. 2015; Košťál et al. 2016), a trait also required for oviposition in honeybees (Hrassnigg et al. 2003). Bakr and Ghoneim (2008) found substantial changes in total protein content following treatment with several CSIs. Fahmy (2014) observed significant variations in total protein levels in *A. ipsilon* after treatment with chlorfluazuron and triflumuron. Our present findings indicate that flufenoxuron treatment in *S. mauritia*, caused significant changes in total protein levels and SDS-PAGE analysis (Reshma and Manogem 2026), which corroborates this study.

Our study found that flufenoxuron induces aberrant reproductive variations in the testes of *S. mauritia*, as evidenced by cellular and biochemical analyses (Reshma and Manogem 2026). Moreover, these impairments can be correlated with the altered haemolymph metabolite profile on flufenoxuron treatment in *S. mauritia*. In an earlier study by Loeb (1986), it was evidenced that the steroids observed in the testicular sheath were sequestered from the haemolymph, suggesting that haemolymph acts as a barrier to the transport of materials. Therefore, the present study suggests that sex-specific haemolymph metabolites in flufenoxuron-treated *S. mauritia* may induce variation in spermatogenesis and reproduction.

5 | Conclusion

The current study demonstrated that metabolomic analysis is an efficient method to examine the changes in the haemolymph metabolome induced by the chitin synthesis inhibitor flufenoxuron. Prolonged exposure to flufenoxuron is anticipated to reduce fitness and survival by depleting lipid and carbohydrate reserves. The pathway analysis further demonstrated that flufenoxuron disrupted essential processes, including glycolysis/gluconeogenesis, starch and sucrose metabolism, the pentose phosphate pathway, steroid biosynthesis, and amino sugar and nucleotide sugar metabolism. These pathways are vital for energy homeostasis, developmental regulation, and hormonal synthesis, highlighting the systemic impact of flufenoxuron exposure. Our model provides insights into the physiological effects of flufenoxuron by elucidating how alterations in metabolite regulation contribute to the stress response observed in the haemolymph.

Author Contributions

V. Reshma: conceptualization, investigation, writing – original draft, methodology, visualization, software. **E. M. Manogem:** conceptualization, supervision, validation, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available on request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: arch70157-sup-0001-S1_supplementary_data.xlsx.