UGC-MRP PROJECT REPORT

"CHARACTERIZATION OF JUVENILE HORMONE ANALOGUE-RESPONSIVE PROTEIN FROM THE LARVAL HAEMOLYMPH OF *SPODOPTERA MAURITIA* (LEPIDOPTERA: NOCTUIDAE)"

Principal investigator: Dr. Kannan V.M.

Professor, Department of Zoology
University of Calicut. kannanvm@yahoo.com

INTRODUCTION

Insects are the largest group in the animal kingdom. Some of them are pests and cause considerable economic loss. *Spodoptera mauritia* or rice swarming caterpillar is a sporadic pest of paddy. It is estimated that the loss in yield caused by larval infestation of *Spodoptera mauritia* range from 10 to 20%. In India it is found in all the rice growing areas especially along the west coast and delta in Kerala and Tamil Nadu (David and Ananthakrishnan, 2004). Pest management is an integral part of any successful agriculture. Application of conventional insecticides poses great threat to the human health and environment. Development of more eco-friendly pest management approaches is of prime importance for food security and health. Use of Insect Growth Regulators (IGRs) for pest management is an alternative as they are target-specific, non-persistent, biodegradable and environmentally benign substances, with less toxicity to non-target organisms. Many IGR's are juvenile hormone or ecdysone agonists.

Protein metabolism plays a key role in rebuilding adult structures during the transformation of larvae/pupae into adult. Hemolymph protein levels generally increase during each instar but decline during moulting. Several hemolymph proteins like insect hexamerins are thought to transport hormones, phenols /or some cuticular proteins to the hypodermis. Typically two to four physico-chemically distinct storage protein species occur. The last larval instar of holometabolous insects has been characterized by active synthesis of arylphorin (aromatic amino acids bearing storage proteins) and pupal storage proteins (Wyatt, 1978) During metamorphosis larval plasma proteins were hydrolyzed to free amino acids and major part being incorporated into new adult proteins. Thus haemolymph proteins are crucial for insect development. In this

study we demonstrate the effect of pyriproxyfen, a Juvenile hormone analogue on larval protein profile of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae) or paddy army worm, a pest of paddy (*Oriza sativa).*

OBJECTIVES OF THE PROJECT (AS GIVEN IN THE PROPOSAL)

1. To identify and characterize the JH analogue-responsive proteins.
2. To identify the site of synthesis of JH analogue-responsive proteins.
3. To understand the regulation of the JH analogue-responsive proteins by JH analogues.

STANDARDIZATION OF THE CULTURING TECHNIQUES OF *SPODOPTERA MAURITIA*

We standardized the conditions for culturing *Spodoptera mauritia*. The adult moths were collected using fluorescent light traps. They were kept in glass beakers and fed with a dilute solution of honey. They were allowed to mate and lay eggs. The egg will hatch within 3-5 days. The larvae hatched out were fed with fresh leaves of the grass *Ischaemum aristatum* and were maintained at room temperature (28^{o}C.)

TOXICITY OF PYRIPROXYFEN TO LARVAE OF *SPODOPTERA MAURITIA*

The average percentage mortality for 3rd, 4th, 5th & 6th instar larvae of *Spodoptera mauritia* treated with different concentrations of pyriproxyfen was calculated (Table 1.) With increase in concentration of pyriproxyfen, the mortality increased in all the instars of larvae tested.

Amount of pyriproxyfen applied/ larva	Average percentage mortality ± SE			
	3rd instar	4th instar	5th instar	6th instar
Control	0	0	0	0
5µg	14.4±2.1	12.5±2.5	…….	…….
10µg	25±5.0	27.5±6.4	……..	…….
25µg	85±2.9	70.8±5.1	15.8±2.0	…….
50µg	96.7±3.3	91.7±4.4	65.8±2.2	…….
100µg	100±0.0	100±0.0	75±2.9	8.13±2.8
125µg	…….	…….	96.7±3.3	13.8±1.3
200µg	…….	…….	……	32.5±2.5
300µg	…….	…….	…….	45±5.0
400µg	…….	…….	…….	73.3±1.7

Table I: Percentage mortality of 3rd 4th 5th & 6th instar larvae of *Spodoptera mauritia* treated with different concentrations of pyriproxyfen

CALCULATION OF LD_{50} VALUE

The LD_{50} value (24 hours) of pyriproxyfen for the 3rd, 4th, 5th & 6th instar larvae of *Spodoptera mauritia* was found out by treatment with different concentrations of pyriproxyfen in acetone and the values were 14.13±2.67, 15.85±3.67, 39.81±2.61and 316.20±2.64 µg/larvae respectively (Table 2)

SL. NO.	LARVAL INSTAR	LD_{50} VALUE (µg) /larva (MEAN ±SE)
1	THIRD	14.13±2.67
2	FOURTH	15.83±3.67
3	FIFTH	39.81±2.61
4	SIXTH	316.20±2.64

Table 2: LD_{50} value (24 hours) of pyriproxyfen for 3^{rd} 4^{th} 5^{th} & 6^{th} instar larvae of *Spodoptera mauritia*

EFFECT OF PYRIPROXYFEN ON HAEMOLYMPH PROTEIN CONCENTRATION

In our initial study we found that treatment of 5^{th} instar larvae with sub lethal concentrations of pyriproxyfen on day 0, led to a statistically significant ($p<0.05$) increase in total haemolymph protein concentration estimated by modified Lowry's method (Sandermann and H, Jr, Stromiger 1972) on day 1 after treatment, compared to control

SL. No.	SAMPLE	AMOUNT OF PROTEIN (µg/µl) ± SE
1	Control	3.02±0.02
2	LD_{10}	3.23±0.03

Table 3: The difference in haemolymph protein concentration on treatment of pyriproxyfen

EFFECT OF PYRIPROXYFEN ON HAEMOLYMPH PROTEIN PROFILE

Fifth instar larvae wer treated with sub lethal concentration (LD_{10}) of pyriproxyfen (on day 0) and haemolymph collected after 24 hours of the treatment. Haemolymph was subjected to SDS-PAGE under reducing conditions (Laemalli UK, 1970). The band intensity of an 83 kDa protein band is increased significantly compared to that of control (Fig. 1). This is a juvenile hormone analogue (pyriproxyfen) responsive protein.

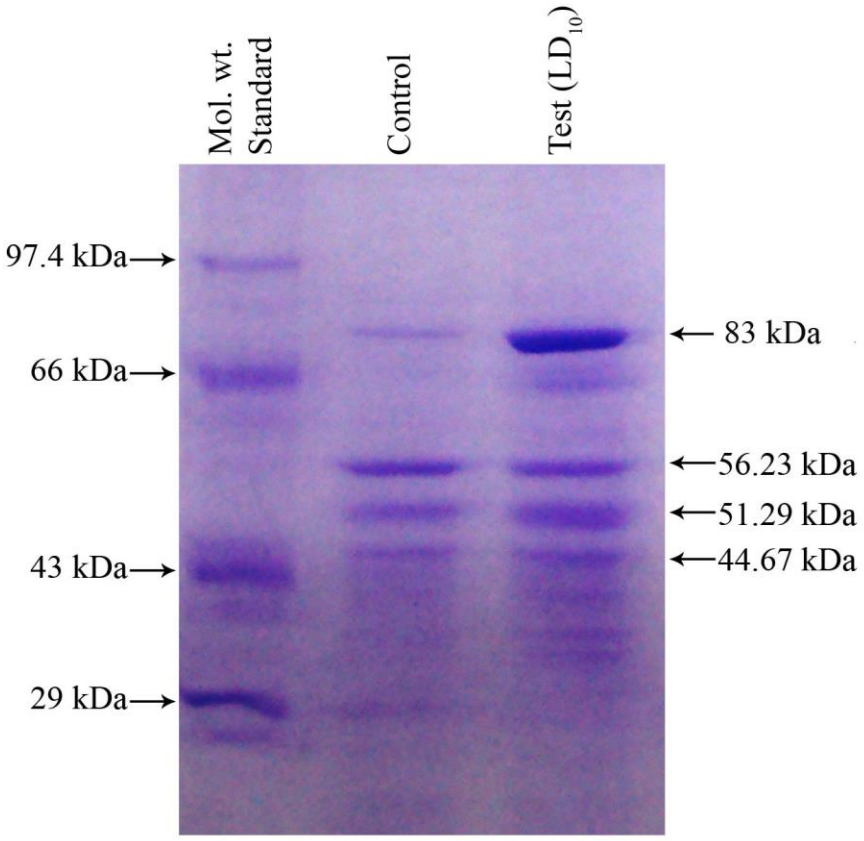

Fig. 1: SDS-PAGE (10%) Gel electrophoresis of haemolymph (3µl) of *Spodoptera mauritia* 5th Instar larvae.

DETERMINTION OF GLYCOSYLATION STATUS OF PYRIPROXYFEN-RESPONSIVE PROTEIN

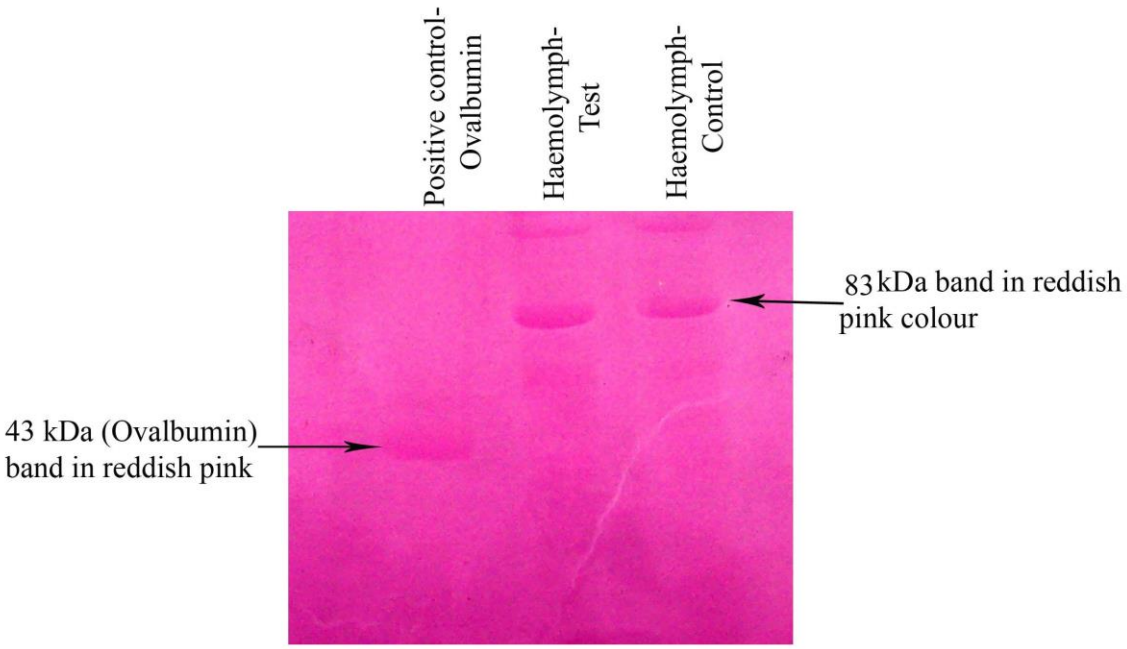

Fig. 2: PAS stained SDS-PAGE (10%) Gel electrophoresis of haemolymph of *Spodoptera mauritia* larvae

The glycosylation status of the identified pyriproxyfen-responsive protein was determined using Periodic Acid- Schiff's (PAS) staining of the SDS-PAGE separated haemolymph proteins (Ref). The band corresponding to the Pyriproxyfen responsive protein (83 kDa) was seen in reddish pink colour which indicates that the identified Pyriproxyfen responsive protein is a glycoprotein.

IDENTIFICATION OF THE SITE OF SYNTHESIS/STORAGE OF PYRIPROXYFEN-RESPONSIVE PROTEIN

The fat body extract of *Spodoptera mauritia* was subjected to 10% SDS-PAGE along with the haemolymph collected from the control larvae. We found a band in the fat body extract which corresponds to the identified Pyriproxyfen responsive protein in molecular weight.

It is possible that the protein identified in fat body may be the JH-analogue responsive protein found in haemolymph. Thus this protein is expressed /localized in fat body. Hence it is possible that one of the sites of synthesis/storage of the identified Pyriproxyfen- responsive protein may be the fat body.

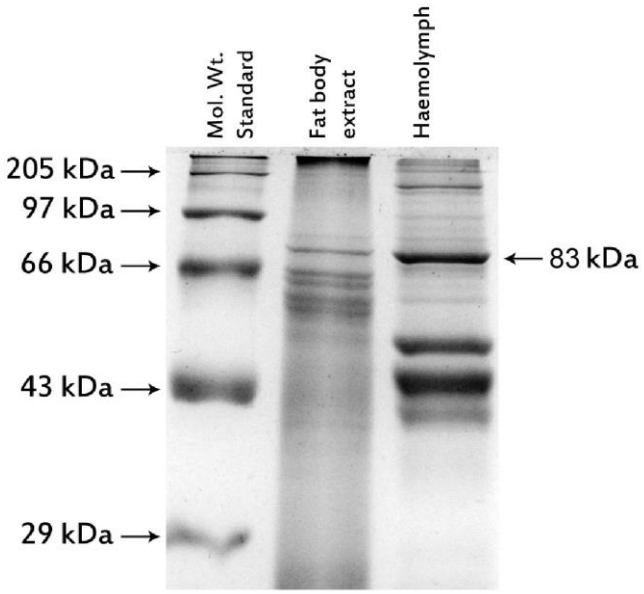

Fig. 3: SDS-PAGE (10%) Gel electrophoresis of Fat body extract and haemolymph of *Spodoptera mauritia* larvae.

DETERMINATION OF THE REGULATION OF THE JH ANALOGUE-RESPONSIVE PROTEIN BY PYRIPROXYFEN

Fifth instar larvae of *S.mauritia* was treated with different concentrations (10µg, 25µg and 100µg) of pyriproxyfen to determine its role in the regulation of the identified protein. The haemolymph of the treated larvae were collected after 24 hours and subjected to SDS-PAGE. The intensity of the identified protein band was increased with the increasing concentration of pyriproxyfen.

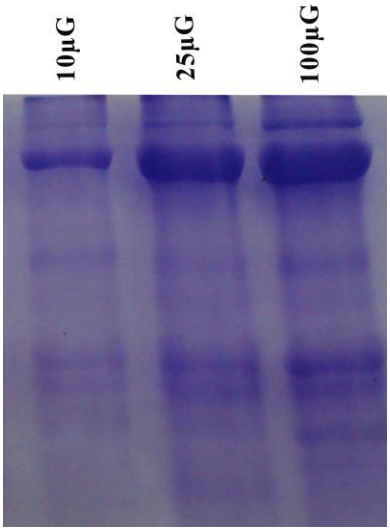

Fig. 4: SDS-PAGE (10%) Gel electrophoresis haemolymph of *Spodoptera mauritia* larvae treated with different concentration of pyriproxyfen

The concentrations of three storage proteins (SL-1,SL-2 and SL-3, hexamers of 70–80 kDa subunits) and two biliverdin-binding proteins (BP-A and BP-B, dimers of 165 kDa) in the haemolymph and fat body during larval and pupal development of *Spodoptera litura* were determined by immunodiffusion tests using polyclonal antisera (ToyoshiYoshiga etal 1997). All these three storage proteins have molecular sizes between 400 and 450 kDa, and are composed of subunit(s) which range in size from 70 to 80 kDa. (Tojo S and Yoshiga T , 1994). Thus the JH-analogue responsive protein reported from the hemolymph of *Spodoptera mauritia* is likely to be a hexamerine, which is a storage protein in insects. The storage proteins are metabolized and the amino acids are utilized for making the new tissues during development. Some of them also bind and transport ligands including hormones. These proteins are over expressed in response to JH analogue (pyriproxyfen) exposure and it will be worth examining the physiological significance of this insecticide induced over expression.

CONCLUSIONS

1) We determined the average percentage mortality of 3rd, 4th, 5th and 6th instar larvae of *Spodoptera mauritia* for different concentrations of Pyriproxyfen (JH-analogue).

2) We determined the L_{D50} of Pyriproxyfen for 3rd, 4th, 5th and 6th instar larvae of *Spodoptera mauritia*.

3) Also we found that exposure of 5th instar larvae to sub lethal concentration of pyriproxyfen led to an increase in haemolymph protein concentration.

4) In addition we showed that the treatment with sub lethal (LD_{10}) concentration of pyriproxyfen to 5th instar larvae leads to increase in intensity of an 83 kDa protein band.

5) The identified JH analogue- responsive protein is a glycoprotein with a subunit molecular mass of 83 kDa.

6) Fat body may be one of the sites of synthesis of the Pyriproxyfen-responsive 83 kDa protein.

7) The native Pyriproxyfen-responsive protein has subunit with a molecular weight of 83kDa.

8) The Pyriproxyfen-responsive protein may be a member of hexamerin family of proteins, which are storage proteins in the haemolymph of insects with subunit molecular weight in the range of 70-80kDa.

9) Hexamerins are major proteins in the haemolymph and they have roles in rebuilding adult structures. But these proteins are over expressed in response to JH analogue (Pyriproxyfen) exposure.

10) As storage proteins are crucial for insect development, it will be worth examining the physiological significance of this insecticide induced over expression of this protein.

PAPERS PUBLISHED

A part of the work is published in

1. International Journal of Agriculture Innovations and Research (2016) Volume 5, Issue 1, ISSN (Online) 2319-1473.

PAPERS PRESENTED IN CONFERENCES

1. Part of the results obtained in the study is presented in the Fourth Biopesticide international conference (BIOCICON-2013) held at Palayamkottai, Tamil Nadu
2. A part is also presented in the international symposium on Comparative endocrinology and integrative physiology (CEIP-2015) held at Thiruvananthapuram, Kerala

AWARDS OBTAINED

1. 'Best paper award' for the presentation in the Fourth Biopesticide international conference (BIOCICON-2013) held at Palayamkottai, Tamil Nadu.

PAPERS COMMUNICATED

1. The Juvenile Hormone mimic, Pyriproxyfen, increases the level of the major haemolymph protein in the larvae of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae) Resmitha C and Kannan Vadakkadath Meethal.

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Dr. Kannan V.M. (PI- UGC MRP)

Professor, Dept. of Zoology,

University of Calicut.

EXECUTIVE SUMMARY OF THE UGC-MRP PROJECT

"CHARACTERIZATION OF JUVENILE HORMONE ANALOGUE-RESPONSIVE PROTEIN FROM THE LARVAL HAEMOLYMPH OF *SPODOPTERA MAURITIA* (LEPIDOPTERA: NOCTUIDAE)"

Principal investigator: Dr. Kannan V.M.

Professor, Department of Zoology
University of Calicut. kannanvm@yahoo.com

1. Determined the average percentage mortality of 3rd, 4th, 5th and 6th instar larvae of *Spodoptera mauritia* for different concentrations of Pyriproxyfen (JH-analogue).
2. Determined the L_{D50} of Pyriproxyfen for 3rd, 4th, 5th and 6th instar larvae of *Spodoptera mauritia*.
3. Also found that exposure of 5th instar larvae to sub lethal concentration of pyriproxyfen led to an increase in haemolymph protein concentration.
4. In addition showed that the treatment with sub lethal (LD_{10}) concentration of pyriproxyfen to 5th instar larvae leads to increase in intensity of an 83 kDa protein band.
5. The identified JH analogue- responsive protein is a glycoprotein with a subunit molecular mass of 83 kDa.
6. Fat body may be one of the sites of synthesis of the Pyriproxyfen-responsive 83 kDa protein.
7. The native Pyriproxyfen-responsive protein has subunit with a molecular weight of 83kDa.
8. The Pyriproxyfen-responsive protein may be a member of hexamerin family of proteins, which are storage proteins in the haemolymph of insects with subunit molecular weight in the range of 70-80kDa.
9. Hexamerins are major proteins in the haemolymph and they have roles in rebuilding adult structures. But these proteins are over expressed in response to JH analogue (Pyriproxyfen) exposure.
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Toxicity of Insect Growth Regulator, Pyriproxyfen, on larvae of *Spodoptera mauritia* Boisd (Lepidoptera: Noctuidae)

C. Resmitha
University of Calicut,
Thenjipalam (PO), Malappuram-673635, Kerala, India
E-mail: rechujan@gmail.com

Vadakkadath Meethal
University of Calicut,
Thenjipalam (PO), Malappuram-673635, Kerala, India
E-mail: kannanvm@yahoo.com
*Author for correspondence

***Abstract* – Insect growth regulators belong to a class of compounds which interfere with normal growth, development and reproduction in insects. Many insect growth regulators are analogues or mimics of insect hormone ecdysone or juvenile hormone. Pyriproxyfen is an insect growth regulator which mimics the action of juvenile hormone. The effects of insect growth regulators on many lepidopteran pests have been studied with respect to its toxicity and developmental alterations. But the toxicity of pyriproxyfen to larvae of *Spodoptera mauritia* is not explored. In this study we treated 3rd 4th 5th & 6th instar larvae of *Spodoptera mauritia* Boisd. with different concentrations of pyriproxyfen (Knack IGR). It was found that the LD_{50} value of pyriproxyfen for the 3,rd 4,th 5,th and 6th instar larvae were 14.13±2.67, 15.85±3.67, 39.81±2.61and 316.20±2.64µg/larvae respectively. Studies are ongoing to understand the effect of sub lethal concentrations of pyriproxyfen on the development of *S.mauritia*.**

***Keywords* – JH Analogue, Insect Growth Regulators, Pyriproxyfen, *Spodoptera mauritia*, LD_{50}.**

I. INTRODUCTION

Insects are the largest group in the animal kingdom. Some of them are pests and cause considerable economic loss. *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae) or rice swarming caterpillar or paddy army worm is a sporadic pest of paddy distributed all over the world. *Spodoptera mauritia* has six larval instars before pupation. The larvae feed on leaves of paddy or alternate host plant such as *Ischaemum aristatum.* In India it is found in all the rice growing areas especially along the west coast and delta in Kerala and Tamil Nadu. [1]. It has attained the status of a major pest of rice in Eastern India, especially in Orissa, Chhattisgarh, Jharkhand and Bihar.

Pest management is an integral part of any successful agriculture. Application of conventional insecticides poses great threat to the human health and environment. Development of more eco-friendly pest management approaches is of prime importance for human health and environment. Use of Insect Growth Regulators (IGRs) for pest management is an alternative as they are more target-specific, non-persistent, biodegradable and environmentally benign substances, with less toxicity to non-target organisms. Insect Growth Regulators belong to a group of compounds which interfere with normal growth, development and reproduction in insects by disrupting hormonally regulated physiological processes of insects. Several such compounds are known and their effects on metamorphosis and reproduction in a number of insect species have been extensively studied.[2,3,4] Advantages of Juvenile hormone analogues (JHAs) include their fast penetrance through the insect cuticle, needed only in low concentrations and get degraded to non toxic compounds in a short time period. Already more than 500 analogues with juvenile hormone (JH) activity have been discovered. Among the well known JHAs are, Epofenonane, Methoprene, Hydroprene, Kinoprene, and Fenoxycarb.[5] The earlier reported JHA of commercial success were Methoprene and Hydroprene.[6] Methoprene is active against dipteran insects and fleas and hydroprene is active against cockroach. These compounds however, were too unstable under field conditions to be used for agriculture.[7] Krysan, J. L. (1990) reported the photostable JH analogue, fenoxycarb was effective not only on household pests but also on agricultural pests such as leaf rollers and codling moth.

Pyriproxyfen is a JH analogue preventing the larvae from developing in to adulthood and thus rendering them unable to reproduce. Most common morphogenetic effect of JHA treatment is the production of extra larval, nymphal or pupal form. The formation of extra larval instar depends on stage and age of the larvae at the time of treatment. Pyriproxyfen has relatively low mammalian toxicity and was first registered in Japan in 1991 for controlling public health pests.[8] Pyriproxyfen is a commonly used insect growth regulator against whiteflies.[9,10] In 1996 it was introduced in US to protect cotton crops against whitefly attack. The utility of pyriproxyfen in whitefly management was demonstrated based on suppression of embryogenesis and adult formation in *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae).[10] and *Trialeurodes vaporariorum* (Westwood) (Homoptera: Aleyrodidae).[11] In a leaf-disk bioassay using pyriproxyfen on the oblique banded leaf roller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), the LC, values for males and females were found to be 2.4 and 4.8 ppm, respectively.[12] Moadeli *et al* (2014)[13] reported that in a leaf dip bioassay with recommended field rate (1000 ppm) of pyriproxyfen, the 1st instar larvae of *Spodoptera exigua*, showed delay in larval development and in turn pyriproxyfen prolonged the feeding period and growth. In their study when compared

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to control the mean generation time was higher in pyriproxyfen treated insects.

Though insecticides containing pyriproxyfen like 'Knack IGR' are used against a variety of pests, the toxicity of pyriproxyfen is not tested on the larvae of *Spodoptera mauritia.* More over determination of the toxicity of pyriproxyfen will be helpful in determining the sub lethal concentration for use in experiments to study effects on development. In the present study we used 'Knack IGR', a pesticide containing pyriproxyfen, as the active ingredient to test its toxicity on the different larval stages of *Spodoptera mauritia*.

II. Materials and Methods

A. Collection, Rearing and Maintenance of the Larvae of Spodoptera Mauritia Boisd.

The moths were attracted by fluorescent lamps during night. They were collected using an insect sweeping net. The adults were kept in glass chimneys closed at both ends with muslin cloth and fed with 10% solution of honey. The adults lay eggs on the cloth covering the container. The caterpillars were fed with fresh, tender leaves of the grass *Ischaemum aristatum*. Larvae were maintained at room temperature (28°C) with relative humidity of 70-80%

B. Treatment to Test Toxicity of Pyriproxyfen

From the laboratory culture the 3rd, 4th 5th and 6th instar larvae were sorted out on the basis of moulting marks. Different concentrations of pyriproxyfen in acetone was applied topically on the dorsal side of the 3rd, 4th, 5th & 6th instar day 0 larvae of *Spodoptera mauritia* using a Hamilton Micro-Syringe in a total volume of 2µL. An equal volume of acetone was applied in the same manner to the control larvae. At least 3 replicates were done for each experiment and the number of larvae per experiment varied from 10 to 15. Mortality was recorded after 24 hours and from the average percentage mortality for different concentrations of pyriproxyfen, LD_{50} value for each instar was calculated from a plot of log concentration versus percentage mortality.

III. Results

Table I: Percentage mortality of 3rd 4th 5th & 6th instar larvae of *Spodoptera mauritia* treated with different conconcentrations of pyriproxyfen

Amount of pyriproxyfen applied/ larva	Average percentage mortality ± SE			
	3rd instar	4th instar	5th instar	6th instar
Control	0	0	0	0
5µg	14.4±2.1	12.5±2.5	…….	…….
10µg	25±5.0	27.5±6.4	……...	…….
25µg	85±2.9	70.8±5.1	15.8±2.0	…….
50µg	96.7±3.3	91.7±4.4	65.8±2.2	…….
100µg	100±0.0	100±0.0	75±2.9	8.13±2.8
125µg	…….	…….	96.7±3.3	13.8±1.3
200µg	…….	…….	……	32.5±2.5
300µg	…….	…….	…….	45±5.0
400µg	…….	…….	…….	73.3±1.7

A. Toxicity of Pyriproxyfen to Larvae of Spodoptera Mauritia

The average percentage mortality for 3rd, 4th, 5th & 6th instar larvae of *Spodoptera mauritia* treated with different concentrations of pyriproxyfen was calculated (Table 1.) With increase in concentration of pyriproxyfen, the mortality increased in all the instars of larvae tested.

B. Calculation of LD_{50} value

The LD_{50} value (24 hours) of pyriproxyfen for the 3rd, 4th, 5th & 6th instar larvae of *Spodoptera mauritia* was found to be 14.13±2.67, 15.85±3.67, 39.81±2.61and 316.20±2.64 µg/larvae respectively (Table 2)

Table 2: LD_{50} value (24 hours) of pyriproxyfen for 3rd 4th 5th & 6th instar larvae of *Spodoptera mauritia*

SL. NO.	LARVAL NSTAR	LD_{50} VALUE (µg) (MEAN ±SE)
1	THIRD	14.13±2.67
2	FOURTH	15.85±3.67
3	FIFTH	39.81±2.61
4	SIXTH	316.20±2.64

IV. Discussion

When the 3rd instar day 0 larvae were treated with 5, 10, 25, 50 and 100 µg per larva of pyriproxyfen, the average percentage mortality after 24 hours was found to be 14.38±2.13, 25±5.0, 85±2.88, 96.67±3.33 and 100±0.0 percentage respectively. The LD_{50} 24 hours, calculated for the 3rd instar larvae is 14.13±2.67µg (Table: 2). In the case of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) 3rd instar larvae, the reported LC_{50} value based on a leaf dip bioassay is 1.223 g L−1. [14]. When pyriproxifen was incorporated in the artificial diet of ten-day-old larvae of Indian meal moth *Plodia interpunctella* (Lepidoptera: Pyralidae) at 0.02, 0.04, 0.08, 0.16, and 0.3 ppm concentrations, it resulted in increased duration of the larval period till the emergence of adults, decreased adult longevity and significant reduction in mean number of eggs laid by adults compared to the controls[15]

In the case of 4th instar day 0 larvae treated with pyriproxyfen, 5, 10, 25, 50 and 100µg per larva, the average percentage mortality after 24 hour was found to be 12.5±2.50, 27.5±6.37, 70.83±5.07, 91.67±4.41 and 100±0.0 percentages respectively. The LD_{50} 24 hour for the 4th instar larvae is 15.85±3.67µg/larva. In the freshly moulted fourth instar larvae of citrus swallowtail *Papilio demoleus* (Lepidoptera: Papilionidae) topical administration of pyriproxyfen (7.5, 15, 30 and 60 µg/1µl/larva) induced a delay in larval– larval ecdysis and subsequent larval–pupal ecdysis. It is also reported that the treatment reduced the frequency of pupation, increased mortality and ecdysial failure, and inhibited adult emergence. [16]

For the 5th instar day 0 larvae there was no mortality for 5µg and 10µg pyriproxyfen per larva. Thus higher amount of pyriproxyfen was applied. The average percentage mortality after 24 hours for 25, 50, 100 and 125µg of pyriproxyfen per larva were 15.83±2.01, 65.83±2.21, 75±2.89 and 96.67±3.33 percentage respectively. The

LD_{50} calculated based on this percentage mortality was found to be 39.81±2.61µg/larva.

Sixth instar day 0 larvae when treated with 100, 200, 300 & 400µg of pyriproxyfen per larvae, the mortality was found to be 8.13±2.77, 13.75±1.25, 32.5±2.5, 45±5.0 and 73±1.67 percentage respectively. On calculation the LD_{50} value was found to be 316.20±2.64µg/larvae. Singh et al (2015)[17] topically administrated sub-lethal doses (0.5, 1.0, 2.5 & 5µg/ µl/larvae) of pyriproxyfen and diofenolan on the 6th instar larvae of *Spodoptera litura* (Lepidoptera: Noctuidae) and reported that these JHAs severely hampered the metamorphosis and development with prolonged larval duration, mortality, ecdysial failure, formation of larval - pupal mosaics, reduced pupation and formation of abnormal pupae, complete suppression of adult emergence and production of adultoids. Significant differences in number and hatchability of eggs, wing abnormalities and morphological ovarian abnormalities were observed when pyriproxyfen was topically applied to *Spodoptera litura* (Lepidoptera: Noctuidae). Application of 0.1 ng of pyriproxyfen to day-1 female pupae and 0.125 µg to day-0 6th stadium larvae reduced the total number of eggs oviposited and hatchability of eggs. Day-1 pupal stage treated with 0.3 ng of pyriproxyfen showed wing abnormalities and about 40% of female adults showed morphological ovarian abnormalities. [18]

As pyriproxyfen is effective at lower concentrations to disrupt the larval development leading to failure in healthy adult emergence, most of the studies are concentrated on sublethal effects. In this study we showed that pyriproxyfen at relatively higher concentration causes death of the larvae in 24 hours after application. The results can be extrapolated to find sub-lethal concentration such as LD_{10} or other sub lethal concentrations for *S. mauritia* for experiments to study its effect on development. Studies are ongoing to find effect of sub lethal concentration of pyriproxifen on development of *Spodoptera mauritia.*

V. Conclusions

At higher concentrations, pyriproxyfen caused the death of the larvae of *S.mauritia* after 24 hours and with increase in concentration of pyriproxyfen, the mortality also increased in 3rd 4th 5th and 6th instar larvae. The LD_{50} value of pyriproxyfen for the 3,rd 4,th 5,th and 6th instar larvae of *Spodoptera mauritia* was found to be 14.13±2.67, 15.85±3.67, 39.81±2.61and 316.20±2.64 µg/larvae respectively.

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AUTHORS' PROFILES

Dr.Kannan Vadakkadath Meethal hails from Ulleary village in Kozikode District of Kerala, India. He completed M.Sc Zoology from University of Calicut and Ph.D from Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. He completed his Post Doctoral Training from University of Michigan,

Mrs. Resmitha C. hails from Thazhecode village of Kozhikode Distrct, Kerala, India and completed M.Sc in Zoology from University of Calicut. She is currently pursuing Ph.D in Zoology and working as a research fellow with Dr.Kannan Vadakkadath meethal in University of Calicut.

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The juvenile hormone mimic, pyriproxyfen, increases the level of the major haemolymph protein in the larvae of *Spodoptera mauritia* Boisd. Lepidoptera: Noctuidae

Resmitha C, Dr. Kannan Vadakkadath Meethal
Department of Zoology, University of Calicut, Thenjipalam (PO), Malappuram, Kerala, India

Abstract
Insect Growth Regulators (IGR's) belong to a group of compounds which interfere with normal growth, development and reproduction in insects by disrupting hormonally regulated physiological processes of insects. Pyriproxyfen is an IGR which mimics the action of Juvenile Hormone (JH). The influence of IGRs on haemolymph protein profile of lepidopteran pests is not well characterized. Exposure of 5th instar larvae of *S.mauritia* to sub lethal concentration (LD_{10}) of pyriproxyfen led to an increase in size of the larvae. Concomitantly haemolymph protein concentration of the treated larvae also increased compared to control. In pyriproxyfen treated larvae, on SDS-PAGE, there was an increase in the intensity of the predominant haemolymph protein band (83kDa) indicating a specific effect of pyriproxyfen on this protein. The pyriproxyfen-responsive protein is a glycoprotein and is synthesized/ stored in fat body. The identified pyriproxyfen-responsive protein is likely to be a member of hexamerins, the major storage protein in the haemolymph of insects.

Keywords: JH-analogue, insect growth regulator, pyriproxyfen, protein profile

1. Introduction
The rice swarming caterpillar, *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae) also known as paddy army worm is a sporadic pest distributed all over the world. In India, earlier it was considered as a minor pest of rice but for the last one decade, it has emerged as serious pest of rice seedlings. In India it is found in all the rice growing areas especially along the west coast and delta in Kerala and Tamil Nadu [1].

Pest management is an integral part of any successful agriculture. Application of conventional insecticides poses great threat to the human health and environment. Insect Growth Regulators (IGR's) belong to a group of compounds which interfere with normal growth, development and reproduction in insects by disrupting hormonally regulated physiological processes of insects. Insect growth regulators adversely affect insect growth and development. Several such compounds are known and their effects on metamorphosis and reproduction in a number of insect species have been extensively studied [2, 3, 4]. Insect growth regulators are more selective in their mode of action and thus less toxic to non-target organisms. An IGR does not necessarily have to be toxic to its target, but instead they may lead to various abnormalities that impair insect survival [5].

Many of the IGRs are mimics of insect hormones, juvenile hormone (JH) or ecdysone. Pyriproxyfen is an IGR which mimics the action of JH. Advantages of IGRs includes species specificity, less or zero toxicity to other animals, fast penetrance through the insect cuticle and they get degraded to non toxic compounds in a short time period. Already around 500 analogues with JH activity have been discovered. Among the well known juvenile hormone analogues (JHAs) are, Epofenonane, Methoprene, Hydroprene, Kinoprene, and Phenoxy carbamate [6]. The first JHA of commercial success were Methoprene and Hydroprene [7]. Methoprene is active against dipteran insects and fleas and hydroprene is active against cockroach. These compounds however, were too unstable under field conditions to be used in agriculture. The photostable JH analogue, fenoxycrab was effective not only on household pests but also on agricultural pest such as leaf rollers, the codling moth and *Psylla pyricola*.[8]

Pyriproxyfen is a juvenile hormone analog with relatively low mammalian toxicity that was registered in Japan in 1991 for controlling public health pests [9]. Timely application of JHs could be employed to control insects because of their ability to disrupt normal physiological functions [10]. Pyriproxyfen mimics the action of JH and maintains the insect in an immature state which inhibit the successful molting of the insect or normal reproduction [11]. Most common morphogenetic effect of JHA treatment is the production of extra larval, nymphal or pupal form. The formation of extra larval instar depends on stage and age of the larvae at the time of treatment. Pyriproxyfen is a commonly used insect growth regulator against homopoteran insect pests, including whiteflies [12, 13]. The utility of pyriproxyfen in whitefly management was demonstrated based on suppression of embryogenesis and adult formation in *Bemisia tabaci* (Gennadius) [14] and *Trialeurodes vaporariorum* [14].

Protein metabolism plays a very important role in rebuilding adult structures during the transformation of larvae/pupae into adult. In general hemolymph protein levels increase during each instar but decline during moulting. In haemolymph typically two to four physicochemically distinct storage protein species occur. Storgae proteins of insects such as hexamerins are also involved in transport of hormones, phenols /or some cuticular proteins to the hypodermis. In holometabolous insects, active synthesis of arylphorins

(aromatic amino acids bearing storage proteins) and pupal storage proteins occur during last instar [15]. Larval plasma proteins were hydrolyzed to free aminoacids and major part being incorporated into new adult proteins during metamorphosis. Thus haemolymph proteins plays a pivotal role in insect development.

In the present study we used Knack IGR, a pesticide containing the active ingredient pyriproxyfen to examine its effect on the haemolymph protein profile of 5th instar larvae of *Spodoptera mauritia* Boisd.

2. Methodology

2.1 Collection, Rearing and Maintenance of the Larvae of *Spodoptera mauritia* Boisd:

The moths were attracted by fluorescent lamps during night. They were collected using an insect sweeping net. The adults were kept in glass chimneys closed at both ends with muslin cloth and fed with 10% solution of honey. Larvae were maintained at 26-28°C. *Spodoptera mauritia* larvae have six larval instars before pupation. The larvae feed on leaves of paddy or alternate host plant such as *Ischaemum aristatum*. The caterpillars were fed with fresh, tender leaves of the grass *Ischaemum aristatum*.

2.2 Exposure of larva to pyriproxyfen for identification of pyriproxyfen responsive proteins

Sub lethal concentration (LC_{10}) of pyriproxyfen determined in our earlier study [16] was selected for the treatment. Pyriproxyfen (Knack IGR) in acetone was applied topically along the dorsal midline of meso and metathorax and to the abdomen of 5th instar larvae of *Spodoptera mauritia* using the Hamilton Micro-Syringe. An equal volume of acetone was applied in the same manner to the control larvae. The haemolymph of both the test and control larvae were collected after 24 hours.

2.3 Collection of Haemolymph

Larvae were anesthetized in a specimen tube using diethyl ether. One of the prolegs of larvae excised with a sterilized scissors and the exuded haemolymph (with haemocytes) from each larva was drawn into separate centrifuge tubes and stored at -20°C.

2.4 Determination of the effect of pyriproxyfen on haemolymph protein concentration

The concentration of haemolymph protein was determined by Modified Lowry's method [17]. For this the haemolymph (with haemocytes) was treated with SDS (1% final) and centrifuged at 5600 g for 5 minutes. The supernatant containing SDS-soluble protein was used for protein estimation using bovine serum albumin (BSA) as standard.

2.5 Electrophoretic Analysis of Haemolymph proteins

The haemolymph was treated with SDS (1% final) and centrifuged at 5600g for 5 minutes. The supernatant containing SDS-soluble protein samples were subjected to SDS-PAGE under reducing conditions using 10% acrylamide in a mini slab gel according to the method described by the Laemmli [18]. Protein profile of the treated larvae was compared with untreated to identify changes in protein band intensity and new appearance/disappearance of polypeptides.

2.6 Determination of glycosylation status of pyriproxyfen-responsive protein

The glycosylation status of the identified pyriproxyfen-responsive protein was determined using Periodic Acid-Schiff's (PAS) staining [19] of the SDS-PAGE separated haemolymph proteins.

2.7 Identification of the site of synthesis/storage of JH analogue-responsive protein

The fat body of 5th instar larvae of *Spodoptera mauritia* was dissected out and homogenized in insect ringer and centrifuged at 5600 g for 5 minutes. After removing lipids, the supernatant is subjected to 10% SDS-PAGE along with the haemolymph collected from the control larvae. Gel was stained to visualize the protein bands.

2.8 Effect of increase in concentration of pyriproxyfen on pyriproxyfen-responsive protein

To determine the effect of increase in pyriproxyfen on the level of pyriproxyfen-responsive protein, fifth instar larvae of *S.mauritia* was treated with different concentrations (10µg, 25µg and 100µg/ larva) of pyriproxyfen. The haemolymph of the treated larvae were collected after 24 hours and subjected to SDS-PAGE to asses change in protein band intensity with change in concentration of pyriproxyfen.

3. Results

3.1 Effect of pyriproxyfen on larval size and haemolymph protein concentration

Exposure of 5th instar day 0 larvae of *S.mauritia* to sub lethal concentrations of pyriproxyfen (LD_{10}) led to an increase in size of the larvae (Fig.1) and a statistically significant ($p<0.05$) increase (7%) in SDS-soluble haemolymph protein concentration after 24 hours compared to control.

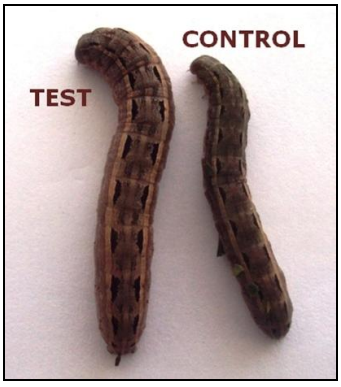

Fig 1: Test and control larvae showing difference in size on exposure to pyriproxyfen

Table 1: The increase in haemolymph protein concentration on treatment with pyriproxyfen

Sl. No	Sample	Concentration of haemolymph protein (µg/µl) ± SE	p value
1.	Control	3.02±0.02	0.03
2.	LD_{10} (4µg/larvae)	3.23±0.03	

3.2 Effect of Pyriproxyfen on haemolymph protein profile

Exposure of 5th instar larvae of *S.mauritia* to sub lethal concentrations of pyriproxyfen (LD_{10}) led to an increase in size of the larvae (Fig.1). Equal volume of haemolymph SDS-soluble protein (processed identically) from treated and untreated larvae were loaded onto 10% SDS -PAGE to assess changes in protein profile. Treatment with pyriproxyfen lead to an increase in intensity of a protein band with molecular weight of 83kDa (Fig. 2)

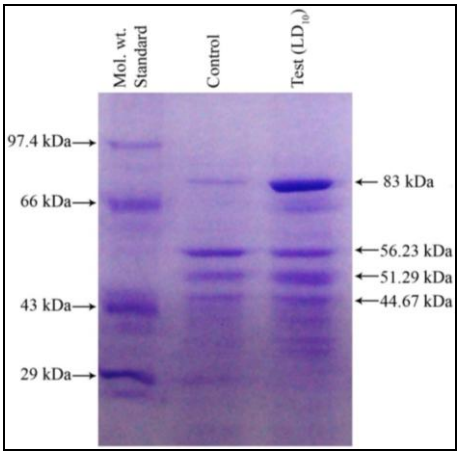

Fig 2: SDS-PAGE (10%) of haemolymph (3µl) of *Spodoptera mauritia* 5th Instar larvae

3.3 Effect of increase in concentration of pyriproxyfen on pyriproxyfen-responsive protein

Fifth instar larvae of *S.mauritia* was treated with different concentrations (10µg, 25µg and 100µg) of pyriproxyfen to determine the effect of increase in pyriproxyfen on the level of pyriproxyfen-responsive protein. The haemolymph of the treated larvae were collected after 24 hours and subjected to SDS-PAGE. The intensity of the pyriproxyfen- responsive protein band was increased with increasing concentration of pyriproxyfen.

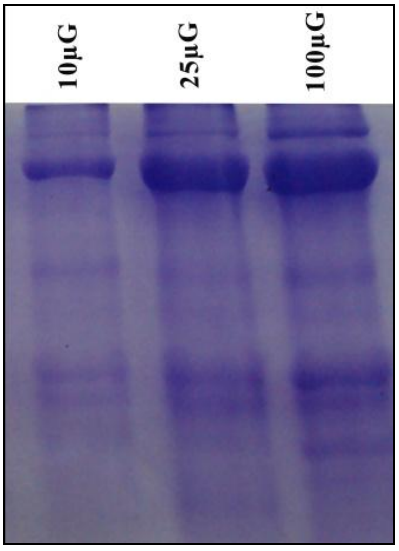

Fig 3: SDS-PAGE (10%) Gel electrophoresis haemolymph of *Spodoptera mauritia* larvae treated with different concentrations of pyriproxyfen

3.4 Glycosylation status of pyriproxyfen-responsive protein

On Periodic Acid- Schiff's (PAS) staining of the SDS-PAGE separated haemolymph proteins, the band corresponding to the JH analogue responsive protein (83 kDa) was seen in reddish pink colour (Fig 4) which indicates that the identified pyriproxyfen- responsive protein is a glycoprotein.

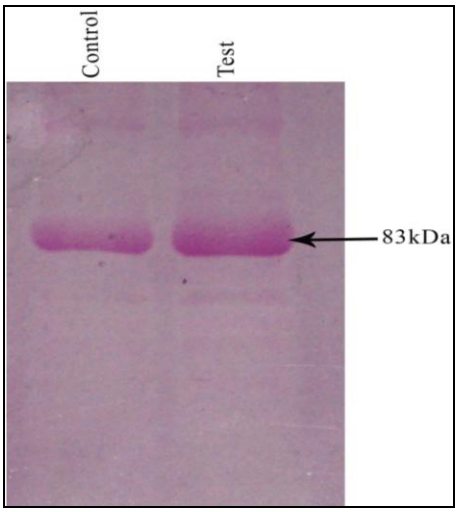

Fig 4: PAS stained SDS-PAGE (10%) of haemolymph proteins of *Spodoptera mauritia* larvae.

3.5 Identification of the site of synthesis/storage of JH analogue-responsive protein

When the fat body extract of *Spodoptera mauritia* was subjected to 10% SDS-PAGE, it is found that there is a protein band in the fat body extract which corresponds to the identified pyriproxyfen- responsive protein in molecular weight (Fig 5). Thus it is likely that the protein identified in fat body may be the pyriproxyfen- responsive protein found in haemolymph indicating that this protein is expressed /localized in fat body. Hence it is possible that one of the sites of synthesis/storage of the identified pyriproxyfen- responsive protein is the fat body.

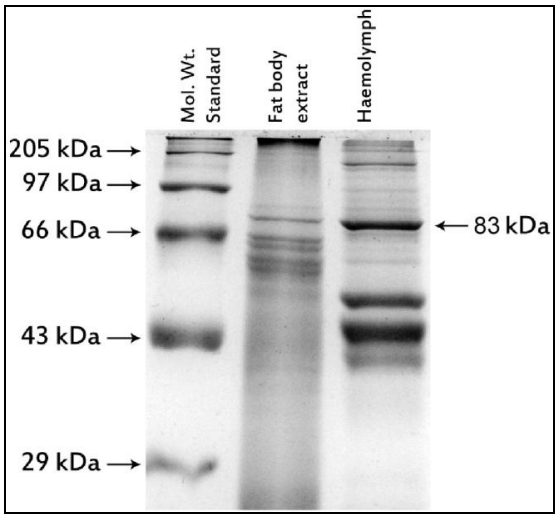

Fig 5: SDS-PAGE (10%) of fat body extract and haemolymph of 5th instar larvae of *Spodoptera mauritia*

4. Discussion

Haemolymph total protein concentration of the larvae treated with LD_{10} concentration (4µg/larvae) of pyriproxyfen increased significantly ($p< 0.05$) compared to control (Table 1) The increase in haemolymph protein concentration may be due to the effect of pyriproxyfen on synthesis or degradation of proteins/peptides in the haemolymph. In the desert locust *Schistocerca gregaria* after 1 day of treatment, pyriproxyfen and lufenuron elevated the protein level in nymphs [20]. When the last larval instar of *Spodoptera littoralis* treated with methoprene, hydroprene or kinoprene, the haemolymph protein concentration increased [21].

When protein profile of the haemolymph of fifth instar (day 0) larvae of *Spodoptera mauritia* treated with LD_{10} concentration of pyriproxyfen (4µg/larvae) were analyzed by SDS-PAGE, there was an increase in intensity of the major protein band (83 kDa) in the treated compared to control after 24 hour of exposure (Figure 2). The SDS-PAGE analysis of haemolymph collected from the larvae treated with different concentrations of pyriproxyfen showed an increase in the pyriproxyfen-responsive protein band with the increasing concentration of pyriproxyfen. Thus the effect of pyriproxyfen on haemolymph protein level is concentration dependent (Fig 3.). In *Trichoplusia ni* there are several hemolymph proteins which increase to high levels during the final larval instar [22]. Treatment of larvae with JH suppresses the levels of these proteins [23]. Larval haemolymph storage proteins also help transport of hormones in insects. From the haemolymph of *Diploptera punctata* a high affinity juvenile hormone binding protein was identified as a lipophorin. The lipophorin was composed of two subunits, apolipoprotein I (230 kDa mol. wt) and apolipoprotein II (80 kDa mol. wt) [24].

To analyze glycosylation status of the pyriproxyfen - responsive protein, SDS-PAGE separated protein were subjected to PAS staining. The identified pyriproxyfen - responsive protein band appeared in pink colour which indicates that the pyriproxyfen - responsive protein is a glycoprotein. Four potential N-glycosylation sites were found in storage hexamerins from *Spodoptera exigua*, *SeHex* (amino acids 75, 209, 479 and 647), and one potential site (amino acid 47) in *SeSP1* [25].

When the protein extract of fat body of *S. mauritia* was subjected to SDS-PAGE and found that there is a band in the fat body extract which corresponds to the identified pyriproxyfen- responsive protein in molecular weight. Thus pyriproxyfen- responsive protein is expressed /localized in the fat body. Hence it is possible that one of the sites of synthesis/storage of the identified pyriproxyfen- responsive protein is the fat body. In the early instars, proteins are synthesized in the fat body (the main site of protein synthesis) and subsequently released into the surrounding haemolymph [26] which, in later instars are sequestered from haemolymph into the fat body. Simmon and Mitchell [27] have suggested that in *Drosophila* amino acids are first incorporated into peptides and later enter into proteins.

Storage hexamerins are composed of six identical or similar subunits of ~80 kDa with a native molecular weight around 500 kDa. They are the most abundant and widely distributed storage proteins that accumulate in the hemolymph or fat body of insects [28]. Storage hexamerins include the hexamerins, juvenile hormone-related protein, riboflavin-binding hexamerin precursor, methionine-rich storage protein (storage protein 1, SP1), very-high-density lipoprotein, tyrosine-rich proteins and hemocyanin-related proteins [29]. Thus the pyriproxyfen-responsive protein identified in this study may be the sub unit of hexamerin family of storage proteins. The subunit molecular weight, glycosylated nature and abundance in the hemolymph are inconformity with this. These proteins are important not only as storage proteins but act as carriers of hormones, and participate in molting, metamorphosis and reproduction. As the storage proteins are crucial for insect development, they are ideal targets for designing better insect control agents. It will be worth examining the role played by these proteins in the physiology of insects on exposure to pyriproxyfen.

5. Conclusions

The exposure of 5th instar larvae of *Spodoptera mauritia* to sub lethal concentration (LD_{10}) of pyriproxyfen led to an increase in size of the larvae and haemolymph protein concentration compared to control. It also leads to a concentration dependent increase in intensity of an 83 kDa protein haemolymph protein band. The identified pyriproxyfen- responsive protein is a glycoprotein and one of the possible sites of synthesis/storage of this protein is the fat body.

6. Acknowledgments

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