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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

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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.

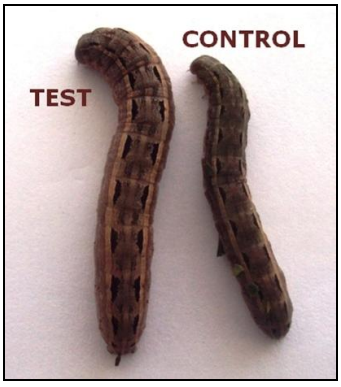

Fig 1: Test and control larvae showing difference in size on exposure to pyriproxyfen

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)

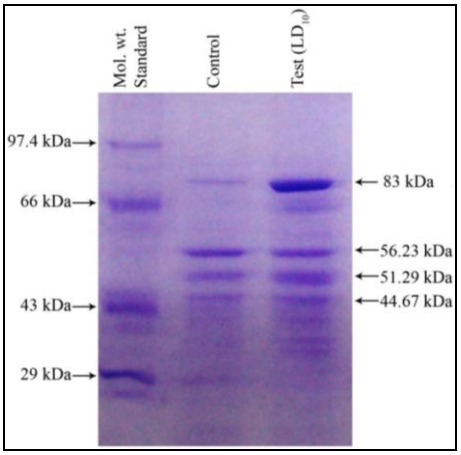

Fig 2: SDS-PAGE (10%) of haemolymph (3µl) of *Spodoptera mauritia* 5th Instar larvae

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.

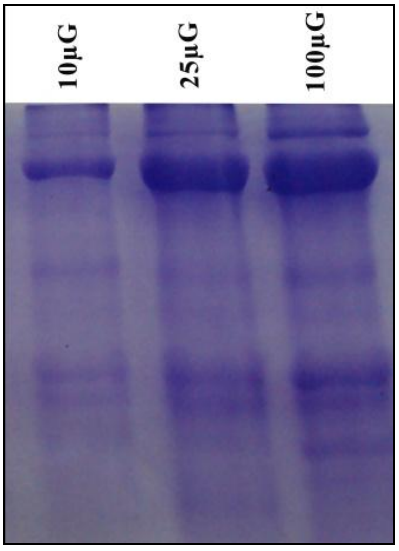

Fig 3: SDS-PAGE (10%) Gel electrophoresis haemolymph of *Spodoptera mauritia* larvae treated with different concentrations of pyriproxyfen

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.

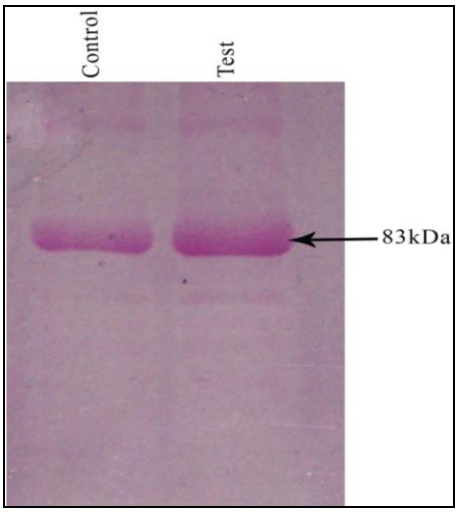

Fig 4: PAS stained SDS-PAGE (10%) of haemolymph proteins of *Spodoptera mauritia* larvae.

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.

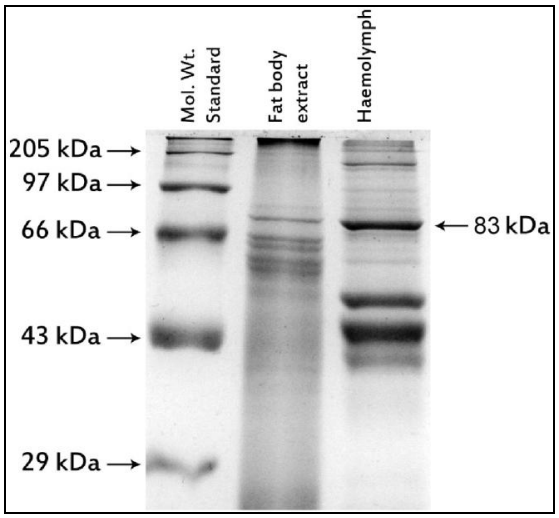

Fig 5: SDS-PAGE (10%) of fat body extract and haemolymph of 5th instar larvae of *Spodoptera mauritia*

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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