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Quantitative analysis of juvenile hormone using electro spray ionization- mass spectroscopy in haemolymph of plumbagin treated *Dysdercus cingulatus* (Heteroptera)

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Abstract
Search for plant products that interfere with insect growth, development, and behavior is the need of the hour. This has encouraged the synthesis of eco-friendly insect control agents, which has also helped poor farmers compete with insect pests. To understand the chemical nature of plant-origin compounds studies on the mode of action are crucial. This will help in establishing structure-relation-activity which will facilitate the formulation of enriched products from plants with insect growth regulator (IGR) properties, for practical application in the field. Juvenile Hormone being involved in crucial physiological processes like morphogenesis, moulting, growth, and reproduction is an essential component to assess the mode of action of any compound. Keeping this in mind, Plumbagin, chemically known as 5-hydroxy-2-methyl-1,4-naphthoquinone was used to understand its effects on the Juvenile Hormone (JH) present in the hemolymph of adult *Dysdercus cingulatus*. Earlier studies on growth and reproduction in plumbagin-treated *Dysdercus sp.* have shown detrimental effects and thus in the present investigation, hemolymph of plumbagin-treated male and female *Dysdercus cingulatus* was analyzed to explore any co-relation. It was observed that juvenile hormone titer in the hemolymph was dose-dependent when *Dysdercus* was treated with Plumbagin. Moreover, with the increase in the post-treatment period, a relative decrease in the amount of JH was noted in the plumbagin-treated group when compared with the control group. Not only this but the results indicate that females are more affected than males. Hence it can be concluded that Juvenile hormone research renders new opportunities for the development of integrated pest management strategies targeted for the disruption of juvenile hormone titer.

Keywords: Plant products, growth, development, metamorphosis, IGR, Plumbagin, Haemolymph, *Dysdercus cingulatus*

Introduction

Hormones play a vital role in the life cycle of insects. Metabolism, moulting, and metamorphosis in insects are under the influence of different hormones secreted by the Neuro Secretory Cells (NSC) of brain, Corpora Cardiaca (CC), Corpora Allata (CA), and Prothoracic Gland (PTG). The peptide hormones are secreted by Neurosecretory cells and are released into hemolymph via CC and amines such as octopamine are released from nerve endings. Lipid hormones namely, Juvenile hormone (JH) and Ecdysteroid are synthesised by CA and PTG respectively and are released into the haemolymph.

Juvenile hormone was discovered as a factor secreted by CA in 1930. It is also called Neotenin, an acyclic sesquiterpenoids that is unique to insects. The structural details of this hormone were determined in 1967 [1,2]. At present, there are eight JH known [3]. The most found Juvenile hormone in insects is JH III [4]. JH III lacks the epoxide group. It is the main effector hormone that helps in the regulation of growth, metamorphosis and reproduction. There is a very tight regulation of JH biosynthesis by CA of insects to ensure proper development and maintenance of reproductive cycles [5]. In certain social insects, JH also is associated with caste differentiation. Besides these functions, JH is also involved in many physiological behaviours such as foraging, diapause, vitellogenesis, vitellogenin uptake by ovaries, maintenance of larval/nymphal stages etc [6]. All tissues in the insect's body are the targets of JH. Literature reveals the fact that there is a marked difference in hormonal titre in different insect groups, but at the same time in the haemolymph, the general pattern of JH titre change during the growth and reproduction follows almost the same pattern. This information has given a cutting edge to researchers working on insect control and encouraged them to look for such compounds that can disrupt the growth and reproduction of insects.

Plumbagin is one such quinone compound which is a secondary metabolite present in Plumbago sps. and has the potential to act as an insect control agent [7]. Many detrimental effects like shrinkage and development of blackish pigmentation over the abdomen, inability to remove excuvia, and reproduction inhibition have been reported in Plumbagin-treated *Dysdercus koenigii* [8]. Further, it was noted that the action of Plumbagin was dose-dependent [9]. In *Dysdercus*, it was also seen that Plumbagin has an adverse effect on digestive enzymes [10]. Thus, it was thought to analyse the effect of Plumbagin on Juvenile hormone.

Here an attempt is made to quantify the JH in plumbagin-treated *Dysdercus cingulatus* using the Electrospray ionization (ESI) method. This method is routinely used to study both the qualitative (structure) and quantitative (molecular mass or concentration) studies of peptides, proteins, carbohydrates, small oligonucleotides, synthetic polymers, and lipids. Hence this study was carried out with a vision that the analysis of juvenile hormone titer will throw some light on the mode of action of Plumbagin.

Materials and Methods

Chemical under investigation

Plumbagin or 5-hydroxy-2-methyl-1,4-naphthoquinone is an organic compound with the formula $C_{11}H_8O_3$ that was purchased from Sigma.

Test organism

The red cotton bug, *Dysdercus cingulatus* Fabr, (Pyrrhocoridae: Heteroptera) has been used in the present study as a test insect. Sensitivity to compounds under investigation and ease of rearing in the laboratory make the red cotton bug, the insect of choice for investigation.

Collection

Dysdercus cingulatus were collected from the field of Rahuri, Ahmednagar Maharashtra. The adult male and female are separated by observing their size.

Rearing

Three pairs of adult *Dysdercus cingulatus* per insect-rearing jar were kept. These rearing jars were layered with an inch of moist soil. They are fed on soaked cotton seeds. These insects were maintained at 28º±2ºC and the humidity was maintained at around 85%. 12 hours of photoperiod was maintained during rearing and experimentation.

Treatment

Freshly moulted 24-36 hours old adults from the culture were selected. These insects were treated topically with the requisite amount of Plumbagin using a Hamilton microliter syringe.

Collection of Haemolymph

Hemolymph from control and treated males and females were collected separately after 24, 96, and 168 hours of treatment [11]. Pooled haemolymph with 70% methanol (1:30) was kept in the refrigerator till further use.

ESI

ESI produces gaseous highly charged ionized molecules directly from a liquid solution which gets finely sprayed as highly charged droplets in the presence of an electric field. It is a very sensitive, robust and reliable tool for studying femto-mole quantities in microliters of samples. With the use of ESI-MS, a neutral compound can also be converted to ionic forms in the solution or in the gaseous phase by either protonation or cationization. Here an attempt is made to quantify the Juvenile hormone in the plumbagin-treated *Dysdercus cingulatus*.

Sample preparation

15µl of haemolymph samples was mixed with an equal volume of 70% methanol and kept at room temperature for 30 min. This mixture was subjected to centrifugation (8500 rpm, 30 min). The upper phase was carefully collected in a new vial and the process was repeated twice. 500 µl Methoprene was added as an internal standard. Further, the mixture was dried to a pellet under a stream of N_2. Dried pellets were dissolved in 10 µl of methanol and subjected to LC-Q-TOF-MS.

Ultra-Performance Liquid Chromatography –H Class with C18 column (50 mm × 2.1 mm× 1.7 µm) were used. Column temperature was maintained at 40°C. Aqueous formic acid (0.1%) was used as mobile phase A whereas 0.1% formic acid dissolved in acetonitrile as mobile Phase B. 15 minutes binary gradient program between mobile phase A and B was run as mentioned below.

Hold 5% B for 0.5 min, ramp to 98% B in 7.5 min, hold 98% B for 3 min, return to 5% B in 0.5 min, hold 5% B for 3.5 min. The flow rate was constant at 0.5 ml/min.

Quadrupole – Time-of-Flight (Q-TOF) mass spectrometer was performed under ESI positive and negative ionization mode and electrospray source parameters were optimized by infusing 1 mg/L solution of each compound in 0.1% formic acid in acetonitrile. Source parameters for both positive and negative modes of ionization were used as follows (Table 1).

Table 1: Parameters for both positive and negative modes of ionization

	Positive mode electrospray capillary	**Negative mode electrospray capillary**
Voltage	3.50 kV	2.50kV
Sample cone	30V	30 V
Extraction cone	1 V	1 V
Source Temperature	350°C	135°C
Gas Flow	Nitrogen	Nitrogen
Gas Cone	50 (L/H)	50 (L/H)
Desolvation gas	900 (L/H)	900 (L/H)

Concentration of Juvenile hormone present in the haemolymph samples was calculated from the chromatogram using the equation:

$$\frac{\text{Concentration of standard}}{\text{Area of sample}} = \frac{\text{Concentration of sample}}{\text{Area of sample}}$$

Statistical Analysis

Two-way Anova analysis was performed in Excel.

Results

Commercially available JH III was analyzed for ESI-MS studies as an internal standard. The limit of detection and quantification was set as 15 minutes and 2-4 ng/10µl respectively (Fig 3.1A). Primarily abundant $[M+H]^+$ ions were noted in JH III The visual inspect of commercially available JH III at m/z 267 are shown in Fig 3.1B. There are prominent 6 ions *m/z* 147,189,217,235, 249 and 507. Following gradient: 0–1 min 60% methanol,1–4 min 60%–80% methanol, 4–6 min 80% methanol, 6–9 min 80%–90% methanol, 9–18 min 90%–100% methanol was used. From the total ion chromatogram, the time of retention was found as 11.58 min and the area of the peak was 268091.

Samples of hemolymph were collected from both control and Plumbagin-treated adult male and female *Dysdercus cingulatus* after 24,96 and 168 hours. As is observed that laboratory-reared *Dysdercus* survived only for 7 days. The collection and loading of the sample were carried out as mentioned in an earlier section.

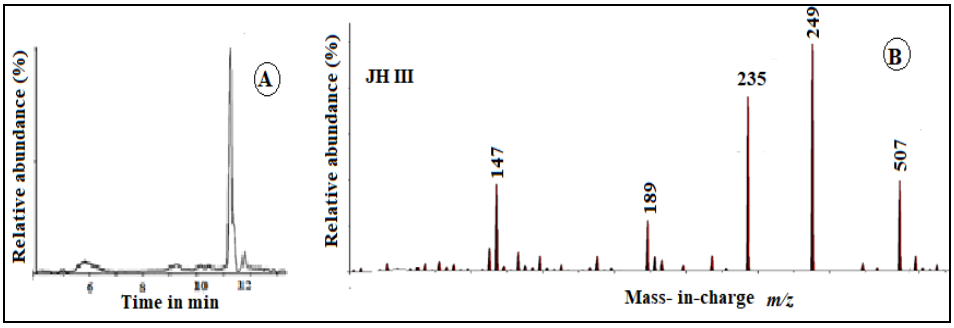

Fig 1: A. Retention Time and B. ESI-MS spectra of the standard JH-III

Haemolymph analysis in the control group of *Dysdercus cingulatus* (Males): Visual inspects of hemolymph samples of male *Dysdercus cingulatus* showed 16 ions *m/z* 41, 91, 110, 120,147, 162, 189, 193, 217, 235, 249, 252, 345, 461, 507 and 551(Fig 3.2B). It was noted that the retention time was 11.11 min and the area of prominent peak was 642 in the control group after 24 hours.

There was no difference noted in the mass of ions and retention time of JH in hemolymph samples after 96 and 168 hours when compared with 24 hours. Interestingly, it was seen that there was a slight increase in the area of the peak after 96 hours in the control group which was found at 714 while the peak area after 168 hours was found at 619. The calculated JH was 2.99 ng/10ml, 3.33ng/10 µl. and 2.89 ng/10µl after 24, 96 and 168 hours respectively.

Haemolymph analysis in the Treated group of *Dysdercus cingulatus* (Males): With all three post-treatment periods (24, 96, and 168 hours) for both the 10 and 12 µg/ml Plumbagin, a total of 14 ions were noted against 16 of the control group in hemolymph samples of male *Dysdercus cingulatus* (Fig 3.2C). Mass of ion *m/z*345 and 461 were missing. There was no difference seen in the retention time.

Immediately after 24 hours of 10µg/ml Plumbagin treatment, in males, the hemolymph samples showed a reduction by 1.9% in total absorption area when compared with control group males of the same. The concentration of JH was found 0.06 ng/10µl less than the control group. In the same manner with 12 µg/ml Plumbagin treatment, after 24 hours of post-treatment period, the maximum absorption area was found as 627 which was 2.39% lesser than the control group and 0.48% less than 10 µg/ml Plumbagin treatment. The JH concentration was found as 2.34ng/10µl.

Increase in the post-treatment period i.e., 96 hours in 10µg/ml Plumbagin-treated males, the maximum absorption peak area was found to be 689 and thus the total calculated hormone concentration was found to be 0.12ng/10µl lesser than the control. With the higher dose of 12µg/ml of Plumbagin, no change in the retention period was noted when compared with the control group. Further reduction in total absorption area by 6% was noted as compared to the previous post- treatment period and was approximately 10% less than the control group for the same post-treatment period. Despite this, it was seen that there was a linear increase in the area of absorption and concentration of JH in the control and treated groups.

After 168 hours of post-treatment period, with 10 and 12µg/ml Plumbagin treatment, further reduction in absorption area by 4.04% and 14.06% respectively was noted when compared with the control group. The JH concentration in10µg/ml dosage was 2.77ng/10µl while that of 12µg/ml was 2.48ng/10µl.

Haemolymph analysis in the control group of *Dysdercus cingulatus* (Females): Female *Dysdercus cingulatus* (Fig3.3)**:** The juvenile hormone in the hemolymph of female *Dysdercus cingulatus* after 24 hours of post-treatment period showed 14 ions differing from the background. The mass of ions were *m/z* 41, 63, 91, 120, 140, 147, 160, 189, 193, 217, 235, 251, 249 and 507 (Fig.3.2D). The total ion chromatogram showed a retention time of 11.09 min which happens to be 2 seconds less than the hemolymph sample from male individuals of the same age.

In treated females, 13 ions were seen in visual inspections. *m/z 91* was absent (Fig 3.2E). The total area of absorption peak was found as 748 in the haemolymph sample of control group females after 24 hours. The concentration of haemolymph was 3.49ng/10 µl. During the next post-treatment period (96 hours), there was a 16.84 % rise in the JH in female haemolymph and 4.07 ng/10 µl. With the highest post-treatment period in the study (168 hours) there was a decline seen in the total area of absorption peak (by 173) and also the concentration of JH dropped by 24.68%.

For all the three post-treatment periods in Plumbagin treated female *Dysdercus cingulatus*, a total of 13 ions were noted differing from the background against 14 of the control group. Mass of ion m/z 63 was missing. After 24 hours of treatment, a 39.17 % of reduction in the total area of absorption was noted in the haemolymph sample of 10µg/ml dosage. The JH concentration was 2.12 ng/10 µl which was less than the control group. Similarly, a further 44.39 % reduction in the absorption area was found with a 12µg/ml dosage and the JH concentration was 1.94 ng/10µl.

With the increase in the post-treatment period of 96 hours,10µg/ml dosage showed the presence of 50.46% lesser absorption area when compared with the control group of the same age. and therefore, the JH concentration was found as 2.02 ng/10µl when compared with the control group of the same PTP. Similarly, with the same post-treatment period, in 12µg/ml Plumbagin treatment, a 59.03% reduction in the peak area of absorption was noted. Thus, here 1.67 ng/10ml of JH was obtained.

Finally, after 168 hours of treatment in females with 10µg/ml of Plumbagin dosage, further reduction in the total area of absorption was noted. 57.06% less area of absorption was found than in the control and the total JH concentration was found as 1.04 ng/10µl of haemolymph sample. With the same post-treatment period, but with a higher dose

(12µg/ml) of Plumbagin, the reduction in the peak area of absorption was found to be 66.62% and JH concentration was 1.09 ng/10µl.

In general, there was a higher level of JH noted in females as compared to males of the same groups but was lesser than in control groups. The results of the area of absorption and concentration of JH in the control and the treated group is summarized in Fig 3.3

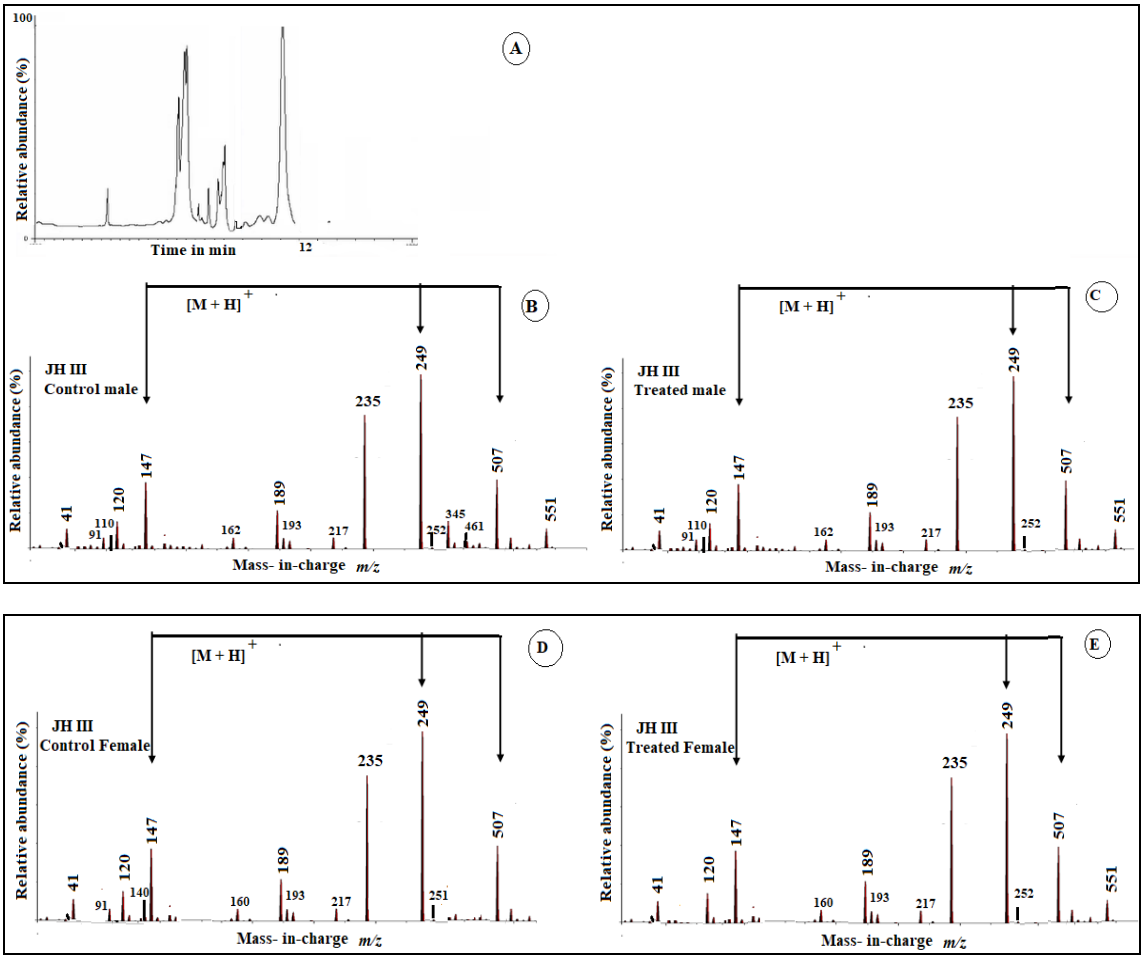

Fig 2: A. Retention time of JH Hormone. B and C: ESI-MS spectra of the JH in the haemolymph of control and treated male *Dysdercus cingulatus.* D and E: ESI-MS spectra of the JH in the haemolymph of control and treated female *Dysdercus cingulatus.*

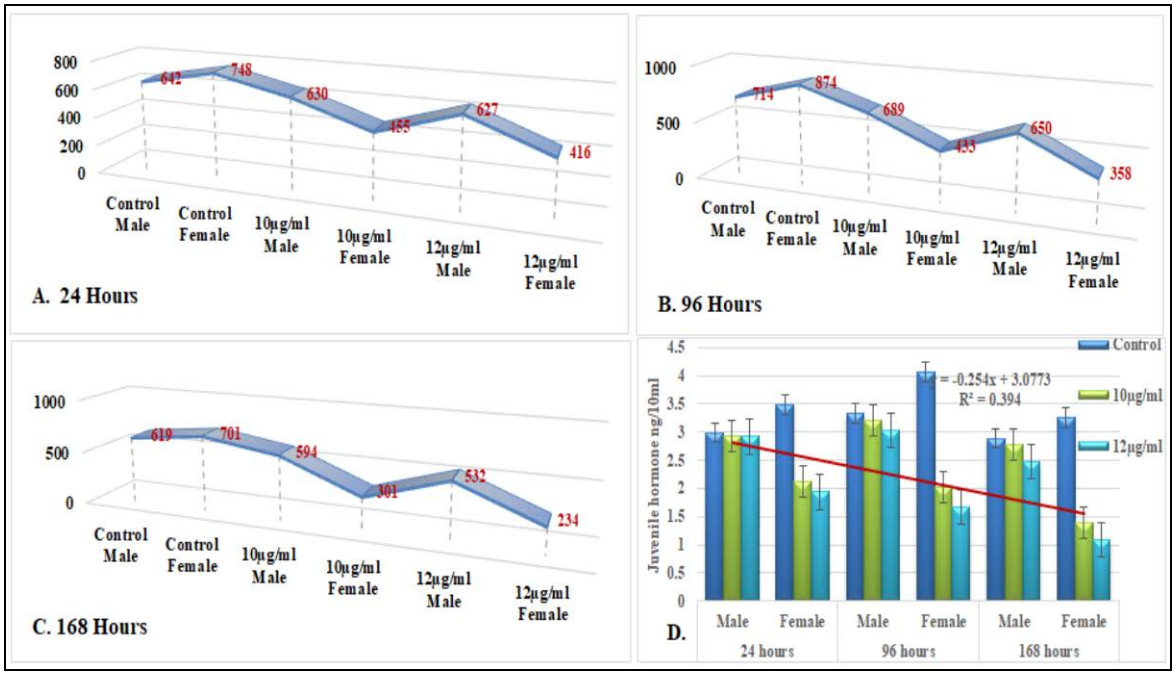

Fig 3: A-C Total area of absorption of prominent peak in haemolymph sample of control and Plumbagin Treated *Dysdercus cingulatus*. D. Total Juvenile hormone concentration in the different post-treatment periods in control and Plumbagin treated *Dysdercus cingulatus.*

ANOVA Analysis

The p-value is 0.013. As this is less than 0.05, it could be stated that the effect of Plumbagin is statistically significant. So, there is a significant difference among the different doses of Plumbagin.

Table 2: Summary of two way ANOVA analysis

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	0.24223	2	0.12112	6.917	0.013002	4.10282
Columns	0.70707	5	0.14141	8.07615	0.002757	3.32583
Error	0.1751	10	0.01751			
Total	1.1244	17				

Discussion

In adult insects, stimulation of yolk production in females and stimulation of the male accessory glands to produce the proteins of seminal fluid and spermatophore is under the influence of JH. JH secretion by the corpus allatum is regulated by two neurohormones, allotropism and allatostatins. The allatotropins stimulate secretion while allatostatins inhibit the production of JH hormone. Splitting the nerve connections or extirpating the neurosecretory cells results in the loss of control [12, 13, 14]. JH is cleared from the hemolymph by JH esterase, which selectively cleaves the methyl ester inactivating the hormone [15, 16]. JH being greatly lipophilic is made soluble by JH binding proteins (JHB) to facilitate transportation to the target sites and also protect it from degradation from nonspecific esterases [17].

In the present study, there is a dose-dependent effect observed in all the treated groups. Also, with the increase in the post-treatment period, there is a relative decrease in the amount of JH noted when compared with the control group. This can be correlated with the reproductive cycle in the life cycle of insects in both male and female *Dysdercus*. As these adults enter into the second and third cycle of the reproductive phase after 96 and 168 hours, an increase in JH concentration in the haemolymph is observed. A similar observation was noted in *Heliothes virescens* during normal reproduction and oviposition [18]. According to studies carried out on adult cabbage looper, *Trichophusia ni*, it is seen that levels of JH titer affect the regulation of egg development [19].

Moreover, earlier studies of Plumbagin, Juglone, and Menadione revealed the fact that these compounds act as chemo sterilants and also adversely affect fecundity, and fertility, and are also known to increase the sterility index in *Dysdercus koenigii* [8]. Now these facts can be used to co-relate the decrease in the JH titer in the Plumbagin-treated *Dysdercus* and its role in being a chemosterilent. This decline in JH could be due to either the inactivation of hormones [15,16] or the degradation of JH from certain nonspecific esterases. There are also chances where it can be stated that a low concentration of Haemolymph could be due to low JHBP (Juvenile Hormone Binding Protein) and probably results in uneven distribution of JH in the body. The reverse is also important for the tissue clearance of JH and its degradation in the haemolymph [20].

For further studies, understanding the nature of binding proteins and their role in transportation is required.

According to Cusson and Palli 2000, interfering with JH endocrinology requires altering the juvenile hormone titer, which means we either artificially increase JH titers at stages of development when titers are normally low or artificially reduce JH titers at stages of development when titers are normally high [21]. This strategy will serve as an integrated pest management tool.

Conclusion

Juvenile hormone research renders new opportunities for the development of integrated pest management strategies targeted at the disruption of juvenile hormone titer.

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