EFFECTS OF TWO INSECT GROWTH REGULATORS ON THE PROTEIN LEVEL AND RNA INTENSITY IN THE LARVAE OF CAMEL NASAL BOT FLY, *CEPHALOPINA TITILLATOR* (CLARK, 1797) (DIPTERA: OESTRIDAE)

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INTRODUCTION

Cephalopina titillator (Family: *Oestridae*) is a serious pest for camels because it is an obligate parasite. *C. titillator* has two generations during the year. The flies deposit their larvae in the nasal cavity of camels where the larvae cause extensive damage of nasopharyngeal tissues before leaving the infested camel to pupate in sand (Zumpt, 1965 and Derhalli *et al.*, 1989). Recently, it was found that the infection by larvae of the nasal bot fly causes changes in neurotransmitters of serum and different brain regions and also decreases thyroid hormones of the thyroid gland (Al Sagair *et al.*, 2004 and El Bassiony *et al.*, 2004)

Controlling this serious pest was achieved by using the systemic organophosphates (Ashetova, 1987 and Ergaliev *et al.*, 1987), especially ivermectin which proved to be effective against *C. titillator* (Lumsden *et al.*, 1992 and Sharma, 1992). Disadvantages of using ivermectin are the lengthy preslaughter withdrawal period and that it should not be used in lactating animals (Jackson, 1989).

Use of conventional chemical insecticides in controlling the camel nasal bot fly carries the risk of ecosystem contamination. No studies have been published on using insect growth regulators (IGRs) against oestrid flies although IGRs were classified as biorationals and offer an alternative to conventional insecticides (Fox, 1990). The use of IGRs during insect development, usually, results in morphological and physiological abnormalities, so they are particularly suited for integrated pest management programs (Sharma, 1992). IGR (dicyclanil) had been successfully used

in the prevention of screwworm infestation in cattle castration wounds (Anziani *et al.*, 1998).

Previous studies showed that pyriproxyfen is a juvenile hormone analogue with relatively low mammalian toxicity (Yokoyama and Miller, 1991). It was previously investigated on order Diptera. It proved to be highly active against Family Muscidae as treatment either by dipping in aqueous solution or by topical application (Kawada *et al.*, 1992 and Bull and Meola, 1993).

Chlorfluazuron is an insect growth regulator proved to be effective as chitin synthesis inhibitor against the larvae of several species of Diptera (Hashizume, 1988). Anti-chitin agents were previously used on blow fly larvae and they inhibited chitin production in the case of myiasis producing flies (Subramanian, 2003).

The present study is undertaken in order to evaluate the effectiveness of the two IGRs, pyriproxyfen and chlorfluazuron, against *C. titillator*. Since this insect is an obligate parasite, no studies can show the effect of IGRs on its life cycle, so biochemical and molecular measures will give us a real picture about the effect of these IGRs on *C. titillator*.

MATERIAL AND METHODS

3rd instar larvae of the camel nasal bot fly, *Cephalopina titillator*, were collected from several camels slaughtered in Cairo, Egypt as described by El Bassiony (2004). The partial rearing of *C. titillator* larvae in the laboratory was carried out according to El-Moursy *et al.* (1993).

Insect Growth Regulators (IGRs) Pyriproxyfen and chlorfluazuron were used in this study. Pyriproxyfen is known as juvenile hormone analogue, whereas chlorfluazuron is a chitin synthesis inhibitor.

Third instar larvae of *C. titillator* were exposed to progressive concentrations of pyriproxyfen or chlorfluazuron. Both IGRs were dissolved in acetone in preliminary tests to determine the range of used concentrations. They were topically applied along the dorsum of the larvae with a dosage 5µl/ larva, using micropipette (socorex 841).

Five groups, 20 larvae per each, were used as replicates for every concentration of both IGRs. Also, five acetone-treated groups (each of 20 larvae) were used as untreated control.

The mortality percentages were recorded after 48 hours and corrected to normal mortality (Abbott, 1925). Data were subjected to probit analysis based on the works of polo-pc (Robertson *et al.*, 1980) and LC_{50} values along with slope and regression lines were determined according to Finney (1971).

Biochemical and Molecular Investigations:

Healthy apparent 3rd instar larvae were topically applied along the dorsum of the larvae with LC_{50} of pyriproxyfen or chlorfluazuron, for further biochemical and molecular investigations.

Total protein concentration of the haemolymph and midgut of control and treated larvae was determined 48h post-treatment using Folin phenol reagent according to the method of Lowry *et al.* (1951). While sodium dedocyl sulphate polyacrylamide gel electrophoresis was used to estimate changes in molecular weight of soluble protein in haemolymph and midgut of controlled and treated larvae of *C. titillator*. Samples from the three groups (control, treated with LC_{50} of pyriproxyfen and treated with LC_{50} of chlorfluazuron) were measured for soluble protein pattern as described by El Bassiony (2001).

Statistical analysis between both control and treated larvae was assessed from Dice's similarity coefficient (Dice, 1945). Similarity coefficient (S) value of 1.0 denotes complete identity in the electrophoretic profile of both groups, while a value of < 1.0 indicates a variation in the identity profile between the two compared samples.

RNA pattern of haemolymph and midgut were detected according to Hassab El-Nabi *et al.* (2001). This procedure was applied to estimate changes in optical density between control larvae and those, treated by pyriproxyfen or chlorfluazuron.

Susceptibility test data and total protein concentration were represented in tables as mean ± standard error. Differences between means were statistically analyzed by using one-way analysis of variance ANOVA and F-test followed by Tukey's multiple range test using the Statistical Package for the Social Sciences (SPSS) version 10.

RESULTS AND DISCUSSION

Susceptibility Test to Studied IGRs:

Based on LC_{50} values for both IGRs, chlorfluazuron achieved superior toxic efficacy against larvae compared with pyriproxyfen. The LC_{50} value of chlorfluazron was 310.07 ppm, whereas, the LC_{50} of pyriproxyfen was 967.53 ppm (Table 1). These results are in agreement with Abdel-Aal (1996) and Shonouda *et al.* (2000), who found that chlorfluazuron was more toxic than pyriproxyfen, when both IGRs were tested against larvae of *Agrotis ipsilon* and *Spodoptera littoralis*, respectively.

Statistically, the difference between the LC_{50} values of both studied IGRs was very highly significant ($P<0.0001$) as indicated by non-overlapping in the respective 95% fiducial limits.

TABLE (I)
Susceptibility of the third larval instar of *C. titillator* to IGRs.

Insect growth regulators (IGRs)	LC_{50} (ppm)	95% Fiducial limits (ppm)		Slope ± S.E
Pyriproxyfen	967.53	840.76	1109.99	2.61 ± 0.33
Chlorfluazuron	310.07	270.27	356.82	2.12 ± 0.59***

*** Very highly significant at $P<0.0001$

Effect of Pyriproxyfen and Chlorfluazuron on Protein concentration:

Total protein:

Data indicated that treatment with LC_{50} of pyriproxyfen induced a very highly significant decrease ($F=85.19$, $P<0.0001$) in total protein level of haemolymph of treated larvae (Table, 2). This can be evidently observed from the value calculated as a percentage of change, which was found to be -53.28%. However, with the treatment of LC_{50} of chlorfluazuron, the total protein level of haemolymph showed a significant decrease ($F=7.18$, $P<0.01$). The value (expressed as a percent of change) was -11.36%. Furthermore, the total protein content of midgut exhibited a very highly significant decrease of -75.81% calculated as percentage of change ($F=108.18$, $P<0.0001$) in larvae treated with LC_{50} of pyriproxyfen. In contrast, a non-significant decrease were observed in the content of the midgut total protein of the third instar larvae of *C. titillator* after chorfluazuron treatment ($F=0.92$, $P>0.05$). The value (expressed as a percentage of change) was 3.10%. Our findings are in accordance with Soltani and Mazouni (1992), Abdel-Aal

(1996), Shaurub *et al.* (1998), Shoukry and Hussien (1998) and Mittal & Navpreet (2000).

TABLE (II)

Effects of IGRs on the total protein content of haemolymph and midgut of the third larval instar of *C. titillator*

Tissue	Treatment	Mean ± S.E.	percentage of change
Haemolymph	Pyriproxyfen	7.92 ± 0.26	—
	Chlorfluazuron	3.70 ± 0.21	- 53.28***
	Control	7.02 ± 0.31	- 11.36*
Midgut	Pyriproxyfen	6.45 ± 0.27	—
	Chlorfluazuron	1.56 ± 0.28	- 75.81***
	Control	6.25 ± 0.20	- 3.10

* Significant at P<0.01

*** Very highly significant at P<0.0001

Changes of protein content in 3rd larval instar of *C. titillator*, under the effect of IGRs, may reflect a diversion of energy under the toxic effects of pyriproxyfen and chlorfluazuron. Firling (1977) discussed the changes of the protein content in the haemolymph of developing stages of *Chironomus tetans* and its involvement in metabolism and concluded that it probably reflected the balance between synthesis, storage, transport and degradation of structural and functional protein which is a response to particular physiological conditions. Also, the decrease recorded in the protein level, especially in the larvae treated with pyriproxyfen, may be due to the decrease in the rate of protein biosynthesis under the effect of IGRs (Patel and Madhaban, 1969).

Protein pattern (total protein fractionation):

On electrophoregrams, the protein fractions were numerated according to increasing electrophoretic mobilities. They usually start from fraction of higher molecular weight (103.82 KDa in control haemolymph and 100.83 KDa in control midgut).

The soluble protein fractionation (for control larvae, treated larvae with LC_{50} of pyriproxyfen and treated larvae with LC_{50} of chlorfluazuron), which were analyzed on 10% SDS-PAGE, indicated that, the haemolymph protein was separated into 20 different fractions (Table 3), while the midgut protein was separated into 19 different fractions (Table 4). The relative front ranged from 0.04 to 0.92 for control haemolymph and from 0.05 to 0.96 for control midgut. The number of bands recorded in control haemolymph and control midgut was 16 and 17 bands

respectively. Mustafa (2003) detected 12 protein bands in salivary gland of 3rd larval instar of *C. titillator*.

TABLE (III)

Electrophoretic scanning pattern of haemolymph proteins extracted from control and treated 3rd larval instar of *C. titillator* by pyriproxyfen and chlorfluazuron

Fraction number	Optical density			M.W. in KDa			Relative front (Rf)			Relative percent		
	Control	Treated with pyri	Treated with chlor	Control	Treated with pyri	Treated with chlor	Control	Treated with pyri	Treated with chlor	Control	Treated with pyri	Treated with chlor
1	0.63	0.69		103.82	103.82		0.04	0.04		1.27	5.24	
2	0.60			102.19			0.05			3.06		
3	0.68		0.44	97.66		97.51	0.07		0.09	1.87		1.02
4	0.70	0.68		93.13	91.66		0.10	0.11		6.93	4.72	
5	0.65		0.54	83.91		81.74	0.16		0.19	3.93		19.40
6	0.62		0.52	77.69		70.88	0.20		0.22	3.79		11.60
7	0.60		0.44	70.16		66.81	0.26		0.23	3.61		3.50
8	0.45		0.33	67.82		58.24	0.28		0.29	5.89		3.71
9	0.78	0.76	0.26	54.14	53.94	52.31	0.43	0.43	0.43	17.20	12.10	3.09
10	0.55	0.55	0.21	47.53	47.71	48.91	0.52	0.52	0.53	2.81	2.62	1.80
11	0.45	0.46	0.15	44.19	43.55	44.38	0.56	0.57	0.57	3.46	2.07	1.60
12		0.47	0.15		39.60	38.22		0.60	0.60		3.47	1.70
13	0.43	0.52	0.15	34.34	34.09	35.16	0.65	0.65	0.64	5.20	4.11	1.40
14	0.24	0.43		28.31	30.89		0.71	0.69		1.38	2.10	
15		0.37	0.14		26.06	25.54		0.72	0.73		2.13	1.90
16	0.41		0.16	19.45		20.80	0.77		0.79	2.96		1.07
17	0.31	0.40	0.12	17.98	17.32	17.24	0.81	0.82	0.85	1.80	2.02	1.09
18		0.40			16.68			0.84			1.26	
19		0.41	0.11		14.22	8.88		0.90	0.94		2.24	1.80
20	0.36	0.43	0.14	13.74	13.53	8.21	0.92	0.92	0.95	2.48	2.53	1.80
Sum										67.64	46.61	56.48
(S)											0.45	0.63

pyri = pyriproxyfen Chlor = chlorfluazuron (S) = the similarity coefficient

In the present study, it can easily be noticed that protein fractions 9,10,11,13,17 and 20 were permanent in haemolymph of both control and treated groups, while fractions 6,9,11,12,15,17 and 19 were permanent in midgut of both control and treated groups (Fig 1, 2 and 3).. However, these permanent fractions showed changes in their relative percentages and intensities (optical densities) between control and treated tissues. The change in the relative percentages and intensities of the permanent fractions can be referred to the material treatment and

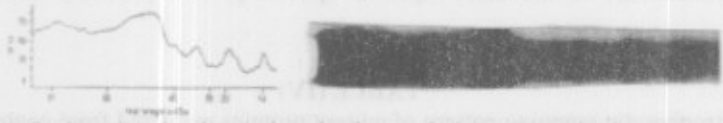

Fig (1): Densitometric tracing of protein electrophoretic pattern for the control haemolymph

Fig (2): Densitometric tracing of protein electrophoretic pattern for treated haemolymph by pyriproxyfen

Fig (3): Densitometric tracing of protein electrophoretic pattern for treated haemolymph by chlorfluazuron

Fig (4): Densitometric tracing of protein electrophoretic pattern for the control midgut

Fig (5): Densitometric tracing of protein electrophoretic pattern for treated midgut by pyriproxyfen

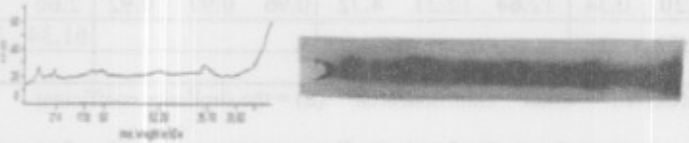

Fig (6): Densitometric tracing of protein electrophoretic pattern for treated midgut by chlorfluazuron

also characteristic to the insect specific species protein (Shoukry and Hussien, 1998).

TABLE (IV)

Electrophoretic scanning pattern of midgut proteins extracted from control and treated 3rd larval instar of *C. titillator* by pyriproxyfen and chlorfluazuron

Fraction number	Optical density			M.W. in KDa			Relative front (Rf)			Relative percent		
	Control	Treated with pyri	Treated with chlor	Control	Treated with pyri	Treated with chlor	Control	Treated with pyri	Treated with chlor	Control	Treated with pyri	Treated with chlor
1	0.17		0.19	100.83		102.48	0.05		0.06	2.53		3.00
2	0.20		0.19	96.79		93.15	0.08		0.10	1.44		4.11
3	0.24			93.12			0.10			3.15		
4	0.16			78.23			0.21			2.72		
5	0.16		0.20	69.35		66.20	0.28		0.27	3.01		3.10
6	0.43	0.13	0.20	56.30	58.61	54.41	0.41	0.38	0.31	11.60	4.72	3.14
7	0.19		0.19	51.16		51.25	0.47		0.44	2.13		4.05
8		0.10	0.18		48.49	44.68		0.51	0.51		2.05	3.49
9	0.27	0.11	0.18	44.68	46.49	40.49	0.56	0.54	0.54	5.40	3.45	3.69
10		0.12			37.99			0.62			2.34	
11	0.21	0.13	0.20	35.28	33.70	35.98	0.65	0.67	0.64	3.30	3.59	3.52
12	0.22	0.13	0.18	31.52	30.64	32.66	0.69	0.70	0.70	2.79	2.89	3.67
13	0.21		0.17	28.00		25.17	0.72		0.73	2.18		2.95
14	0.20	0.12		22.37	21.62		0.75	0.76		2.23	2.61	
15	0.29	0.17	0.20	18.17	18.17	18.41	0.81	0.81	0.82	4.53	4.00	2.50
16	0.30	0.16		17.34	17.23		0.83	0.83		3.56	2.76	
17	0.26	0.14	0.19	15.65	14.71	13.85	0.87	0.89	0.86	3.27	3.05	2.88
18	0.24		0.26	14.04		7.08	0.91		0.89	4.84		1.66
19	0.23	0.20	0.34	12.64	12.21	4.72	0.96	0.97	0.92	2.66	1.49	2.21
Sum										61.34	32.95	43.97
(S)											0.47	0.72

pyri = pyriproxyfen Chlor = chlorfluazuron (S) = the similarity coefficient

A number of protein fractions disappeared as a result of treatment with IGRs. Protein fractions reached 13 and 15 fractions in treated haemolymph with pyriproxyfen and chlorfluazuron, respectively; against 16 fractions in control ones. However, in midgut, Protein fractions reached 11 and 15 fractions in treated larvae with pyriproxyfen and chlorfluazuron, respectively; against 17 fractions in control ones. Disappearance of some protein bands may be related to the loss of certain enzymes which inhibited by the two studied IGRs and affected the metabolic processes (Chun *et al.*, 1994 and Shoukry and Hussien, 1998). Our results are in

accordance with the findings of Taha *et al.* (1989); Maruniak *et al.* (1990); Jabbar *et al.* (1991); Begum *et al.* (1993) and Shoukry and Hussien (1998).

The haemolymph and midgut, of both pyriproxyfen and chlorfluazuron treated larvae had a completely different identity of protein compared to control larvae, whereas the similarity coefficient (S) or the degree of identity recorded 0.45 and 0.47 for the haemolymph and midgut of pyriproxyfen treated groups respectively. However, similarity coefficient (S) recorded 0.63 and 0.72 in haemolymph and midgut of chlorfluazuron treated larvae respectively. This means that pyriproxyfen treated groups had a completely different identity of protein compared with control larvae.

Effect of Pyriproxyfen and Chlorfluazuron on Electrophoretic Pattern of RNA:

The intensity of electrophoretic pattern of RNA reflects the activity of gene expression in haemolymph and midgut. The RNA pattern is considered as a sensitive technique to detect any type of stress on living organism, where there is a correlation between gene expression and the effect of pollutants or stress on organism (Hassab El-Nabi *et al.*, 2001). Some chemicals alter gene expression through inhibition or activation of active or silent genes, respectively (Leveau *et al.*, 1999).

Treatment with LC_{50} of pyriproxyfen and chlorfluazuron showed an observable correlation between the treatment with IGRs and the electrophoretic pattern of RNA in haemolymph and midgut of 3rd instar larvae of the nasal bot fly. Data obtained were shown in table (5) and Fig (7a & 7b). Lanes 1, 2, 3 and 4 in Fig (7) represented ladder, haemolymph treated with pyriproxyfen, haemolymph treated with chlorfluazuron and control haemolymph respectively. However, lanes 1, 2, 3 and 4 in Fig (8a & 8b) represented control midgut, midgut treated with pyriproxyfen, ladder and midgut treated with chlorfluazuron respectively.

Analysis of haemolymph in control larvae of *C. titillator* revealed that the released RNA was separated into two main bands. The first band was located at 406 bp. However, upon treatment with IGRs, this band was separated at 497 and 512 bp for the haemolymph of larvae treated with pyriproxyfen and chlorfluazuron respectively. The second band was separated at 122 bp for all tested groups (control and treated ones).

The intensity of RNA (expressed as optical density) in haemolymph, was decreased by treatment with both IGRs. The optical density of haemolymph of control larvae recorded 16.05 and 34.12 for the 1st and 2nd bands respectively (Table

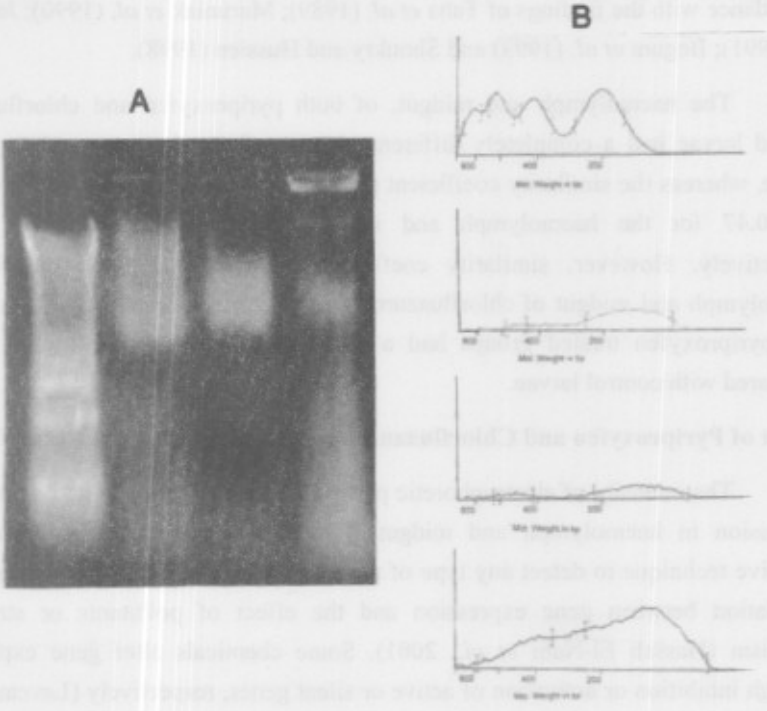

Fig (7a): RNA fingerprint of haemolymph of 3rd instar larvae of *C. titillator* treated with pyriproxyfen and chlorfluazuron. Lane1: ladder, lane2: haemolymph treated with pyriproxyfen, lane3: haemolymph treated with chlorfluazuron and lane4: control haemolymph
Fig (7a): Chart represents lane 1,2,3, and 4 in Fig (7a)

5). However, these intensities were decreased recording of 4.43 and 11.91 in the haemolymph of larvae treated with pyriproxyfen and 4.86 and 9.12 in the haemolymph of larvae treated with chlorfluazuron, for the 1st and 2nd bands respectively.

The electrophoretic pattern of RNA in the midgut of control larvae was separated into three bands located at 406, 122 and 71 bp. However, RNA pattern of the midgut in the treated larvae with both IGRs (pyriproxyfen or chlorfluazuron) was separated into two bands at 406 and 71 bp.

The intensity of RNA bands in the midgut of control larvae recorded 22.53 for the 1st band. The intensity of this band was sharply decreased with values of 5.52 and 7.59 in pyriproxyfen and chlorfluazuron treated larvae respectively. On the other hand, the optical density of the 2nd band, in the midgut, recorded 43.6 in

TABLE (V)

The optical density (OD) of RNA in control and treated haemolymph and midgut of the 3rd larval instar of *C. titillator* using gel pro-program

Nucleic acid	No. of bands	OD of RNA in Haemolymph			OD of RNA in Midgut		
		Control	Treated by pyriproxyfen	Treated by chlorfluazuron	Control	Treated by pyriproxyfen	Treated by chlorfluazuron
RNA	1st band	16.05	4.43	4.86	22.53	5.52	7.59
	2nd band	34.12	11.91	9.12	54.91	43.60	80.14
	3rd band				18.09		

pyriproxyfen treated larvae and recorded 80.14 in chlorfluazuron treated larvae against 54.91 in the midgut of control larvae. The intensity of RNA 3rd band in the midgut of control larvae recorded 18.09, when expressed as optical density. This band was completely disappeared by treatment with both IGRs. The reduction in the intensity of RNA bands could be explained through repression of some genes and starting side effect by IGRs. This conclusion was supported by Zhao *et al.* (1999), who declared that some active genes like IL-2 were repressed by herbicides at low exposure. Moreover, the reduction in RNA level of *C. titillator* larvae treated with pyriproxyfen and chlorfluazuron may be attributed to the direct interference of these two compounds with cell division. Das *et al.* (1964) stated that decrease in RNA synthesis might reflect more increase of an arginine – rich histone that forms a complex with DNA stopping it from acting as a primer for RNA synthesis.

The present results agree with previous work which showed that qualitative reduction in DNA and RNA levels was occurred after treatment of different insects with different treatments (Qadri and Narsaiah, 1978 for *Periplaneta americana* nymphs treated by azadirachtin; Shourub *et al.*, 1998 for *S. littoralis* treated by pyriproxyfen and extract of *Schinus terebinthifolius* and Mittal and Navpreet, 2000 for *Culex sp.* treated by a newly synthesized JHA).

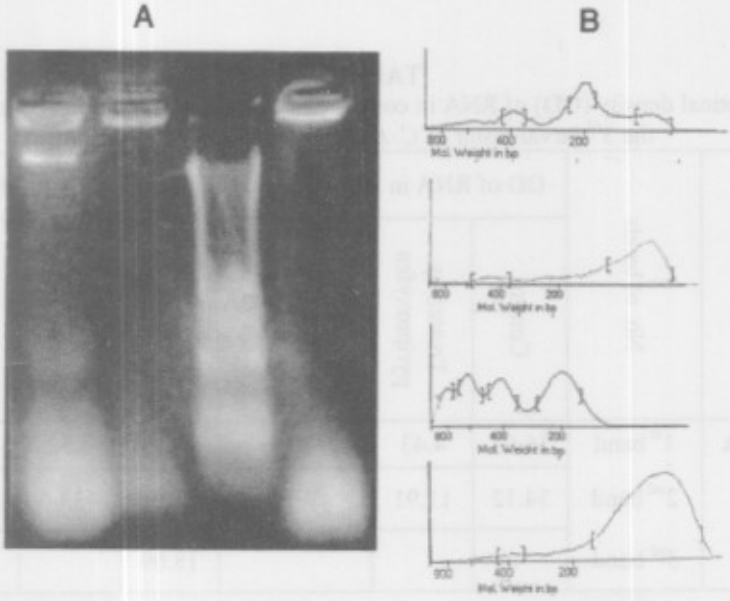

Fig (8a): RNA fingerprint of midgut of 3rd instar larvae of *C. titillator* treated with pyriproxyfen and chlorfluazuron. Lane1: control midgut, lane2: midgut treated with pyriproxyfen, Lane3: ladder and lane4: midgut treated with chlorfluazuron
Fig (8a): Chart represents lane 1,2,3,4 in Fig (8a)

From the above observations, we may suggest that the reduction in protein and RNA in this study after IGRs treatment may be due to the decrease in protein and RNA synthesis.

The results suggested that pyriproxyfen is more effective than chlorfluazuron in controlling *C. titillator* larvae although Chlorfluazuron was found to be more toxic against 3rd instar larvae than pyriproxyfen. Also the two studied IGRs are recommended to be used for the control of this insect after studying their toxicity on camels.

SUMMARY

Pyriproxyfen and chlorfluazuron were topically applied to 3rd instar larvae of the camel nasal bot fly, *Cephalopina titillator*. Chlorfluazuron was more toxic against 3rd instar larvae than pyriproxyfen, whereas, the LC_{50} values were 310.07 and 967.53 ppm for chlorfluazuron and pyriproxyfen, respectively.

The effect of LC_{50} of pyriproxyfen and chlorfluazuron on total protein level, the electrophoretic protein pattern and the RNA intensity of both haemolymph and midgut were determined.

The total protein level exhibited significant decreases in haemolymph and midgut of 3rd instar larvae of *C. titillator* treated with LC_{50} of pyriproxyfen. However, the decrease of total protein level in chlorfluazuron treated larvae relative to controls was significant and non-significant for haemolymph and midgut respectively. The treatment with LC_{50} of pyriproxyfen and chlorfluazuron produced qualitative and quantitative changes in the permanent fractions of the protein patterns in haemolymph and midgut. Moreover, the treated larvae of *C. titillator* had a completely different identity of protein compared to control larvae, whereas, the similarity coefficient (S) recorded 0.45 and 0.47 for the haemolymph and midgut in larvae treated with pyriproxyfen respectively. On the other hand, similarity coefficient recorded 0.63 and 0.72 when it was calculated in haemolymph and midgut for chlorfluazuron treated larvae respectively.

The RNA electrophoretic pattern of haemolymph and midgut of control and treated 3rd instar larvae of *C. titillator* revealed that, the intensity of RNA (expressed as optical density) in haemolymph and midgut was decreased by treatment with both IGRs.

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