African Journal of Biotechnology Vol. 9(35), pp. 5746-5753, 30 August, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 ©2010 Academic Journals

Full Length Research Paper

Cellular energy allocation of pistachio green stink bug, *Brachynema germari* Kol. (Hemiptera.: Pentatomidae) in relation to juvenoid pyriproxyfen

Faezeh Bagheri, Khalil Talebi* and Vahid Hosseininaveh

Department of Plant Protection, College of Agriculture, University of Tehran, Postal code 31587-77871, P.O.Box 4111, Karaj, Iran.

Accepted 30 July, 2010

The purpose of this study is to determine the effects of pyriproxyfen (an insect growth regulator compound) on cellular energy allocation of a heterometabolous insect, *Brachynema germari* Kol. The fifth instar of this insect was treated with 0, 20, 60, 100, 200 and 500 mg l^{-1} formulation of pyriproxyfen (Admiral 10EC) and the amount of energy available (E_a), energy consumption (E_c) and cellular energy allocation (CEA) were compared. The resulting calculated energy reserves (carbohydrate, glycogen, lipid and protein) showed that total lipid amount varied significantly among doses and between each dose and control. Lipid amounts fell as doses grew to 200 mg l^{-1} and then increased. A significant difference was observed in total carbohydrate between each dose and control ($P < 0.05$); however there were no significant differences among doses. The amount of glycogen and total protein and also the amount of energy consumption did not differ significantly from control. Study results showed that pyriproxyfen at low concentrations, raised cellular energy allocation but decreased it at high concentrations.

Key words: Pyriproxyfen, *Brachynema germari*, cellular energy allocation, lipid, glycogen, protein.

INTRODUCTION

Principal processes in energy acquisition and expenditure of an organism are conducted by physiological energetics. Various environmental conditions show different effects on organism's responses such as growth, reproduction and basal metabolism. The effect and mode of action of many toxicants can be elucidated by physiological energetic as it is assumed that exposure to a contaminant will disturb energy allocation in an organism. Cellular energy allocation (CEA) is a rapid and instantaneous method for measuring energy content of an organism (Widdows and Donkin, 1992; De Coen and Janssen, 1997).

Total energy reserve in an insect body as energy available (E_a) is the total amount of energy acquired from available total lipid, total protein, glucose and glycogen content which can be affected by different factors. Energy consumption (E_c) is measured under saturated substrate conditions and changes in its activity have to be measured by altering enzyme production in an organism. In fact, E_c is the activity of electron transport system (ETS). ETS assay can clarify changes in E_c by interaction of the toxicant with the electron transport system (Spicer and Weber, 1991; Oberdorster et al., 1998). Since there is a paradox at the results of energy content assay, CEA ($CEA = E_a/E_c$) technique have been used as a reliable specific energy parameter that can measure the effect of toxicants on different energy sources as well as a marker of the available energy content of an organism. It is an appropriate method to measure CEA from a physiological point of view. This short time assay is based on changes in energy reserves (total carbohydrate, total protein and total lipid) and energy consumption (electron transport activity) (De Coen and Janssen, 1997; Verslycke et al., 2004).

The "metabolic cost" hypothesis proposes that metabolic changes in an organism can be induced by stress

*Corresponding author. E-mail: khtalebi@ut.ac.ir. Tel: 98(261)2224022-24. Fax: 98(261)2238529.

Abbreviations: Ea, Energy available; **Ec,** energy consumption; **CEA,** cellular energy allocation; **ETS,** electron transport system; **IGRs,** insect growth regulators.

agents (e.g.; toxins) and these can cause the rundown of an organism's energy thereby resulting in adverse effects on reproduction and growth (Calow and Sibly, 1990).

Endocrine system can control the performance of an individual by controlling energy metabolism; because all life processes are dependent on energy (Lorenz and Gade, 2009). Third generation pesticides consisted of hormones and their analogues are biological and biochemical agents for controlling insect pests (Williams, 1967; Slama et al., 1974). Insect hormones and their analogues (insect growth regulators) regulate development and growth in insects (Bowers, 1982; Mian and Mulla, 1982; Staal, 1982; Jennings, 1983). Pyriproxyfen is one of the most potent insect growth regulators (IGRs) that mimics the action of juvenile hormones in many biological and metabolic processes. It has low toxicity for mammals and that it was registered in Japan for control of public health pests in 1991 (Miyamoto et al., 1993). Nowadays, this insecticide is used for the control of different pests and many researches have been done on efficiency of pyriproxyfen and other juvenoids (Liu, 2003; Liu and Stansly, 2004). Juvenile hormone analogues, such as pyriproxyfen, have different effects on morphological, physiological and biological characteristics of an organism. They cause the formation of abnormal forms, sterile adults and some other adverse effects on insects (Pedigo, 2002; Dhadialla et al., 1998). Juvenoids can disturb allocation of energy among different vital activities and treated organism may face the deficiency of specific and sufficient energy reserves for normal growth and development.

The pistachio green stink bug, *Brachynema germari* (Kolenati) (Hemiptera: Pentatomidae), has a great importance in pistachio production due to its quantitative and qualitative damages. Serious infestation of nut pistachio by *B. germari* can make it unpleasant or unmarketable. Piercing of the soft-shelled pistachios by the stylets of the stink bug and sucking the nutrients causes epicarp lesion on the hull in the early season (Mehrnejad, 2001) and kernel necrosis in the midseason as well as transmission of a fungal pathogen, *Nematospora coryli* in pistachio nuts (Mehrnejad 2001; Ershad and Barkhordary, 1974). Although cellular energy allocation technique was primarily developed and validated with *Daphnia magna*, it can also be used with other invertebrates and vertebrates (De Coen and Janssen, 1997). As far as the authors are aware there is no published information on the effects of pyriproxyfen on cellular energy allocation in insects, this study reports the effects of pyriproxyfen on energy reservoirs and cellular energy allocation on *B. germari* Kol. (Hemiptera: Pentatomidae).

MATERIALS AND METHODS

Insect collection and maintenance

Adults of the pistachio green stink bug, *B. germari* Kol. (Hemiptera: Pentatomidae) were collected from pistachio orchards in Kerman province, Iran. They were maintained and reared under controlled conditions of 16:8 L: D, 25 ± 2°C and 45 ± 10% relative humidity.

Treatment

The fifth instars (three to four days old) of *B. germari* were treated in different concentrations (0, 20, 60, 100, 200 and 500 mg l^{-1}) of pyriproxyfen. The concentrations were selected so that they caused morphological abnormalities from 10 to 90%. One µl of each concentration was placed on abdominal surface of the insect subjects using a microaplicator (Burkard, UK). They were maintained at rearing condition for three days after treatment and the nymphs were then homogenized in appropriate buffer (0.1 M Tris-HCl; pH 8.5, 15% polyvinilpyrrolidone, 0.55 mg ml^{-1} $MgSO_4$, 0.2%(v/v) Triton X-100) for the subsequent experiments.

Biochemical analysis

Assay for CEA was performed according to van Handel and Day, (1988), Yuval et al. (1998) and Verslycke et al. (2004) with minor modifications. CEA was calculated after determination of E_a and E_c according to the following formula:

$E_a = \sum$ (glucose, total lipid, glycogen and total protein) (mj mg^{-1} wet wt h^{-1})
E_c = ETS activity (mj mg^{-1} wet wt h^{-1})
CEA = E_a/E_c

Assessment of the amount of energy reserves; total lipid, glucose and glycogen, were carried out after homogenization of the individual insects in 400 µl of 2% Na_2SO_4 using a hand-held homogenizer (Glass homogenizer, Kontes, New Jersey, USA). After addition of 1300 µl of chloroform: methanol (1:2, V:V) to the mixture, it was centrifuged at 8000×g for 10 min. Resulted supernatant was used for assessing total lipid and glucose and pellet for glycogen content.

Homogenization buffer for protein and ETS assay was a solution mixture of 0.1 M Tris HCl pH 8.5, 15% (w/v) polyvinyl pyrrolidone, 153 µl $MgSO_4$ and 0.2% (w/v) triton X-100. After centrifugation of the homogenates, the resulted supernatant was used for assessing the amount of total protein and electron transport system activity (Van Handel and Day, 1988; Yuval et al., 1998; Verslycke et al., 2004).

Lipid assay

Fifty µl of the resulted supernatant was pipetted out and dried at 90°C. After 10 min, 50 µl of concentrated H_2SO_4 was added and the mixture was heated at 90°C for 10 min. An aliquot of 15 µl was taken from each replicate and was added to 135 µl of vanillin reagent in each microplate well and shaken for 30 min at room temperature. Optical density was then read at 530 nm using a microplate reader model ELX 808^{TM} Bio-Tek. (Vanillin reagent was made by dissolving 600 mg vanillin in 100 ml distilled water and 400 ml 85% H_3PO_4). Standard curve for lipid assay was plotted using cholesterol as the standard (Yuval et al., 1998).

Glucose assay

Glucose assay was performed by addition of 30 µl of the supernatant to 20 µl distilled water and 100 µl anthron reagent. The sample was then heated in boiling water for 15 min. After cooling, the absorbance was read at 630 nm. Glucose was used as the standard (Yuval et al., 1998).

Glycogen assay

The pellets resulting from centrifuged samples were used for glycogen assay. For eliminating the effects of glucose, pellets were washed by 400 µl 80% methanol, 250 µl distilled water was then added to each sample and heated at 70°C for 5 min. The mixture was centrifuged at 8000×g for 5 min. The anthron reagent was then added to the supernatant. The samples were heated at 90°C for 1 min and then cooled down. The absorbance was read at 630 nm. Anthron reagent was made by dissolving 0.006 g anthron in 3 ml 85% H_2SO_4. Glucose was used as the standard (Yuval et al., 1998).

Protein assay

Protein content of the samples was determined according to Bradford (1973) with bovine serum albumin as standard.

Electron transport activity assay

The mixture of 12.5 µl homogenization buffer, 75 µl buffered substrate solution (0.13M Tris HCl, 0.3% (w/v); Triton X100, pH 8.5), 25 µl of mixture of nicotinamide adenine dinucleotide (NADH) (1.7 mM) and nicotinamide adenine dinucleotide phosphate (NADPH) (250 µM) and 50 µl INT (8 mM iodo nitro tetrazolium; 8mM) was added to 12.5 µl of the sample. Absorbance of the reaction was measured continuously at 400 nm (Van Handel and Day, 1988; Yuval et al., 1998; Verslycke et al., 2004).

E_c calculation

Different components of energy reserves were transformed to energetic equivalents. Equivalent of these reserves are combustion energy which consisted of 17500 mj mg^{-1} glycogen and sugar, 24000 mj mg^{-1} protein and 39500 mj mg^{-1} lipid. E_c was calculated by the following formula based on electron transport activity to equivalent of energy consumption. From a theoretical point of view, formation of 2 µl formazan will use 1 µmol of O_2 (King and Pakard, 1975).

$$mol\ O_2/org.\min = \frac{OD \times V_r\,\mu l \times (\frac{V_h\,\mu l}{V_a\,\mu l})}{(15900 \times 10^6 \frac{\mu l}{mol\ formazan \cdot cm}) \times Optical\ path\ cm \times (2 \frac{mol\ formazan}{mol\ 0_2}) \times \min}$$

Where, V_r = Final reaction volume; V_h = original homogenate (total volume of homogenate); V_a = aliquot of the homogenate (homogenate used in the reaction mixture)

Statistical analysis

Samples preparation was carried out three times and each data had its own three replicates. The statistical software Statgraphics Plus 5.1 was used for analysis of variance (one way ANOVA) and necessary statistical analysis. Calculations were performed using Excel and Sigma Plot software.

RESULTS

A significant decline was observed in the amount of total lipid in treated insects in all concentrations of pyriproxyfen except control. The lowest amount of total lipid was observed at the concentration of 200 mg l^{-1} of pyriproxyfen ($P < 0.01$, df = 5, $F = 35.78$) (Figure 1). On the other hand, pyriproxyfen caused significant increase in total glucose in all concentrations ($P < 0.01$, df = 5, $F = 5.79$), but there was no significant differences among different concentrations (Figure 2). Also, there was no significant changes in glycogen ($P = 0.90$, df = 5, $F = 0.29$) and protein ($P = 0.92$ df = 5, $F = 0.27$) contents in the insects treated with pyriproxyfen (Figures 3, 4). In the study of pyriproxyfen effects on E_a amount, a decreasing trend was observed with increased concentration of pyriproxyfen. Minimum significant amount of E_a was obtained at the concentration of 200 mg l^{-1} ($P < 0.01$, df = 5, $F = 5.17$) (Figure 5). In the study of the ETS activity in the E_c assay, there was no significant difference among all applied concentrations of pyriproxyfen ($P = 0.088$, df = 5, $F = 2.52$) (Figure 6). In contrast to the E_c, CEA was increased as the concentration increased and a maximum CEA was obtained at 100 mg l^{-1}, followed by a minimum at 200 mg l^{-1} ($P<0.01$, df=5, $F=6.77$) (Figure 7).

DISCUSSION

Results of the present research showed that pyriproxyfen caused certain biochemical changes in *B. germari*. Among the energy reserves of the *B. germari* adults, only total lipid and total carbohydrate was significantly affected by pyriproxyfen treatment (Figures 1, 2, 3 and 4).

Insect fat bodies serve as valuable energy reserves that use or store energy reservoirs in the body, depending on the insect's physiological conditions. Lipid turnover in insects is regulated by neuro-endocrine controlled feedback loops (Miranda et al., 2003). Significant decrease in lipid content may be induced by metabolic activity for detoxification process, poor nutrition in the treated *B. germari,* fat utilization or its metabolism by applied juvenoid. Lipid content decrease is accompanied with an increase in fatty acids accumulation in haemolymph (Cymborowiski, 1992). On the other hand, some research showed that juvenile hormone and its analogues influence lipid synthesis rather than lipid consumption. For example, after allatectomy of *Melanopus differentialis* lipids were accumulated in the fat bodies (Cymborowiski, 1992), whereas our results may suggest that juvenile hormone analogues, such as pyriproxyfen, did not cause lipid accumulation in the fat bodies of *B. germari*.

In our experiments, pyriproxyfen did not cause a significant difference in energy consumption. On the other hand, fatty acids resulting from lipid oxidation act as an uncoupling factor in electron transport chain. Thus it can be concluded that ATP cannot be synthesized and the energy will dissipate (Cymborowiski, 1992). Application of pyriproxyfen on *Aulacophora nigripennis* also resulted in an increase in insect respiration rate (Watanabe and Tanaka, 2009).

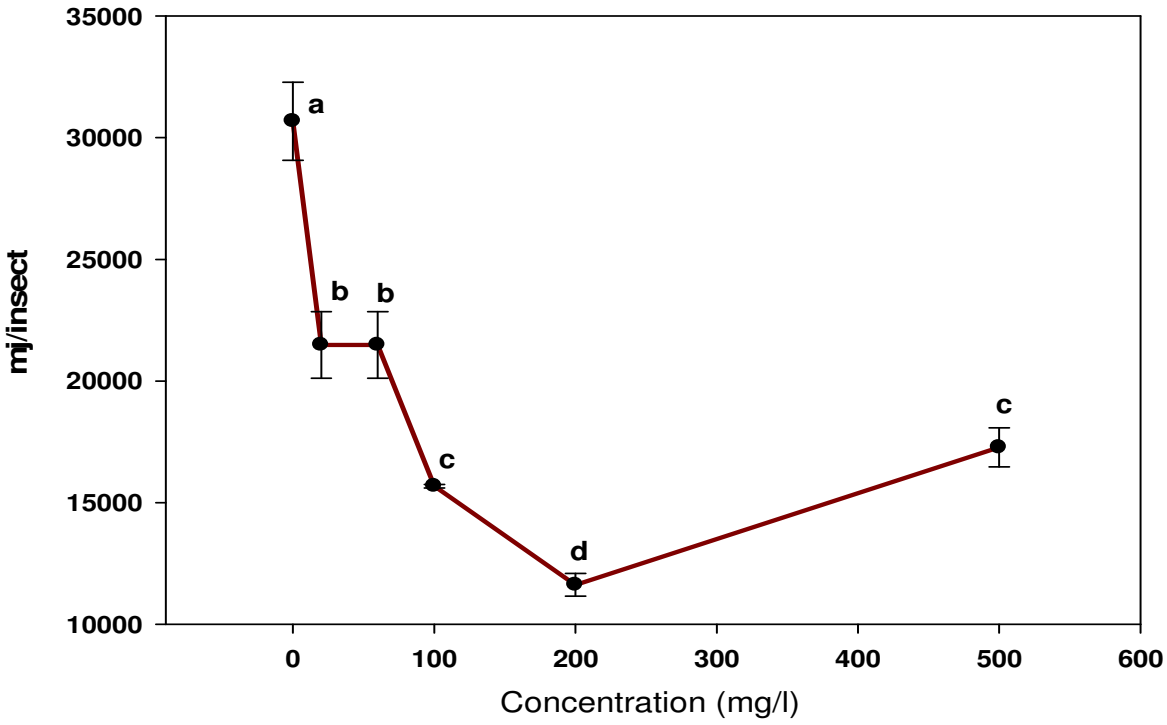

Figure 1. Lipid content in *B. germari* treated with different concentrations of pyriproxyfen. The points labeled with similar letters are not significantly different) $p < 0.05$).

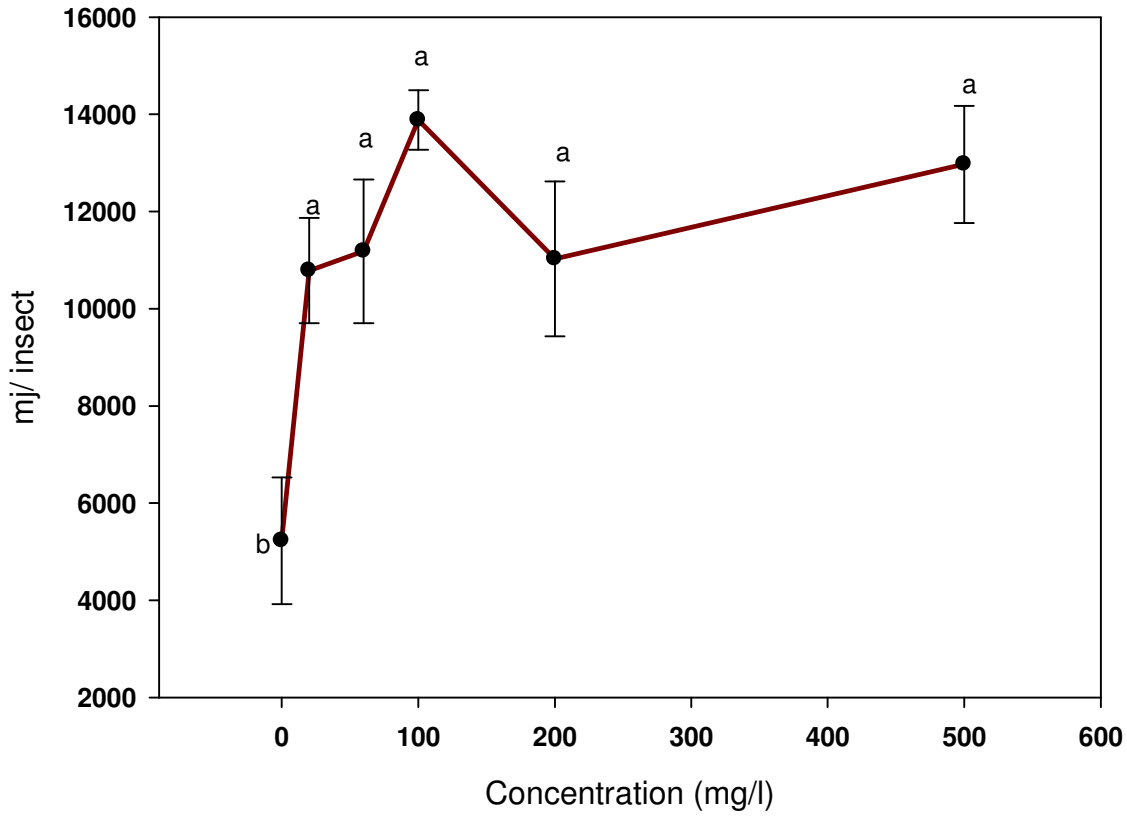

Figure 2. Total glucose content in *B. germari* treated with different concentrations of pyriproxyfen. The points labeled with similar letters are not significantly different ($p < 0.05$).

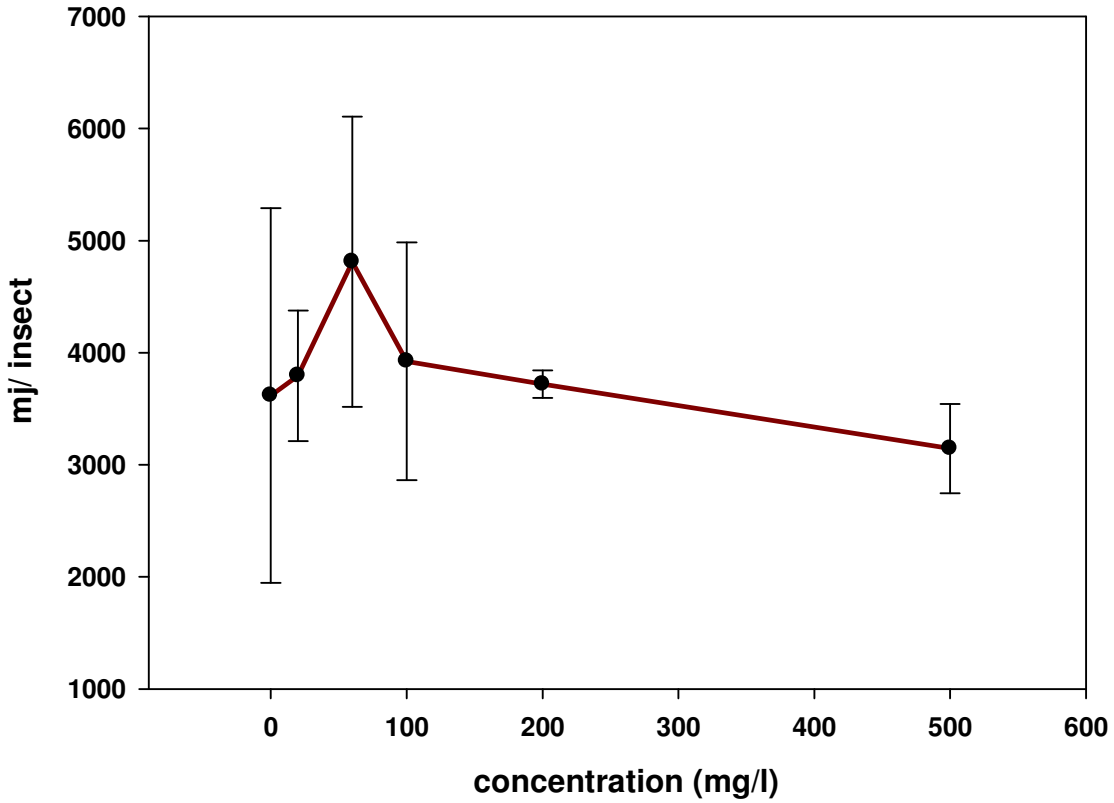

Figure 3. Glycogen content in *B. germari* treated with different concentrations of pyriproxyfen.

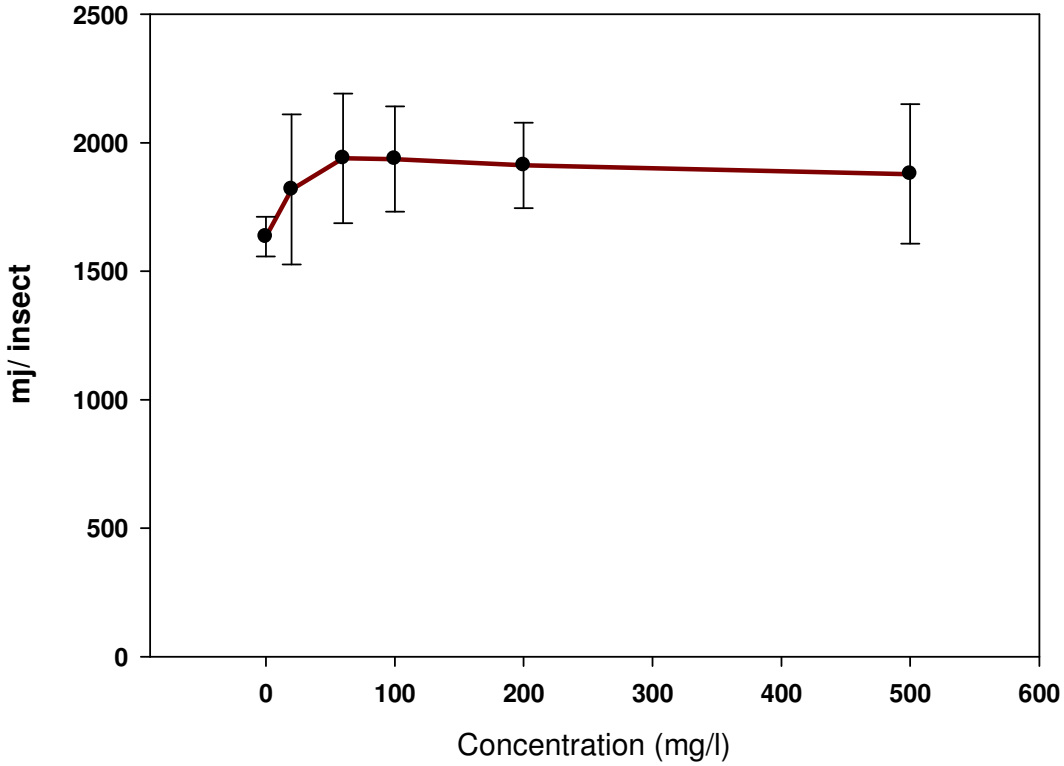

Figure 4. Total protein content in *B. germari* treated with different concentrations of pyriproxyfen.

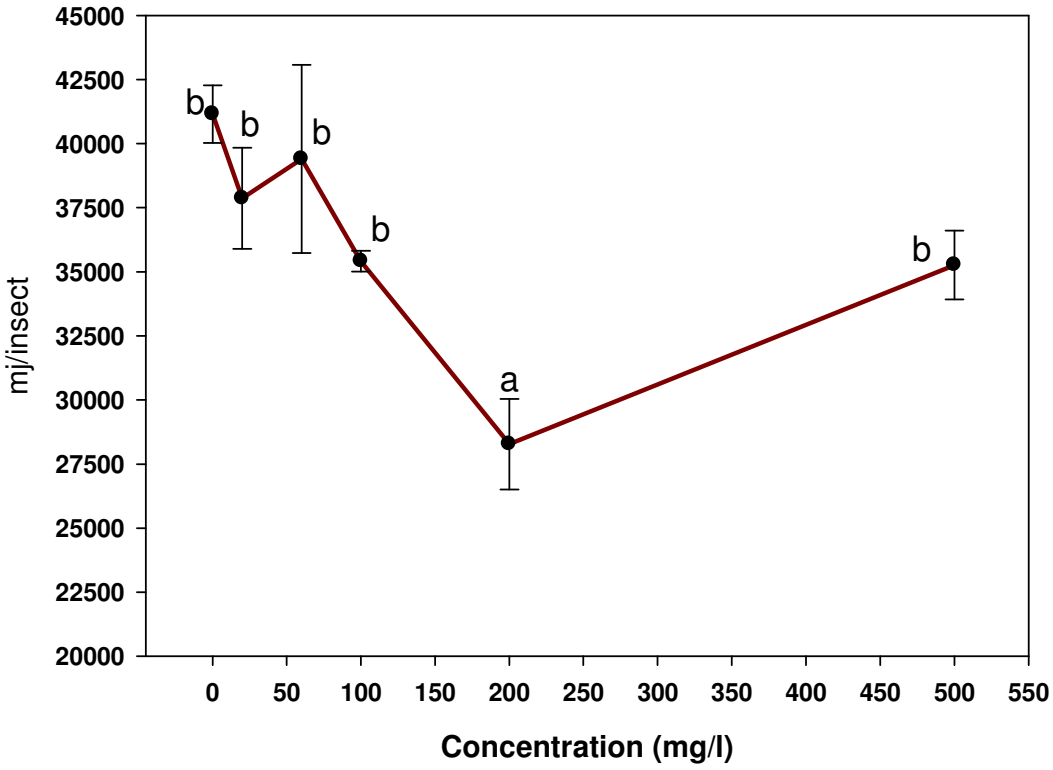

Figure 5. E_a in *B. germari* treated with pyriproxyfen. The points labeled with similar letters are not significantly different ($p < 0.05$).

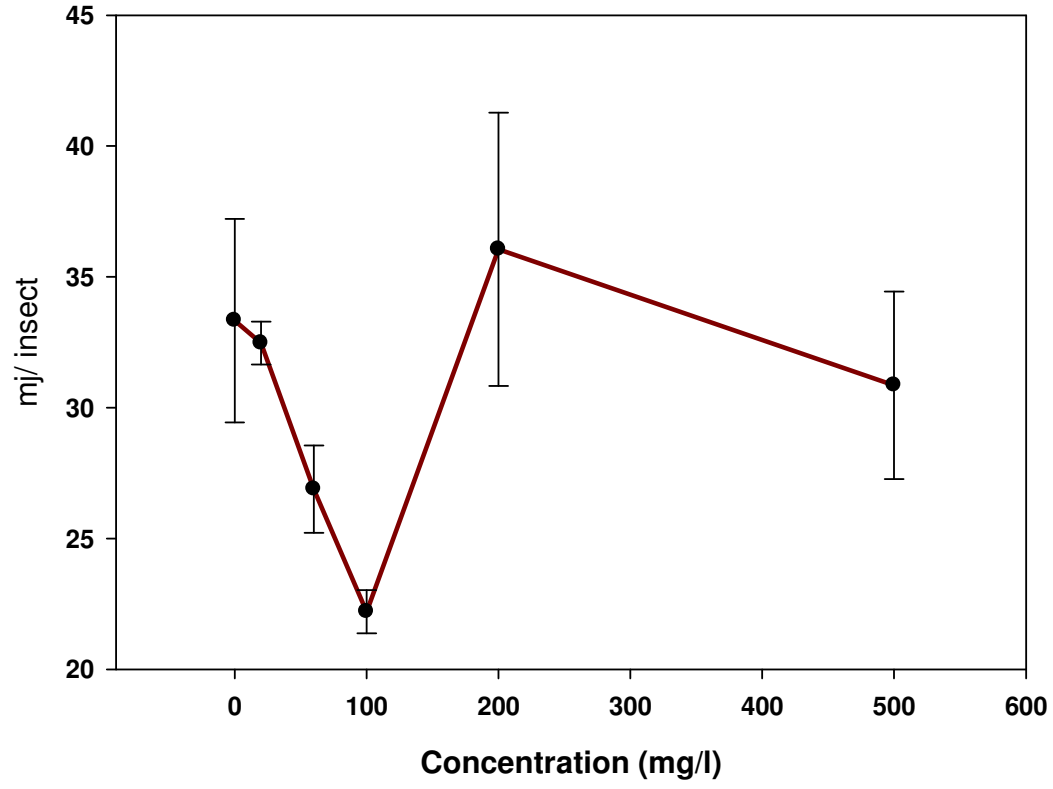

Figure 6. E_c in *B. germari* treated with pyriproxyfen.

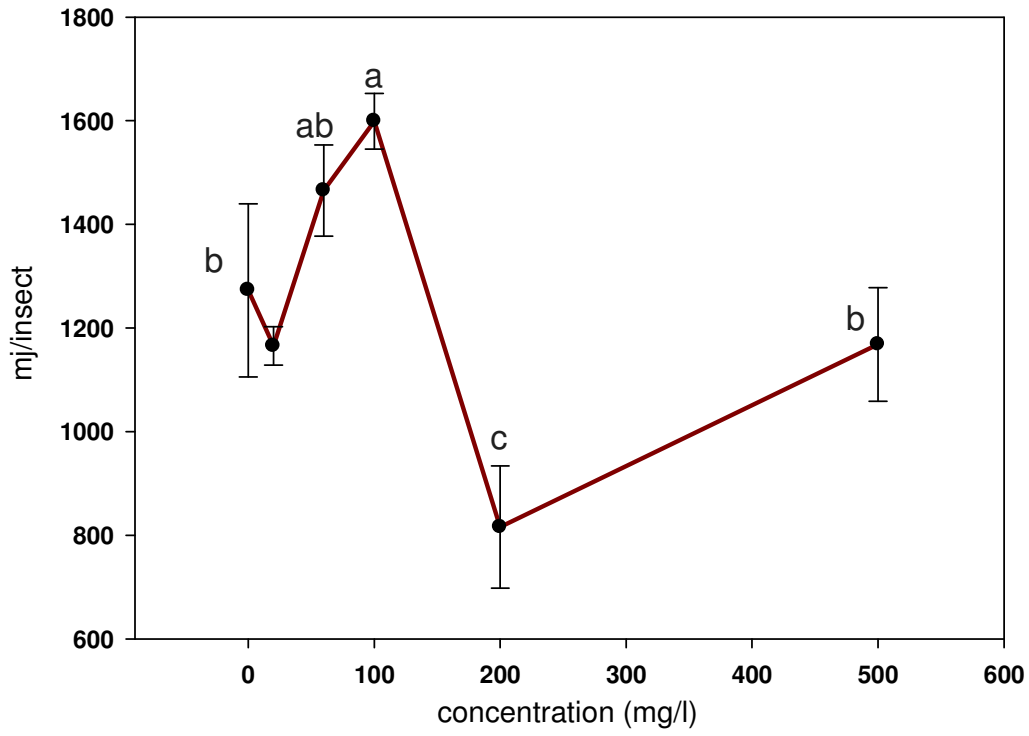

Figure 7. CEA in *B. germari* treated with pyriproxyfen. The points labeled with similar letters are not significantly different ($p < 0.05$).

Depending on insect species, pyriproxyfen may act on energy content in a different way. Cymborowski (1992) has demonstrated that changes in metabolic pathways and not increase in food consumption cause an increase in lipid accumulation. Some scientists have cited the importance of the lipid fraction for developing embryos lipid being one of the vital components of egg formation (Cowgill et al., 1984; Elendt, 1989).

Pyriproxyfen-treated normal and abnormal insects neither produced any egg, nor showed abnormality in their ovaries (unpublished data). So, it seems that the disruption of oviposition can be the result of lipid deficiency but not abnormality.

The results showed that there was no significant decrease or increase in glycogen content. This means that the sugar reservoir, glycogen, was not affected by pyriproxyfen treatment in this insect. Hence, the increase in glucose amounts was not the result of glycogen analysis. Increase in carbohydrate content was suggested doubtfully by Beament et al. (1967) to be the result of carbohydrates transfer from cuticle to haemolymph. In our study also, it seems that carbohydrate content did not convert to lipid, but rather accumulated in haemolymph. The absence of significant difference in carbohydrate content among different treatments means that the lowest dose was enough to maximize carbohydrate content. Therefore, high doses of pyriproxyfen could not increase the carbohydrate content significantly. All biochemical measurements were taken three days after the treatment and the results may depend on the time interval between treatment and biochemical measurements. Treatment of *Bombyx mori* with pyriproxyfen lowered glucose and cholesterol content 24 h after treatment, but these substances showed an increase in some doses after 120 h (Etebari et al., 2007)

Similar to glycogen results, there was no significant difference in protein content among different treatments. One possible reason to this observation may be difference in innate response of treated species; however more experiments are needed before concluding on this observation.

In this experiment, changes in CEA at different doses were consistent with changes in energy reserves, but not energy consumption. As can be seen in Figures 1 and 7, the minimum amount of lipid completely conforms to minimum amount in CEA. On the other hand, due to an increase in carbohydrate amount in 100 mg l^{-1} and no decrease in lipid content in this dose, CEA amount rose in 100 mg l^{-1}. Furthermore significant drops co-occurred at 200 mg l^{-1} dose in the E_a curve and lipid content.

Depending on life stage, age and species of treated insects and the type of pesticide, observed effects will be different. Gadenne et al. (1990) reported fenoxycarb effects varied with dose and timing of application. Elsewhere, the sugar and lipid content in Mediterranean fruit fly depended significantly on the time of the day and insect

activity (Warburg and Yuval, 1997).

Overall pyriproxyfen could affect the cellular energy allocation and energy reserves at some concentrations. As indicated earlier, in this study the determining factors in measured energies were lipid and sugar, in that order. Here, the importance of lipid is obvious in all significant responses. To summarize, lipid content can serve as a marker of dose-dependent effects of pyriproxyfen and likely other juvenoids on this heterometabolous insect.

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