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Effect of Insect Growth Regulators, Temperature and Overwintering on Larvae of Pistachio Leaf White Borer (*Ocneria terebinthina*)

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ABSTRACT

Pistachio leaf white borer, *Ocneria terebinthina* (Lepidoptera: Lymantriidae), is a minor pest of pistachio trees. In this study, effects of two insect growth regulators (pyriproxyfen & chlorfluazuron), temperature and overwintering were investigated on physiology and morphology of the larvae. In chlorfluazuron and pyriproxyfen treatments, longevity of the last larval instars with 20 and 30 days, respectively was significantly longer than the control with 9 days. Most of treated larvae molted to malformed prepupae and supernumerary larvae, respectively. No significant differences in total sugar and lipid were observed between pyriproxyfen, chlorfluazuron and control treatments, but glycogen content in chlorfluazuron treatment (5.78±0.66 mg/g fresh weight) was significantly lower than pyriproxyfen and control treatments (10.62±0.66 & 10.66±0.66 mg/g fresh weight, respectively). Protein content in pyriproxyfen treatment (8.75±0.24 mg/g fresh weight) was significantly higher than chlorfluazuron and control treatments (7.01±0.26 & 7.16±0.24 mg/g fresh weight, respectively). No significant differences in total sugar and lipid of the larvae reared at 25°C and 35°C were observed, but glycogen and protein contents in the larvae reared at 25°C (12.21±0.81 & 8.8±0.48 mg/g fresh weight, respectively) was significantly different from the larvae reared at 35°C (15.89±0.89 & 6.41±0.48 mg/g fresh weight, respectively). No significant difference in total body sugar and protein of non-overwintering and overwintering larvae were observed, but glycogen content in non-overwintering larvae (12.22±0.95 mg/g fresh weight) was significantly lower than overwintering larvae (38.62±1.02 mg/g fresh weight). In non-overwintering larvae, total body lipid (11.13±0.93 mg/g fresh weight) was significantly lower than overwintering larvae (15.06±1.14 mg/g fresh weight). The data suggest that pyriproxyfen, chlorfluazuron, temperature and overwintering significantly affected some physiological aspects of *Ocneria terebinthina* larvae.

Key Words: Chlorfluazuron; *Ocneria terebinthina*; Overwintering; Pyriproxyfen

INTRODUCTION

The pistachio leaf white borer, *Ocneria terebinthina* Strg., is a multivoltine and minor pest of pistachio trees. *O. terebinthina* has been distributed throughout the most of pistachio growing regions of Rafsanjan, which is the main pistachio producing area of the world. This pest passes the winter as fourth larval instar under the loose bark on the trunks of pistachio trees. The young larvae feed on the leaf parenchyma and lower epidermis but older larvae feed on the whole leaf and leave only the mid-veins (Behroozi, 2010).

Most of the insecticides currently used for the control of insect pest of crops are neurotoxic compounds with several adverse and side effects on environment and non-target organisms. In the last two decades, investigations have been focused on the development of compounds that have more selectivity and short residual life. Consequently, a number of insecticides having novel modes of action are developed. These include new class of chitin synthesis inhibitors, juvenile hormone (JH) analogous, ecdysone agonists, neo-nicotinoids and botanical insecticides such as azadirachtin. In view of the above, it is essential to evaluate these new compounds in the laboratory to obtain data on their efficacy as well as their possible use in integrated pest management. Since these molecules have different modes of action and are non-neurotoxic, it is essential to realize their effects on the insect development, reproduction and other physiological processes (Sun & Barrett, 1999). Pyriproxyfen is a pyridine based juvenile hormone analogue pesticide, which is found to be effective against a variety of insect pests, preventing larvae from developing into adulthood and thus rendering them unable to reproduce. Chlorfluazuron is one of the benzoylphenilurea insect growth regulators

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(IGRs), inhibiting chitin synthesis and disturb the molting process of the insect larvae and found to be effective against a variety of the insect pests especially in the order of Lepidoptera.

It is well known that temperature has a pervasive effect on insects. Nearly every aspect of the insect's life is influenced by temperature, from direct effects on the kinetics of enzyme reactions, to defining the limits of physiological function and behavior, and ultimately to shaping of evolutionary pathways. As a group, insects have evolved more than any other eukaryotic taxon, not only to survive but also to flourish in a wide variety of thermal environments. Insects respond to low temperature in essentially two ways: either they survive by enter into dormant (diapause) or quiescent state or they remain active. Insects that enter a dormant state, exhibit tolerance to a grater range of low temperatures than those that do not (Lee, 1991). There are two major physiological problems faced by insects during overwintering dormancy. Firstly, maintaining a stable metabolic condition throughout a period of starvation and secondly, avoiding damage due to the adverse effects of low temperatures. Many insects of temperate habitats accumulate energy reserve (mostly lipids in the form of triacylglycerols) prior to the onset of cold weather and reduce metabolic activity and energy consumption. These insects synthesize and accumulate low molecular weight sugars and polyols during overwintering (Asahina, 1969; Valder *et al.*, 1969; Dortland & Esch, 1979; Beenakkers *et al*., 1981; Somme, 1982; Pullin & Bale, 1989; Storey & Storey, 1991; Ramlov, 2000; Khani *et al*., 2007; Han *et al*., 2008). Accumulated sugars (e.g. trehalose and/or glucose) and polyols (e.g., glycerol, sorbitol or inositol) function as colligative and/or non-colligative cryoprotectants, enhancing the level of cold hardiness and thus increasing the chances of winter survival (Gekko & Timasheff, 1981; Zachariassen, 1985; Storey & Storey, 1991; Lee, 1991; Kostal *et al*., 2001, 2004). These carbohydrates and specially glycogen are also assumed to have a role as fuel for basal metabolism during the overwintering period. Role of glycogen is more complex and it may acts as a precursor for cryoprotectants such as glycerol (Storey & Storey, 1986; Kimura *et al*., 1992).

The first purpose of the present study was to compare the chemical composition and morphological changes of overwintering and non-overwintering pistachio leaf white borer larvae to determine what factors might be associated with cold hardiness and winter survival of this pest. Second purpose was to investigate the effects of pyriproxyfen, chlorfluazuron and two different temperatures on the chemical compositions and the morphological changes of non-overwintering larvae.

MATERIALS AND METHODS

Insects: Overwintering larvae (fourth instar) of *O. terebinthina* were collected from infested trees of a pistachio garden in Rafsanjan from October to March 2009. For rearing of non-overwintering larvae, egg batches were collected from infested trees and maintained in two separate climate chambers set at 65±5% RH with a photoperiod of 16 h light/8 h dark and 25±1˚C and 35±1˚C, respectively. For IGRs evaluation, larvae were reared at 65±5% RH with a photoperiod of 16 h light/8 h dark and 25±1˚C.

Insecticides: Commercial formulation of pyriproxyfen (Admiral, 10% EC, Sumitomo, Japan) and chlorfluazuron (Chlorfluazuron 5% EC, Osaka, Japan) were used. All tests were done with 2500 ppm fresh solutions of commercial pesticides prepared with methanol. 2 µl of each pesticide was topically applied to the dorsal region of early 7^{th} instar larvae of *O. terebinthina* using automatic microsyringe pump (Stoelting, USA). Methanol was used as control.

Scoring of the insect response: Development of the treated insects was monitored until adult emergence. Data were collected on larval mortality, larval-pupal intermediates, prepupal mortality, defective pupae, pupal mortality, abnormal adults and normal adults. The adults that were morphologically identical with their counterparts emerging in the control and were capable of mating were regarded as normal. This experiment was repeated 3 times for each treatment with 10 larvae.

Preparation of the Whole Body Homogenates for Chemical Analysis

Total body sugar: Total body sugar was measured by a method described by Warburg and Yuval (1997). Larvae were carefully brushed to remove contaminating particles, weighed and homogenized in 200 µL of 2% Na_2SO_4. 1300 µL of chloroform: methanol (1:2) was added to the homogenate to extract the total sugar of the larvae. Individual homogenates were centrifuged for 10 min at 7150g. To determine the amount of sugar in each larva, 300 µL of supernatant was taken and mixed with 200 µL of distilled water. The sample was reacted with 1 mL of anthrone reagent (500 mg anthrone dissolved in 500 mL concentrated H_2SO_4) for 10 min at 90°C. Absorbance was measured at 630 nm on a spectrophotometer (T60U). The amount of total sugar was determined from standard curve using glucose (Sigma) as standard. This experiment was repeated 6 times for each treatment with individual larva.

Glycogen: Glycogen content was determined from the pellet resulting from the centrifugation mentioned above. The pellet was washed in 400 µL of 80% methanol, thus removing possible remnants of sugar. To extract the glycogen, 250 µL distilled water was added to the washed pellet, and the mixture was heated for 5 min at 70°C. Subsequently, 200 µL of the solution was removed and reacted for 10 min at 90°C with 1 mL anthrone reagent (600 mg anthrone dissolved in 300 mL concentrated H_2SO_4). The optical density was read at 630 nm on a spectrophotometer (T60U). The amount of glycogen in the sample was determined from a standard curve by using glycogen (Sigma) as standard. This experiment was repeated 6 times with individual larva.

Lipids: Lipids were measured using the method of Folch *et al*. (1957) but with some modification as described by Goto *et al.* (1998). Each larva was homogenized with 80% ethanol, and the resultant insoluble residue was centrifuged at 2600 × *g* for 5 min. The supernatant was removed; the residue was extracted in a chloroform–methanol (2:1) mixture; and an aliquot of the lower phase was evaporated to dryness and assayed by the Bragdon (1951) oxidation method. The absorbance was measured at 580 nm on a spectrophotometer (T60U). The amount of lipid was determined from a standard curve, using Triolein (Sigma) as standard. This experiment was repeated 6 times in with individual larva.

Proteins: The residue from the lipids assay was resuspended in a solution of 1% SDS containing 0.4% sodium hydroxide, 2% sodium carbonate and 0.18% sodium-potassium tartarate and left overnight to solubilise the protein. After centrifugation, the protein content was estimated using the Lowry method (Markwell *et al*., 1978) with some modification. Bovine serum albumin was used as standard. This experiment was repeated 6 times for each treatment with individual larva.

Statistical analysis: The chemical contents data were analyzed by one-way analysis of variance (ANOVA) with a post-hoc Tukey test using SPSS (version 16.00). Student's *t*-test was used to compare the means of two groups when necessary. The results were expressed as mean±SE and considered significantly different at $P < 0.05$.

RESULTS

In this study, effects of two IGRs were investigated on morphology, longevity and formation of supernumerary larvae. Longevity of the last larval instars treated with chlorfluazuron (20 days) was significantly longer than the control larvae (9 days). After 22 days, all larvae pupated and adults emerged in the control, however, in chlorfluazuron treatment, only 30% of the larvae pupated and others died after molting to malformed prepupae with a piece of the old cuticle attached to the new cuticle. Some pupae emerged to abnormal adults with short wings. Treatment of prepupae with chlorfluazuron resulted in formation of smaller and blackened pupae, which were not able to form pupal case and never emerged as adult. In pyriproxyfen treatment, longevity of the last larval instars (30 days) was significantly longer than the control larvae (9 days). Most of these larvae molted in to malformed supernumerary larvae. These larvae were not able to feed and eventually died. Treatment of prepupae with pyriproxyfen resulted in formation of smaller and blackened pupae which were not able to form pupal case and never emerged as adult.

The effects of pyriproxyfen and chlorfluazuron were also investigated on some physiological changes of the last instar larvae of pistachio white leaf borer by measuring total body sugar, glycogen, lipid and protein contents of larval body. As it is evidence from Fig. 1, no significant difference

Fig. 1: Comparison between carbohydrate contents of *O. terebinthina* larvae under pyriproxyfen, chlorfluazuron and control treatments

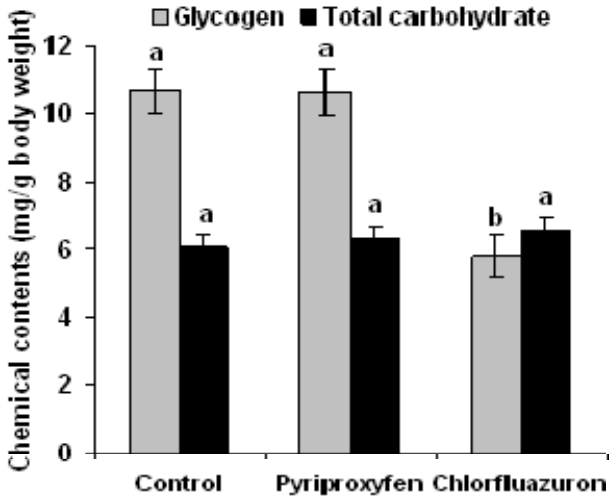
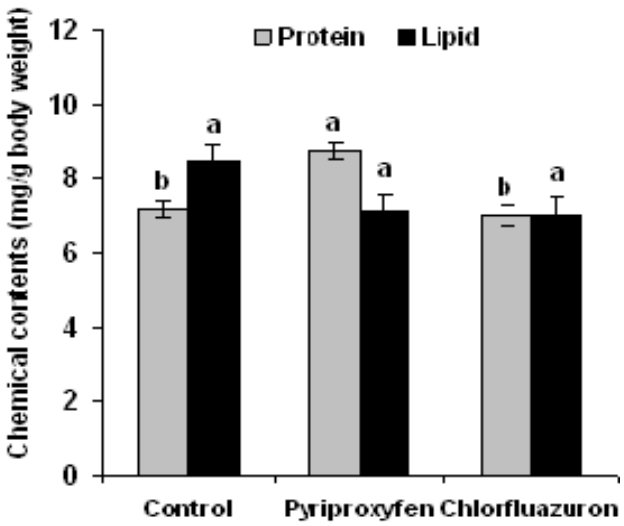


Fig. 2: Comparison between lipid and protein contents of *O. terebinthina* larvae under pyriproxyfen, chlorfluazuron and control treatments



in total body sugar of larvae ($F_{2,14} = 0.409$, $P = 0.672$) was observed between pyriproxyfen, chlorfluazuron and control treatments, but in chlorfluazuron treatment, glycogen content (5.78±0.66 mg/g fresh body weight) was significantly different from larvae treated with pyriproxyfen (10.62±0.66 mg/g fresh body weight) and control (10.66±0.66 mg/g fresh body weight) ($F_{2,12}$=18.212, P=0.000). As it is shown in Fig. 2 there were no significant differences in total body lipid ($F_{2,14}$=3.238, P=0.070) of different treatments but in pyriproxyfen treatment, protein content (8.75±0.24 mg/g fresh body weight) was significantly different from chlorfluazuron (7.01±0.26 mg/g fresh body weight) and control treatments (7.16±0.24 mg/g fresh body weight) ($F_{2,14}$=15.596, P=0.000).

Fig. 3: Comparison between carbohydrate contents of *O. terebinthina* larvae at two different temperatures

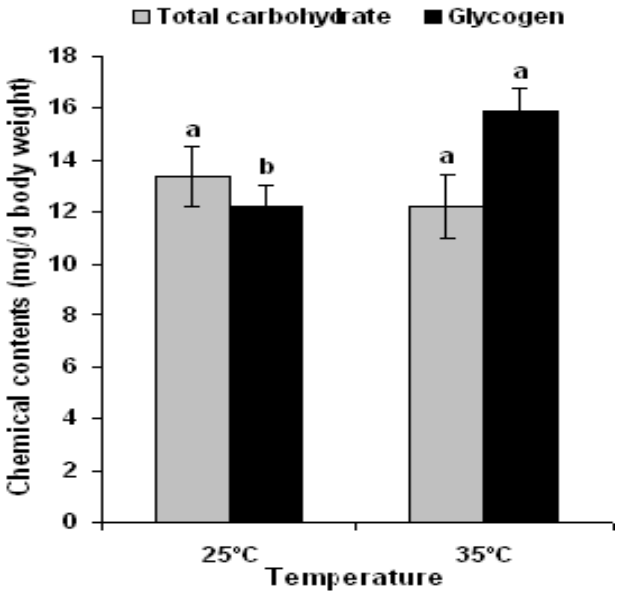
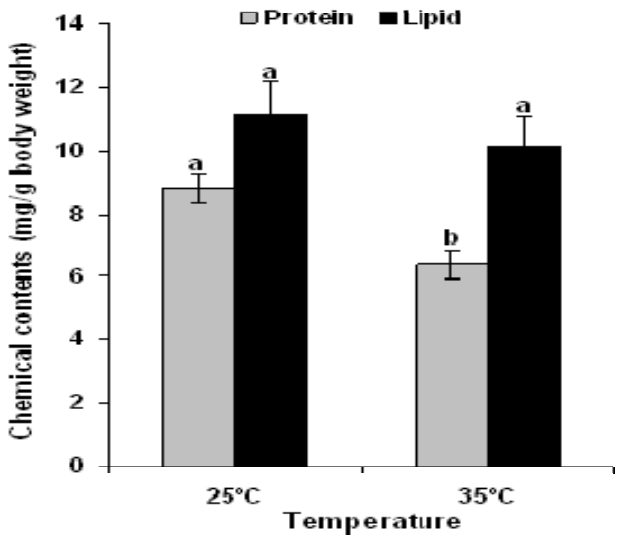


Fig. 4: Comparison between lipid and protein contents of *O. terebinthina* larvae at two different temperatures



Some physiological changes in two different temperatures (25°C & 35°C) were also investigated in fourth instar larvae of pistachio white leaf borer by measuring of total body sugar, glycogen, lipid and protein contents of the larvae. Results from this experiment revealed that there was no significant difference in total body sugar of larvae reared in two different temperatures (t = -0.671, P = 0.516), but glycogen content in larvae reared at 35 °C (15.89±0.89 mg/g fresh body weight) was significantly higher than larvae reared at 25°C (12.21±0.81 mg/g fresh body weight) (t=3.080, P=0.010) (Fig. 3). In addition, there was no significant difference in total body lipid (t=-0.702, P=0.500) in larvae reared at 25°C and 35°C but protein content in larvae reared at 25°C (8.8±0.48 mg/g fresh body weight) was significantly higher than the larvae reared at 35°C (6.41±0.48 mg/g fresh body weight) (t=-3.585, P=0.004) (Fig. 4).

Furthermore, in this study, some physiological and morphological changes were monitored in the field collected early overwintering fourth instar and the laboratory reared non-overwintering larvae of pistachio white leaf borer by measuring of total body sugar, glycogen, lipid and protein contents of larval body as well as their body color and weight. No significant difference was observed in the total body sugar of larvae (t=1.814, P=0.103), but glycogen content in non-overwintering larvae with 12.22±0.95 mg/g fresh body weight was significantly lower than overwintering larvae with 38.62±1.02 mg/g fresh body weight (t = -18.917, P = 0.000) (Fig. 5). There was no significant difference in total body protein of the larvae reared at two different temperatures (t = 0.0729, P = 0.484) but total body lipid in non-overwintering larvae with 11.13±0.93 mg/g fresh body weight was significantly lower than overwintering larvae with 15.06±1.14 mg/g fresh body weight (t = -3.011, P = 0.015) (Fig. 6). Overwintering larvae in relation to non-overwintering larvae had darker color and longer hairs but there was no significant difference between weight of the larvae reared at two different temperatures (t=0.462, P=0.648).

DISCUSSION

Molting and metamorphosis are two critical and important physiological events in the life of insects. All insects molt periodically in order to grow and all but a very few go through either gradual or complete metamorphosis to become an adult. These two events are regulated by the steroid 20-hydroxyecdysone and the sesquiterpenoid juvenile hormone (Nation, 2001). It is obvious that any interference with the homeostasis of these two hormones with exogenous sources of the hormones or synthetic analogs can be exploited as novel insecticide target to disrupt normal development of target pest insect (Aribi *et al*., 2006). Chlorfluazuron as a chitin synthesis inhibitor induces morphological disruptions at molt. In this study, treatment of early last instar larvae with chlorfluazuron resulted in long longevity of these larvae and formation of malformed prepupae, which possess both larval and pupal characters. Investigations of Omatsu *et al*. (1991) showed that five days after treatment of the common cutworm, *Spodoptera litura* larvae with chlorfluazuron various degrees of malformations in the integuments at/after molting were observed. Significant abnormalities were also found in adults of Phlebotomine sand flies treated with chlorfluazuron as third instars larvae (Quesada & Montoya-Lerma, 1994). These results are consistent with our findings. In this study, treatment of last instar larvae with chlorfluazuron significantly reduced glycogen content of

Fig. 5: Comparison between carbohydrate contents of overwintering and non-overwintering larvae of *O. terebinthina*

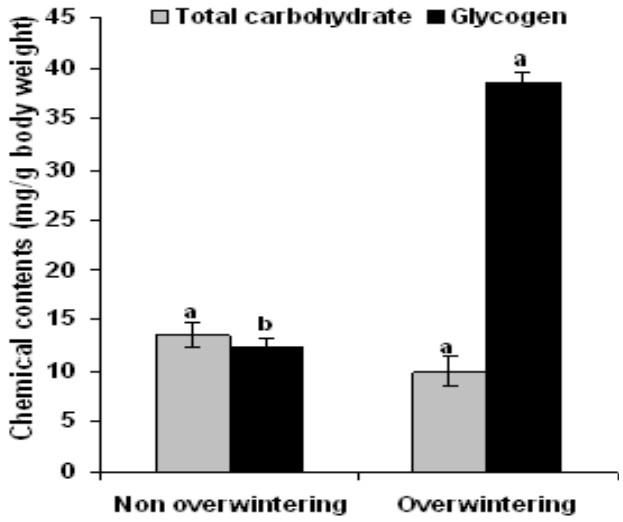
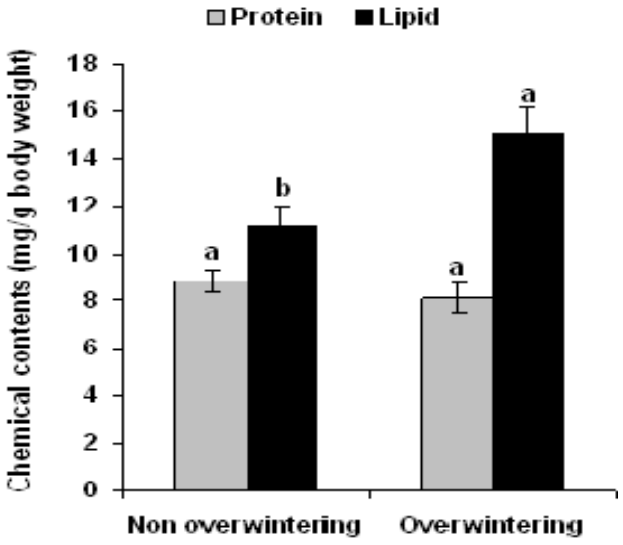


Fig. 6: Comparison between lipid and protein contents of overwintering and non-overwintering larvae of ***O. terebinthina***



larval body. One of the important constituents of insect cuticle is chitin. The starting point for synthesis of this polysaccharide is glucose, which may comes from a storage form such as trehalose or glycogen. Chlorfluazuron as a chitin synthesis inhibitor is known to disrupt chitin synthesis pathway resulting reduction of glycogen content. Our results indicate that chlorfluazuron has no significant effect on the level of total body sugar, protein and lipid. Treatment of the early fourth instar larvae of *Aedes aegypti* with methoprene did not significantly affect the amount of fat body but caused a significant elevation in the hemolymph carbohydrate and reduction in hemolymph protein concentration (Gordon & Burford, 1984). In the present study, pyriproxyfen as a juvenile hormone analogue, significantly enhanced longevity of the last larval instar and resulted in formation of malformed supernumerary larvae. Most of these larvae failed to shed the old cuticle and turned black and eventually died. Aribi *et al.* (2006) showed that pyriproxyfen caused a blockage of the ecdysteroid production by using it against pupae of *Tenebrio molitor*. Decrease in ecdysteroid titer was coincided with a complete inhibition of adult cuticle deposition. In the present study, pyriproxyfen significantly increased protein level of last instar larvae. Increase in protein level showed a direct correlation with pupal cuticle deposition. This finding is in agreement with the results of Aribi *et al*. (2006). The decrease in ecdysteroid titer inhibits a new cuticular cycle, thus, the protein accumulation in treated larvae could be due to the inhibition of the new cuticle secretion.

Results of this study revealed that fourth instar larvae of pistachio white leaf borer reared at 25°C had a lower level of glycogen and a higher level of protein in comparison with those larvae reared at 35°C. Overwintering larvae compare to non-overwintering larvae had a higher glycogen and lipid content. Glycogen content in the early overwintering larvae was about 3 times more than non-overwintering larvae. Glycogen is storage form of energy, therefore temperature rise leads to increasing metabolic rate, decreasing the development period and causing an increase in stored foods. In temperate insects, which have been examined for seasonal changes in carbohydrate content, glycogen is at the highest level in the beginning of overwintering and rapidly depleted and converted to sugar-alcohol or sugars in late autumn or early winter (Shimada *et al*., 1984; Storey & Storey, 1986; Hoshikawa, 1987; Richards *et al*., 1987; Rojas *et al*., 1991; Goto *et al*., 1998; Izumi *et al*., 2005). Most probably, overwintering larvae reserve glycogen and lipid to maintain the development of overwintering. Lipid and glycogen utilize during winter as fuel and/or cryoprotectant to survive harsh conditions. Large amounts of metabolic reserves i.e., glycogen and lipids accumulated prior to diapause and decrease concomitant with overwintering development (Tsumuki, 1990). In conclusion, our results revealed threefold increase in longevity of larvae treated with pyriproxyfen. Overwintering larvae in relation to non-overwintering larvae had higher lipid and glycogen contents. Protein content in larvae reared at 25ºC was significantly higher than those reared at 35ºC, but glycogen content in the larvae reared at 35ºC was more than larvae reared at 25ºC.

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