VOL. 6, NO. 5, MAY 2011 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science

www.arpnjournals.com

ENERGY ALLOCATION CHANGES DURING DIAPAUSE IN OVERWINTERING LARVAE OF PISTACHIO TWIG BORER, *Kermania pistaciella* AMSEL (LEPIDOPTERA: TINEIDAE) IN RAFSANJAN

Hamzeh Izadi, Mohamad Amain Samih, Ehsan Behroozy, Firuzeh Hadavi and Kamran Mahdian
Department of Plant Protection, Faculty of Agriculture, Vali-e- Asr University, Rafsanjan, Iran
E-Mail: izadi@vru.ac.ir

ABSTRACT

Pistachio twig borer**,** *Kermania pistaciella,* is a devastating pest of pistachio trees in pistachio producing zone of Rafsanjan, overwintering as last larval diapausing instar. In this study, energy allocation changes in relation to environmental changes were investigated in field collected larvae of pistachio twig borer by measuring total body sugar, glycogen, lipid and protein contents. Glycogen content decreased with decrease in ambient temperature. Decrease in glycogen content was proportional to increase in total body sugar content. In December with mean ambient temperature of 8.5˚C, glycogen content with 26.2 mg/g fresh body weight was at lowest level whereas total body sugar with 22.6 mg/g fresh body weight was at highest level. In the same time, whole body protein content with about 6.7 mg/g fresh body weight was also at lowest level. Total body sugar content increased as temperature decreased from 19˚C to 8.5˚C. Total body lipid decreased during diapause and reached lowest level in full developed diapause larvae. In conclusion, low molecular weight carbohydrates may play a role in winter surviving and adaptation of pistachio twig borer to cold and provide the cryoprotection and most probably, diapausing larvae of pistachio twig borer have ability to reserve energy in the form of lipid and utilize it during over wintering.

Keywords: pistachio pest, *kermania pistaciella*, energy allocation, trehalose, glycogen.

INTRODUCTION

The pistachio twig borer**,** *Kermania pistaciella,* is one of the most important pests of pistachio trees in Iran. This species is widely distributed throughout most of pistachio growing regions of Iran and is a key pest in Rafsanjan. In autumn the last instar larvae enter diapause in the pistachio twigs and over winter. This pest has a monovoltine life cycle in all parts of Iran. In early autumn with mean ambient temperature of 20˚C the last instar larvae enter diapause. Over wintering larvae terminate diapause at the end of winter with mean ambient temperature of 22˚C and get pupae on the surface of twigs and stem of pistachio trees. Adult moths emerge at beginning of the spring; lay eggs individually on the twigs, the hatched larvae enter the twig of pistachio trees and feed inside along the twig (Samih *et al*., 2005).

Diapause is an adaptation assisting in survival of insects through periods of harsh environmental conditions and adverse seasons (Kostal and Simek, 1995). Diapause is a genetically determined and an endocrine-mediated dormancy that occurs at a specific developmental stage and the expression of diapause is subject to both environmental and genetic factors (Den linger, 1985, 1991, 2002; Tauber *et al*., 1986, Goto *et al*., 2001a; Han *et al*., 2008;). Many insects distributed in the temperate zones enter winter diapause to survive seasonal environmental stresses (Tauber *et al*., 1986; Denlinger, 1991; Leather *et al*., 1993). The physiology of diapause state has been thoroughly studied in many insects (Lees, 1956; Salt, 1961; Mansingh, 1971; Mansingh and Smallman, 1972; Storey and Storey, 1988; Denlinger, 1991; Goto *et al*., 1993, 1998, 2001; Kostal *et al*., 1998; Watanabe and Tanaka, 2000; Khani *et al*., 2007; Han *et al*., 2008). Diapause in insects represents a complex dynamics process characterizing by several specific physiological and behavioral features (Tauber *et al*., 1986; Den linger, 1991; Kostal, 2006). Diapausing larvae cease their development and feeding activities and lower their metabolism to save energy reserve such as lipid and glycogen stored in fat body (Danks, 1987; Watanabe and Tanaka, 2000; Han *et al*., 2008). Several biochemical and molecular studies have investigated these aspects of diapause induction and development. Lipids, especially triacylglycerols, are usually the main energy reserve for overwintering whereas glycogen is mainly used for the trehalose and polyol synthesis (Yaginuma and Yamashita, 1978; Beenakkers *et al*., 1981; Storey and Storey, 1983). Numerous studies have documented the impact of accumulation of low molecular weight sugars and polyols on diapause initiation in many species of insects (Chino, 1957, 1958; Tsumuki and Kanehisa, 1978; Furusawa *et al*., 1982; Han *et al*., 2005, Khani *et al*., 2007; Han *et al*., 2008).

The main purpose of the present study is to investigate the chemical compositions of overwintering pistachio twig borer larvae to determine what factors may be associated with cold hardiness and winter survival of this pest. Providing more insight into the physiological aspects of *K. pistaciella* is essential for the development of efficient control programs targeting this pest.

MATERIALS AND METHODS

Insects

Over wintering larvae (last instars) of *K. pistaciella* were collected from infested trees of a pistachio garden in Rafsanjan at the end of each month from October 2009 to February 2010.

Low temperature survival

Determination of low temperature survival was carried out by a method described by Khani *et al*., (2007). In this method feeding non-diapausing and diapausing larvae were collected at the end of summer and middle of winter (n = 15), respectively. The collected larvae transferred to a programmable refrigerated test chamber and the temperature lowered at the rate of 0.5˚C/min from 20˚C to the desired treatment temperature (-15±1˚C). The larvae were held at this temperature for 24 h and then slowly (0.5˚C/min) heated to 25˚C and held at this temperature for 24 h. the total number of live and dead larvae were recorded. The larvae showing no movement were considered as dead.

Preparation of whole body homogenates for chemical analysis

Low molecular weight carbohydrates

Low-molecular-weight carbohydrates were measured using a method described by Warburg and Yuval (1997). Each larva was carefully brushed to remove contaminating particles, weighed, and homogenized in 200 µL of 2% Na_2SO_4. An additional 1300 µL chloroform-methanol (1:2) was added to the homogenate to extract the sugars of the larvae. Individual homogenates were centrifuged for 10 min at 7150 × *g*. To determine the amount of sugars in each larva, 300 µL was taken from the supernatant and mixed with 200 µL distilled water. The sample was reacted for 10 min at 90°C with 1mL of anthrone reagent (500 mg anthrone dissolved in 500mL concentrated H_2SO_4). Absorbances were measured at 630 nm on a spectrophotometer (T60U, Harlow Scientific, USA). The amount of component was determined from a standard curve by using trehalose (Sigma) as standard. This experiment was repeated 6 times in each month 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 1mL anthrone reagent (600mg anthrone dissolved in 300mL concentrated H_2SO_4). The optical density was read at 630 nm on a spectrophotometer (T60U, Harlow Scientific, USA). 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 in each month 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, Harlow Scientific, USA). The amount of lipid was determined from a standard curve, using Triolein (Sigma) as standard. This experiment was repeated 6 times in each month 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 tartarate and left overnight to solubilise the protein. After centrifugation, the protein content was estimated using a modification of the Lowry method (Markwell *et al*., 1978). Bovine serum albumin (Sigma) was used as standard. This experiment was repeated 6 times in each month with individual larva.

Meteorological data

Environmental temperature data were obtained from Data Processing Center of Iran Meteorological Organization (IMO) (Figure-1).

Statistical analysis

The chemical content data were analyzed using one- way analysis of variance (ANOVA) followed by Tukey's test (P = 0.05). The results were expressed as mean ± SE and considered significantly different at $P<0.05$

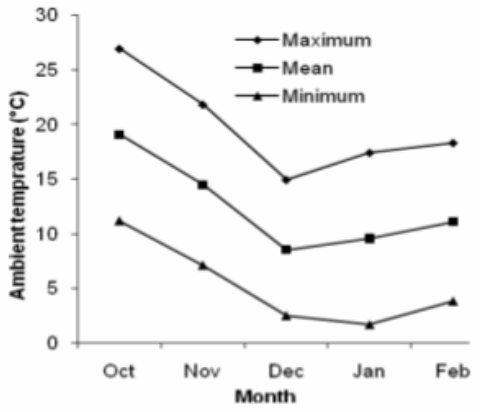

Figure-1. Seasonal changes in average, minimum and maximum ambient temperature (ºC) in Rafsanjan between October to February 2009-2010.

Low temperature survival

Results revealed that the cold hardiness of diapausing larvae was obviously higher than that of non-diapausing larvae. All non-diapausing larvae exposed to -15˚C died after short time of exposure whereas 100% of diapausing larvae survived at this temperature.

Carbohydrate content

Major sugars in *K. pistaciella* larvae were found to be low molecular weight carbohydrates (trehalose) and glycogen. As shown in Table-1, significant differences in the levels of low molecular weight carbohydrates and glycogen were detected in different months of over wintering. The trehalose content increased ($F_{4, 24}$ = 3.392, P < 0.05) from 15.44 mg/g fresh body weight in October as mean temperature decreased below 19˚C and reached a plateau of 22.61 mg/g fresh body weight in December as mean temperature reached to 8.5˚C. Trehalose content dropped to 17.84 and 20.40 mg/g fresh body weight in January and February as ambient temperature increased and reached to 9.5˚C and 11˚C, respectively. Glycogen content ($F_{4, 26}$ = 19.790, P < 0.05) began to decrease from October as temperature fell below 19˚C, and reached the lowest level (24 mg/g fresh body weight) in February.

Lipid content

The larval body lipid content ($F_{4, 14}$ = 2.898, P > 0.05) steadily decreased during over wintering period. Lipid content decreased by 42% from the beginning of diapause in October (159.3 mg/g fresh body weight) to the end of diapause in February (91.3 mg/g fresh body weight) but no significant difference in the lipid level was detected among the various months of over wintering (P > 0.05). It is evident that lipids are the main source of energy for larvae during over wintering.

Protein content

Protein level in the coldest month of year, December, reached the lowest level but no significant difference in the level of protein was detected ($F_{4, 27}$ = 2.119, P > 0.05) (Table-1).

Table-1. Chemical contents of diapausing larvae of pistachio twig borer in 2009-2010.

Time	Chemical components			
	Trehalose	**Glycogen**	**Protein**	**Lipids**
October	15.44 ± 1.65b	38.20 ± 1.84a	6.80 ± 0.48a	159.30 ± 13.23a
November	19.41 ± 1.35ab	36.90 ± 1.50a	7. 90 ± 0.34a	155.70 ± 13.23a
December	22.61 ± 1.25a	26.20 ± 1.39b	6.70 ± 0.36a	133.50 ± 13.28a
January	17.84 ± 1.25ab	27.00 ± 1.39b	7.17 ± 0.36a	123.80 ± 15.23a
February	20.40 ± 1.48ab	24.00 ± 1.39b	7.67 ± 0.39a	91.30 ± 13.23a
F value	$F_{4, 24}$ 3.392	$F_{4, 26}$ 19.790	$F_{4, 27}$ 2.119a	$F_{4, 14}$ 2.898
Probability	0.0246	0.0000	0.1210	0.0612

[1]Means ± SE; means within a column followed by the same letter are not significantly different (P>0.05, Tukey test).

DISCUSSIONS

Diapause's is essential component of adverse conditions survival for most insects in temperate zone and diapausing insects, in general, have stronger tolerance to low temperature in comparison with non-diapausing insects (Tauber *et al*., 1986; Den linger, 1991). Many over wintering insects increase their cold hardiness over the winter (Lee, 1991; Li *et al*., 2000; Goto *et al*., 2001a). The survival at subzero temperatures is a reliable index of cold tolerance in pistachio twig borer larvae. Gradually decreasing temperatures and enhancement of cold hardiness that was demonstrated by increased low temperature survival of diapausing larvae, suggest that over wintering larvae of pistachio twig borer could adjust their cold hardiness to the environmental conditions. The results of this study were similar to those observed by Kelty and Lee (1999), Goto *et al*., (2001a), Powell and Bale (2004), Wang and Kang (2005), Khani *et al*., (2007), and Behroozi (2010). During diapause stage, trehalose content increased and reached the maximum level in the coldest month of year, December. The increase in trehalose content is most likely a response to a temperature cue and acts as a cryoprotectant. This finding is in agreement with the results of Goto *et al*., (2001a), Khani *et al*., (2007), Han *et al*., (2008), and Behroozi (2010). With this result, we considered trehalose as a dominant sugar accumulated in the over wintering larvae of *K. pistaciella* and as a cryoprotectant plays an important role in the over wintering strategy of this pest. This sugar has previously been reported as a cryoprotectant in some insect species (Shimada *et al*. 1984, Storey and Storey, 1991; Kostal and Simek, 1995, 1998; Goto *et al*., 1998, 2001; Khani *et al*., 2007; Han *et al*., 2008). The results strongly suggest the interconversion between glycogen and trehalose in diapausing larvae of *K. pistaciella* under field conditions. The trehalose content changed during over wintering period with a concomitant decrease in glycogen content. Decreasing in ambient temperature in October to

December was associated with a remarkable decrease in glycogen and increase in trehalose contents. Trehalose reached to their highest level in December with ambient temperature of 8.5˚C. This result is consistent with results of Hayakawa and Chino (1981, 1982) Shimada *et al*., (1984), and Goto *et al*., (2001a). Accumulation of the reserves prior to the onset of over wintering is a common feature of some insect. Lipid and glycogen are two major forms of energy reserve and their pattern of utilization can differ during diapause (Mansingh, 1971; Masaki, 1980; Han and Bauce, 1998). Our results showed substantially decrease in lipid content of overwintering pistachio twig borer, *K. pistaciella.* This suggests that diapausing larvae of pistachio twig borer have ability to reserve energy in the form of lipid and utilize it during diapause. Lipid has been reported as energy resource during diapause of some other insects (Li *et al*., 2002; Ding *et al*., 2003; Han *et al*., 2008). Goto *et al*., (1998) demonstrated that lipid content of over wintering larvae of *E. leucotaniella* did not differ at various acclimation temperatures. Kostal (1998) demonstrated that glycogen content decreased substantially toward the end of diapause of Mediterranean tiger moth, *Cymbalophora pudica* whereas decrease in lipid content was not significant. They considered glycogen as main metabolic fuel during diapause and lipid as sources of energy and constituent for larval-pupal and pupal-imaginal metamorphoses. Protein and amino acid contents are greater in diapausing larval blood than in non-diapausing larvae, which mean that diapausing larvae reserve nutrition to maintain the development of diapause (Den linger, 1991). In this study, protein content of whole larval body changed during over wintering period and reached the lowest level in December but this was no significant.

In conclusion, our results documented some biochemical adaptations for winter survival in larvae of pistachio twig borer, *K. pistaciella*. Large amount of metabolic reserves (glycogen and lipid) accumulated prior to diapause and decreased concomitant with diapause development. Since the loss of cryoprotectants in spring is linked to the termination of diapause in many insects (Tsumuki, 1990) so, decreasing in trehalose content of overwintering larvae of pistachio twig borer and in the other hand increasing in glycogen content in the onset of spring may indicate diapause termination.

ACKNOWLEDGEMENTS

This research was supported by the Research Vice Presidency, Vali e Asr University of Rafsanjan; grant number Agro-86, p. 304.

REFERENCES

Baust J.G. and Miller L.K. 1972. Influence of low temperature acclimation on cold hardiness in *Pterostichus brevicornis*. Journal of Insect Physiology. 18: 1935-1947.

Beenakkers A.M.T., Van der Horst D.J. and Van Marrewijik W.J.A. 1981. Role of lipids in energy metabolism. In: Energy Metabolism in Insects, Eds. R.J.H. Downer. Plenum Press, New York, USA.

Behroozi E. 2010. Study on the nutritional reserves and cold hardiness of overwintering larvae of pistachio white leaf borer, *Ocneria terebintina* (Lepidoptera: Lymantriidae) in Rafsanjan township and physiological evaluation of some IGRs against the larvae. M.Sc. thesis. Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran.

Bragdon J. 1951. Colorimetric determination of blood lipids. The Journal of Biological Chemistry. 190: 513-517.

Chino H. 1957. Conversion of glycogen to sorbitol and glycerol in the diapause egg of the *Bombyx* silkworm. Nature. 180: 606-607.

Chino H. 1958. Carbohydrate metabolism in the diapause egg of the silkworm *Bombyx mori*. Journal of Insect Physiology. 2: 1-12.

Danks H.V. 1987. Insect dormancy: an ecological perspective. Biological Survey of Canada (Terrestrial Arthropods), Monograph Series No. 1, p. 439.

Denlinger D.L. 1985. Hormonal control of diapause. pp. 353-412. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, Eds. G.A. Kerkut and L.I. Gilbert.: Pergamon Press, Oxford.

Denlinger D.L. 1991. Relationship between cold hardiness and diapause. pp. 174-198. In: Insect at Low Temperature, Eds. R.E. Lee and D.L. Denlinger. Chapman and Hall, New York, USA.

Denlinger, D.L. 2002. Regulation of diapause. Annual Review of Entomology 47: 93-122.

Ding L., Li Y. and Goto M. 2003. Cold physiological and biochemical changes in summer and winter diapause and non-diapause pupae of the cabbage armyworm, *Mamestra brassicae* L. during long-term cold acclimation. Journal of Insect Physiology. 49: 1153-1159.

Folch J., Lees M. and Solane Stanley G.H. 1957. A simple method for isolation and purification of total lipids from animal tissues. The Journal of Biological Chemistry. 226: 497-509.

Furusawa T., Shikata M. and Yamashita O. 1982. Temperature dependent Sorbitol utilization in diapause eggs of the silk worm, *Bombyx mori*. Journal of Comparative Physiology. 147: 21-26.

Goto M., Takahashi K. and Suzuki C. 1993. Ecological study on the barnyard grass borer, *Enosima leucotaeniella*

(Lepidoptera: Pyralidae): X. Seasonal changes of carbohydrate contents in overwintering larvae. Applied Entomology and Zoology. 28(4): 417-421.

Goto M., Fuji M., Suzuki K. and Sakai M. 1998. Factor affecting carbohydrate and free amino acid content in over wintering larvae of *Enosima leucotaeniella*. Journal of Insect Physiology. 44: 87-94.

Goto M., Li Y.P., Kayaba S., Outani S. and Suzuki K. 2001a. Cold hardiness in summer and winter diapause and post-diapause pupae of the cabbage armyworm, *Mamestra brassicae* L. under temperature acclimation. Journal of Insect Physiology. 47: 709-714.

Goto M., Sekine Y., Outa H., Hujikura M. and Suzuki K. 2001b. Relationship between cold hardiness and diapause and between glycerol and free amino acid contents in overwintering larvae of the oriental corn borer, *Ostrinia furnacalis*.
Journal of Insect Physiology. 47: 157-165.

Han E.N. and Bauce E. 1998. Time of diapause initiation, metabolic changes and overwintering survival of the spruce bud worm *Choristonura fumiferana*. Ecological Entomology. 23: 160-167.

Han R.D., Ge F., Yardim N.E. and He Z.H. 2005. The effect of low temperatures on diapause and non-diapause larvae of pine caterpillar *Dengrolimus tabulaeformis* (Lepidoptera: Lasiocampidae). Applied Entomology and. Zoology. 40: 429-435.

Han R.D., Gan Y.L., Kong X.H. and Ge F. 2008. Physiological and endocrine differences between diapausing and non-diapausing larvae of the pine caterpillar *Dengrolimus tabulaeformis* (Lepidoptera: Lasiocampidae). Zoological Studies. 47(1): 96-102.

Hayakawa Y. and Chino H. 1981. Temperature-dependent activation or inactivation of glycogen Phosphorylase and syntheses of fat body of silk worm, *Philosamia cynthia*. The possible mechanism of the temperature-dependent inter conversion between glycogen and trehalose. Insect Biochemistry. 12: 361-366.

Hayakawa Y. and Chino H. 1982. Temperature-dependent interconversion between glycogen and trehalose in diapausing pupae of *Phylosamia cynthia ricini* and *pryeri*. Insect Biochemistry. 11: 43-47.

Kelty J.D. and Lee R.E. 1999. Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. Journal of Insect Physiology. 45: 719-726.

Khani A., Moharamipour S. and Barzegar M. 2007. Cold tolerance and trehalose accumulation in overwintering larvae of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). European Journal of Entomology. 104: 385-392.

Kostal V. 2006. Eco-physiological phases of insect diapause. Journal of Insect Physiology. 52: 113-127.

Kostal V. and Smiek P. 1995. Dynamics of cold hardiness, supercooling and cryoprotectants in diapause and non-diapause pupae of the cabbage root fly, *Delia radicum* L. Journal of Insect Physiology. 41: 627-634.

Kostal V., Sula J. and Smiek P. 1998. Physiology of drought tolerance and cold hardiness of the Mediterranean tiger moth, *Cymbalophora pudica* during summer diapause. Journal of Insect Physiology. 44: 165-173.

Leather S.R., Walters K.F.A. and Bale J.S. 1993. The ecology of insect overwintering. Cambridge University Press, Cambridge. p. 255.

Lee R.E. 1991. Principles of insect low temperature tolerance. pp. 17-46. In: Insect at low temperature, Eds. R.E. Lee and D.L. Denlinger. Chapman and Hall, New York,

Lees A.D. 1956. The physiology and biochemistry of diapause. Annual Review of Entomology. 1: 1-16.

Mansingh A. 1971. Physiological classification of dormancies in insects. Canadian Entomologist. 103: 983-1009.

Mansingh A. and Smallman B.N. 1972. Variation in polyhydric alcohol in relation to diapause and cold-hardiness in the larvae of *Isia Isabella.* Journal of Insect Physiology. 18: 1565-1571.

Markwell M.A.K., Haas S.M., Bieber L.L. and Tolbert N.E. 1978. A modification of the Lowry procedure to simplify determination in membrane and lipoprotein samples. Annals of Biochemistry. 87: 206-210.

Masaki S. 1980. Summer diapause. Annual Review of Entomology. 25: 1-25.

Ordin J.H., Cui A. and Yin C.M. 1984. Cold-induced glycerol accumulation by *Ostrinia nubilalis* larvae is developed regulated. Journal of Insect Physiology. 30: 563-566.

Powell S.J. and Bale J.S. 2004. Cold shock injury and ecological costs of rapid cold hardening in the grain aphid *Sitobion avenae* (Hemiptera: Aphididae). Journal of Insect Physiology. 50: 277-284.

Samih M.A., Alizadeh A. and Saberi R. 2005. Pistachio pests and diseases in Iran and their IPM. Jahad-e-daneshgahi, University of Tehran. p. 301.

Salt R.W. 1961. Principles of insect cold-hardiness. Annual Review of Entomology. 6: 55-74.

Shimada K., Sakagami S.F., Honma K. and Tsusui H. 1984. Seasonal changes of glycogen/trehalose contents, supercooling points and survival rate in mature larvae of the overwintering soybean pod borer *Leguminivora glycinivorella*. Journal of Insect Physiology. 30: 369-373.

Storey J.M. and Storey K.B. 1983. Regulation of cryoprotectant metabolism in the overwintering gall fly larva, *Eurosta solidaginis*: Temperature control of glycerol and sorbitol levels. Journal of Comparative Physiology. 149: 495-502.

Storey K.B. and Storey J.M. 1988. Freeze tolerance in animals. Physiological Review. 68: 27-84.

Storey K.B. and Storey J.M. 1991. Biochemistry of cryoprotectants. pp. 64-93. In: Insect at Low Temperature, eds.

R.E. lee and D.L. Denlinger. Chapman and Hall, New York.

Tauber M.J., Tauber C.A. and Masaki S. 1986. Seasonal adaptation of insects. Oxford University Press, Oxford. p**.** 411.

Tsumuki H. 1990. Environmental adaptations of the rice stem borer, *Chilo supressalis* and the blue alfalfa aphid, *Acyrthosiphon konodi* to seasonal fluctuations. pp. 273-278. In: Advances in Invertebrate Reproduction, eds. M. Hoshi and O. Yamashita. Elsevier, Amsterdam.

Tsumuki H. and Kanehisa K. 1978. Carbohydrate content and oxygen uptake in larvae of rice stem borer, *Chilo supressalis* Walker. Brichte des Ohara Institute Fur Landwirtschaftliche Biologie, Okayama University. 17: 95-110.

Wang H. and Kang L. 2005. Effect of cooling rates on the cold hardiness and cryoprotectant profiles of locust eggs. Journal of Insect Physiology. 51(2): 220-229.

Warburg M.S. and Yuval B. 1997. Effect of energetic reserves on behavioral patterns of Mediterranean fruit flies (Diptera: Tephritidae). Oecologia. 112: 314-319.

Watanabe M. and Tanaka K. 1998. Adult diapause and cold hardiness in *Aulacophora nigripennis* (Coleoptera: Chrysomelidae). Journal of Insect Physiology. 44: 1103-1110.

Watanabe M. and Tanaka K. 2000. Hormonal control of diapause and over wintering traits in leaf beetle, *Aulacophora nigripennis*. Physiological Entomology. 25: 337-345.

Yaginuma T. and Yamashita O. 1978. Polyol metabolism related to diapause in *bombyx* eggs: different behavior of sorbitol from glycerol during diapause and post - diapause. Journal of Insect Physiology. 24: 347-354.