



Differences in Accumulation of Nutrients in Pre-Reproductive and Pre-Diapause Female Adults of *Riptortus pedestris* (Hemiptera: Alydidae)

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ABSTRACT

Diapause offers a series of physiological changes to sustain the successful existence of insects under adverse environmental conditions. To synchronize with the environmental changes, insects suppress their reproductive development and store a large quantity of energy reserves to meet the energy demands during diapause period. However, the differences in the energy reserves in diapausing and pre-reproductive insects remain to be understood. With this aim, we compared the major energy nutrient reserves between the pre-diapause and pre-reproductive adult females. In this study, we measured the total lipids, triglyceride (TG), glycogen and the total protein contents in pre-diapause and pre-reproductive adult females of the *Riptortus pedestris*. Moreover, we also compared the fresh and dry weights, and the water content retained by pre-diapause and pre-reproductive females. The results indicated that the weights in both groups of females were increased over the time period of adult emergence, while water content remained significantly high in pre-reproductive adult females. The pre-diapause females stored more reserves of the total lipids, triglyceride and glycogen contents to maintain the diapause period whereas, the pre-reproductive females increased the total protein contents to mature their reproductive development. These findings suggested that the different mechanisms are involved in regulating the developments of pre-diapause and pre-reproductive females, which provide the basic knowledge for further molecular research on these mechanisms in insects.

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Key words

Riptortus pedestris, Pre-Diapause, Pre-Reproductive, Lipids, Triglyceride, Glycogen, Protein

INTRODUCTION

Diapause is a genetically predetermined phase of an insect's life. It offers a series of physiological changes to synchronize the life cycle of the insects under unfavorable environmental conditions (Bale *et al.*, 2002). Among all the abiotic factors, temperature and photoperiod are well known prime factors (stimulus), which cause the significant effects on physiological processes that take place during diapause and post-diapause phases of the insects (Košťál, 2006; Wu *et al.*, 2019). Short day photoperiod with low temperature are the most commonly reported factors involved in diapause induction and

termination mechanism of the insects (Numata, 1987; Galka and Brust, 1987). Particularly in overwintering diapause, photoperiod is the major factor which provides the most accurate information about the seasonal changes in autumn when temperature and day-night cycle become highly variable for the insects (Jung and Lee, 2018). In many insect species, the diapause inducing photoperiod is also influence the diapause intensity as, in *Riptortus clavatus* adults, the longer nights induce more intense diapause as compared to the shorter nights (Numata and Hidaka, 1982; Nakanura and Numata, 2000).

Eco-physiologically, the diapause period is subdivided into three consecutive phases as pre-diapause, diapause and post-diapause phase (Košťál, 2006). Generally, the diapause inducing signals interact during the pre-diapause phase which is a sensitive and genetically predetermined period in the life cycle of an insect. Insects collect all upcoming information from the environment and store it into their brains then make the decisions of either they undergo diapause period or continue their reproductive development (Broufas, 2001; Hahn and Denlinger, 2011). In response to diapausing signals, adult insects suppress their reproductive developmental pathway

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and accumulate a sufficient amount of energy nutrients to spend the diapause period (Tan *et al.*, 2016). However, the insects that are unable to accumulate a sufficient amount of energy nutrients prior to diapause have limited strategies to manage a lengthy diapause either they die during diapause period or avert the diapause and produce one more generation before the depletion of energy reserves. Moreover, they may terminate the diapause and resume their feeding, in order to compensate this deficiency. Insects use very common strategies to minimize the energy cost during the diapause period, building up of energy reserves and reduce the metabolic rate at its low level (Hahn and Denlinger, 2011). During diapause, insects suppress their reproductive development and store large quantities of lipids, amino acids, and carbohydrates to meet the energy demands. Reproductive insects, on the other hand, also store similar types of nutrients which are used primarily for their reproduction instead of energy storage (Michaud and Denlinger, 2007). Thus, the regulation of diapause and reproduction programs are subjected to different mechanisms. Therefore, it remains important to determine the accumulation of nutrients between pre-diapause and pre-reproductive adults to understand the mechanisms involved in diapausing and reproducing insects.

The bean bug, *Riptortus pedestris* (Hemiptera: Alydidae), is a highly destructive pest of soybean crop in temperate regions of Japan, Korea, and China. This species is a polyphagous in nature that feeds on various types of leguminous plants including soybean (*Glycine max*), kidney bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*) and pea (*Pisum sativum*) (Kim *et al.*, 2014; Rahman and Lim, 2017). Being multivoltine, adults adopt a facultative diapause under leaf litters in response of shorter day-night cycle of late autumnal season. However, after overwinter, the first generation of *R. pedestris* adults emerged in early summer when day-night cycle begin longer to promote the reproductive development of this species (Jung and Lee, 2018). In response to diapause inducing photoperiod, the newly emerged adult females suppressed their ovarian development and store a sufficient quantity of fat bodies in their abdominal cavity. In comparison, the females reared under long-day (16L:8D) photoperiod at 25°C, continuously matured their reproductive development and began reproduction within a week of adult emergence (Numata and Hidaka, 1982, 1984; Hafeez *et al.*, 2020).

In our previous studies, the internal reproductive development of pre-reproductive and pre-diapause adult females of *R. pedestris* was investigated, but there was a need to explore the differences between the accumulation of nutrient reserves participating in pre-reproductive and pre-diapause females of this species. With this aim, we

compared the fresh and dry weights, and water contents in both pre-diapause and pre-reproductive adult females of *R. pedestris*. Moreover, we analyzed the total lipid content, triglyceride, glycogen and total protein content in the pre-diapause and pre-reproductive adult females.

MATERIALS AND METHODS

Insect rearing and sample preparation

The adults of *R. pedestris* were captured from the soybean crop field of Huazhong Agricultural University (30°27'56"N 114°21'30"E), Wuhan, China, in 2017. For consecutive generations, this wild population was bred under reproductive conditions of 25±1°C, R.H. of 70±5% and long-day photoperiod (16L:8D) in an incubator (HP-250-GS, Wuhan Ruihua Instrument and Equipment, Wuhan, China). Insects were regularly provided with fresh soya bean pods (*Glycine max*) and water (supplemented with 0.05% ascorbic acid solution). For this experiment, newly molted third instar nymphs were divided into two groups as long-day (16L:8D) and short-day (12L:12D) photoperiods at 25°C, and only female adults were assigned for investigations.

Fresh, dry weight and water content

The fresh weight (FW) of newly emerged 20 pre-diapause and pre-reproductive adult females were recorded by using an electronic balance (OHAUS AR2140, Shanghai, China). These females were dried at 100°C under a dry oven for 24 h. This loss in weight regarded as total water content. However, the dry weight (DW) of adult females was calculated as the difference between the FW and total loss in weight.

Determination of the major nutrients in pre-reproductive and pre-diapause R. pedestris females

To quantify the nutrient reserves, we measured the total lipids, triglyceride (TG), glycogen and total protein contents in pre-diapause and pre-reproductive adult females of *R. pedestris* by following methods as described below.

Sample collection

We used 9 adults of pre-diapause and pre-reproductive females after each experimental day (1, 3, 5, 7 and 9) for the measurement of triglyceride (TG), glycogen and protein contents. These 9 females were further subdivided into 3 biological replications. The fresh weight (FW) of each biological replicate was recorded in 1.5 mL microcentrifuge tubes by using electronic balance accurate to 0.1 mg. For instant storage of the samples, we used chilling liquid nitrogen to avoid further metabolism and

stored at -80°C till the measurement day.

Total lipid content

Folch *et al.* (1957) described chloroform-methanol (2:1) lipid extraction method for measuring the lipid content. In brief, 20 weighted females were dried for 24 h at 100°C in a dry oven (SFG-01; Hengfeng Medical Instrument, Huangshi, China), and their initial dry weights were measured. The females were immersed in a 3 mL solution of chloroform and methanol (2:1) for 24 h at room temperature. The dry weight was calculated, followed by a 6 h extraction at room temperature in a chloroform and methanol solution. Total lipid content is defined as the difference between the initial dry weight (IDW) and the end dry weight (EDW).

$$\text{Total lipid content (\%)} = \frac{\text{IDW} - \text{EDW}}{\text{IDW} - W}$$

Here, W, total weight; IDW, initial dry weight, EDW, end dry weight.

Triglyceride (TG) content

The TG level of the whole female was determined using a glycerol-3-phosphate oxidase-phenol + aminophenazone (GPO-PAP) kit method (code F001-2; Jiancheng Bioengineering Institute, Nanjing, China). In brief, three female samples were weighted in a 1.5 mL microcentrifuge tube and centrifuged at 6000 rpm for 5 min at 4°C in absolute ethanol (sample weight (g): volume (mL) = 1:9). In 250 μL of reaction solution, 2.5 μL of supernatant was calculated and incubated for 10 min at 37°C . The TG content of the sample was determined using a microplate reader (Bio-Rad, Xmark, Berkeley, CA, USA) at 510 nm absorbance.

$$\text{Triglyceride (TG) content } (\mu\text{g mg}^{-1}) = \left[\left(\frac{\text{S.O.D} - \text{B.O.D}}{\text{St.O.D} - \text{B.O.D}} \right) \times \text{St.C} \right] / (\text{FW}/\text{St.D})$$

Here, OD, optical density; S.OD, sample OD; B.OD, blank OD; St.OD, standard OD; St.C, standard concentration; St.D, standard dilution; St.Q, standard quality; FW, fresh weight.

Glycogen contents

The anthrone-sulfuric acid (ASA) method established by (Dubois *et al.*, 1951) was used to determine the glycogen content. In brief, the samples were weighted and homogenized in 400 μL of 10% trichloroacetic acid ($\text{C}_2\text{HCl}_3\text{O}_2$) in precooling phosphate-buffered saline (PBS) (sample weight (g): volume (mL) = 1:9). 1 mL homogenized solution was transferred in the new 1.5 μL tubes and centrifuged at 5000 rpm at 4°C . The supernatant was then diluted ten times in PBS solution. In glass tubes, 250 μL of diluted solutions were combined with 1000 μL of 0.2% anthrone reagent (100 mg anthrone dissolved in

100 mL concentrated H_2SO_4) and heated at 100°C in a water bath for 10 min before cooling for 20 min on ice. The reaction solutions were poured into 96-well microplates, and the absorbance of each sample was measured using a microplate reader (Bio-Rad, Xmark, Berkeley, CA, USA) at 628 nm. The glycogen content of the samples was determined using a standard curve that was adjusted for the different glycogen dilution ratios.

$$\left(\text{Glycogen concentration } (\mu\text{g mg}^{-1}) = \left(\frac{\text{Dilution Times}}{\text{fresh weight}} \right) \times X \right)$$

Here, $X = (Y - 0.07)/(0.003)$ and Y = sample OD

Total protein content

The total protein contents of a female was determined using the bicinchoninic acid (BCA) technology and the manufacturer's procedure (code A045-3; Jiancheng Bioengineering Institute). Each weighted sample of three adult females was homogenized in cooled PBS solution (sample weight (g): volume (mL) = 1:9) and centrifuged for 10 min at 4°C at 2500 rpm. Only 2.5 μL of supernatant was utilized for 250 μL of reaction solution, which was incubated at 37°C for 30 min. The absorbance at 450 nm was measured after 200 μL of the reaction solution was put into a 96-well microtiter plate.

$$\text{Protein content } (\mu\text{g mg}^{-1}) = \left[\left(\frac{\text{S.O.D} - \text{B.O.D}}{\text{St.O.D} - \text{B.O.D}} \right) \times (K.C \times D.T) \right] / \text{FW}$$

Here, S.OD, sample OD; B.OD, blank OD; St.OD, standard OD; K.C, kit concentration; D.T, dilution times; FW, fresh weight.

Statistical analysis

All statistical analyses were carried out using the Statistics Software SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The independent t-test with a 95% confidence level was used to calculate the significant differences in nutrient accumulations. All the graphs were created using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Fresh weight, dry weight, and water content of female adults

The fresh weights (FW) of both groups of females were increased continuously at first, and no significant difference in FW was observed between pre-reproductive and pre-diapause bugs ($t=3.523$, $df=38$, $P=0.001$), but after 5 days, the pre-reproductive females gained significantly higher FW than the pre-diapause females ($t= 3.278$, $df= 38$, $P= 0.001$) (Fig. 1A). However, after 7 days of adult emergence, FW declined ($t= 1.510$, $df= 38$, $P= 0.140$), while pre-reproductive females regained FW after 9 days

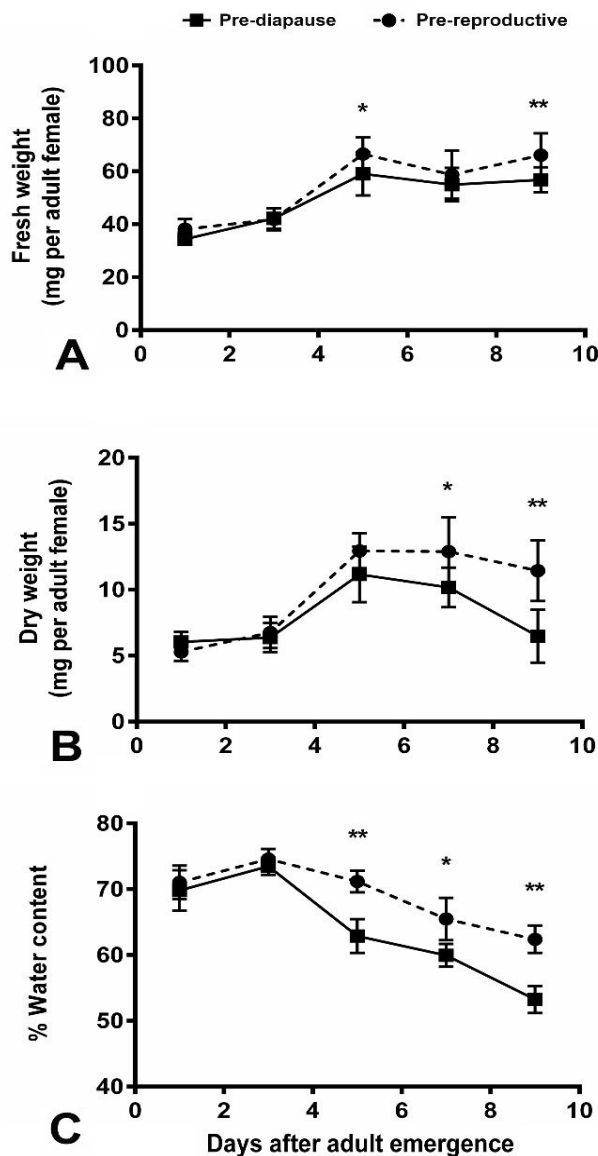


Fig. 1. Variations in fresh weight (A), dry weight (B), and water content (C) stored in pre-diapause and pre-reproductive adult females. Horizontal line of each graph shows the different days of observation while, perpendicular line shows amount of nutrient stored in pre-diapause and pre-reproductive adult females of *R. pedestris*.

($t = 4.349$, $df = 38$, $P = 0.000$). However, the dry weights (DW) of both pre-reproductive and pre-diapause females increased over the emergence period, and after 7-9 days of adult emergence, the pre-reproductive females have significantly higher DW than the pre-diapause females (7 days $t = 4.283$; 9 days $t = 8.054$, for both $df = 38$, $P = 0.010$) (Fig. 1B). Water content stored by insects, as opposite to weights (FW and DW), is a key component in estimating

nutrition accretion between reproductive and diapausing females. We found that the water storage capacity in both pre-diapause and pre-reproductive adults was reduced at the start of the study, but after 5 days of adult emergence ($t = 12.162$, $df = 38$, $P = 0.001$), the pre-reproductive females stored more water content than that of pre-diapause females and it remained high throughout 9 days of ($t = 13.996$, $df = 38$, $P = 0.000$) (Fig. 1C).

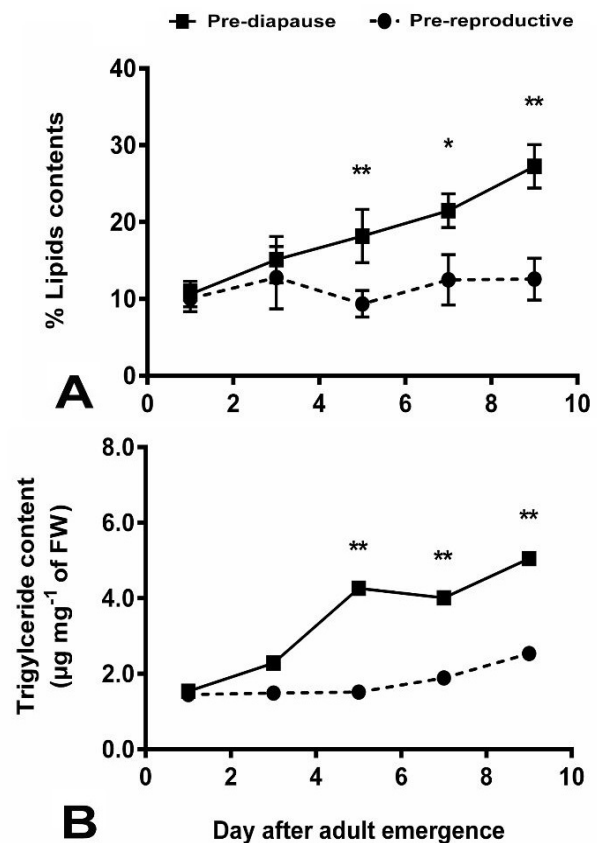


Fig. 2. Differences between lipid (A) and triglyceride (B) stored in pre-diapause and pre-reproductive adult females. Horizontal line of each graph shows the different days of observation while, perpendicular line shows amount of nutrient stored in pre-diapause and pre-reproductive adult females of *R. pedestris*.

Total lipids and triglyceride (TG) contents

The lipid contents of pre-diapause and pre-reproductive females were not significantly different after the 3 days of newly adult emergence ($t = 1.075$, $df = 38$, $P = 0.29$), but after 5 days of emergence, the lipid content stored by pre-diapause females became significantly higher than that of pre-reproductive females ($t = 10.164$, $df = 38$, $P = 0.000$). Following that, the lipid content in pre-diapause females increased steadily and remained considerably

greater than that of pre-reproductive females after 7-9 days (7 day: $t = 10.242$, 9 days: $t = 16.744$, for both $df = 38$, $P = 0.000$) (Fig. 2A). Similarly, the triglyceride (TG) content was not substantially greater in pre-diapause females of *R. pedestris* compared to pre-reproductive females ($t = 3.645$, $df = 4$, $P = 0.22$). Later, after 5-7 days of adult emergence (5 day; $t = 29.665$, 7 days; $t = 64.638$, both $df = 4$, $P = 0.001$), the TG content in both groups of females were increased. At 9 days of observation, the TG content in pre-diapause females abruptly increased, and the difference in TG accumulation in both groups became quite evident ($t = 37.927$, $df = 4$, $P = 0.000$) (Fig. 2B). These findings suggested that the principal nutrients accumulated in pre-diapause females before they enter the diapause condition are lipid and TG levels.

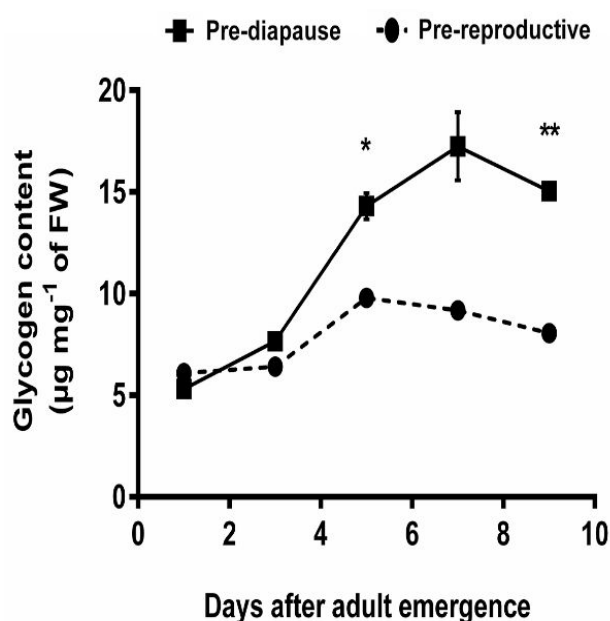


Fig. 3. Differences between glycogen content stored in pre-diapause and pre-reproductive adult females. Horizontal line of each graph shows the different days of observation while, perpendicular line shows amount of nutrient stored in pre-diapause and pre-reproductive adult females of *R. pedestris*.

Glycogen content

Glycogen content in newly emerged adults of pre-reproductive was higher than that of pre-diapause females ($t = 2.068$, $df = 4$, $P = 0.107$), but after feeding for 3 days, the glycogen content was increased in pre-diapause females ($t = 3.378$, $df = 4$, $P = 0.028$). As the adult emergence days increased, the glycogen content was also increased in pre-diapause females and after 7 days of emergence, the concentration of glycogen became higher in pre-diapause

females ($t = 4.402$, $df = 4$, $P = 0.026$). Although increased in concentration, a sudden decreased in glycogen content was observed in pre-diapause females after 9 days of adult emergence ($t = 18.545$, $df = 4$, $P = 0.000$) (Fig. 3).

Total protein contents

Similar to other energy nutrients, the total protein contents were also increased in both groups of females of pre-reproductive and pre-diapause adults. After 3 days of adult emergence, the total protein contents were slightly increased in pre-reproductive females than that of pre-diapause females ($t = 0.283$, $df = 4$, $P = 0.110$), but this difference was not significant. However, the total protein contents was continuously increased in pre-reproductive females and a significant difference was observed between the pre-reproductive and pre-diapause females after 7-9 days of adult emergence (7 day: $t = 5.531$, 9 days: $t = 4.952$, for both $df = 4$, $P = 0.001$) (Fig. 4).

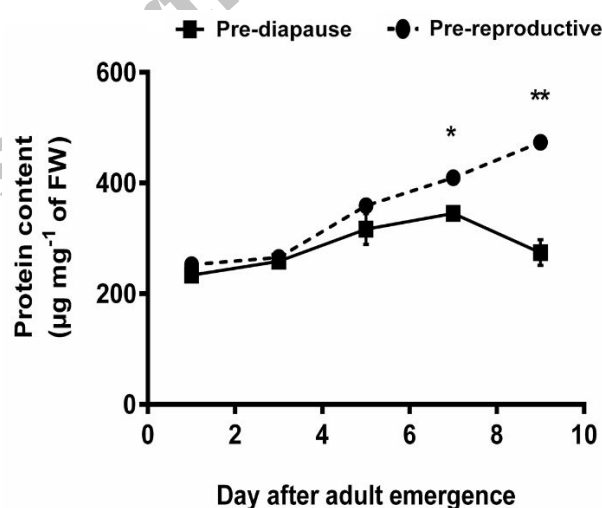


Fig. 4. Differences between protein content stored in pre-diapause and pre-reproductive adult females. Horizontal line of graph shows the different days of observation while, perpendicular line shows amount of reserves stored in pre-diapause and pre-reproductive adult females of *R. pedestris*.

DISCUSSION

Diapausing insects differ physiologically and biochemically from non-diapausing insects. The most noticeable feature of the diapause insects is that they stop reproducing and store more energy nutrients (Hahn and Denlinger, 2007, 2011; Tan *et al.*, 2016). Diapausing insects appear to be relatively basic and metabolically slower than nondiapausing insects, but investigations revealed that they have their own metabolic demands

and entered into an alternate developmental pathway (Hahn and Denlinger, 2011; Tachibana *et al.*, 2020). During diapause phase, a decrease in metabolic rate is accompanied with a decrease in total body water content. Overwintering insects, on the other hand, fulfill their water requirement from lipids metabolism (Denlinger, 2002). The water content of *R. pedestris* pre-diapause and pre-reproductive females were reduced after the adult emergence, but remained higher in pre-reproductive females. Similarly, the water content of diapause larvae in the pine caterpillar *Dendrolimus tabulaeformis* was lower than that of non-diapause larvae. The abdominal water content of the potato beetle *Leptinotarsa decemlineata* reduced during the diapause preparation period (Lehmann *et al.*, 2012). *Culex pipiens* diapausing females has larger dry masses than non-diapause females, and their water content was considerably lower than that of non-diapause females, indicating a higher rate of water loss (Benoit and Denlinger, 2007).

In many insect species, the weight difference between the diapausing and nondiapausing individuals is significant, as the weight gain in diapause pupae of *Helicoverpa armigera* is believed to reflect the higher food reserves (Chen *et al.*, 2014). Insects with larger bodies are assumed to have more storage capacity for the nutritional reserves needed to endure the adverse environmental circumstances (Shin *et al.*, 2012). However, this strategy is not shared by all insect species. We discovered that the fresh and dry weights of *R. pedestris* pre-reproductive females were substantially higher than those of pre-diapause females. Our findings suggested that the weight gain is not the most essential approach for energy storage in female *R. pedestris*. Another possibility is that the pre-diapause females of the *R. pedestris* stored more energy nutrients and lowered their moisture contents to improve the ability to withstand against the effects of adverse environmental circumstances.

Even though, the size differences are not obvious, the diapausing insects often stored a substantial amount of energy reserves in the form of lipid contents. For example, the pre-diapause adult females of the lady bird beetle, *Harmonia axyridis*, stored around 28% of their total body weight, but the pre-reproductive female adults stored only 11% of their total body weight (Gao *et al.*, 2019). Furthermore, the diapausing adults of *C. pipiens* mosquitoes retained around twice as much lipid as non-diapausing adults of the same age (Shin *et al.*, 2012). We found that the pre-diapause females of *R. pedestris* stored 30% of their total body weight in lipids content, but the pre-reproductive females accumulated only 9% of their total body weight in lipids.

During diapause, the lipids are stored in the form of

triglyceride content, which can be obtained directly from the fat bodies and produce more calories per gram than the carbohydrate or protein oxidation (Arrese and Soulages, 2010). Even within a species, the triglyceride content prior to diapause would differ from non-diapause individuals despite feeding on the same food (Khani *et al.*, 2007; Cakmak *et al.*, 2008). The triglycerides in diapausing individuals are unsaturated fatty acids which provide quick source of energy at low temperature, whereas the non-diapause individuals store more saturated fatty acids. We found that the pre-diapause females of *R. pedestris*, stored a large amount of triglyceride contents than that of pre-reproductive adults. Moreover, it has been noticed that the triglyceride contents in pre-reproductive adults were depleted with maturing the reproductive development of females. In the diapausing adult females of *Colaphellus bowringi*, stored 2.5 times more triglyceride contents than that of reproductive individuals (Tan *et al.*, 2016). The storing capacity of triglyceride contents is correlated with the diapause intensity and species of insects. The diapausing females of *Drosophila melanogaster* has deep diapause under cold condition, stored more triglyceride contents compared to *D. tufa* and *D. lutescens* species which have less intense diapause under same conditions (Ohtsu *et al.*, 1992).

It is well known that insects must store greater reserves of triglyceride and glycogen contents before they induced the diapause to sustain them throughout diapause period (Lorenz and Anand, 2004; Wang *et al.*, 2007). Glycogen is a polymeric form of glucose that can be available on degradation of polysaccharides carbohydrates to meet the glycolytic fuel (Steele, 1982). However, the accumulation of glycogen contents are most reported in overwinter diapause insects and the main function of glycogen is to protect the insects against low temperature (Wang *et al.*, 2007; Bemani *et al.*, 2012; King *et al.*, 2016). In current studies, we found that the pre-diapause females accumulated more glycogen contents than that of pre-reproductive females, but after few days of accumulation, the glycogen contents were decreased in pre-diapause females of *R. pedestris*. The diapausing pupae of the pistachio fruit hull borer accumulated a greater quantity of low molecular weight of carbohydrates such as trehalose, sorbitol and myo-inositol that were responsible for decrease in glycogen content (Amiri and Bandani, 2013; King *et al.*, 2016).

In many species of insects, protein is the major reserve stored by reproductive females. It is believed that the accumulation of protein content has direct relation with the reproductive development of an insect. In some previous studies, the reproductive adult females of *Cotesia vestalis*, *Colaphellus bowringi* and *Harmonia axyridis*

stored a significantly higher amount of protein content than those of diapause adult females (Hao *et al.*, 2012; Tan *et al.*, 2016; Gao *et al.*, 2019). Similarly, the pre-diapause females of *R. pedestris* slow down the development under stress conditions which ultimately decreased the production of protein but, the pre-reproductive females continuously developed and store more protein contents. However, accumulation of protein content strategy is not similar in diapausing insects. In response to cold temperature, the diapause insects upregulate the heat shock proteins (Hsps) in order to protect the damaged being caused by stress tolerance (King and Macrae, 2014). It has been noted that the majority of the Hsps were up-regulated in diapausing females of *Liriomyza sativa*, *Liriomyza huidobrensis* and *Sarcophaga crassipalpis* (Huang and Kang, 2007). Hsps were up-regulated when insects perceived the diapausing signal and remained high throughout the overwintering period. The up-regulation of Hsps appears to be quite prevalent in diapause that occurs at distinct developmental stages (embryo, larva, pupa, adult), as well as in species from multiple insect orders, including Diptera, Lepidoptera, Coleoptera, and Hymenoptera (Rinehart *et al.*, 2007). These data strongly suggested that protein content is also a crucial component which is being stored by both reproductive and diapause adult females, but the role of this nutrient might be different, depending on the environmental conditions. However, more research is needed to understand the molecular mechanisms behind the accumulation of these nutrients in *R. pedestris* females during diapause and reproduction.

CONCLUSION

In this study, the lipid and triglyceride contents are the major energy reserves stored by adult females of the bean bug, which may play an important role in the diapause preparation mechanism. Despite the storage of a substantial amount of glycogen contents, pre-diapause females also stored a modest amount of total protein contents, which would be up-regulated in response to diapause stress conditions. Hence it can be concluded that, two different mechanisms are involved in the allocation of the identical nutrient reserves in pre-diapause and pre-reproductive females, which provides the basic knowledge for further molecular research on the mechanisms regulating the reproductive development and nutrients reserves in *R. pedestris* and other insects.

A model of different nutrient reserves participating during pre-diapause and pre-oviposition periods of *R. pedestris*. Pre-diapause females were reared at 25 °C and L12:D12 photoperiod, while pre-reproductive females were reared at 25 °C and L16:D8 photoperiod. Insects

were fed on fresh soya bean pods with water. Females under the short-day photoperiodic conditions stored a subsequent amount of lipids and triacylglycerol (TG) contents as the major nutrients for longer diapause period whereas, the females under the long-day photoperiodic conditions stored the protein contents for continuing their ovarian development.

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Statement of conflicts of interest

The authors have declared no conflict of interest.

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