Effect of thermoperiod on diapause intensity in *Pyrrhocoris apterus* (Heteroptera Pyrrhocoridae)

P. Kalushkov a, b, M. Hodková b, O. Nedvěd b, c, I. Hodek b,*

* Institute of Zoology, Bulgarian Academy of Sciences, Blvd. Tzar Osoboditel, 1000 Sofia, Bulgaria
b Institute of Entomology, Academy of Sciences, Braníčská 31, 370 05 České Budějovice, Czech Republic
c Faculty of Biological Sciences, University of South Bohemia, Braníčská 31, 370 05 České Budějovice, Czech Republic

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Abstract

The intensity of adult diapause in *Pyrrhocoris apterus* was measured in two series of experiments as the duration of pre-oviposition period at a constant temperature of 25°C after transfer from short (12L:12D) to long day conditions (18L:6D). Higher diapause intensity was induced with a thermoperiod than at constant temperatures. After the induction throughout larval instars 3–5 and during 4 weeks of adult life at short days and a thermoperiod of 25/15°C the pre-oviposition period was 30±4 and 26±3 days. After induction at constant 25°C the pre-oviposition period was 22±3 and 23±4 days, while after induction at constant 20°C it was 17±4 and 19±4 days. Induction at a lower constant temperature of 20°C was thus followed by a less intense diapause than the induction at a higher constant temperature of 25°C. These counterintuitive results are discussed. The oxygen consumption rate measured at experimental temperatures prior to transfer from short to long days was higher at thermoperiodic conditions than at constant temperatures and it was similar at constant 20 and 25°C. Thus, the oxygen consumption rate measured prior to the transfer was highest (indication of the least intense diapause) in the insects that showed later, after the transfer to long days, the longest pre-oviposition period (indication of the most intense diapause). Within the first two days after transfer to constant 25°C, oxygen consumption rate was similar in all groups. Cold hardiness was not correlated with diapause intensity. The low lethal temperature in diapausing insects was correlated with the night temperature during diapause induction. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Pyrrhocoris apterus*; Diapause intensity; Pre-oviposition period; Metabolic rate

1. Introduction

The intensity of diapause has been defined as the time required under given conditions before some measure of diapause end is observed (Danks, 1987). Thus, in adult females, the duration of the pre-oviposition period has usually been used to describe the intensity of diapause.

Great variation in diapause intensity can be recorded in most insects and it has been attributed both to genetic and exogenous factors. In many studies variation in photoperiod was revealed as one of the factors inducing variation in diapause intensity (Tauber et al., 1986; Danks, 1987). Thus diapause was, e.g. less intense in the bug *Riptortus clavatus* when induced by photoperiods near the critical value (Numata and Hidaka, 1983).

Temperature is another source of variation in diapause intensity, but the literature data are ambiguous. Sieber and Benz (1980) in *Laspeyresia pomonella* and Denlinger and Bradfield (1981) in *Manduca sexta* recorded that higher temperatures produced more intense diapause than lower temperatures. On the contrary, the adult diapause of *Bruchidius atrolineatus* males was more intense after rearing at 23°C/10°C than at 40°C/25°C (Glitho et al., 1991). Similarly the 3rd instar larvae of the chrysopid *Mallada flavifrons* had a diapause twice as long after the induction in the 2nd instar at 15°C than at 25°C (Principi et al., 1990).

In late summer/early autumn, outdoor *P. apterus* are often exposed during the night to low temperatures near zero which could modify the intensity of diapause. It has
been already recorded how much *P. apterus* responds to only short exposures to cold (5–12°C); diapause was induced in 30 to 75% of the females in spite of diapause-preventing long days (Hodek, 1971b). Similar to diapause incidence, the intensity of diapause could be affected by low temperatures in this insect.

Our aim was to determine whether a more intense diapause can be induced under conditions simulating outdoor temperatures, i.e. thermoperiod with low scotophase temperature, than at comparable constant temperatures.

Varjas and Sáringer (1998) suggest that the level of oxygen consumption is a better criterion of diapause intensity. Thus the rate of oxygen consumption was also recorded, together with several other parameters: duration of pre-oviposition period, incidence of oviposition, fecundity, and parameters of cold hardiness.

### 2. Material and methods

#### 2.1. Experimental animals and treatment

The above mentioned question was repeatedly addressed in two series of experiments (I and II). The animals in both series were F1 offspring of adults collected near České Budějovice (49°N, 14°E), South Bohemia. The larvae were reared under long day conditions (18L:6D), at 25°C until the mid 3rd instar (series I) or mid 4th instar (II). They were subsequently placed into diapause-inducing short day photoperiod (12L:12D) and experimental temperatures where they completed larval development and spent four weeks of adult life before measurements or further transfer (see Section 2.2). The highest diapause intensity was recorded at this age in earlier studies (Hodek, 1983). Experimental temperatures were: constant temperatures 25 and 20°C, and thermoperiod 25/15°C.

#### 2.2. Pre-oviposition period and fecundity

After the diapause-inducing treatment, a portion of the diapausing adults were transferred to long day conditions and constant temperature (25°C) for photoperiodic activation (i.e. diapause development accelerated by diapause averting photoperiods). Fifty individual pairs were observed daily in series I (35 in series II) to record the time until first oviposition. In series I, the total number of eggs laid until death was counted. The incidence of ovipositing females was calculated.

#### 2.3. Oxygen consumption

The metabolic rate was measured as oxygen consumption manometrically with a Warburg respirometer (Slama, 1960). Respiratory vessels of about 10 ml volume were employed with sodium calcite to absorb carbon dioxide and with a small piece of wet cotton to maintain constant pressure of water vapour. The recorded value of oxygen consumption of each individual at rest represented an average from three 0.5 h readings.

Oxygen consumption was measured at experimental temperatures (20 or 25°C during photophase and 15, 20 or 25°C during scotophase) one day before transfer to constant 25°C and long days or short days (series II). After the transfer, both photophase (both series) and scotophase (series II) measurements were performed at 25°C on the day of transfer (both series), two days after transfer and at subsequent seven-day intervals (series II). The oxygen consumption was measured 4–8 h after lights on in both series and at subjective midnight in series II.

#### 2.4. Cold hardiness

In adults reared at three diapause-inducing conditions (series I) supercooling points were measured according to Nedvěd et al. (1995) in 16 individuals per treatment. Survival was observed after exposure to −8.5°C for 5 days (30 individuals). Mortality caused by freezing was then estimated as the proportion of individuals having a supercooling point above −8.5°C, the rest was attributed to non-freezing mortality.

In series II, cold hardiness was measured as survival after exposure to subzero temperatures (above mean supercooling point). Thirty individuals were exposed to constant temperatures of −5, −7, or −10°C; after 10 days they were transferred to laboratory temperatures and survival was calculated after 24 h. Lethal temperature for 50% of the animals was computed using logistic regression (Statistica software, nonlinear estimation, Statsoft, 1997; see Nedvěd et al., 1998). The pairs of values with their standard error were compared using t-test.

### 3. Results

#### 3.1. Pre-oviposition period and fecundity

Duration of the pre-oviposition period was significantly longer in females induced to diapause by thermoperiod than by constant temperatures (Table 1, Table 2). The difference was smaller in series II, where the experimental treatment began in older larvae and was thus shorter. The females reared during diapause-inducing conditions at a lower constant temperature of 20°C showed in both series significantly shorter pre-oviposition periods than those reared at 25°C.

The incidence of ovipositing females was high and similar in all experiments (Table 2) in series II. In series
Table 1
Parameters of diapause intensity and cold hardness in Pyrrhocoris apterus, measured after transfer to long days and 25°C (series I)*

<table>
<thead>
<tr>
<th>Experimental temperature (°C)</th>
<th>Incidence of ovipositing females (IO) (%)</th>
<th>Pre-oviposition period (days)</th>
<th>Fecundity (eggs/♀)</th>
<th>Oxygen consumption on day 0 (μl/g/h)</th>
<th>Supercooling point (°C)</th>
<th>Survival (%)</th>
<th>Freeze mortality (%)</th>
<th>Non-freeze mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>82</td>
<td>22±3.2 a</td>
<td>23±140</td>
<td>279±91 a</td>
<td>−11.7±4 a</td>
<td>37</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td>20</td>
<td>62</td>
<td>17±4.1 b</td>
<td>152±83</td>
<td>404±80 b</td>
<td>−12.6±4 a</td>
<td>67</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>25/15</td>
<td>38</td>
<td>30±4.1 c</td>
<td>271±195</td>
<td>446±99 bc</td>
<td>−12.4±4 a</td>
<td>60</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>n</td>
<td>50</td>
<td>50.0±100</td>
<td>50.0±100</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Duration of pre-oviposition period, incidence of ovipositing females, fecundity, oxygen consumption rate on the day of transfer, supercooling point, survival after 5 days at −8.5°C, freeze mortality and non-freeze mortality. Standard deviations and indication of different values (letters a–c; α=0.05) according to Tukey’s HSD test are given.

Table 2
Parameters of diapause intensity and cold hardness in Pyrrhocoris apterus, measured after transfer to long days and 25°C (series II)*

<table>
<thead>
<tr>
<th>Experimental temperature (°C)</th>
<th>Incidence of ovipositing females (IO) (%)</th>
<th>Pre-oviposition period (days)</th>
<th>Lethal temperature (LT₅₀) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>62</td>
<td>23±4 b</td>
<td>−7.2±0.2</td>
</tr>
<tr>
<td>20</td>
<td>62</td>
<td>19±4 a</td>
<td>−7.6±0.1</td>
</tr>
<tr>
<td>25/15</td>
<td>60</td>
<td>26±3 c</td>
<td>−8.1±0.2</td>
</tr>
<tr>
<td>n</td>
<td>35</td>
<td>50.0±100</td>
<td>3±30</td>
</tr>
</tbody>
</table>

* Duration of pre-oviposition period (±S.D.), incidence of ovipositing females, lower lethal temperature after 10 days exposure (LT₅₀). Indication of different values (letters a–c; α=0.05) according to Tukey’s HSD test is given.

I, the insects had lower viability. Some of the females reared at the thermoperiod died early after the transfer into LD, and thus only a lower oviposition incidence was recorded (Table 1); in insects from constant temperatures the incidence was higher.

A great variance in fecundity of P. apterus has always been recorded (e.g. Hodek, 1988), and thus the differences between individual samples are rarely significant. This was also the case here; although the average fecundity values were much higher in females reared at higher temperatures (Table 1), the difference was not significant (ANOVA).

3.2. Oxygen consumption

3.2.1. Oxygen consumption before transfer (Series II)

Oxygen consumption rate was measured one day before transfer from experimental temperatures and short-day conditions. The oxygen consumption rate was always higher at photophase than at scotophase (Fig. 1A, B values on day −1). The scotophase oxygen consumption rate was similar in all temperature regimens, although scotophase temperatures were different (15, 20, 25°C) (Fig. 1B, values on day −1). In contrast, the photophase oxygen consumption rate was dependent on the temperature regimen (Fig. 1A, values on day −1). Thus the photophase oxygen consumption rate was higher in bugs reared at a thermoperiod of 25/15°C than in bugs reared at constant 25°C, although the photophase temperature was 25°C in both cases. At constant 20°C, the oxygen consumption was similar to that at constant 25°C.

3.2.2. Oxygen consumption after transfer to constant 25°C

The insects were transferred from experimental temperatures and short-day conditions to 25°C and either short-day or long-day conditions.

3.2.2.1. Short-day conditions (Series II)

The control group kept permanently at constant 25°C maintained a similar oxygen rate consumption during the whole experiment (Fig. 1A, B). Insects transferred from constant 20°C to constant 25°C showed an important transient increase in photophase oxygen consumption rate on the day of transfer (Fig. 1A, values on day 0). On day 2 after transfer, the oxygen consumption rate decreased to the original level found before transfer (Fig. 1A, B). The photophase oxygen consumption rate of insects transferred from the thermoperiod of 25/15°C did not change on the day of transfer, but it decreased on day 2 after transfer (Fig. 1A). The scotophase oxygen consumption rate did not change after transfer (Fig. 1B). From day 2 after transfer, the oxygen consumption rate remained low and similar in all groups. The scotophase values (Fig. 1B) were always lower than the day values (Fig. 1A).

3.2.2.2. Long-day conditions (Series II)

Conditions on days −1 and 0 were identical for insects transferred to short-day and long-day conditions. Therefore the oxygen consumption rate shown for days −1 and 0 in Fig. 1 (transfer to short-day conditions) are identical with those given in Fig. 2 (transfer to long-day conditions). The oxygen consumption rate in all groups gradually increased under long-day conditions. The increase was
Fig. 1. Oxygen consumption rates in females of *P. apterus* measured under short-day conditions and experimental temperatures and after transfer to short-day conditions and 25°C (series II). Measurement at photophase (A), at scotophase (B). Experimental temperatures: constant 25°C (squares), 20°C (circles), thermoperiod of 25/15°C (triangles). Arrows indicate the day of transfer. Females were transferred during photophase or scotophase from 20°C and during photophase from 25/15°C. Each point represents mean ± S.E.M., *n*=20. Twelve comparisons were made among the data plotted in Fig. 1 and Fig. 2. The significance level was thus adjusted from 0.05 to 0.0042. There was a significant difference in the starting values (day 21, or day 0 in bugs from 25°C) measured during the day (photophase), uncovered by ANOVA (*F*=7.4, *P*=0.0014). Tukey’s HSD test indicated the value in the bugs from thermoperiod (25/15°C) as different from the other two conditions (20°C, 25°C). There was no such difference in the starting values measured during night (scotophase) (*F*=1.89, *P*=0.16). There was a significant increase in oxygen consumption rate from day 21 to day 0 in the bugs from 20°C in measurements held during photophase (*F*=29, *P*=4.10-6) but not during scotophase (*F*=7, *P*=0.01). There was a significant decrease in oxygen consumption rate from day −1 to day 2 in the bugs from thermoperiod (25/15°C) in measurements undertaken during the photophase (in short day: *F*=20, *P*=5.10−3; in long day: *F*=26, *P*=10−3), but not during scotophase (in short day: *F*=4.3, *P*=0.045; in long day: *F*=1.6, *P*=0.22). There was not a significant difference between the three treatments on the day 2, in both photoperiods and day phases (in short day, photophase: *F*=0.4, *P*=0.68; scotophase: *F*=0.3, *P*=0.77; in long day, photophase: *F*=2.6, *P*=0.08; scotophase: *F*=5.8, *P*=0.0052).

Fig. 2. Oxygen consumption rates in females of *P. apterus* measured under short-day conditions and experimental temperatures and after transfer to long-day conditions and 25°C (series II). For explanations and statistics see Fig. 1.

3.3. Cold hardiness

The supercooling point was similar in all three treatments of series I (ANOVA). Survival after 5-day cold exposure differed among treatments but there was no clear relation to the intensity of diapause measured by the other variables. The samples reared under the thermoperiod or constant 20°C had lower proportions of non-freeze mortality compared to those from constant 25°C (Table 1).

The lethal temperature, LT<sub>50</sub>, was similar (Table 2) in all three conditions of series II. However, the values were different at the 5% level (*t*-test of pairs of treatments). The lethal temperature of diapausing insects was correlated (*P*=0.04) with the night temperature during diapause induction.

4. Discussion

4.1. Diapause intensity after induction at constant temperatures (20 vs 25°C)

Low temperatures and short-day photoperiods have generally been reported as diapause inducing cues in hibernation diapause of “long-day” insects (Danks, 1987; Tauber et al., 1986). Low temperatures increase thus the incidence of diapause. As diapause incidence is
mostly linked to diapause intensity one would expect that the action of low temperatures at induction would strengthen diapause. Such effects were often recorded (e.g. Glitho et al., 1991; Principi et al., 1990—see Introduction for details).

However, we found in both experimental series a significantly longer pre-oviposition period in \textit{P. apterus} adults reared at short days in higher constant temperature. While rearing at 25°C was followed by pre-oviposition periods of 22 and 23 days, rearing at 20°C produced pre-oviposition period of 17 and 19 days. Although it appears counterintuitive, such findings are not unique.

Thus in the codling moth, \textit{Carpocapsa (=Laspeyresia) pomonella}, induction of larval diapause at short days and 19 or 21°C resulted (after transfer to long days and 26°C) in a short photoperiodic activation period of 20–30 or 30–40 days, respectively. Induction at a higher temperature of 26°C was followed by much longer activation period of 70–80 days. Such a longer activation period was considered as an indication of a more intense diapause (Sieber and Benz, 1980). Also in \textit{Teleogryllus emma} crickets (Masaki, 1962) and in the Hessian fly, \textit{Mayetiola destructor}, higher temperatures induced a more intense diapause (Wellso, 1991).

In the corn borer, \textit{Ostrinia nubilalis}, Beck (1989) induced at 22°C a more intense larval diapause than at 19°C: the pupation delay was 5 days later. In his explanation, the larvae from 19°C were transferred too early to the activating conditions; they had not yet completed the intensification of diapause. Those results are similar to a thorough analysis made in the tobacco hornworm, \textit{Manduca sexta} (Denlinger and Bradfield, 1981). It was clearly demonstrated that the intensity of diapause depended on the length of the induction period, i.e. on the number of photoperiod cycles perceived. At lower temperatures of 20°C the larvae developed much longer (44–50 days) and were thus exposed to many short-day cycles; diapause lasted about 50 days. At 30°C the larvae developed in only 16–18 days and diapause lasted about 80 days. When autumn pupae have a shorter diapause than the earlier pupae the start of post-diapause development is better synchronized (Denlinger and Bradfield, 1981).

While in \textit{M. sexta} and some other species the above model of synchronization appears adaptive, in \textit{P. apterus} such a type of synchronization does not seem to be needed. Diapause development is completed in December/January (Hodek, 1971a) and even the insects with longest diapause have enough time for synchronization with earlier individuals before the reproduction is started due to the appropriate temperature increase in April/May (Table 2 in Hodek, 1988). Neither does Beck’s intensification hypothesis seems plausible for our results as the four-week induction period in adults from all three temperature conditions is certainly satisfactory.

We assume that the increase in temperature by 5 degrees at the transfer of females from diapause-inducing 20°C to activation conditions might represent an important stimulus overriding the impact of deeper diapause. When the insects live at 25°C both before and after transfer no such stimulus is perceived. Our explanation seems to hold also for the results on \textit{C. pomonella}. Sieber and Benz (1980) reported that even a mere increase in temperature from 19 to 26°C, without any change in photoperiod, terminated diapause within 35 days in most larvae.

Apparently the stimulating (or inhibiting) effect of temperature change cannot be disregarded in the studies of diapause intensity.

4.2. Effect of thermoperiods

In both experimental series longer pre-oviposition periods were recorded after diapause induction by thermoperiod than by constant temperature. The values achieved by induction at laboratory thermoperiod (26 and 30 days) were similar to those found in September samples of four populations that experienced outdoor thermoperiods (aver. 25, 26, 30 and 31 days) (Hodek, 1971a). The difference between the thermoperiod of 25/15°C and the comparable constant temperature of 20°C (Table I and Table 2) amounted to 13 days in series I and 7 days in series II. The lower value in series II, where older larvae were exposed to diapause inducing conditions, indicates a high sensitivity of larvae of \textit{P. apterus} to the effect of low temperatures of 15°C during scotophase. Sensitivity of \textit{P. apterus} young adults to low temperatures in the range of 5–12°C was previously recorded in experiments where the incidence of diapause induction was measured (Hodek, 1971b).

The effect of thermoperiod (10/30°C) was compared to constant high temperature (30°C) also in prepupal diapause of the alfalfa leaf cutting bee \textit{Megachile rotundata}, and much longer pre-emergence time resulted from incubation at 30°C (Rank and Rank, 1990). However, the pre-emergence time cannot be taken as adequate measure of diapause intensity as it contained not only diapause of prepupae but also development of pupae. In addition, diapausing pupae were exposed to the two experimental temperatures and not the pre-diapause stage; the authors do not provide data on diapause induction and sensitive stage. Thus, in fact, the effect of thermoperiod on diapause development was measured, rather than on the induced intensity.

4.3. Cold-hardiness

The supercooling points (around −12°C) and the mortality caused by freezing were similar after the induction of diapause at all three experimental temperatures (Table 1). On the other hand, non-freeze mortality and the low
lethal temperature were lower in insects reared under lower night temperatures during the induction period (Table 1, Table 2). Thus, lower night temperatures in the field during late summer and autumn might enhance survival of *P. apterus* during winter. Our earlier experiments showed that acclimation of laboratory *P. apterus* to a thermoperiod of 20/5°C or constant 15°C resulted in a decrease in the supercooling point from −11°C to −15°C (Hodkova and Hodek 1994, 1997). In field *P. apterus*, the supercooling point decreased from −12°C to −17°C in January and February when minimum temperatures were below 0°C (Hodkova and Hodek, 1997). Thus it seems that acclimation to temperatures lower than those used in the present experiments are needed for a decrease of freeze mortality and optimum survival of winter conditions.

4.4. Oxygen consumption

4.4.1. Effect of temperature on oxygen consumption rate

The photophase oxygen consumption rate was highest at the thermoperiod of 25/15°C, most probably due to the stimulation by the daily temperature increase from 15 to 25°C. The decrease in oxygen consumption rate after the transfer to constant 25°C was apparently due to the disappearance of the temperature stimulation. On the other hand, similar oxygen consumption rates at constant 20 and 25°C may be explained by temperature compensation of metabolism. Therefore, the transfer from 20 to 25°C caused only a transient increase of the oxygen consumption rate. Both temperature stimulation and temperature compensation of metabolism are well documented in ectothermic organisms (Precht et al., 1973).

4.4.2. Relationship between pre-oviposition period and oxygen consumption rate

Apart from measuring oviposition delay as a criterion of diapause intensity in adults the relationship between the pre-oviposition period and the oxygen consumption rate was analysed. The intensity of diapause is generally thought to be inversely proportional to the metabolic and developmental rates (Beck, 1980). If so, there should be an inverse relationship between the duration of pre-oviposition period and the oxygen consumption rate in the case of adult diapause. However, no such relationship was found. Prior to transfer to constant 25°C, the photophase oxygen consumption rate was higher at thermoperiodic conditions (25/15°C) than at constant 20 or 25°C, although the pre-oviposition period was longest after the induction at thermoperiod. Under constant temperatures, the oxygen consumption rate was similar at 20 and 25°C, although the pre-oviposition period was shorter after the induction at 20°C. Therefore, if the duration of pre-oviposition period is a measure of diapause intensity, the oxygen consumption rate measured prior to the transfer to constant 25°C does not seem to be related to diapause intensity. A similar oxygen consumption rate at constant 20 and 25°C might be caused by deeper diapause suppression of the metabolic rate at 25°C. However, the presumably deeper diapause did not prevent a daily increase in oxygen consumption rate under thermoperiodic conditions.

Two days after transfer to constant 25°C, the oxygen consumption rate was similar in all three groups. However, the change of oxygen consumption rate within 2 days after the transfer to constant 25°C seems to be inversely proportional to pre-oviposition period. The shortest pre-oviposition period was associated with a transient increase in oxygen consumption rate (after transfer from 20°C to 25°C), the longest pre-oviposition period was associated with a decrease in the photophase oxygen consumption rate (after transfer from 25/15°C to constant 25°C), and the intermediate pre-oviposition period was associated with constantly low oxygen consumption rate at 25°C (Fig. 3). Gradual increase in oxygen consumption rate later, after the transfer (>7 days)

![Fig. 3. Schematic description of the relationship between oxygen consumption rate and pre-oviposition period in *P. apterus*. (A) Duration of pre-oviposition period after transfer from experimental temperatures and short days to 25°C and long days. (B) Photophase oxygen consumption rate at experimental temperatures and after transfer to 25°C; arrows indicate the time of transfer. Transient increase in oxygen consumption rate after transfer from 20°C to 25°C is associated with the shortest pre-oviposition period, maintenance of low oxygen consumption rate at constant 25°C is associated with intermediate pre-oviposition period, decrease in oxygen consumption rate after transfer from 25/15°C to constant 25°C is associated with the longest pre-oviposition period.](image3)
to long-day conditions, is most probably related to ovarian maturation rather than to the photoperiodic activation itself.

Photoperiodic activation of diapause is mediated by the neuroendocrine system (Denlinger, 1985). It can be hypothesized that the change of metabolism acts as a signal to the neuroendocrine system; the photoperiodic activation may be delayed or accelerated by the decrease or transient increase of metabolism, respectively. Alternatively, the neuroendocrine system and the oxygen consumption rate may be independently inhibited or stimulated by temperature change. As suggested in Section 4.1, the effect of temperature change at the onset of photoperiodic activation may be another factor influencing the pre-oviposition period, in addition to the initial intensity of diapause and to the conditions acting during the subsequent activation.

Acknowledgements

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