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Physiology of diapause in pupae of *Pieris brassicae* L. (Lepidoptera: Pieridae)

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Abstract. Respiration patterns, water loss and heart activity were investigated during the first three months of diapause in non-acclimated pupae of *Pieris brassicae*. To observe and record diverse events during pupal diapause, a complex apparatus was used: a micro-calorimeter, an electrolytic respirometer, a fibre-optical oxygen sensor, a flow-through respirometer (infrared gas analyzer), an infra-red actograph and a thermocouple cardiograph. Most of the pupae (about 80%) reared in 2004 were characterised as long-cycle individuals whose discontinuous gas exchange cycles (DGCs) were very regular and lasted 26 hours on average. The remainder of the pupae studied were short-cycle individuals displaying irregular DGCs lasting less than 2 hours. Standard metabolic rates (SMR) measured during the first month of diapause between long- and short-cycle pupae did no differ significantly, being about 0.018 ml O_2 g^{-1} h^{-1}. At the same time, water loss rate (WLR) in long- and short-cycle pupae differed significantly, being 1.07 and 1.61 mg g^{-1} day^{-1}, respectively. During the first three months of diapause, the values of SMR and WLR did not change significally in the long-cycle pupae. In the short-cycle individuals, SMR and WLR thereupon increased gradually during the months, but the values of SMR never reached the levels characteristic of pharate adult development. The heartbeat reversal was characteristic for both the long- and short-cycle individuals but heart pauses in the first pupal group were regular, lasting 20–30 min, whereas in the second group the heart pauses were shorter and irregular.

From the results we concluded that the intensity of pupal diapause varied individually despite the apparently similar developmental conditions of the individuals, however, some hidden factors were obviously involved in diapause induction. The primary cause of the enhanced water loss in the short-cycle pupae was obviously the disturbing of the water conserving mechanisms due to the irregular gas exchange.

Key words: *Pieris brassicae*, pupal diapause, discontinuous gas exchange cycles, (C)FV and (C)FO cycles, standard metabolic rate, water loss rate, heart activity, cold tolerance

INTRODUCTION

Diapause was earlier defined as a state of arrested growth and development (Lees, 1956). As the term “arrest” allows no possibilities for different degrees of intensities of diapause, Beck (1980) identified diapause as a state of suppressed developmental rate, recognising that some developmental changes must occur during diapause. The pupal diapause of lepidopterous species always involves a suppression of metabolic and

developmental rates. Metabolic rate is measured by oxygen consumption or by the rates of carbon dioxide release. The intensity of diapause is usually estimated by the duration and stability of diapause (Danks, 1987). Diapause intensity was also expressed as the period required for adult emergence (Li et al., 2001). However, the intensity of pupal diapause is generally thought to be inversely proportional to metabolic rate and developmental rate. Great variation in diapause intensity can be recorded in most insects during the photoperiod; temperature as well as the food-plants of larvae have been found to influence the apparent intensity of pupal diapause in a number of different species (Hunter & McNeil, 1997). Food constituents are one of the several interacting determinants of diapause induction in the lepidopterous pupae (Morris, 1967; Metspalu et al., 2003). However, diseases have been found as a common factor inhibiting the processes of diapause induction in the pupae of *P. brassicae* (Metspalu, 1976; Metspalu & Hiiesaar, 1984).

The physiological state and health of individual insects are usually estimated and compared by measuring their standard metabolic rate (SMR), which is defined as a value measured at a particular temperature when an insect is quiet and inactive, not digesting a meal and exposed to any stress (Withers, 1992). Thus, in pupae the values of SMR can be measured easily as their muscular activity is essentially restricted.

In addition to the standard metabolic rate (SMR), the pattern of discontinuous gas exchange cycles (DGCs) has been often used for characterising the physiological state of an insect, while the several stress factor including the chemical ones can abolish or disturb DGC (Kestler, 1991). Each cycle of gas exchange consists of three periods according to the spiracular movements: closed (C), open (O) and flutter (F). During the O period, a burst of carbon dioxide release occurs. The flutter, observed between the bursts, i.e. during the interburst period, means that the spiracles opened only for a fraction of a second (fluttering), but most of the time they were closed. During the flutter, air is sucked passively into the tracheae, referred to as passive suction ventilation (PSV). The DGC is termed as a CFV cycle when the O phase is associated with active, i.e. muscular ventilation, whereas in the CFO cycle the active ventilation is lacking (Kestler, 1985; Tartes, 1995; Lighton, 1996; for reviews). Usually, the gas exchange patterns in diapausing pupae of lepidopterous species have been studied in non-acclimated individuals kept at room temperatures during the winter months (see Schneiderman & Schechter, 1966).

The respiration of dormant individuals and their rates of water loss are closely linked, because together with bursts of carbon dioxide water vapour can be released (Danks, 2000). Intermittent respiration, and essentially PSV, has been supposed to conserve water by keeping the spiracles shut most of the time (Kestler, 1985).

Pupal diapause in many insects, including *P.brassicae*, is terminated only after a prolonged exposure to relatively low temperatures (–5 to 10ºC), referred to as cold acclimation. The process of diapause reactivation in *P. brassicae* can not at all occur without preliminary exposition of pupae to low temperatures (Metspalu, 1976). According to Metspalu & Hiiesaar (1980, 1984), the mean supercooling point (SCP) in non-acclimated *P. brassicae* was –23.5±2.1ºC, however, cold tolerance was not studied. Usually the cold hardiness or cold tolerance is estimated as the survival rate after exposure to low temperatures for a certain period of time (Ding et al, 2003).

Earlier there have been investigated the relations between patterns of gas exchange and water loss in pupae of *P. brassice* that have spent 4–6 months at a

diapause state (Jõgar et al., 2004). However, there are very little data about the physiological characteristics at the time of deep diapause of lepidopterous pupae, i.e. during the first 1–3 months of diapause when metabolic rate is maximally suppressed. The aim of the present paper was to investigate DGC, SMR, water loss rate or WLR, heartbeat patterns and cold tolerance, i.e. the essential parameters of the physiological state, during the first months of the pupal diapause of *Pieris brassicae.*

MATERIALS AND METHODS

Insects and weighing

The eggs of *P. brassicae* (second generation) were collected in cabbage fields near Tartu (Estonia) in July and August 2004. The insects were reared at a laboratory in short-day conditions (12L: 12D, at 20–22ºC). The pupae (n=55) were not acclimated at low temperatures and were kept during the winter months at a state of "permanent" diapause in Petry dishes at a room temperature (20–22ºC) and ambient air humidity (55–65% RH). Experiments were undertaken during the first three months of diapause (October to December). The pupae were weighed weekly with an analytical balance to 0.1 mg. In the experiments we used pupae whose body mass ranged from 370 to 510 mg (mean 415±34 mg) measured 2–3 weeks after pupation i.e. at the outset of diapause.

Water loss measurements

Body mass loss was measured gravimetrically. The gravimetric method used assumes that body mass loss and water loss are equivalent at the pupal stage when there is no intake of food or water (Hadley, 1994) and the RQ is nearly that of the ratio of the molar masses of O_2 to CO_2 0.727 (Kestler, 1985). Hence, assuming fat metabolism, pupal mass loss will be referred to below as water loss. The water loss rate (WLR) was calculated not on the individual surface area but on body mass, assuming that the WLR was mostly due to the respiratory and not the cuticular transpiration (see Edney, 1977; Loveridge, 1980).

Calorimetric measurements

Calorimetry was the only method for continuous recording of DGCs for weeks in the long-cycle individuals without evoking the stress by handling and adjusting the apparatus. Early there was demonstrated that micro-calorimeters are useful for long-term measurements of cyclic gas exchange in insects (Kuusik et al., 1995; Harak et al., 1996; Harak, 1997; Harak et al., 1998; Lamprecht & Schmolz, 1999). For this study, a simple twin differential calorimeter was constructed of vessels made from copper foil (0.1 mm) connected with copper-constantan thermocouples, while a micro-nano-voltmeter and a recorder were used (see Harak et al., 1999). The volumes of both the insect and reference vessels were 0.5 ml and the sensitivity of the calorimeter was 50 $\mu V\ mW^{-1}$ with a detection limit of 4 μW (twice the background noise). The calorimeter was calibrated electrically by the Joule effect (Hemminger & Höhne, 1984). The calorimeter was sufficiently sensitive to record the carbon dioxide release by burst and abrupt air intakes into the tracheae in pupae of *P. brassicae*.

Electrolytic respirometry

A differential electrolytic microrespirometer-actograph was used for the sensitive recordings of respiratory patterns (Kuusik et al., 1991; Tartes & Kuusik, 1994; Tartes et al., 1999, 2000, 2002). This closed-system and constant volume micro-respirometer allowed simultaneous recording of metabolic rate, discrete CO_2 releases (bursts), rapid intakes of air into the tracheae, referred to as passive suction ventilation (PSV) in micro-cycles, and active abdominal movements. The rates of generation of oxygen by electrolysis are indicated on graphs as oxygen flux `$FO_2$ (STPD ml $O_2$ $h^{-1}$)`. They represent also the recorded transient ml rate changes of CO_2 release and air intake as indicated.

The respirometer ensures continuous replacement of consumed oxygen by electrolysis-produced oxygen. The insect itself plays an active role in this self-regulating system. Rapid changes of pressure in the insect chamber, caused by active body movements of the insect, or other rapid events, will lead to corresponding rapid changes in the electrolysis current reflected as spikes on recordings. Carbon dioxide release causes a rise of the liquid meniscus on the left side of the U-shape capillary (Fig. 1), thus the photodiode is screened from the light beam. This event causes temporary decrease in the electrolysis current and oxygen generation. In this way, CO_2 bursts are not measured but only indicated on the respirogram as the clear downward peaks lasting several minutes, and these peaks we refer to as bursts of carbon dioxide. A 40% potassium hydroxide solution was used to absorb the CO_2 and to guarantee about 60% RH inside the insect chamber (see Tartes et al., 1999). Temperature inside the insect chamber was continuously recorded with a thermocouple Datalogger Thermometer (TES Electrical Electronic Corp.).

Respirometry by means of an oxygen sensor

Constant volume respirometry by means of a fibre-optical oxygen sensor was used as a simple express method for preliminary estimation of the oxygen consumption level in single individuals, in 6–8 pupae per hour. A selection of pupae with high and low SMR was made using this oxygen sensor. A hermetically closed insect chamber with an inner volume of 2 ml was the same used in our other respirometry systems (Fig. 1). It was assumed that the pupal body mass of 1 g was adequate to a volume of 1 ml. The humidity (%RH) and temperature inside the insect chamber were continuously recorded on the PC monitor using the Humidity and Temperature Display Instrument for digital HygroClip probes (HygroPalm, Rotronic Company) referred to as hygrometer.

The fibre-optical oxygen sensor was a laboratory prototype built in the Institute of Physics of the University of Tartu. The sensor is based on the effect of quenching the luminescence of dye molecules by molecular oxygen (Lübbers & Opitz, 1975), and it measures the intensity of the optode luminescence excited by a violet LED. The fibre-type optode of the sensor was dip-coated by a thin film of polymethylmetacrylate, doped with Pd-tetraphenylporphyrin dye. The properties of this sensor material have been recently studied (Õige et al., in print). It has been found that the luminescence intensity follows exactly the linear Stern-Volmer law, which assures a linear response of the sensor. For achieving a high stability of the calibration (drift of the sensitivity less than 0.1% per month), the optode was properly pre-aged before using it in the

measurements. The calibration of the sensor was checked before and after the measurements of breathing.

Flow-through respirometry

The infrared gas analyser, or IRGA (Infralyt-4, VEB, Junkalor, Dessau), was used to prove that the presumed CO_2 signals, i.e. the downward peaks on the recording of the electrolytic respirometer, were actually due to CO_2 bursts and to measure them quantitatively. IRGA was calibrated at the different flow rates by means of calibration gases (Trägergase, VEB, Junkalor, Dessau) and with gas injection. Air flow rates from 3.6 to 10.8 l per h were used; the lower air flow rates gave higher sensitivity. The insect chamber could be switched either to the IRGA or to the electrolytic respirometer (Fig. 1) without disturbing the insect as described earlier (see Martin et al., 2004).

Simultaneous measurements of water loss rate (WLR) and CO_2 release

The cyclic releases of CO_2 and WLR were simultaneously recorded by means of flow-through respirometry (IRGA) combined with a hygrometer (HygroPalm, Rotronic Company) inserted into the insect chamber.

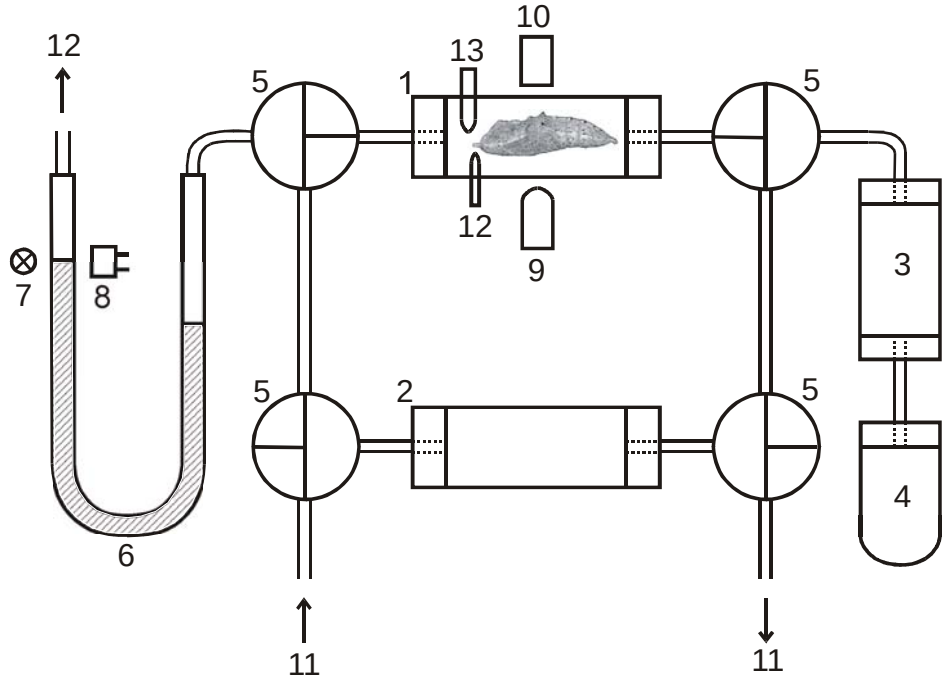

Fig. 1. Schematic diagram of an experimental set-up for respirometry. 1 – An insect chamber that may be switched from still-air electrolytic respirometry to the flow-through respirometry system of the infrared gas analyser (IRGA); 2 – reference chamber of IRGA; 3 – vessel for potassium hydroxide; 4 – electrolysis unit; 5 – three-way taps; 6 – glass capillary with ethanol; 7 – light source; 8 – photodiode; 9 – infrared emitter diode; 10 – infrared sensor diode; 11 – connections to the fow-through system; the double lines denote the polyethylene tubings; 12 – fibre-optic oxygen sensor; 13 – hygrometer.

Infrared actography

The electrolytic respirometer and the IRGA both were combined with an infrared (IR) cardiograph of insects, which we refer to as the IR actograph (IRA), because it records not only heart pulses but also all other abdominal contractions, including the muscular ventilating. An IR-emitting diode was placed on one side of the chamber near the ventral side of the abdomen, while an IR-sensitive diode was placed on the opposite side of the chamber (see Metspalu et al., 2001, 2002; Kuusik et al., 2002) (Fig. 1). The light from the IR-diode was modulated by the contractions of the heart and skeletal muscles. The level of output voltage reflected the vigour of the muscular contractions of the insect (see Hetz, 1994; Hetz et al., 1999). Abdominal contractions and heart systoles, resulting in downward spikes, muscular relaxations and heart diastoles, were directed upward.

Thermocouple cardiography

The direction of heart peristalses was observed and recorded by the method of differential copper-constantan thermocouples or a contact thermography, which in principle was described earlier (Tartes et al., 2000). Here we used an improved thermocouple method for recording the heartbeat reversals. One welding of the thermocouple was fixed to the metathorax above the dorsal vessel using a slip of adhesive tape, the other welding was fixed to the second abdominal segment (Fig. 2). A glass thermistor was contacted between the two thermocouples for the local heating of the pupal body about 2ºC above the ambient temperature. The forward directed peristalses of dorsal vessel, referred to as anterograde heartbeats, resulted in warming of the forward thermocouple, while the backward thermocouple was cooled at the same time. When the peristalses were directed backward (retrograde heartbeat), on the contrary, the forward thermocouple was cooled and the other warmed. The insect with fixed thermocouples was inserted into a special insect chamber and switched to the flow-through respirometry system. Thus heartbeat reversals and bursts of carbon dioxide release (only qualitatively measured) were recorded simultaneously.

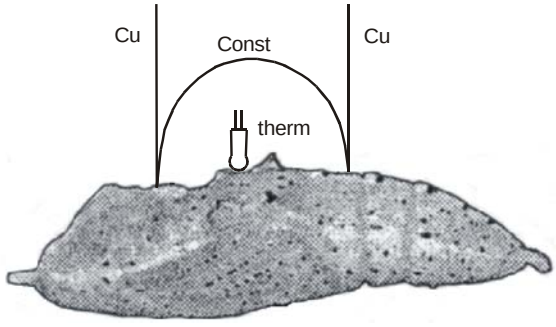

Fig. 2. A scheme of contact thermography by means of the differential thermocouples for registrating the direction of heart peristalses. A thermistor (therm) is used as a heater of the abdominal segment located between the weldings of the thermocouples.

Evaluation of cold tolerance

For evaluation of cold tolerance the novel thermocouple method was used. Single specimens were contacted to a fine thermocouple inside a foam plastic holder. Low temperature (freezing) exotherm of the super cooled pupa was recorded using a Li-Cor data logger thermometer (TES Electrical Electronic Corp.). Also the exposition time was estimated, allowing the survival of the pupae at controlled low temperatures. Usually, survival rates of pupae were determined by adult emergence, by active abdominal movements or by heart activity (see Lee et al., 2001; Ding et al., 2003). However, these mentioned methods were not acceptable for us as there occurred long heart pauses in diapausing pupae of *P. brassicae*, and often the living pupae did not exhibit any muscular response to the prodding. In the present study, the survival of a pupa was judged already during 1– 2 minutes by its metabolic activity using the fibre-optical sensor.

Data acquisition and statistics

Computerised data acquisition and an analysis were performed using DAS 1401 A/D hardware (Keithley, Metrabyte, USA) with a 10 Hz sampling rate. The four bipolar channels allowed the recording of four events simultaneously. The mean metabolic rate was automatically calculated by averaging data over a period involving at least 3 periods of activity or at least 12 cycles of gas exchange, i.e. a period lasting at least 1 hour.

Probabilities of $P < 0.05$ were considered significant. Tests were performed using the statistic package StatSoft ver. 6, Inc./USA. Values are shown as means±standard deviations. Statistical comparisons were performed with the Student's *t*-test, one-way ANOVA or repeated measures ANOVA (after testing for homogeneity of variance), followed by the Turkey's multi-range test.

RESULTS

Gas exchange in the long-cycle individuals

The calorimetric measurements revealed gas exchange cycles lasting 18 to 30 hours (mean 26.3±4.3 hours; 6 pupae with at least 6 cycles each) (Fig. 3). The flutter (F) phase can be observed in the calorimetric curve as a slow decrease of the heat production rate due to the endothermic effect of water evaporation. The burst of the carbon dioxide release (O period) results in a steep decrease of the heat flux of the calorimeter as the water evaporates into the insect chamber. Thus short-term endothermic transients, due to active water loss, were observed. Characteristic was an exothermic spike prior to the steep endothermic curve during the burst of carbon dioxide, and this spike was obviously due to the release of relatively warm carbon dioxide from the tracheae not yet mixed with the water vapour. The recording shows well that the stress state due to handling and apparatus lasted one to two days. Only during the third day of the measurements, the DGCs acquired stable intervals lasting about one day. The O period, i.e. the bursts of carbon dioxide, constitutes less than 10% of a whole gas exchange cycle.

The constant volume respirometry revealed that all long-cycle pupae had very low values of SMR: 0.015 to 0.021 (mean 0.018) ml O_2 g^{-1} h^{-1}, and during the three first

months of diapause the SMR did not change essentially (repeated measures ANOVA, $F_{2,190}$ = 16.2; $P > 0.05$; Tukey test $P > 0.05$).

For this group of pupae, the bursts of carbon dioxide were associated with active ventilation observed as abdominal rotating movements (Fig. 4). Thus the CFV cycle of gas exchange was typical of the long-cycle pupae.

Respiratory patterns in the short-cycle pupae

After a longer cycle lasting about 1 h, a series of shorter cycles, lasting 2–4 minutes, followed. Active ventilation was not observed during the bursts and, therefore, CFO cycles were characteristic of these pupae. The frequencies of gas exchange cycles in pupae varied essentially, but no obvious difference in the pattern of DGC was found between the three first months of diapause. Of the overall time, only about 60% constitutes the flutter period while during the remained time the bursts of carbon dioxide occurred (Fig. 5). The values of SMR gradually increased during the first, second and third months of diapause: 0.021±0.005, 0.036±0.011 and 0.051±0.016 ml O_2 g^{-1} h^{-1}, respectively, showing significant difference between the months (repeated measures ANOVA $F_{2,18}$ = 15.3; $P < 0.01$; Tukey test $P < 0.01$).

Discontinuous uptake of oxygen and passive suction ventilation (PSV)

The most pupae of *P. brassicae* of both observed groups displayed a clear pattern of discontinuous uptake of oxygen at the beginning of most of the bursts of carbon dioxide (Fig. 4). However, there also occurred individuals of continuous uptake of oxygen. Between the bursts, i.e. during the interburst periods, miniature passive suction ventilation or PSV occurred. The PSV are seen in the recording of the electrolytic respirometer as sharp spikes (Fig. 5). The spikes due to PSV were followed in the long-cycle pupae by more or less regular intervals lasting 4–10 minutes. In the short-cycle pupae, these miniature inspirations were more frequent and irregular. Often the discontinuous uptake of oxygen was accompanied by the abdominal rotating movements (Fig. 4). It was also remarkable that these miniature inspirations due to the passive suction ventilation (PSV) were not associated with micro bursts of carbon dioxide. The micro bursts of carbon dioxide occurred separately of the miniature air intakes (Fig. 5). It is clearly seen on the recordings that the rapid upward spikes were really due to the PSV as they were not synchronised with spikes due to the muscular contractions (Fig. 5).

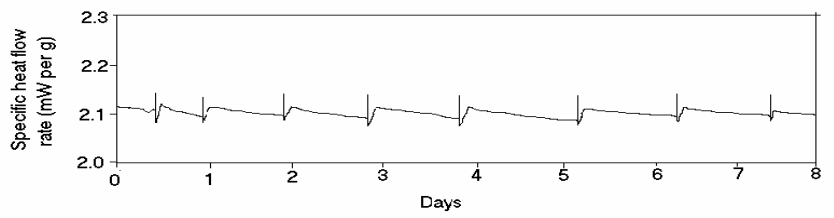

Fig. 3. A calorimetric recording of heat production in a diapausing long-cycle pupa (380 mg) of *P. brassicae*. The peaks are caused by the bursts of carbon dioxide release. The exothermic (upward) spikes recorded at the beginning of bursts transformed into endothermic signals due to the cooling effect of transpiratory water vapour.

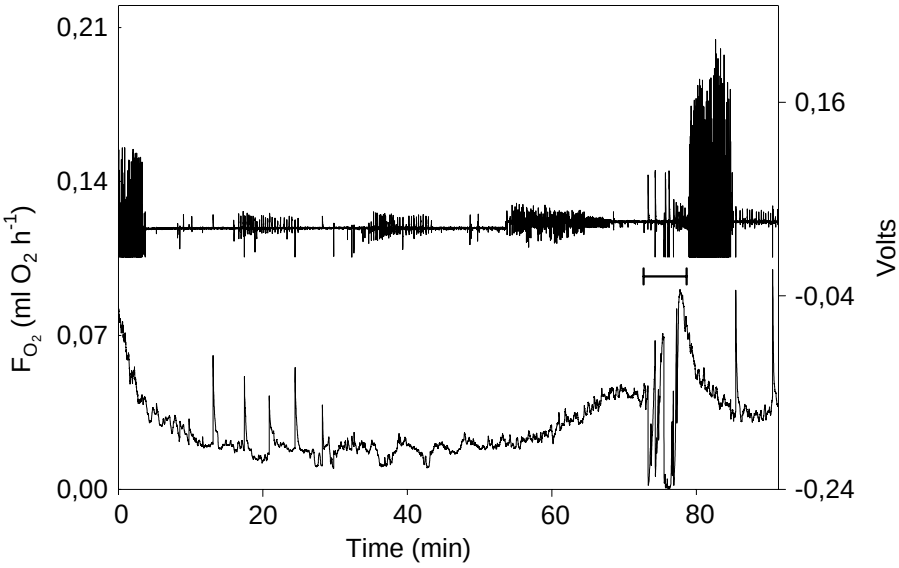

Fig. 4. On the recording of electrolytic respirometer there is represented the pattern of oxygen uptake associated with abdominal rotating movements (horizontal bar), occurring together with a burst of carbon dioxide (lower trace, left axis). Upper trace is a synchronous recording of infrared actograph showing three periods of retrograde heartbeats occurring between two periods of anterograde heartbeats.

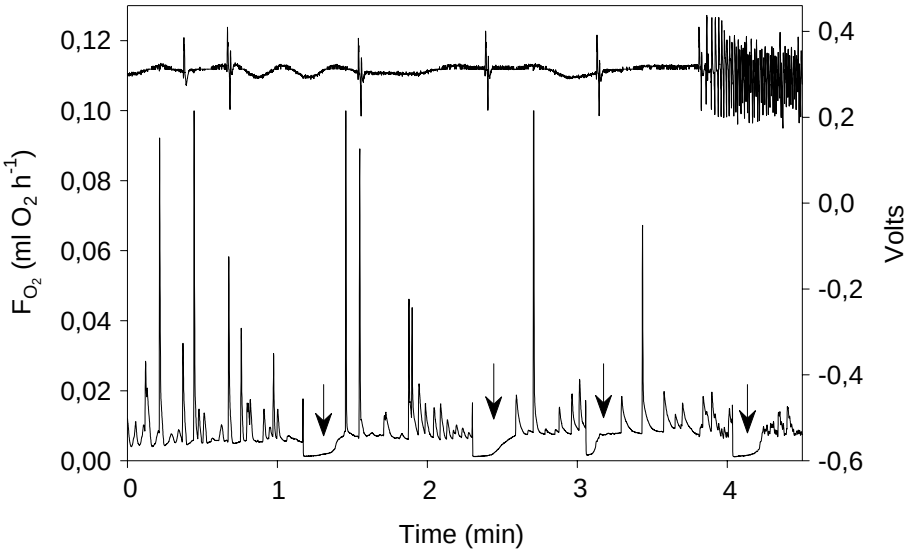

Fig. 5. A typical pattern of discontinuous gas exchange in a diapausing short-cycle pupa of *P. brassicae* (423 mg) simultaneously recorded by electrolytic respirometer (lower trace, left axis) and infrared actograph (upper trace, right axis). A period of anterograde heartbeats is seen in the right part of the upper trace, the rare spikes are the retrograde heartbeats. The bursts of carbon dioxide are indicated by arrows, the upward spikes between bursts are due to passive suction ventilation or flutter (lower trace).

Heartbeat patterns

The heartbeat patterns varied individually to a large extent both in the long- and short-cycle individuals. In the long-cycle pupae, a period of anterograde heartbeat was followed by a heartbeat pause, lasting usually 20–30 min, and then there was observed a period of retrograde heartbeat. One cycle of heartbeat patterns, including the three mentioned events, lasted usually at least 2 h. In the short-cycle individuals, the periods of anterograde heartbeat, heart pause and retrograde heartbeat also alternated, but these periods were shorter than in the long-cycle pupae.

In about 50% of the pupae, a clear correlation between the pattern of heartbeat and gas exchange cycles was observed in the both groups of pupae. The period of anterograde heartbeat was initiated during the larger bursts of carbon dioxide or soon after the burst (Figs 4 & 6). It was remarkable also that the heart pause was usually terminated after the abdominal rotating movements and a period of anterograde heartbeat started (Fig. 4).

The frequencies of heart systoles during the anterograde and retrograde heartbeats were 11–15 (12.3±3.4) per min and 2–3 (2.1±0.32) per min, respectively (Fig. 7).

Intermittent transpiration

The water was not released continuously, and clear cycles were observed instead. The most of the body water was released as vapour during each burst of carbon dioxide, as it is seen on the simultaneous recording of these bursts and transpiratory water loss (Fig. 8). Only a small fraction was released vapour during the interburst or flutter period. The similar pattern of water loss rate (WLR) and cyclic gas exchange was observed both in the long- and short-cycle individuals, however, it was easy to demonstrate that the shorter flatter periods, i.e. the frequent DGCs, caused greater water losses, and the longer gas exchange cycles and longer flutter periods resulted, on the contrary, in lesser water loss rates.

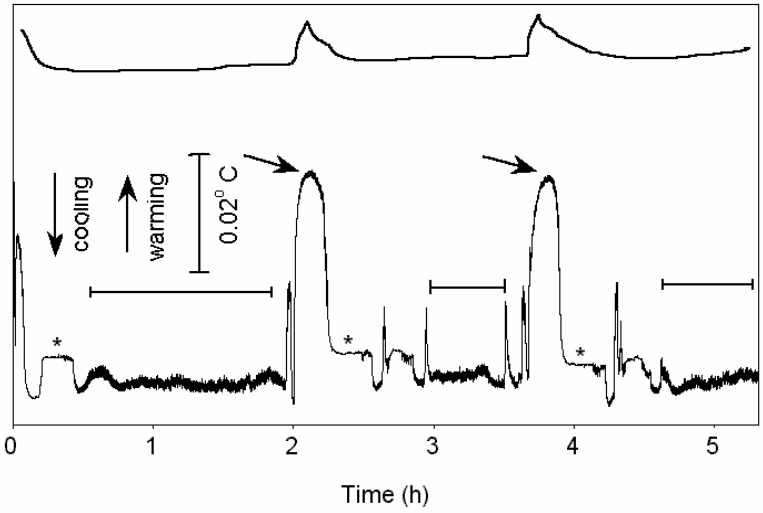

Fig. 6. A typical recording of heartbeat reversals in a diapausing pupa of *P. brassicae*, by using the contact thermography. Anterograde heartbeats are indicated with the inclined arrows, heart pauses with asterisks, the retrograde heartbeats with horizontal bars.

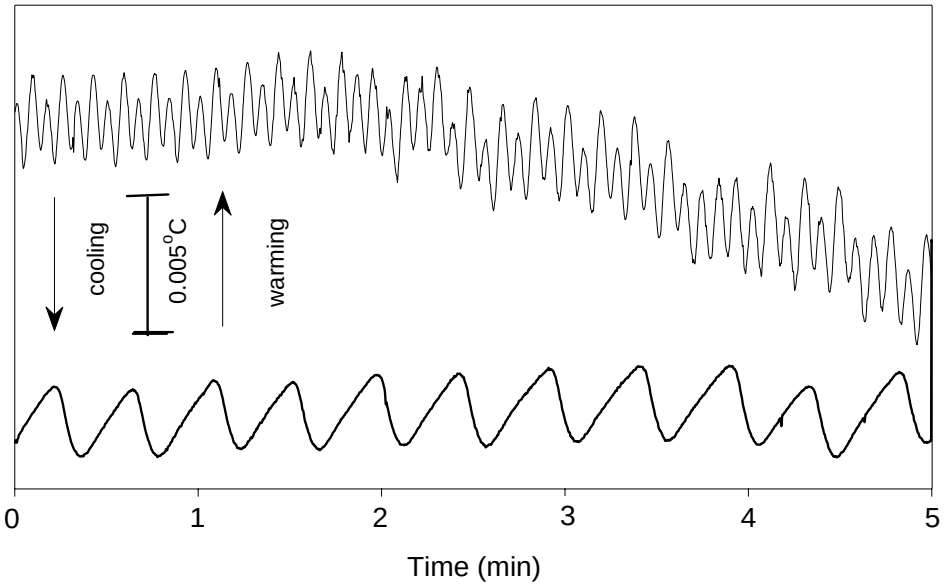

Fig. 7. A high resolution detail of upper figure showing the faster anterograde heartbeats (upper trace) and slower retrograde heartbeats (lower trace). The systoles are directed downward, the diastoles upward.

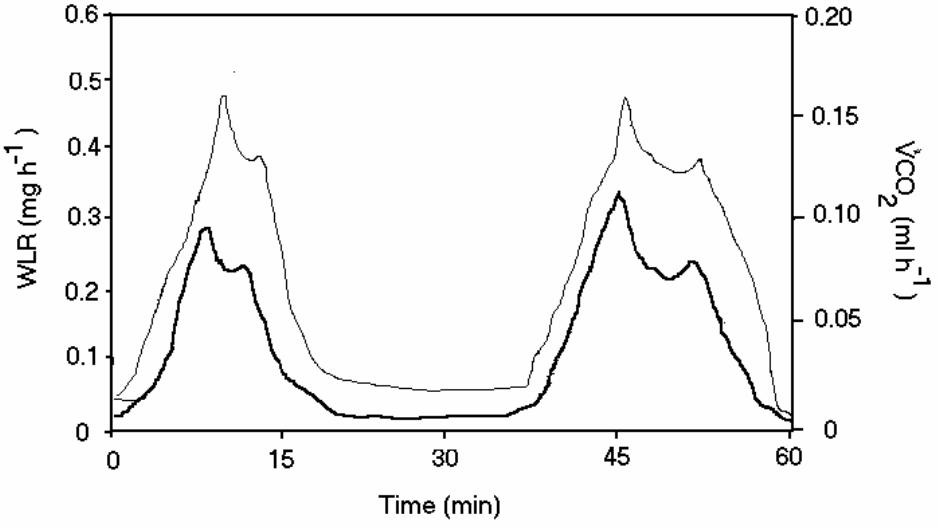

Fig. 8. Two bursts of carbon dioxide recorded by the infrared gas analyser (thick trace, right axis) in parallel with the recording of water loss rate (WLR) made by means of the humidity indicator or hygrometer (thin trace, left axis) in a diapausing pupa of *P. brassicae*. Note that most of the transpiratory water is lost at the time of the bursts of carbon dioxide.

Water loss rate (WLR) and body mass

The long-cycle individuals did not reveal any significant changes in WLR during the three first months of diapause: 1.07±0.42, 0.98±0.5 and 1.14±0.66 mg g^{-1} day^{-1}, respectively (repeated measures ANOVA $F_{2,190}$ = 2.40*; P* = 0.093; Tukey test *P* >0.05). The short-cycle pupae, on the contrary, showed an essential increase of WLR during the three first months of diapause: 1.61±0.53, 2.07±0.41 and 2.46±0.43 mg g^{-1} day^{-1}, respectively (repeated measures ANOVA $F_{2,18}$ = 17.2*; P* < 0.01; Tukey test *P* < 0.01). Thus, the WLR between the long-cycle and short-cycle pupae differed significantly already in the first month of diapause, and the discrepancy became greater in the two subsequent months (Fig. 9).

The initial body masses of the long-cycle pupae did not differ from the body mass of the short-cycle individuals: 438 and 422 mg, respectively (Student *t*-test; *t* = 1.09, *df* = 19*; P* >0.05). After three months, the mean body mass has decreased in the long-cycle pupae only by 22 mg but, at the same time, the short-cycle pupae had lost averagely 70 mg of their body mass (see Fig. 10).

Cold tolerance

All non-acclimated three-month-old diapausing pupae of *P. brassicae* (*n* = 20) survived after exposure to –16ºC for 48 h., but decreased to 20% after exposure to –20ºC for 24 h. No pupae (*n* = 12) survived after 8 h exposure at –22ºC. However, after cold acclimation at –5ºC for 7 days all pupae (*n* = 8) survived after exposure to –20ºC for 24 h. It was remarkable that, after the mentioned short time of cold acclimation, the signs of pharate adult development were observed, because SMR and WLR increased at least three times. The dependence of cold tolerance on the gas exchange patterns was not studied.

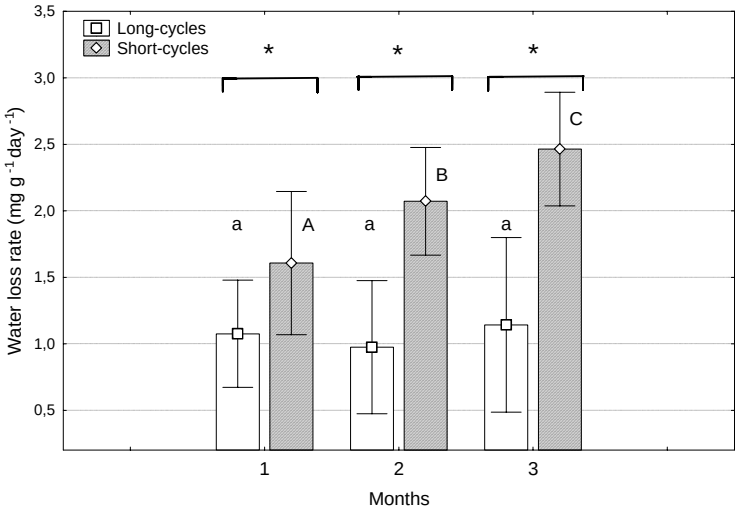

Fig. 9. Water loss rate in long- and short-cycle diapausing pupae. The different letters indicate significant differences (*P* < 0.05) between the months: capital letters – short-cycle pupae, the common letters – long-cycle pupae (repeated measures ANOVA, Tukey test). The asterisks indicate the significant differences between the two mentioned groups of pupae in each month (Student's *t*-test).

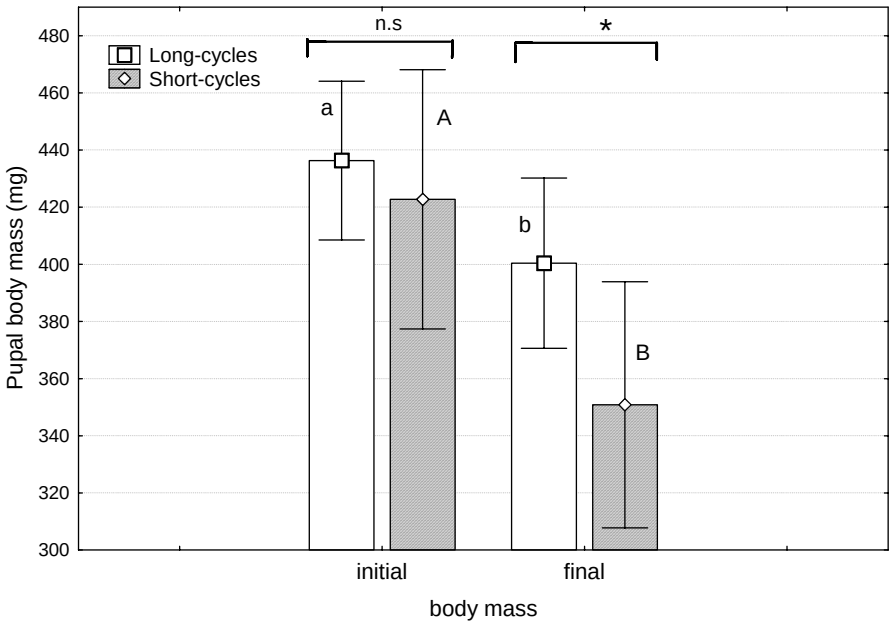

Fig. 10. Pupal initial body masses and final body masses (measured after three months). The different letters indicate significant differences ($p<0.05$) between initial and final body mass (Student's paired *t*-test): capital letters – short-cycle pupae, the common letters – long-cycle pupae. The asterisks indicate significant differences between pupal groups (one-way ANOVA).

DISCUSSION

The present study revealed that the respiration patterns in diapausing pupae of *P. brassicae* varied essentially between individuals, and roughly two groups were separated: the long-cycle and the short-cycle pupae. In the first group of pupae, SMR was maximally suppressed during the first three months. In the second group of pupae, SMR gradually increased but never reached the values characteristic of pharate adult development, i.e. over 0.1 ml O_2 g^{-1} h^{-1}. These individuals with relatively high SMR, WLR and frequent DGCs during the first months of diapause indicated on certain exclusive events occurring during the period of diapause induction in the last two larval instars (e.g. photoperiods, temperature, food). About 70% of these pupae died during spring months due to enhanced transpiratory water loss.

The flow-through respirometry based on the measurements of carbon dioxide release is the most widely used respirometry at the present time, however, this method does not enable recording of the body active movements and the PSV. We used constant volume respirometry to record the bursts of carbon dioxide, the level of SMR, body movements and miniature air intakes due to the PSV. The C period, when spiracles are tightly closed, did not exist in *P. brassicae* pupae: there was only a period after the burst when no carbon dioxide was released. The miniature inspirations after a burst were not associated with micro bursts of carbon dioxide. Thus flutter occupied all the interburst period.

Usually the oxygen uptake was thought to be continuous and not influenced by the gas exchange cycles in diapausing lepidopterous pupae (Miller, 1981). The present results demonstrated that both the carbon dioxide release and oxygen uptake in diapausing *P. brassicae* pupae are usually discontinuous. Similar patterns of intermittent O_2 uptake have also been recorded from pupae of *G. mellonella* (Kuusik et al., 1996) using the same type of electrolytic respirometer. Intermittent O_2 uptake has also been reported from adults of the ant *Formica polyctena* (Martin et al., 2004; Kuusik et al., 2004) and in some species of Coleoptera (Lighton, 1988).

The results revealed that heartbeat pauses were longer in the long-cycle pupae and shorter in the short-cycles individuals, while heartbeat reversals occurred in both pupal groups. According to some earlier data, the heartbeat pauses may be used as an indicator of diapause intensity (Metspalu et al., 1982). Heartbeat reversals is a common event in lepidopterous pupae (Wasserthal, 1996), but in this paper it was demonstrated for the first time that in diapausing pupae of *P. brassicae* the heartbeat pauses and reversals are strongly coordinated with the gas exchange cycles, however, the physiological function of this interrelation is unclear at present time. It is very likely that the active movements of the abdomen act as triggers of the anterograde heartbeats in a pupa, quite similar as described in pupae of *Leptinotarsa decemlineata* (Kuusik et al., 2001).

The discontinuous gas exchange was thought to be an adaptation for conserving the water in insects, however, experimentally this theory has never been verified by comparing WLR in an insect displaying different patterns of cyclic gas exchange. The present experiments demonstrated that WLR was extremely low in the long-cycle pupae of *P. brassicae*, whose flutter period contributed over 90% of a cycle. In the short-cycle pupae, whose F period contributed 50% and less of a cycle, there was observed an enhanced loss of water due to transpiration. In addition to that, the present paper showed that water was lost mainly during a burst of carbon dioxide, and only a negligible fraction of water was released during the interburst period. Passive air suction into the tracheae or PSV was thought to be the key factor for conserving the water in insects (Kestler, 1985).

From the results of present study it was concluded that for observing the diverse events occurring during the pupal diapause of P. *brassicae*, a complex of methods must be used: calorimetry, still-air and flow-through respirometries, oxygen analyser, infra-red actography combined with respirometry, while the relative humidity (RH%) and temperature must be continuously controlled inside the insect chamber.

CONCLUSIONS

Calorimetric measurements was the only acceptable method to record the long gas exchange cycles for several weeks, but it was also demonstrated that the stress state of pupae usually lasted 4–5 days, after which a stable frequency of DGC was reached.

The simultaneous recording of CO_2 release and WLR showed that water loss in pupae was mainly due to the respiratory transpiration, while most of the water was released during a burst of carbon dioxide.

The results suggest that short and irregular cycles of gas exchange were the main cause for enhanced transpiratory water losses in diapausing pupae.

Elucidation of the factors inducing the individual variability of respiration patterns in *P. brassicae* will be a task of further investigations.

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