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RESEARCH ARTICLE

Oxygen convective uptakes in gas exchange cycles in early diapause pupae of *Pieris brassicae*

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SUMMARY

Oxygen convective uptakes in gas exchange cycles were directly recorded in early diapause pupae of *Pieris brassicae* L. (Lepidoptera; Pieridae) by means of O_2 coulometric respirometry. This method was combined with flow-through CO_2 respirometry, the two systems being switchable one to the other. During recording with both systems, measurements were also taken with infrared actography. The pupae displayed short discontinuous gas exchange cycles lasting 40–70 min. No true C phase was found by flow-through measurements; instead, flutter opening of the spiracles with discrete convective O_2 uptakes began shortly after the O phase whereas CO_2 release was suppressed by the inward directed passive suction ventilation. The F phase was characterized by a series of small CO_2 bursts (flutter events). Between these bursts, novel sub-phase 'miniflutter' was observed, which consisted of six to 10 miniature inspirations without any CO_2 emission. During the flow-through measurements, oxygen convective uptakes were indirectly recorded by the infrared actograph as sudden extensions (lengthening) of the abdominal segments at each spiracular microopening.

Key words: oxygen uptake, *Pieris brassicae,* gas exchange cycle, butterfly, flutter, coulometric respirometry.

INTRODUCTION

Breathing in many insects is characterised by discontinuous gas exchange cycles (DGCs), during which carbon dioxide is released periodically. To date, the focus of studies of DGCs has been on CO_2 release as measured by flow-through CO_2 respirometry, whereas O_2 consumption during the DGC has been little studied.

Classically, the DGC consists of three phases (Schneiderman, 1960; Lighton, 1996; Chown and Nicolson, 2004). During the open (O) phase, the spiracles are open and CO_2 is released in a burst. The O phase is followed by the facultative closed (C) phase, when the spiracles are closed and little or no gas exchange occurs. Within the C phase, sub-atmospheric pressure is created in the tracheae because of O_2 consumption by the tissues and CO_2 buffering by means of bicarbonates in the tissue and haemolymph (Wobschall and Hetz, 2004). After the C phase, the flutter (F) phase occurs, during which the spiracles open and close rapidly in succession (fluttering). When open, air is sucked through the spiracles into the tracheae by convection along the negative pressure gradient. This is known as passive suction ventilation (PSV); thus PSV occurs in the absence of muscular movement (see Miller, 1974; Miller, 1981). PSV during the F phase has been described as a mechanism for restricting water loss in insects (see Kestler, 1978; Kestler, 1982; Kestler, 1991). DGC is regarded as CFO cycles when CO_2 bursts are not actively ventilated by abdominal pumping, but as CFV (V, ventilation) cycles when the CO_2 bursts are associated with pumping movements (Kestler, 1985; Kestler, 2003).

Discontinuous O_2 uptakes and CO_2 release during the O phase has been demonstrated in some Carabidae beetles and in pupae of the Cecropia moth, *Hyalophora cecropia*, using heat conductivity detectors or diaferometers (Punt et al., 1957). Simultaneous measurements of O_2 uptake and CO_2 release by flow-through respirometry in the tok-tok beetle, *Psammodes striatus*, showed a peak of O_2 consumption at the beginning of the O phase, together with a burst of CO_2 release (Lighton, 1988). Oxygen uptake also peaked during the release of CO_2 in the giant burrowing cockroach, *Macropanesthia rhinoceros* (Woodman et al., 2007).

The F phase cannot be detected by flow-through O_2 respirometry without a significant diffusive component, because the inward bulk flow of air into the tracheal system is functionally equivalent to a minute and probably undetectable reduction in the flow rate of air through the respirometer chamber (Lighton, 1988; Lighton, 1994). Thus, single microopenings of the spiracles and air convective uptakes into the tracheae cannot be detected by flow-through respirometry.

Special techniques are required to record PSV during microopening of the spiracles in the F phase. Schneiderman used cannulated spiracles to measure partial pressure and thus described the rhythms of passive air uptake in silkworm pupae (*H. cecropia*) (Schneiderman, 1960). Sláma recorded a sawtooth pattern of abdominal retractions with contact transducers in lepidopteran pupae (including the large cabbage white *Pieris brassicae*) (Sláma, 1984; Sláma, 1988). This pattern was caused by the microopening of the spiracles and passive inspirations. A similar pattern of passive inspirations was recorded by Sláma and Neven in young pupae of the codling moth, *Cydia pomonella* (Sláma and Neven, 2001). Hetz et al. used miniaturized amperometric sensors to make direct O_2 measurements within the tracheal system of lepidopteran pupae (Hetz et al., 1994). Wobschall and Hetz recorded O_2 uptake directly

in diapausing Atlas moth (*Attacus atlas*) pupae by simultaneous measurements of tracheal pressure and volume changes (plethysmometry) in the tracheal system, while combining CO_2 measurements by flow-through respirometry (Wobschall and Hetz, 2004). Coulometric (volumetric-manometric) respirometry has been used to directly record O_2 convective uptakes in diapausing 2–5 month old pupae of the cabbage moth, *Mamestra brassicae* (Jõgar et al., 2007), and *P. brassicae* (Jõgar et al., 2004; Jõgar et al., 2005; Jõgar et al., 2008). However, there is a lack of information about the gas exchange patterns, including O_2 convective uptake during the initiation phase of diapause (early diapause) (see Koštál, 2006; Belozerov, 2009).

Coulometric respirometry was combined with flow-through CO_2 respirometry. We suppose that the flutter events observed by coulometric O_2 measurements can usefully be directly compared with flow-through CO_2 respirometry.

The main aim of the present investigation was to describe the pattern of O_2 convective uptakes and associated body movements in young pupae of *P. brassicae*. To achieve this, coulometric O_2 respirometry was combined with flow-through CO_2 respirometry, and discrete O_2 discrete uptake was simultaneously recorded indirectly using an infrared (IR) actograph.

MATERIALS AND METHODS

Insects

For laboratory experiments, eggs of *Pieris brassicae* (Linnaeus 1758) (second generation) were collected from cabbage fields near Tartu, Estonia (58°23′N, 26°41′E), during July and August 2009. They were reared in a laboratory under short-day conditions (12 h:12 h light:dark) at 21±1°C and ambient air humidity (55–65% relative humidity). The larvae that hatched from the eggs were fed on leaves cut from cabbage plants, which were replaced with a fresh supply daily. After pupation, each pupa was placed in an Eppendorff tube and kept in laboratory conditions.

For the experiments, twenty-five 2 week old (14±2 days) pupae were used. Each pupa was weighed to 0.1 mg with an analytical balance before experimentation (Explorer Balances, max. 62 g; Ohaus Corporation, Nänikon, Switzerland). Pupal body mass ranged from 0.383 to 0.411 g (Table 1). During respiratory measurements, temperature and humidity conditions were recorded using a digital HygroClip probe (HygroPalm, Rotronic Company, Basserdorf, Switzerland). All measurements were made at 21±1°C and ambient air humidity (50–55% relative humidity). The Eppendorff tube with pupa was used as the insect chamber in the respiratory systems; this avoided handling stress. The respiratory measurements of the first 30 min were discarded; recordings in both systems lasted at least 3 h. Tests with each individual were made twice. We confirmed, with preliminary experiments, that the switch from still air to flowing air (120 ml min^{-1}) and *vice versa* did not significantly change the frequency of the DGC. Body movements were visually observed under a stereomicroscope (SZ-ZTW, Olympus, Japan).

Table 1. Characteristics (mean ± s.d.) of the discontinuous gas exchange cycle in 2 week old pupae of *Pieris brassicae* (*N*=25) in the initiation phase of diapause

Body mass (g)	0.397±0.014
Metabolic rate	
V_{CO_2} (ml g^{-1} h^{-1})	0.042±0.0057
V_{O_2} (ml g^{-1} h^{-1})	0.051±0.0027
Discontinuous gas exchange cycle	
Frequency (mHz)	0.26±0.0002
Period (min)	58.25±11.72
Small bursts of CO_2 during flutter	
Number	25.06±3.69
Frequency (mHz)	12.20±0.32
Period (s)	90.75±13.19
Oxygen uptakes between two flutter bursts	7.49±2.63

Coulometric respirometry

Coulometric respirometers usually work in an interrupted regime (on–off) of electrolysis (e.g. Heusner et al., 1982). By contrast, our coulometric respirometry (a volumetric manometric system) was characterised by a continuously (uninterrupted) O_2-compensating system (Kuusik, 1977; Kuusik et al., 1996; Tartes et al., 1999; Tartes et al., 2002). This setup has also been described by Lighton (Lighton, 2008). This respirometer ensures continuous and adequate replacement of consumed O_2 with electrolytically produced O_2. The insect itself plays an active role in this self-regulating system. The rates of O_2 production and O_2 consumption by the insect are indicated on graphs as V_{O_2} (ml h^{-1}). The system also records transient changes in the rate of release of CO_2. In our respirometer, we did not use the switching electrodes of electrolysis; instead, the electrolysis current was directly connected with a photoelement. High sensitivity of the respirometer to pressure changes in the respiration chamber was achieved by replacing the standard photodiode with the photosensitive element of a transistor (KT302A, Semitronics, Freeport, NY, USA), which has a very small photosensitive area (approximately 0.5 mm^2). In this way, the smallest movement in the meniscus of ethanol inside the U-shaped capillary was reflected as a signal on the recording trace (Fig. 1). The electrolysis current depended on the intensity of the light falling on the phototransistor. The ethanol meniscus in the glass capillary served as a shutter to screen the photosensitive area from light. The electrochemical equivalent of O_2 generation has been reported as 209.5 µl O_2 mA^{-1} h^{-1} (Taylor, 1977). This value was used to convert

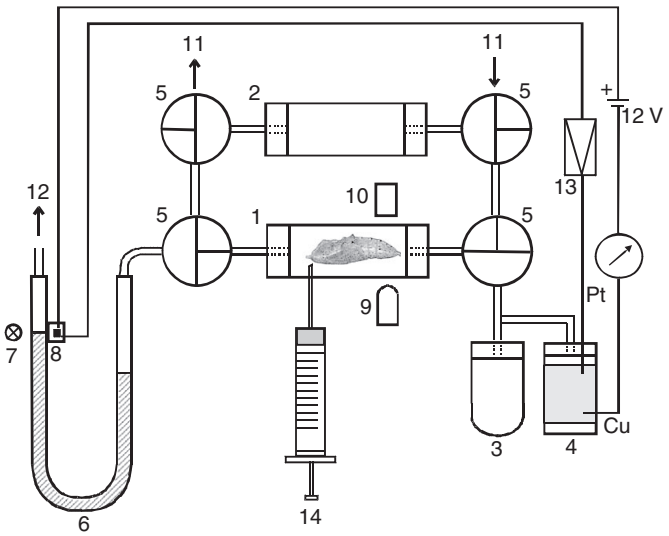

Fig. 1. Design of the electrolytic continuously O_2-compensating differential volumetric-manometric closed-system respirometer. 1, Insect chamber with pupa; 2, empty insect chamber; 3, vessel with potassium hydroxide solution; 4, electrolysis unit, $CuSO_4$ solution with platinum (Pt), and copper (Cu) electrodes; 5, taps for switching from volumetric-manometric respirometry to flow-through CO_2 respirometry; 6, glass capillary half-filled with ethanol; 7, light source; 8, photo transistor; 9, infrared (IR) emitter diode; 10, IR sensor diode; 11, connections to the flow-through respirometry system; 12, compensating vessel; 13, current amplifier; 14, microsyringe.

the readings of the event recorder to O_2 consumption values ($\mu l\,O_2\,h^{-1}$ or $\mu mol\,O_2\,h^{-1}$).

The coulometric respirometer allowed simultaneous recording of O_2 consumption, sudden O_2 (air) uptake (known as PSV) by convection into the tracheae at microopenings of the spiracles, discrete CO_2 releases by bursts, abdominal pumping movements and heartbeat patterns (see Jõgar et al., 2004; Jõgar et al., 2007). Rapid changes in pressure (lasting seconds) in the insect chamber, caused by active body movements of the insect or other rapid events, were not compensated and led to corresponding rapid changes in the electrolysis current, reflected as spikes on recordings. Thus, our coulometric respirometer also served as an activity detector.

A rapid O_2 convective uptake resulted in adequate air volume decrease in the insect chamber and the ethanolic meniscus shifted down by a fraction of a millimetre. As a result, more light fell on the sensitive area of the transistor and an upward signal was recorded (Fig. 2). Downward signals indicated CO_2 release by bursts (Tartes et al., 1999). The volume of air uptake was estimated by extracting air from the insect chamber with a microsyringe (1 µl volume, Agilent Technologies, Espoo, Finland). The calibration of convective air uptake is shown in Fig. 2.

Flow-through CO_2 respirometry

The infrared gas analyser or flow-through CO_2 respirometer (Infralyt-4, Saxon Junkalor GmbH, Dessau, Germany) was used to confirm that the presumed CO_2 signals, i.e. the downward spikes on the recording trace of the electrolytic respirometer, were actually due to CO_2 bursts, and to measure them quantitatively. The respirometer was calibrated at different flow rates by means of calibration gases (Trägergase, Saxon Junkalor GmbH) and with gas injection. An air flow rate of $120\,ml\,min^{-1}$ was used. The insect chamber could be switched either to the flow-through CO_2 respirometer or to the coulometric respirometer without disturbing the insect (Fig. 1). During the measurements with coulometric respirometry, the empty respiration chamber served to determine the baseline of the measurements.

IR actography

Both the coulometric (electrolytic) respirometer and the flow-through respirometer were combined with an IR insect cardiograph (opto-cardiography); we refer to this as the IR actograph, because it records not only heartbeats but also all other abdominal contractions, including muscular ventilation. An IR-emitting diode was placed on one side of the respirometer chamber near the ventral side of the abdomen, while an IR-sensitive diode (TSA6203, Mikrotechna, Prague, Czech Republic) was placed on the opposite side of the chamber (see Metspalu et al., 2001; Metspalu et al., 2002). The light from the IR diode (BP104, Mikrotechna) was modulated by contractions of the heart and skeletal muscles. The level of output voltage reflected the vigour of the muscular contractions of the insect (Hetz et al., 1999). Sudden extensions (lengthening) of abdominal segments (PSV) are recorded as relatively long upward spikes synchronous with microopenings of the spiracles (Figs 3, 4). Weak regular muscular contractions of the abdomen resulted in two-phased relatively short spikes on the recording traces we refer to as abdominal pulsations (Fig. 5). Regular, high-frequency, low-amplitude signals were interpreted as heartbeats (Fig. 3).

Data acquisition and statistics

Computerised data acquisition and analysis were performed using DAS 1401 A/D hardware and TestPoint software (Keithley, Metrabyte, Cleveland, OH, USA) with a sampling rate of 10 Hz. Four bipolar channels allowed simultaneous recording of four events. Mean (±s.d.) standard metabolic rate was calculated automatically using STATISTICA (version 8, StatSoft, Tulsa, OK, USA). Statistical comparisons were made with one-way ANOVA (analysis of variance). Significant ANOVAs were followed with the Fisher's least significant difference (LSD) test. The significance level was set at $P<0.05$.

RESULTS

At the initiation phase of diapause (14±2 days old), *P. brassicae* pupae displayed DGCs lasting 40–70 min (Table 1), whereas the duration of CO_2 release by burst was 2–6 min (3.1±0.1). Recordings by flow-through respirometry showed a typical pattern. After the O-phase CO_2 emission had ceased, the C phase began, which was followed by the F phase with small bursts of CO_2 release (Fig. 3). No true C phase was found by flow-through respirometry. Shortly after the end of the O phase, coulometry revealed convective O_2 uptake. During this time, CO_2 release was suppressed by the inward-directed PSV. This convective O_2 uptake indicates an earlier beginning of the F phase than detectable with the flow-through

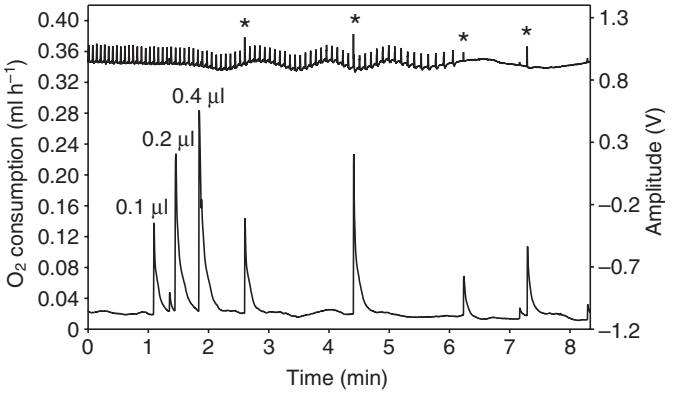

Fig. 2. Calibration with different volumes of air extracted from the insect chamber by means of coulometric measurements (lower trace, three left spikes). The other four spikes are caused by sudden oxygen uptake by *Pieris brassicae* pupae, which are synchronous with abdominal extensions (asterisks) (upper trace, IR probe actograph). By these measurements, the volume of first oxygen uptake was 0.1 µl and the second was 0.2 µl.

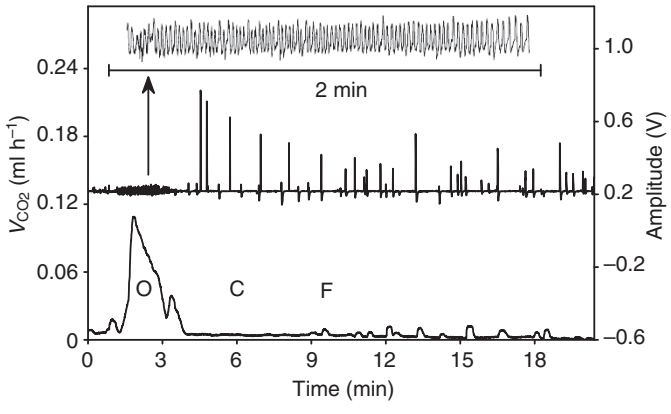

Fig. 3. Discontinuous gas exchange in *P. brassicae* pupa recorded with CO_2 respirometry. Note the discrete small bursts of the F phase (lower trace). The upper trace shows a series of abdominal extensions or lengthening (upper spikes) due to microopening of the spiracles and O_2 uptakes (IR probe actograph); the abdominal movements during O phase were identified as heartbeats.

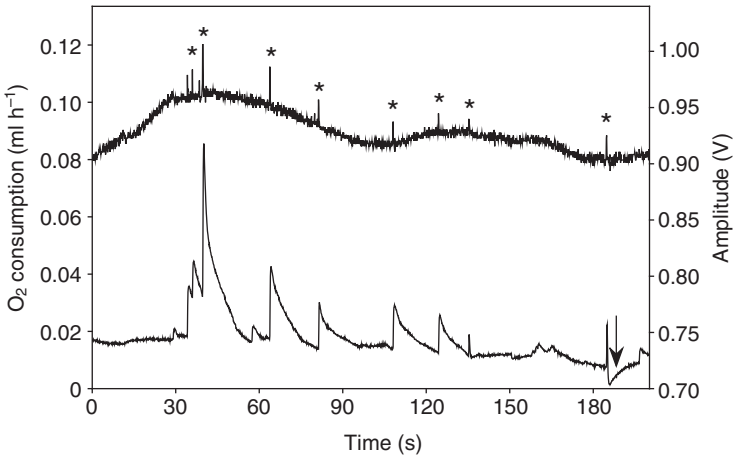

Fig. 4. Typical pattern of convective O_2 uptake by coulometric respirometry recorded in *P. brassicae* pupa at the time that corresponded to the closed phase by the flow-through CO_2 respirometer. The spikes are gradually shortened due to the pressure rises inside the trachea. Asterisks (upper trace) indicate the extensions (lengthening) of abdominal segments. Arrow indicates a CO_2 release by miniburst.

system. Thus the F phase lengthened on account of the C phase. At each microopening of the spiracles and passive convective oxygen uptake event, signals of abdominal lengthening were recorded (Figs 3, 4). Recordings of coulometric respirometry showed clear, gradually shortened signals due to convective oxygen uptake (Fig. 4). Each of the two to three first microopenings and O_2 uptakes lasted less than 0.5 s. Oxygen convective uptakes during the interburst period increased metabolic rate by 5–6% compared with the metabolic rate when these uptakes were absent. Flow-through measurements also indicated abdominal lengthening concurrent with the small bursts of CO_2 during flutter (Fig. 5).

Between two large CO_2 bursts, a series of small bursts of CO_2 were recorded by coulometric respirometry (Fig. 6A). Each small burst started with a brief uptake of air into the tracheae, recorded by the IR actograph as a sudden extension of the abdomen, indirectly indicating air (O_2) uptake (Fig. 5). Between two consecutive small CO_2 bursts, a series of air uptakes (miniature inspirations) were recorded, which we considered as 'miniflutter' (Fig. 6A,B; Table 1). These uptakes were irregular with respect to spike height and interval. During such miniflutters, no emissions of CO_2 were recorded.

Simultaneous recording with the IR actograph during flow-through CO_2 respirometry indicated that pupae differed in the type of body movement associated with the respiratory patterns of CO_2 release. In some pupae, CO_2 bursts were always concurrent with abdominal ventilating movements (CFV cycles) (always group, N=9) (Fig. 7A), whereas in others (occasionally group, N=10), only some CO_2 bursts were concurrent with abdominal ventilating movements (Fig. 7B). In a few pupae (never group, N=6), CO_2 bursts occurred without active ventilation (CFO cycles) (Fig. 7C). Ventilating movements (amplitude 1–2 V) associated with CO_2 bursts were visible externally as twisting abdominal movements.

Active ventilation during the bursts of CO_2 showed individual variation in the vigour of contractions and their number (from one to 15). In pupae with only one to five muscular (active) ventilating movements accompanying the burst, as well as in those lacking active ventilation, a relatively low level of CO_2 release was observed. In contrast, pupae with vigorously ventilated bursts showed a significantly higher level of CO_2 release (Fig. 7A). Each burst lasted 3–6 min in the always group of pupae, but 2–2.5 min in the occasionally and never groups. Statistical comparison of CO_2 release frequency (ANOVA, $F_{24.2}$=41.8, P>0.05) did not show a significant difference. The energy cost of muscular ventilation during a burst was not studied.

Abdominal two-phase regular contractions (5–7 min^{-1}) of low amplitude (0.2–0.3 V) (referred to as abdominal pulsations) (Fig. 5, Fig. 6B) occurred periodically; these were not visible externally.

In some pupae showing no active ventilation during CO_2 release by bursts, very regular low amplitude (0.1–0.2 V) pulsations (57–70 min^{-1}) were recorded; these we interpreted as heartbeats (Fig. 3).

DISCUSSION

Our results showed that, in the initiation phase of diapause, *P. brassicae* pupae display relatively short DGCs (40–70 min), with CO_2 bursts lasting 2–6 min. This contrasts with earlier studies, using

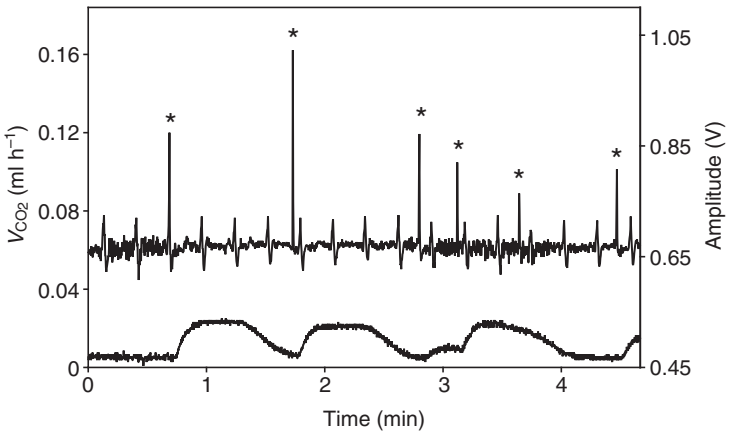

Fig. 5. Three small discrete bursts of CO_2 (lower trace) during flutter in a *P. brassicae* pupa, recorded with flow-through respirometry. The upper trace is a simultaneous recording from the IR probe actograph showing abdominal pulsations as two-phase signals (smaller spikes) and abdominal extensions (spikes indicated by asterisks). At the beginning of each small burst, abdominal extension (lengthening) occurred due to microopening of the spiracles and air convective uptake.

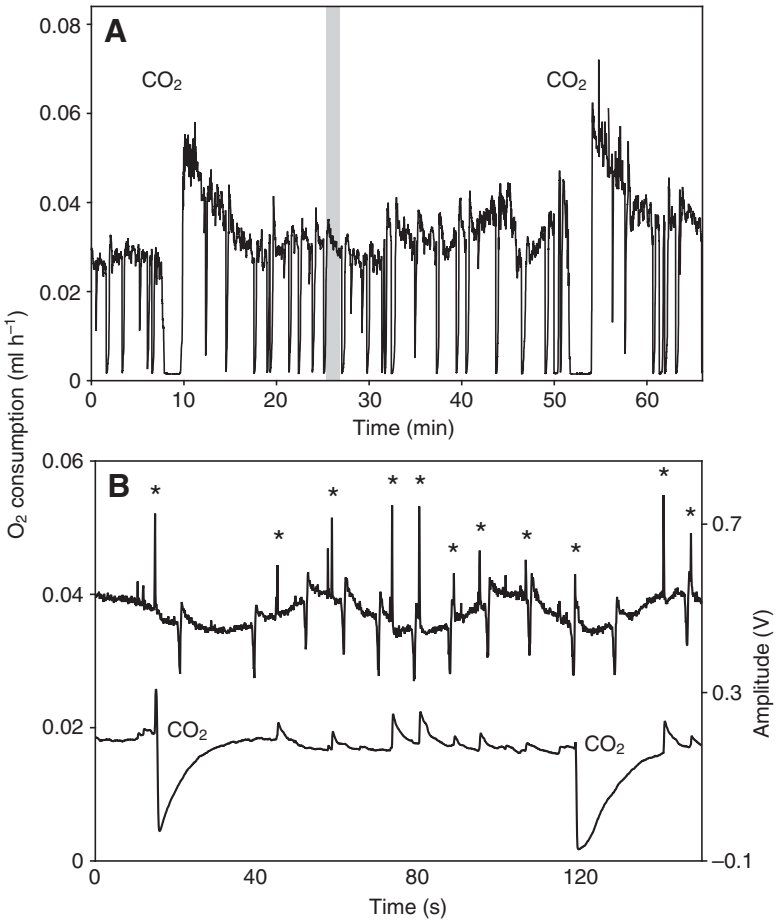

Fig. 6. (A) A discontinous gas exchange cycle in a *P. brassicae* pupa recorded by coulometric respirometry, showing an interburst period with small O phases (small CO_2 bursts). (B) Detail of the shaded area in A. The lower trace (coulometric respirometry) shows the convective uptake of air at the microopening (mini-flutter) of the spiracles between two small bursts of CO_2, recorded in pupae of *P. brassicae*. The upper trace is a simultaneous recording from the IR probe actograph showing abdominal pulsations (downward spikes) and sudden abdominal extension (lengthening) at each microopening of the spiracles (upward spikes indicated by asterisks).

P. brassicae pupae more than 2 months old, which displayed longer DGCs (8–23 h) (Harak et al., 1999; Jõgar et al., 2004; Jõgar et al., 2005) with CO_2 bursts lasting 13–18 min (Harak et al., 1999; Kuusik et al., 1980; Tartes et al., 1999). The young pupae we used with their short DGCs were convenient for studying flutter events. They had a relatively high metabolic rate; in 2–3 month old pupae metabolic rate is at least two times lower (12–28 ml O_2 g^{-1} h^{-1}) (Kuusik 1977; Jõgar et al., 2004; Jõgar et al., 2005).

Commonly, after the O phase, a period with no CO_2 release occurs (C phase); later, the CO_2 level was marginally elevated (F phase) (Chown et al., 2006). The present study revealed that in young *P. brassicae* pupae, the DGC measured with flow-through respirometry was characterised by a C phase, at the end of which a series of O_2 convective uptakes was found. Thus, the C phase in those pupae was not as closed as previously thought. Between two large CO_2 bursts, almost regular small CO_2 bursts were recorded. Each small burst started with sudden uptake of air into the trachea (PSV). The main finding in the present study was a series of irregular microopenings of the spiracle(s) with convective O_2 uptakes (mini-flutter) found between small bursts. During the mini-flutter, no recordable CO_2 emission occurred.

There are several examples where the interburst period consists of discrete small CO_2 bursts. Such bursts were described by Lighton (Lighton, 1988) in the tok-tok beetle, *P. striatus*; in this beetle, each burst was accompanied by active abdominal movement. Discrete CO_2 emissions during the F phase have also been reported by Duncan et al. in the tenebrionid beetle, *Pimelia grandis* (Duncan et al., 2002), and by Kovac et al. in resting honeybees *Apis mellifera* (Kovac et al., 2007). However, spiracle openings within the F phase were commonly observed to be irregular with respect to frequency and amplitude, if inferred from the CO_2 release pattern (e.g. Wobshall and Hetz, 2004).

Our flow-through CO_2 measurements showed no CO_2 release for a short time after the O phase. The coulometric respirometry and IR actographic recordings showed rapid and clear uptakes of air shortly after the O phase, indicating the beginning of the F phase. An earlier study by Tartes et al. revealed that air convective uptakes began immediately after the O phase (Tartes et al., 2002). Air convective uptakes, shortly after large CO_2 bursts, also occurred in old diapausing *M. brassicae* pupae (Jõgar et al., 2007). We suggest that, at the beginning of the flutter, air uptakes were convective but later were diffusive-convective. These results concur with the plethysmometry flow-through measurements of Wobschall and Hetz (Wobschall and Hetz, 2004), revealing that the convective uptakes of O_2 dominate at the beginning of the flutter phase but that, in the later F phase, diffusion takes over from convection as the chief mechanism of O_2 uptake. Wobschall and Hetz showed that, in diapausing moth pupae (*A. atlas*), uptake of air into the tracheal system at the beginning of the F phase along the negative hydrostatic pressure gradient may initially inhibit CO_2 release from the tracheae (Wobschall and Hetz, 2004). We suppose that, at the beginning of the F phase of *P. brassicae* pupae, CO_2 emission was also inhibited. One may suppose that water is conserved only at the beginning of the F phase when clear convective O_2 uptakes (PSV) occur, but not later when diffusion is the dominating mechanism of the fluttering period.

The duration of the F phase may be underestimated, as the F phase may start before the CO_2 measurements can detect it (Hetz

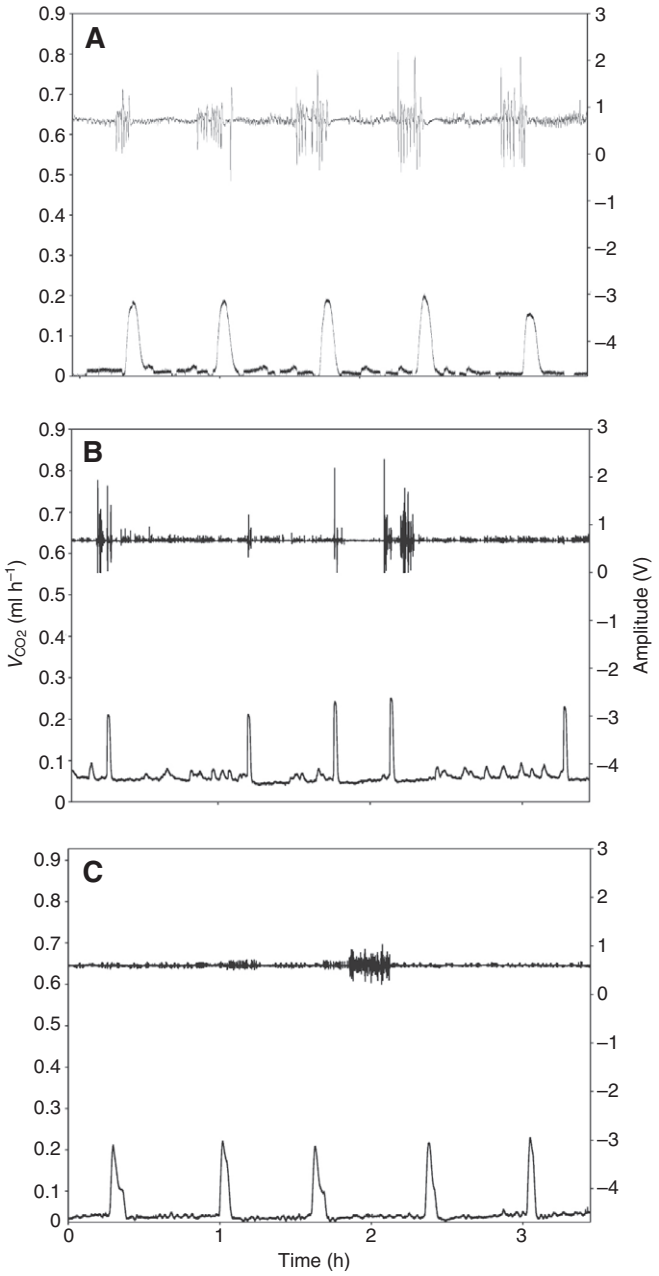

Fig. 7. Examples of individual variation in early diapause pupae of *P. brassicae* in the types of gas exchange patterns taking in account visible body movements: (A) CO_2 release by bursts accompanied by active ventilation (twisting abdominal movements) (CFV cycles); (B) CO_2 release by bursts with only some accompanied by active ventilation; (C) CO_2 release by bursts with no active ventilation (CFO cycles). The upper trace shows reccordings of IR probe actograph and the lower trace recordings of flow-through respirometry.

et al., 1994; Wobschall and Hetz, 2004). In pupae of *P. brassicae*, the duration of the F phase may also be longer than estimated by the flow-through system, as far as CO_2 release was prevented at the beginning of the F phase by convective air uptakes. Wobschall and Hetz showed that small volume and pressure decreases occurred between the microopenings in the F phase (Wobschall and Hetz, 2004). This confirmed a small but significant contribution of suction ventilation during each microopening (see also Kestler, 1985). In our measurements in *P. brassicae* pupae, each miniature inspiration was synchronised with rapid extension of the abdomen, confirming that a convective component was always present in O_2 uptakes. We observed in *P. brassicae* a relatively longer flutter phase compared with that in some other insects, such as the carabid *Pterostichus niger* (Kivimägi et al., 2011) and the bumblebee *Bombus terrestris* (Karise et al., 2010).

Gas exchange patterns are known to vary between and within individuals (see Chown, 2001; Chown et al., 2002; Marais and Chown, 2003). In the present study, variation was found between individuals in the duration of CO_2 release and metabolic rates, but not in DGC frequency. Individuals displayed different gas exchange cycles. Most showed DGCs with all CO_2 bursts actively ventilated, a few with no bursts actively ventilated, and others with only some bursts actively ventilated.

Beside active ventilation, some other types of body movements, differing in frequency and amplitude, were observed in the present study. The regular but periodically occurring abdominal pulsations in *P. brassicae* pupae correspond, in our opinion, to the extracardiac hemocoelic pulsations described in lepidopteran pupae (Sláma, 1984; Sláma, 1999). These pulsations and other abdominal movements play an important role in the regulation of pupal respiration and haemolymph circulation (Sláma and Neven, 2001). In *P. brassicae* pupae, we interpreted high-frequency but low-amplitude signals, accompanied by CO_2 release in bursts, as heartbeats. In a previous study using thermographic measurements, we demonstrated heartbeat reversal, correlated with gas exchange cycles and twisting abdominal movements in diapausing *P. brassicae* pupae (Jõgar et al., 2005). Heartbeat reversal correlated with gas exchange cycles has also been reported in saturnid moth pupae (Wasserthal, 1996; Hetz et al., 1999; Sláma, 2003).

Manometric O_2 respirometry methods have been criticized and their readings mistrusted because these methods usually do not allow separation of active and resting metabolism (see Van Voorhies et al., 2008). Nevertheless, some volumetric manometric methods, including our coulometric respirometry, are regarded as useful (Klok and Chown, 2005). We are convinced that in gas exchange studies of insects, coulometric respirometry supplemented by the flow-through method has clear advantages. Lighton pointed out that coulometric continuously recording respirometry deserves to be more widely used (Lighton, 2008).

In summary, in this study we have shown that the pattern of gas exchange in *P. brassicae* pupae may be effectively investigated by the combined use of coulometric respirometry and flow-through CO_2 systems by switching the same respiration chamber from one system to the other without disturbing the insect. By combining IR actography in parallel with both types of respirometry, it was possible to record rapid air uptakes. Thus, on our recording traces, the patterns of microopenings of the spiracles were clearly indicated.

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