

Influence of environmental conditions on the breathing rhythms of the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae)

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Abstract. The metabolic rate and respiratory patterns of adults of the large pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae) was compared in hydrated and dehydrated conditions using a constant volume electrolytic respirometer-actograph combined with an infrared actograph.

The weevils displayed continuous pumping movements in hydrated conditions (hydrated individuals) while the cyclic gas exchange was entirely lacking or only unclear small releases of CO₂ (bursts, B) were observed. However, when a short period of quiescent state (tonic immobility) was artificially induced by a mechanical stimulus, 3–6 clear cycles of gas exchange appeared, with the frequency of 14–18 cycles per hour, i.e. the discontinuous gas exchange cycles or DGCs were displayed. These were recognized as CFO (closed, flutter, open) type of gas exchange cycles known by closed-flutter-open phases of spiracular movements without active ventilation, i.e. pumping movements, during the bursts. At 0°C, when the muscular activity was suppressed, all the hydrated weevils showed CFO cycles, nearly one burst per hour.

When *H. abietis* adults were kept in dry conditions without food for 3 days (dehydrated state), CFV (closed, flutter, ventilation) cycles appeared with the frequency of 5–6 cycles per hour, while the bursts were associated with the pumping movements of the abdomen. Dehydration caused lengthening of the flutter period nearly two times. During the flutter, regular miniature inspirations were recorded.

After being in the dehydrated state for 5 days, the adults of *H. abietis* displayed CFO and CFV cycles with a frequency of about one cycle per hour, while during the extremely long interbursts periods clear inspiration movements were recorded.

Metabolic rate in the hydrated weevils was 0.36 ± 0.05 ml O₂ g⁻¹ h⁻¹, but after they had been kept in dehydrated conditions without food for 5 days, metabolic rate decreased essentially, being only 0.16 ± 0.02 ml O₂ g⁻¹ h⁻¹.

The authors suggested that in hydrated *H. abietis* adults the lacking of clear cyclic gas exchange was due to the almost continuous pumping movements, or active ventilation, externally not observable. The active ventilation without DGC was obviously a normal mode of respiration in the hydrated weevils. However, the hydrated weevils were able to display the cyclic gas exchange during the forced quiescent periods but the artificially evoked tonic immobility was considered as a stress state. Thus our results supported the hypothesis that DGC is a water conserving mechanism in beetles.

Key words *Hylobius abietis*, large pine weevil, hydration state, gas exchange cycles, CFO, CFV, muscular ventilation

INTRODUCTION

The large pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae), is a widespread, abundant and economically important insect pest in reforestation areas in boreal coniferous forests extending from Northern and Western Europe to Eastern Siberia and Japan (Eidmann, 1974; Gourov, 2000). The adults damage seedlings of several coniferous species, including Scots pine *Pinus sylvestris* L. and Norway spruce *Picea abies* (L.), during the first few years after cutting a conifer stand. They inflict significant economic damage by destroying a large proportion of seedlings (von Sydow, 1997; Leather et al., 1999; Örlander & Nilsson, 1999; Hannerz et al., 2002; Långström & Day 2004).

Chemical control measures have been used to reduce the damage caused by *H. abietis*. Restrictions on the use of chemical insecticides in forests in many countries have stimulated the experiments with alternative control methods against this forest pest (Luik et al., 2000; Sibul et al., 2001; 2002; 2005). At the same time, application of new control methods and introduction of biological insecticides require profound knowledge of the ecology, behaviour and physiology of forest pest insects (Merivee et al., 1998; Sibul, 1999). Estimation of the physiological state of harmful insects is therefore an inevitable task.

In early spring, weevils become active when the diurnal temperature is 8–9°C. More active locomotion of weevils starts generally when the diurnal temperature is 13–16°C (Eidmann, 1974). Usually in Northern Europe, mass flight occurs from the middle or end of May until the second half of June (Långström, 1982; Lekander et al., 1985). According to Christiansen & Bakke (1968, 1971), the feeding and oviposition activities of pine weevils culminated at temperatures between 18 and 28°C. Sibul et al. (1999) showed that a decrease in the locomotor activity of *H. abietis* occurs under natural conditions when the air temperature on the ground is above 26°C. The feeding intensity of the pine weevil at the shaded side of a clear-cut area was found to be strongly correlated with global radiation and soil temperature in a study by Nordlander et al. (2003). Several authors (Havukkala & Selander, 1976; Sibul et al., 1999) have shown that *H. abietis* is also influenced by air humidity. The feeding activity of adult weevils peaks approximately at 25°C and 60% relative humidity (Christiansen & Bakke, 1968; Pohris, 1983). Under the conditions of high relative humidity (85–95% R.H.), weevils' movement is very active, while in lower relative humidity conditions it decreases significantly (Sibul et al., 1999).

Usually, the metabolic rate of insects is studied at room temperatures, mostly at 22–25°C. The physiological state of an insect is commonly estimated by the standard metabolic rate (SMR), defined as the value measured at a particular temperature when an animal is quiet, inactive, not digesting a meal, and not exposed to any stress (Withers, 1992).

Many insects in the pupal and adult stages display a specific respiratory pattern, the discontinuous cycles of gas exchange (DGCs), which means that carbon dioxide is released in bursts and oxygen uptake is often discontinuous. The DGC consists of three phases or periods consistent with spiracular behaviour: the closed (C) phase, the flutter (F) phase, and the open (O) or burst (B) phase, while the C and F phases together are

called the interburst period (see reviews by Miller, 1981; Kestler, 1985; Slama, 1988; Lighton, 1994, 1996; Wasserthal, 1996).

The DGCs are termed the CFO (closed, flutter, open) cycles when during the bursts no ventilation movements occur, and the CFV cycles when active ventilation occurs during the bursts (see Kestler, 1971, 1985; Tartes, 1995). According to Kestler (1991), cyclical release of carbon dioxide is a highly sensitive indicator of physiological stress in insects, as the sub-lethal and lethal effects of stressors (e.g. handling, heat and toxicants) are characterized by loss of discontinuous cycles of gas exchange (Möbius et al., 1996).

It was proposed that discontinuous gas exchange may have evolved as a means of reducing respiratory water loss in insects (for reviews see Kestler, 1985; Hadley, 1994). Both the closing of the spiracles in the C and V periods between pumping and a fluttering in the F periods reduce water loss (Kestler, 1978, 1980, 1982). Rapid release of carbon dioxide should minimize the time the spiracles are open and, therefore, reduce water vapour loss. Some arid-dwelling beetles reduce respiratory water loss by using DGCs, but they use the mesothoracic spiracle as the major route for cyclic emission of carbon dioxide (Duncan & Byrne, 2000; Duncan, 2002, 2003).

According to another hypothesis, the DGCs may have evolved originally to enhance gas exchange efficacy under hypoxic (insufficiency of oxygen) and hypercapnic (superabundance of carbon dioxide) conditions (see Lighton, 1998; Chown & Holter, 2001). According to the third hypothesis (Hetz & Brdley, 2005), insects breath discontinuously to avoid oxygen toxicity.

Adult *H. abietis* exhibit regular CFV cycles at rest when dehydrated (Sibul et al., 2004a, 2004b). The influence of air humidity and the hydration state of *H. abietis* on its respiratory patterns has not yet been investigated.

The aim of the present study was to compare the gas exchange patterns in hydrated and dehydrated pine weevil *H. abietis* adults at 22°C, and hydrated individuals at 0°C, taking into consideration the possible influence of the abdominal pumping movements on gas exchange.

MATERIALS AND METHODS

Insects

For experiments, *H. abietis* adults were collected in May and June 2003 from pitfall traps (25 × 40 × 50 cm) in a fresh clear-cutting area in an intensively managed forest area in the forest district of Rāpina, Southern Estonia (latitude 58°09' N, longitude 27°08' E, altitude 55 m a.s.l.), where the abundance of *H. abietis* was very high (Sibul, 2000; Voolma et al., 2003). Clear-cuttings of former pine-dominated stands (90% of *P. sylvestris* and 10% of *P. abies*) on dry soil stand in a *Vaccinium-Myrtillus* site type. The weevils were stored in one-litre glass containers at 2–3°C supplied with food and water. The body mass of the weevils used in the experiments ranged from 126–258 mg (mean 198 ± 32 mg). The weevils were weighed before and after the respirometer measurements.

The weevils kept for three days at 22°C with access to food (Scots pine twigs) and water (prior to experiments) are referred to as hydrated individuals. The weevils kept

for three days without food and water under dry conditions at 22°C are referred to as the dehydrated individuals.

Respirometry

In order to study the gas exchange rhythms in parallel with the abdominal contractions of *H. abietis*, a closed-system constant-volume respirometer, the electrolytic differential micro-respirometer was used, referred to as the respirometer-actograph. Such a respirometer allows the simultaneous monitoring of the level of oxygen consumption, gas exchange cycles and microcycles, as well as body movements (see Kuusik et al., 1996, Tartes et al., 1999; 2002).

The respirometer measures any volume changes occurring in the insect chamber. Rapid pressure changes (lasting a few seconds) in the respiration chamber, caused by insect active body movements or other rapid events, are not compensated and will lead to corresponding rapid changes in the electrolysis current output, reflected as peaks on the recordings. Single pumpings are recorded by the O₂ uptake volley. The abdominal pumping movements and other contractions were reflected as the sharp spikes on the respirograms (Figs 1, 2). The bursts of carbon dioxide release were recorded as the downward peaks lasting 3–10 minutes (Figs 3,4). The weevil in the chamber was observed also visually using a stereomicroscope.

The respirograms display the rates of electrolytic generation of oxygen, noted as arbitrary units 'FO₂ (ml h⁻¹)' in the graphs. The mean metabolic rates (oxygen consumption) were automatically calculated by averaging the data over a period involving at least 10 CFB cycles or at least 100 pumping movements of the abdomen.

An Eppendorff conical test tube (volume 1.5 ml) was used as the insect chamber. The respirograms indicated whether the beetle was struggling (escape behaviour), or whether there occurred regular pumping movements (see Figs 1, 2). As many adults of *H. abietis* were highly active in the insect chamber for many hours at room temperature, their immobility was achieved by a method described earlier: use of mechanical stimulus – shaking of the insect chamber (see Metspalu et al., 2002). The measurements were performed in a thermostat at 22 ± 0.2°C. A dry KOH was used as CO₂ absorbent when a dehydrated weevil was in the insect chamber. In the case of hydrated weevils, a 10% KOH water solution was exploited, besides a slit of wet filter paper was inserted into the insect chamber.

In order to carry out the measurements at 0°C, the insect chamber was put into a thermos containing crushed ice slush. By means of a thermocouple thermometer (RS-232/datalogger thermometer, TES Electrical Corporation) the exactly measured temperature inside the insect chamber was 0.08–0.09°C we referred to as 0°C.

To ascertain whether the peaks on the respirograms of the electrolytic respirometer were really due to discharge of carbon dioxide, the insect chamber was switched by three-way taps from the still-air system to the gas tubin system of flow-through respirometry based on an infrared gas analyser of IRGA (Infralyt-4, Dessau) (see Martin et al., 2004).

Infra-red (IR) actographic measurements

The respirometric device was combined with an infra-red (IR) cardiograph referred to as the IR actograph as it recorded not only heartbeats but also abdominal pumping movements. Thus the gas exchange patterns and the abdominal contractions were simultaneous. An IR-emitting diode (TSA6203) and an IR-sensor were attached to the sides of the insect chamber. Light from the IR-diode was modulated by the contractions of the heart or the skeletal muscles. The output voltage reflected the vigour of the muscular contractions of the insect (see Hetz, 1994; Hetz et al., 1999; Wasserthal, 1996; Kuusik et al., 2001, 2002; Metspalu et al., 2002 for detail).

Data acquisition and statistics

The measurements were computerized using a data acquisition board (DAS 1401, Keithley-Metrabyte, USA) with the TestPoint software (Capital Equipment Corp., Billerica, MA, U.S.A.). The sampling rate was usually 10 Hz.

The means are accompanied by standard deviation and the number of observations (N). The means were compared using Student's *t*-test or the repeated measures ANOVA, after testing for homogeneity of variance. The level of significance was $P \leq 0.05$ for all tests.

RESULTS

Respiration of the hydrated weevils

At the beginning of every measurement, there occurred a struggling period, or escape behaviour, lasting 15–30 minutes externally well observable. After the ending of the struggling, continuous pumping, i.e. regular abdominal contractions were recorded (Fig. 1).

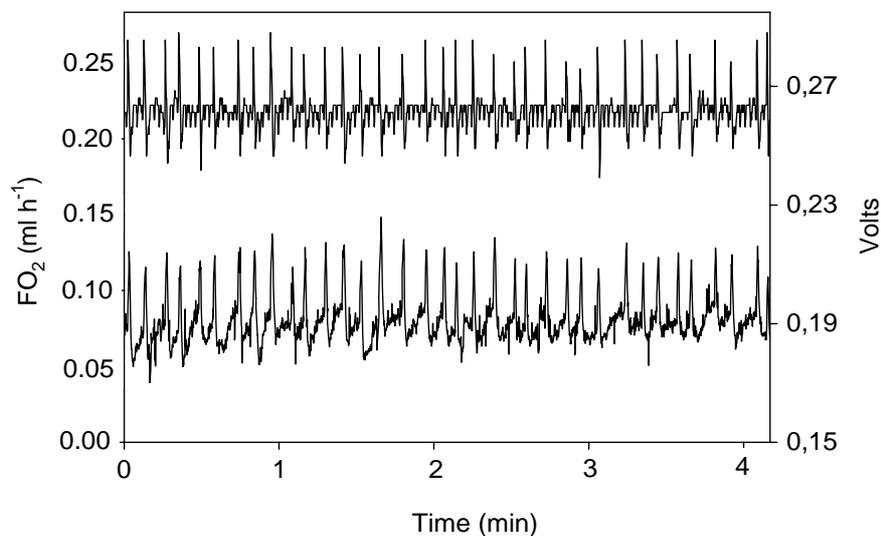


Fig. 1. A typical pattern of continuous pumping movements of the abdomen simultaneously recorded with an infra-red actograph (upper trace) and a respirometer-actograph of a hydrated adult of *Hylobius abietis* (167 mg).

The regular pumping we regard as continuous muscular or active ventilation. In about 90% of the hydrated beetles, abdominal pumping lasted at least 5–8 hours, which means that in these beetles continuous respiration occurred without discrete bursts of carbon dioxide.

When in the hydrated beetles, displaying continuous pumping for long periods, pumping was interrupted by a mechanical stimulus (Fig. 2), a short quiescent period with 3–6 CFO cycles appeared, while one cycle lasted 3–4 minutes. Further regular pumping was renewed and cyclic gas exchange was abolished. As abdominal pumping occurred under the elytra, the beetles were externally fully motionless, and only regular spikes on the recording traces of the respirometer-actograph (Fig. 2) indicated the ventilating movements of the abdomen.

There occurred beetles showing escape behaviour or struggling for hours. In about 30% of the struggling beetles the struggling was stopped by means of a mechanical stimulus (tapping the insect chamber), after which a short quiescent period occurred, lasting 5–12 minutes. During this period of muscular inactivity, the *H. abietis* adults displayed clear CFO cycles (Fig. 3).

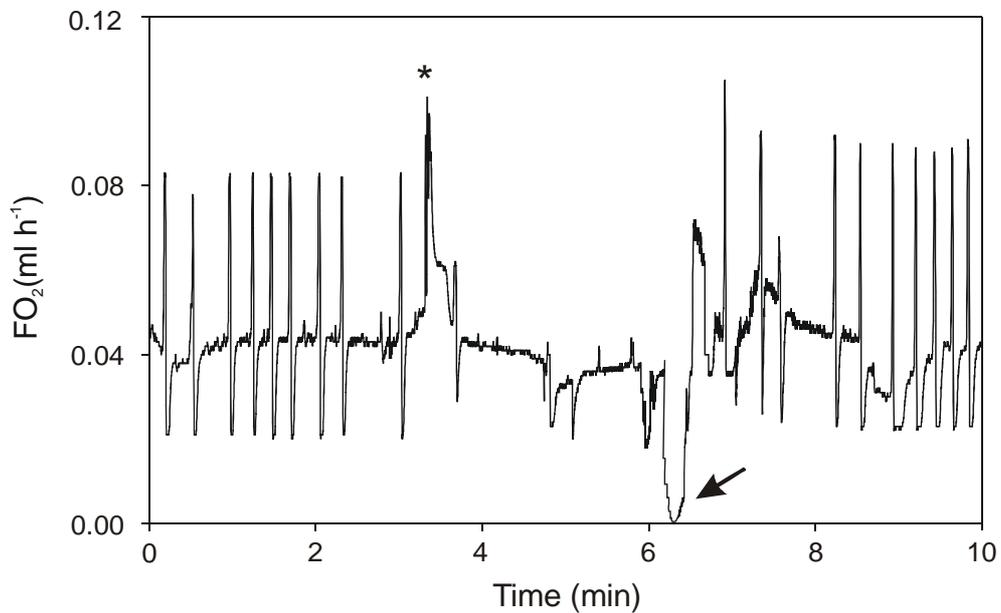


Fig. 2. A respirometer recording representing continuous pumping movements of the abdomen of a *Hylobius abietis* (130 mg) hydrated for 3 days, interrupted by a mechanical stimulus (tapping) (asterisk). Note that, during immobility, a burst of carbon dioxide release occurred (arrow).

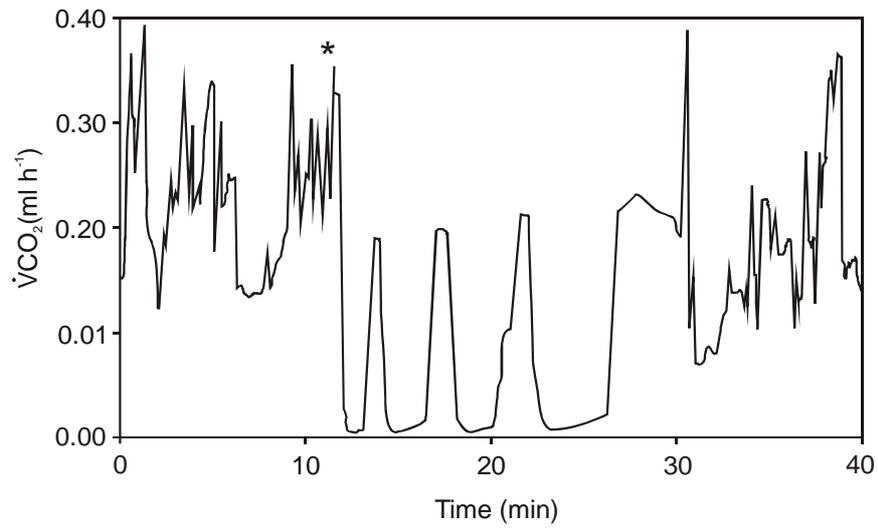


Fig. 3. CFO cycles evoked by a mechanical stimulus (asterisk) in a hydrated *Hylobius abietis* (246 mg).

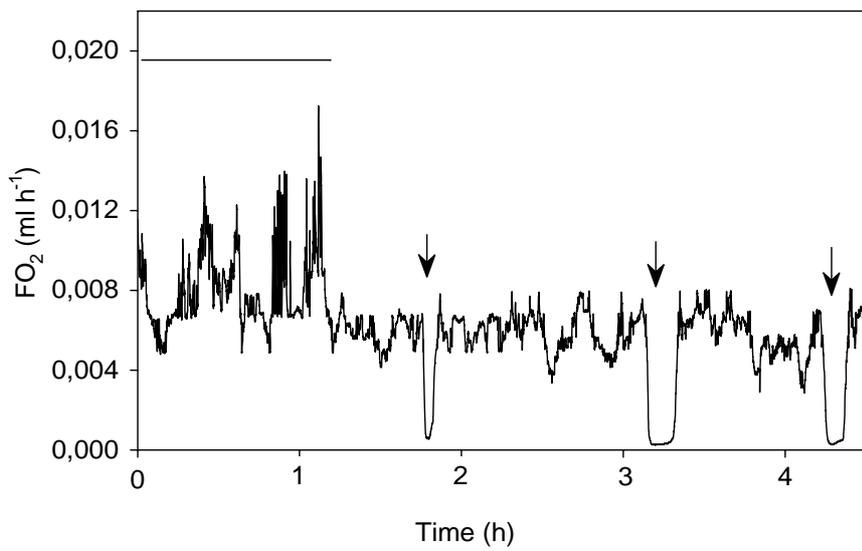


Fig. 4. Release of carbon dioxide by bursts (arrows) of a hydrated *Hylobius abietis* adult, recorded by means of electrolytic respirometer at 0°C. The horizontal line indicates a period of activity.

The metabolic level during continuous pumping of hydrated adults of *H. abietis* was significantly higher than the metabolic level measured during the quiescent periods with clear CFV cycles ($0.33 \pm 0.07 \text{ ml O}_2 \text{ g}^{-1} \text{ h}^{-1}$ and $0.23 \pm 0.05 \text{ O}_2 \text{ g}^{-1} \text{ h}^{-1}$, respectively; Student's *t*-test, $t = 4.04$, $df = 18$, $P < 0.01$). All beetles showed nearly similar differences in the metabolic rate (25–30%) between the pumping and the quiescent periods. Thus active ventilation caused an essential rise of metabolic level.

At 0°C all hydrated *H. abietis* adults displayed clear CFO cycles with the frequency of 1.2 ± 0.3 ($n = 8$ individuals) cycles per hour. The bursts at 0°C were associated with the active ventilating movements or did not remain unclear because we could not use the IR actograph at this temperature (Fig. 4).

Respiration patterns in the dehydrated weevils

When a hydrated weevil was dehydrated for 3 days, the patterns of gas exchange changed essentially. First, the struggling periods were shorter, lasting only 3–5 minutes at the beginning of the measurements. Secondly, the long pumping periods were lost and replaced by resting periods with CFV cycles (Fig. 5). Thus regular pumping movements in the dehydrated weevils were observed only during the bursts of carbon dioxide. The common frequency of CFV was 3–4 cycles per hour ($3.4 \pm 0.5 \text{ cycles h}^{-1}$, $n = 10$ individuals).

After the *H. abietis* adult had been kept in dry conditions without food for 5 days, the frequency of the cycles decreased drastically, and less than one cycle per hour was observed ($0.45 \pm 0.09 \text{ cycles h}^{-1}$, $n = 6$ individuals).

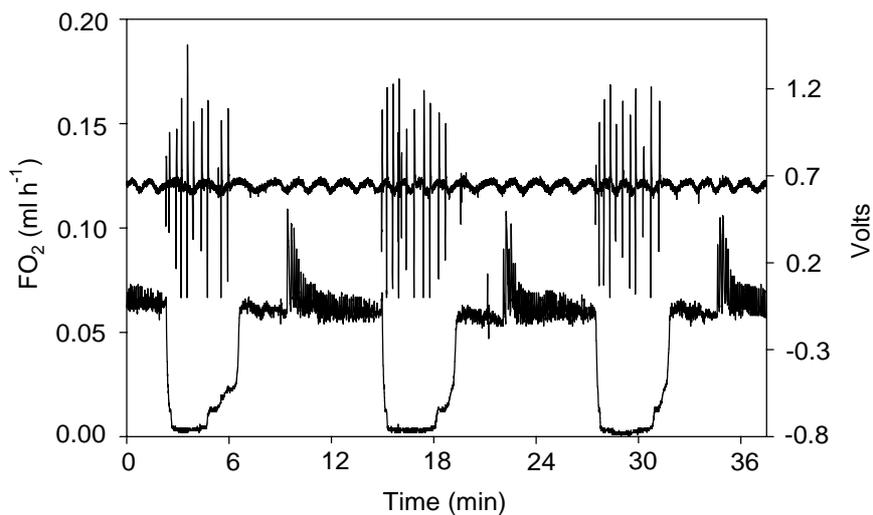


Fig. 5. A simultaneous recording of infra-red actograph (upper trace) and electrolytic respirometer (lower trace) showing three CFV cycles of an *Hylobius abietis* adult (130 mg), dehydrated for 3 days. The bursts of carbon dioxide release (respirogram) are associated with the abdominal pumping (IRA recording).

During the long interburst periods, lasting 50–100 minutes, clear spikes, caused by the to micro-opening of the spiracles, and miniature oxygen uptakes (flutter) appeared on the respirometric recording trace (Fig. 6A, 6B). The spikes due to the heartbeats on the IRA recordings were only slightly modulated by the regular spiracular movements (Fig. 6C).

During dehydration, the metabolic rate decreased essentially. In the hydrated weevils, the metabolic rate was $0.36 \pm 0.05 \text{ ml O}_2 \text{ g}^{-1} \text{ h}^{-1}$, but after dehydration for 3 days and for 5 days, it was $0.21 \pm 0.05 \text{ ml O}_2 \text{ g}^{-1} \text{ h}^{-1}$ and $0.16 \pm 0.02 \text{ ml O}_2 \text{ g}^{-1} \text{ h}^{-1}$, respectively (repeated measures ANOVA, HSD test; $df = 18$, $F = 45$, $P < 0.01$). Thus after being dehydrated for several days, the metabolic rate in the weevils decreased about twice.

DISCUSSION

Our result shows that continuous abdominal pumping was the main reason why hydrated adults *H. abietis* displayed continuous respiration without cyclic release of carbon dioxide. Nevertheless, continuous pumping, i.e. active or muscular ventilation, was assumed to be a normal mode of respiration and not a stressed state in *H. abietis* when hydrated.

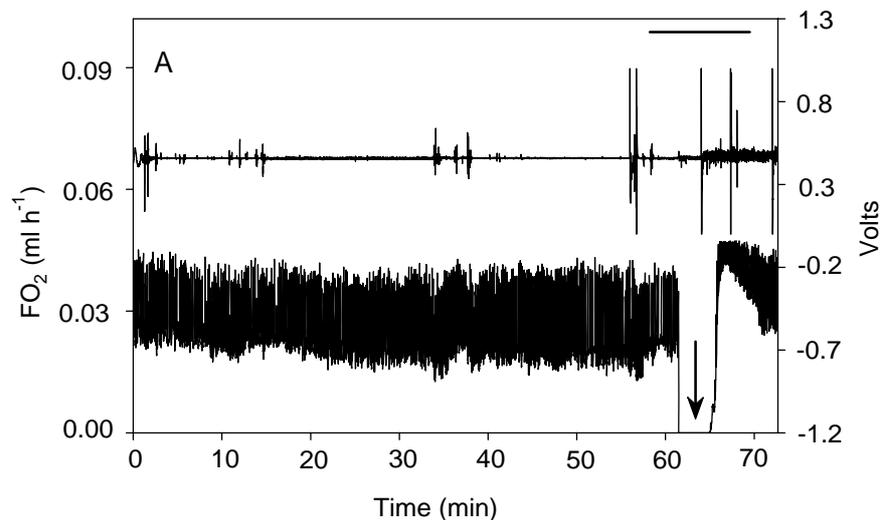


Fig. 6 A – Miniature inspirations and a burst of carbon dioxide (arrow) in a *Hylobius abietis* dehydrated for 5 days (lower trace, recording of electrolytic respirometer). The simultaneous recording of the infra-red actograph (upper trace) shows rare pumping movements (high spikes) and a pattern due to heartbeat (short spikes). The horizontal bar indicates a detail shown on the lower graphic at higher resolution.

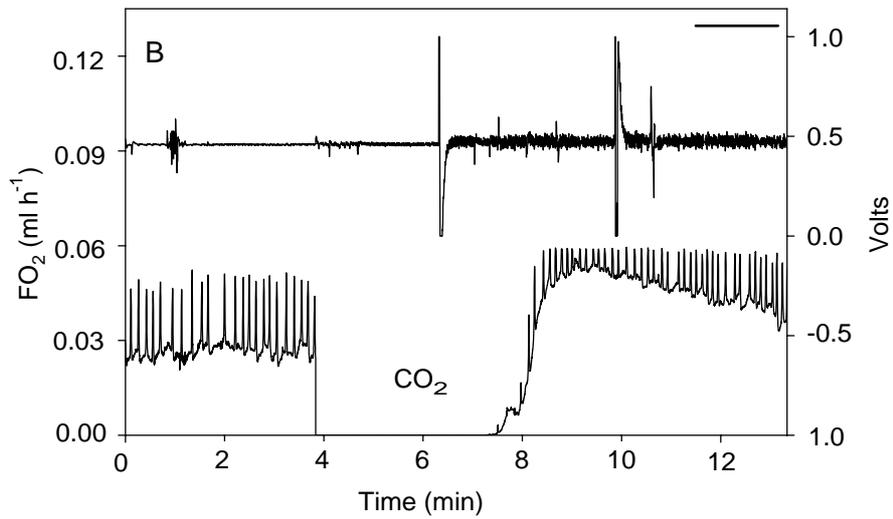


Fig. 6 B – A detail of **A** recorded at higher resolution. Horizontal bar indicates a detail represented on lower graphic at higher resolution.

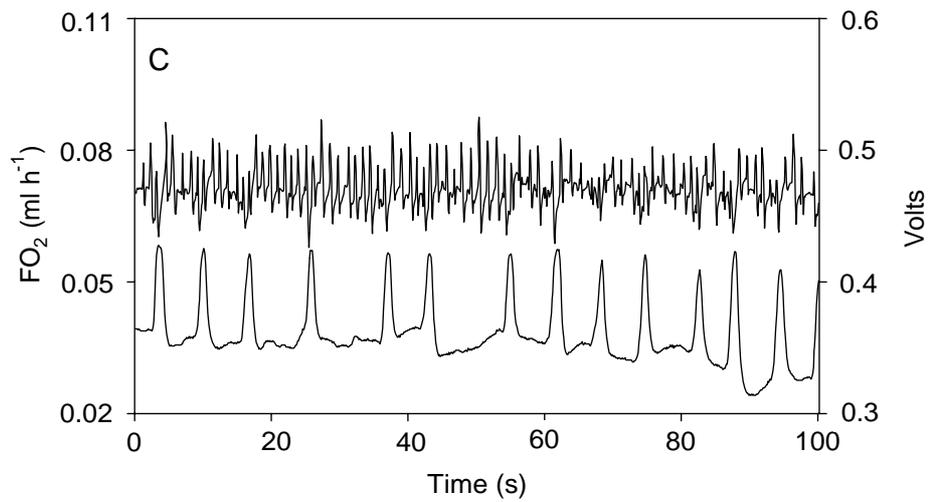


Fig. 6 C – Miniature inspirations (lower trace) and pattern due to heartbeat (upper trace). Note that heartbeats are slightly modulated by miniature inspirations.

When pumping in the hydrated weevils was interrupted by a mechanical stimulus (tapping), CFO cycles followed until pumping began anew and the gas exchange cycles were lost.

The interburst or flutter periods in the individuals *H. abietis* were short when hydrated, but they became far longer, lasting nearly 1 hour, when the weevils were dehydrated. During the flutter, highly regular micro-openings and closings closing, i.e. miniature oxygen uptakes, were recorded in this weevil. Duncan et al. (2002) demonstrated that the beetle *Pimelia grandis* (Coleoptera: Tenebrionidae) uses DGC when dehydrated, whereas the beetles, given access to food and water, show a form of continuous gas exchange, while the dehydrated beetles have far longer flutter periods than the hydrated individuals. Thus water-stressed beetles should have long flutter periods. A long and conspicuous flutter period is characteristic of some arid dwelling insects (Lighton et al., 1993).

The flutter (F) period of cyclic gas exchange was considered an important means for reducing respiratory water loss in insects. During the flutter (F) period, the spiracles are slightly not fully open (Kestler, 1971), the rapid spiracular movements (fluttering) allowing oxygen uptake through the spiracles by diffusion and convection, while little water vapour is lost (Kestler, 1978, 1980, 1982, 1985; Lighton & Garrigan, 1995). Some beetle species produce discrete bursts of carbon dioxide (miniature openings of the spiracles) during the flutter (Lighton, 1991; Duncan et al., 2002).

In our observations, the hydrated weevils displayed only CFO cycles when quiescent, whereas the dehydrated individuals always showed CFV cycles. Thus there occurred no pumping movements in *H. abietis* during the bursts of carbon dioxide release when hydrated; however, after dehydration, the bursts were always associated with pumping movements. Abdominal pumping has been regarded as a possible mechanism for reducing respiratory water loss during the burst of carbon dioxide release (V period) because the muscular contractions are coupled with the spiracular movements (see Kestler, 1985; Duncan & Dickman, 2001).

Metabolic rate in *H. abietis* decreased drastically after being dehydrated. It was suggested that a decrease in the metabolic rate is a method by means of which the beetles could reduce overall water loss rates (for review see Chown, 2002). According to Duncan et al. (2002), the overall metabolic rate for the tenebrionid beetle *Pimelia grandis* in the dehydrated state was lower than that in the hydrated state.

During continuous abdominal pumping, the beetles were externally immobile, however, the metabolic rate during pumping, i.e. at the time of muscular activity, was 25–30% higher than in the quiescent periods when cyclic gas exchange was recorded. These observations support the opinion that regular cycles of discontinuous gas exchange are the best sign of the quiescent state in an insect, when real SMR may be measured (see Kestler, 1971, 1985, 1991; Lighton, 1994; Duncan & Newton, 2001).

The results of this study suggest that cyclic gas exchange is a general property, or the `default state` of adult *H. abietis* when continuous abdominal contractions are absent. However, the tonic immobility evoked by a mechanical stimulus we consider as a stress state in hydrated beetles. These results support the hypothesis that DGC is a useful mechanism for reducing water losses in dehydrated weevils that have spent a long time in dry conditions. In the hydrated state of beetles the DGC was lost, but nevertheless it appeared when tonic immobility was evoked.

According to Vanatoa et al. (2006), the Colorado potato beetle *Leptinotarsa decemlineata* exhibits DGCs irrespective of its hydration state and temperature. Thus, *H. abietis* is not an exceptional beetle species who is able to display DGCs both in dehydrated and hydrated states, but only on conditions that, during the hydrated state, the tonic immobility must be evoked.

We suppose that several hypothesis of DGC origin are not so antagonistic as described in literature. There is likely to be a strong selection both for low metabolic rate, to conserve energy resources, and for any mechanism that might reduce water loss, irrespective of its origin (see Chown, 2002).

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