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DISCONTINUOUS VENTILATION IN THE RHINOCEROS BEETLE *ORYCTES NASICORNIS*
Direct and indirect calorimetry

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Discontinuous gas exchange cycles (DGCs) are frequently observed with insects, i.e. oxygen take up and carbon dioxide release occur interrupted by periods of a few minutes up to many hours.

The paper presents direct and indirect calorimetric experiments on DGCs of the scarabid rhinoceros beetle *Oryctes nasicornis*. A direct/indirect calorimetric experiment is presented. Total and specific heat production rates amount to 0.56 mW and 0.42 mW g^{-1} in the first period without DGCs and to 0.43 mW (0.32 mW g^{-1}) in the second phase, resp. The mean DGC amplitude is 0.184 mW and thus between 33 and 66% of the total turnover.

Keywords: *calorimetry, discontinuous gas exchange cycles (DGC), insects, metabolism, respirometry*

Introduction

Periodic ventilation is a widespread trait among vertebrates and invertebrates [1]. The discontinuous gas-exchange cycle (DGC) of arthropods was described first fifty years ago [2] as release of carbon dioxide in short periodic bursts, often with a remarkably stable frequency over longer periods of time. It is thought to have evolved independently in at least five orders of insect, and is observed in flighted, non-flighted, subterranean, non-subterranean, arid and xeric species [3]. While early studies [2] and recent comparative work [4] supported the role of DGCs in reducing water loss, the origin and adaptive significance of DGCs remains controversial [5, 6].

The DGC consists of three phases: closed (C), flutter (F) and open (O) [7]. During the C-phase, intratracheal pressure declines as tissues consume oxygen and carbon dioxide is buffered by bicarbonate in body liquids (e.g. haemolymph). When the internal O_2 tension reaches ~2–4 kPa, the spiracles start to flutter (F-phase) and air enters the trachea following the pressure gradient. There is nearly no water loss against the inward convective movement of air during this phase. When the internal CO_2 tension reaches ~3–6 kPa the spiracles open (O-phase), and CO_2 escapes as a burst together with water. Thus, the majority of respiratory water loss occurs during the O-phase [8, 9].

This calorimetric study is a preliminary examination of the relationship between CO_2 and water loss during DGCs of flower beetles. The aim was to relate the amount of CO_2 lost, evaluated by CO_2 respirometry, with spiracular evaporative water loss, evaluated by direct calorimetry of the heat of evaporation of water. For the present calorimetric investigations, the O-phase with CO_2 burst and main water loss is the most interesting part of the DGC. Water loss means evaporation which is connected with a large endothermic heat uptake of 2.4 kJ g^{-1} of liquid water at room temperature. Because tissue respiration is more or less constant during the DGC, but respiratory gases and water are exchanged discontinuously, one has to expect a more or less constant heat production rate by the insect in the calorimeter, structured by smaller peaks of locomotor activities and abdominal ventilatory movements and of longer lasting broad downward peaks of the O-phase. As internal water is essential for the beetles to keep appropriate osmotic pressures in hemolymph and tissue, the loss will be as small as possible and no big effects can be expected.

Experimental

The experiments were performed during April and May 2008 at the North coast of the Greek island Crete

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Fig. 1 Male rhinoceros beetle *Oryctes nasicornis* from a Cretian olive grove. Pay attention to the horn that gave name to the beetle

in Panormos (near Rethimno). Most plant and beetle samples were collected in the surrounding olive groves and along the roads. The object of the present determinations was a local rhinoceros beetle (*Oryctes nasicornis*, Linnaeus 1758). The male beetle used here had a mass of 1.330 g, measured with a Tanita model 1210 balance (Fig. 1).

Calorimetry

Two isothermal twin calorimeters of the TAM Air type were used [10]. These consist of ampoule holders mounted on heat flow sensors that are fixed on an aluminium heat sink. Each calorimeter is surrounded by an aluminium temperature shield. The mass of each calorimeter is about 1500 g. The sensitivities of their heat flow sensors were 286 and 313 μV mW^{-1}, respectively, determined by electrical calibration with resistors inside the beetle ampoules. Each of their four compartments housed a glass vessel of 24 mm diameter and 65 mm height. Because of the changing beetle masses the reference vessels remained empty.

Both calorimetric units were placed side by side, but separated by an insulating layer to avoid cross-talk, in a tight metallic box which was on the outside covered with a further layer of insulation. In this way temperature fluctuations introduced by the regulator system were reduced to insignificant levels for the beetle experiments. This metallic box was placed in a box (40×31×20 cm, walls of 3 cm) of extruded polystyrene. An air-air thermoelectric thermostat (AA-100-24 from SuperCool, Göteborg, Sweden) controlled by an NTC thermistor and a temperature regulator (PR-59, SuperCool) was placed in one side of the box. During the experiments, the controller was used in the PI mode. The temperature inside the calorimeter box was stable to about ±0.02 K, but in the calorimeters (inside the inner insulation) it was one magnitude better. Baseline stability and signal-to-noise-ratio were good enough for biological measurements in a range of 0.05 to 0.2 mV.

Calorimetric output signals were logged by a modified (increased amplification) eight-channel data logger (TC-08 Thermocouple Logger, Pico Technology Ltd., St. Neots, UK) as mV with a resolution of 0.1 μV.

The calorimetric mean values (in mW or as specific value divided by the mass of the beetle in mW g^{-1}) were obtained by integrating the area under the power–time curve in the given borders of time divided by the time of this period. They were determined for the pre-period without DGC and for the following DGC time.

Respirometry

Rate of carbon dioxide release was measured by pumping dry atmospheric air through a flow meter (Sable Systems SS-3, Las Vegas, Nevada, USA), the reference cell of a CO_2 analyser (Li-Cor Li-7000, Li-Cor Inc., Lincoln, Nebraska, USA), a 25 mL chamber containing the animal, and finally the sample cell of the Li-7000. The temperature of the animal chamber was regulated using a custom built 'Doug-o-Therm' Peltier effect constant temperature cabinet stable to better than ±0.1 K, and measured with a thermocouple calibrated against a precision mercury thermometer. The Li-7000 was interfaced via USB with a notebook computer, which recorded the CO_2 concentrations in both the sample and reference cells, as well as the voltage output of the SS-3 (i.e. flow rate). Flow rates were 200–250 mL min^{-1}.

Simultaneous calorimetry and respirometry

True simultaneous determinations of heat flow and CO_2 release were carried out on *O. nasicornis*. In this experiment an air flow of 25 mL min^{-1} was drawn from the air-thermostat volume at first through the calorimetric ampoule and from there via a tube (3 mm inner diameter, 200 cm length, total volume 14 mL) through the sample cell of the Li-7000. The 34 s from the calorimeter to the analyzer could be taken as 'simultaneous' compared with the much longer periods between the CO_2 bursts. The calorimeter was hermetically sealed and permanently flushed with outside air of atmospheric CO_2 concentration at a slight overpressure. Air was simultaneously drawn from within the air-thermostat and passed through the reference cell of the Li-7000.

Results and discussion

In addition to the direct calorimetric curves CO_2 release was monitored and used as indirect calorimetry. At 14.9°C, the rhinoceros beetle had a DGC highly constant frequency of 0.22 mHz (period length 1.33 h) and a metabolic rate of 0.42 mW (assuming an energy

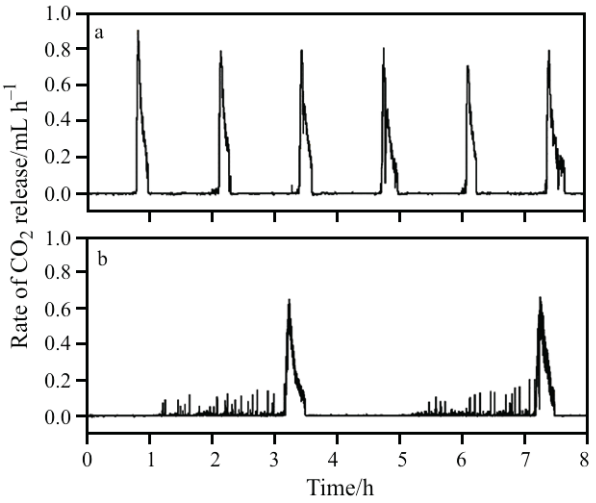

Fig. 2 Discontinuous gas exchange cycles of rhinocerous beetles recorded at a – 14.9 and b – 10.3°C using indirect calorimetry

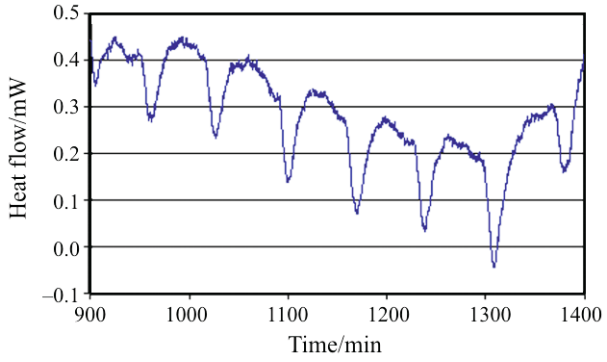

Fig. 3 Second part of the power–time curve of *O. nasicornis* with the sequence of DGCs at 20°C. The calorimetric baseline is at –0.1 mW

equivalence of CO_2 of 24.65 kJ L^{-1}) (Fig. 2). At 10.3°C, DGC frequency dropped to 0.074 mHz (period length 3.75 h), and the metabolic rate to 0.18 mW.

The power–time (*p–t*) curve of the rhinoceros beetle with its experimental baseline at –0.1 mW started with a period of about 12 h, coined by locomotor activities (not shown here). The mean energy turnover during this period (see above) amounted in total

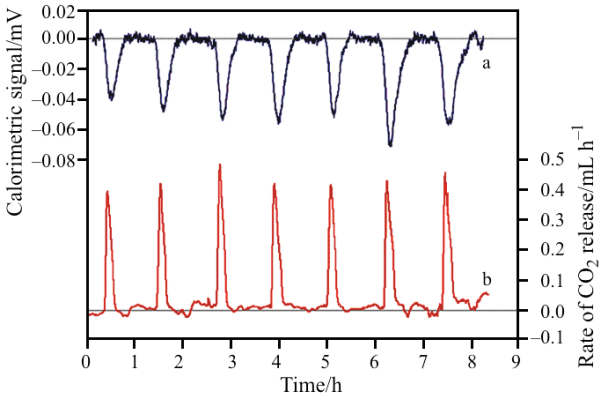

Fig. 4 Perfect synchronicity between the a – endothermic water peak and b – CO_2 release detected calorimetrically. For better comparison, the curved calorimeter base was transformed into a horizontal line

to 0.56 mW or mass specifically to 0.42 mW g^{-1}. With the beginning of the night the *p–t* curve dropped to a lower level and revealed the negative endothermic peaks of DGCs (Fig. 3). In this period, the corresponding mean data were 0.43 mW (0.32 mW g^{-1}). However, because the peaks were not on a stable baseline, the data were subjected to a procedure that determined the baseline and subtracted it to flatten it out. The essential values of the DGC peaks are compiled in Table 1. It indicates that the amplitudes were in the mean 184 μW and thus equal to 33% of the total output in the beginning of this phase and to 60% in its minimum. Comparing the sum of the DGC areas with the total area under the power–time curve in this period tells that the evaporation enthalpy consumes about 10% of the metabolic heat output.

When the curved base of this DGCs part is transformed into a horizontal line and exposed against the CO_2 release peaks one obtains a perfect synchronicity of both curves (Fig. 4). Comparing the areas of the corresponding calorimetric and respirometric peaks a good and significant (p=0.02) correlation appears (Fig. 5). Larger peaks of CO_2 release correspond to larger peaks of enthalpy consumption. The gradient of this straight line renders a value equal to the evaporation enthalpy of water: estimation

Table 1 Specific data of the endothermic DGC peaks in Fig. 3

Peak No.	1	2	3	4	5	6	7	MV	SD
Peak area/mJ	152	145	164	213	176	222	174	178	30
Peak height/μW	149	160	183	190	190	235	179	184	27
Base width/min	34	29	26	41	32	35	32	32.7	4.8
Half maximum width/min	15	13	13	13	13	13	15	13.6	1.0
Time to preceding/min	54	66	74	71	69	71	71	68.0	6.6

MV – mean value; SD – standard deviation. A peak was approximated as an inverted bell-shaped figure, characterized by its area, its height of the maximum, the width at 50% of the maximum, the width of its base and the time between two maxima

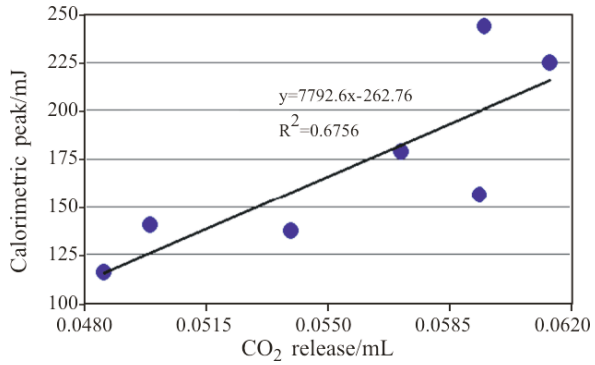

Fig. 5 Relationship between the CO_2 release peaks and the endothermic calorimeter peaks

shows that the mean value of the 7 endothermic peaks amount to 178±30 mJ. With a mean water loss of 70 mg per peak (C. W. and P. M., unpublished results) one calculates an enthalpy of 2.44 kJ g^{-1} water evaporated during the peak in good agreement with textbook values. Assuming a specific heat capacity of 3.2 J g^{-1} K^{-1} for a beetle and thus a total heat capacity of 4.26 J K^{-1} for this 1.33 g specimen, the mean 178 mJ per peak would be sufficient to cool the beetle by 0.04 K.

Discontinuous ventilation is an interesting entomologic–physiologic phenomenon that is usually studied via respirometry: oxygen consumption and carbon dioxide production (e.g. [3]). As the CO_2 bursts are connected with water release and therefore with a strong endothermic evaporation, it made sense to apply direct calorimetry also.

The present study demonstrates that simultaneous calorimetric and respirometric measurement of the discontinuous gas exchange of insects is technically feasible, but also demonstrated some potential pitfalls that remain to be overcome. The close temporal correspondence of calorimetric and respirometric peaks is encouraging (Fig. 4), as is the significant correlation between in magnitude of these peaks (Fig. 5). Thus, we are confident that, when used together, each of the techniques has sufficient resolution to detect cycle-by-cycle variation in discontinuous gas exchange. However, the combination of the techniques resulted in a significant decrease in the CO_2 resolution of the respirometry system (compare, for example, Figs 2 and 4). It was therefore not possible to resolve the fine details of the DGCs when calorimetric and respirometric measurements were made simultaneously. Two factors account for the reduction of resolution observed when the methods were combined.

Firstly, during combined calorimetry-respirometry, the ampoule was not supplied with dry air, and the sample and reference cells of the CO_2 analyser were connected in parallel. Thus, each cell was supplied with air drawn independently from within the air-thermostat volume. Imperfect mixing of atmospheric air could therefore result in differences in observed CO_2 concentration between the reference and sample cells, and these variations will be superimposed over the CO_2 release of the animal. In the respirometry-only configuration, in contrast, dry air flowed sequentially through the reference cell, the chamber containing the animal, and then the sample cell. Thus, any changes in CO_2 concentration between the reference and sample cell can be unequivocally attributed to respiration of the animal. In future work, this problem could be overcome by passing air of known CO_2 concentration (either zero, or measured using the reference cell) through the ampoule prior to measurement of CO_2 concentration.

Secondly, the low flow rate of the calorimetry–respirometry configuration compared to the respirometry-only system limits the temporal resolution with which variation in CO_2 release can be quantified. This reduces the ability of the system to detect fine-scale variation in CO_2 release, and almost certainly precludes detection of the F-phase. Potentially, this problem could be overcome by increasing the flow rate, but this introduces the additional problem of convection removing heat from the ampoule and preventing detection by the calorimeter. 'Instantaneous' correction of the measured changes in CO_2 concentration could be used [11], but these techniques require a stable baseline, and therefore require that problem 1, above, is also solved. Ultimately, combined respirometry-calorimetry may be limited to separation of only the O- and CF-phases. This may not represent a significant limitation, however, as the C- and F-phases are often difficult to distinguish, particularly when flow rate is low [12].

DGC was observed for the first time by Punt *et al*. [13] as a discontinuous release of CO_2 and studied more intensively in the following years [14–16]. The astonishing effect was that these bouts occurred with a remarkably stable frequency over longer times. It has evolved independently in the Chelicerata, Myriapoda, and five orders of insect (Blattodea, Orthoptera, Lepidoptera, Coleoptera and Hymenoptera) [1, 3], and is frequently observed in species that have to cope with hypoxic or hypercapnic environmental conditions, such as burrowing carabid beetles [8, 9].

Usually it is assumed that the discontinuous gas exchange in insects was developed (*i*) to reduce respiratory water loss and (*ii*) to deal with hypoxic as well as hypercapnic conditions in underground species. In 2005 Hetz and Bradley proposed a new explanation that oxygen is of course necessary for aerobic metabolism, but at the same time toxic for tissues even when at rather low concentrations [17]. The authors conclude

that DGC thus serves to get the respiratory system free of CO_2 and to keep the O_2 level low.

Although the total water losses are often mentioned in the literature, more recent observations showed that the main water loss happens cuticularly so that only small losses occur via the CO_2 bouts. Values from a few up to about 20% are found in the literature depending on the insect and the strength of its cuticule [14–16] so that one may assume that 80 to 90% of water loss occur independently of DGCs. Nevertheless, respiratory water loss is an essential component of the water balance in many insects [18–20].

Conclusions

The present results show that direct calorimetry is well suited to study DGCs in beetles sized to fit in commercial isothermal twin instruments with ampoules of about 20 mL. Ventilation by a thermally equilibrated air flow is advantageous to avoid the anaesthetizing/poisoning effect of CO_2. Often, perforated ampoule lids are sufficient at these low metabolic rates. Direct calorimetry allows monitoring of body movements and thus of activities that are tightly coupled with beetle respiration.

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