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The role of the subelytral spiracles in respiration in the flightless dung beetle *Circellium bacchus*

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Summary

The role of the subelytral cavity in flightless beetle species as an adaptation to water saving in arid habitats is still in dispute. We found that relatively little CO_2 was released from the subelytral cavity of a large apterous beetle *Circellium bacchus* during simultaneous measurements of CO_2 emission from the anterior mesothoracic spiracles and posterior body, which included the subelytral spiracles. However, when we sampled air directly from inside the subelytral cavity, we discovered that this pattern was reversed. A discontinuous gas exchange cycle (DGC) was recorded from the posterior body half, revealing a flutter phase that had been absent from the anterior mesothoracic DGC. The anterior mesothoracic and posterior subelytral spiracles act in synchrony to maintain high CO_2 and water vapour levels inside the subelytral cavity. In addition, the O_2 concentration of the air within the subelytral cavity is lower than the air around the elytral case, irrespective of the time of sampling. These findings lead us to conclude that the subelytral spiracles work in a coordinated fashion with the anterior spiracles to create a DGC, which allows us to extend the hypothesis of the function of the subelytral cavity as a respiratory water-saving device.

Key words: discontinuous gas exchange cycle, Scarabaeidae, *Circellium bacchus*, beetle, spiracle, subelytral cavity.

Introduction

Many arid-dwelling beetles are wingless and have evolved a hermetically sealed air space beneath the wing covers. This space is called the subelytral cavity and is created by the fusion of the elytra to each other along their midline and a tight seal to the dorsal tergites by means of minute hairs around the perimeter of the elytra (Gorb, 1998). The presence of a subelytral cavity is widespread among desert beetles, with representatives found in the tenebrionids, carabids and scarabs, which supports an ecological rather than phylogenetic explanation for its existence. These currently range from providing space for abdominal expansion during feeding or egg maturation to the more satisfactory explanation that it creates an area of high humidity over the posterior spiracles (Draney, 1993). This reduces the concentration gradient of water vapour between the body and the ambient atmosphere, which will reduce water loss during respiration (Ahearn and Hadley, 1969; Zachariassen, 1991). Flightless dung beetles appear then to have traded the capacity of flight, which must be adaptive in searching for a scattered and ephemeral food resource, for enhanced resistance to desiccation *via* the subelytral cavity. This hypothesis is supported by the fact that many flightless species are desert dwellers (Roff, 1990).

The water-saving role of the subelytral cavity is an attractive hypothesis (Cloudsley-Thompson, 1964) with circumstantial evidence to support it, such as the air inside the cavity having a high water content (Zachariassen, 1991; Cloudsley-Thompson, 2001). However, the exact mechanism by which it works is still unclear. Ahearn (1970) suggested that there is unidirectional, retrograde airflow in which the thoracic spiracles are used for inspiration and the subelytral spiracles are used for expiration. After air has been expelled from the spiracles, the CO_2 that has accumulated in the subelytral space is eliminated to the atmosphere by lifting the elytra, creating an opening above the anus through which CO_2 could be expelled on an intermittent basis (Nicolson et al., 1984), thus resulting in the discontinuous gas exchange cycle (DGC) measured from desert beetles (Lighton, 1991). However, although the flightless dung beetle *Circellium bacchus* (Coleoptera, Scarabaeidae) has a marked DGC (Duncan and Byrne, 2000), we found CO_2 emission to take place predominantly through one anterior mesothoracic spiracle, and the airflow through the beetle to be predominantly anterograde (or possibly tidal) when at rest (Duncan and Byrne, 2002).

The DGC is an intermittent discontinuity in external gas exchange that typically consists of three periods (Miller, 1981; Kestler, 1985; Lighton, 1994). First, there is a closed period, where the spiracles are shut, which inhibits gas exchange and respiratory water loss. Oxygen levels in the tracheae drop

while CO_2 is largely buffered in the tissues and haemolymph. This is followed by the flutter period during which slight opening of the spiracles on an intermittent basis allows some normoxic O_2 uptake through the spiracles by diffusion and convection, but little CO_2 or water vapour is lost. The final CO_2 burst period is triggered when the accumulation of CO_2 from respiring tissues causes some or all of the spiracles to open widely or triggers a bout of active pumping movements. The rapid unloading of CO_2 minimises the time that the spiracles are open and therefore reduces water loss (Kestler, 1985).

In our previous study (Duncan and Byrne, 2002), the DGC was only found at one anterior, mesothoracic spiracle, outside of the subelytral cavity, and showed only the closed and burst periods. The flutter period was largely reduced or absent. We detected no large, intermittent CO_2 bursts from outside the elytral case, which would correspond to the lifting of the elytra to expel accumulated CO_2. Water loss also occurred mainly *via* the mesothoracic spiracle when it opened, and 90% of the CO_2 expelled was eliminated through this spiracle (Duncan, 2002). Because the small amount of CO_2 emitted was measured from outside the subelytral cavity, we could not determine what role, if any, the subelytral spiracles had in respiration.

There are seven pairs of spiracles that open into the subelytral cavity in *C. bacchus* (a single metathoracic pair and six abdominal pairs; Fig. 1). The shape and position of the spiracles within the Scarabaeoidea are so variable that they have little utility as a taxonomic character (Richter, 1969); nevertheless, the Scarabaeidae have them positioned under the elytra. Because these spiracles represent a significant surface area for gaseous exchange, we would expect them to be involved in respiration. The forward airflow (anterograde) from the subelytral cavity to the anterior spiracles found in *C. bacchus* implies that the subelytral cavity could act as a humidity chamber but in a different manner to that previously proposed.

The large size and quiet nature of *C. bacchus* allowed simultaneous measurement of respiratory gas exchange from the anterior and posterior body half of the beetle at rest. By drilling holes in the elytra, we were also able to sample gas from the subelytral cavity at different stages of the DGC. Because CO_2 release rate is proportional to the degree of opening of the spiracles (Kestler, 1985), we were able to examine the role of the subelytral spiracles in respiration by infrared gas analysis and to conclude that they are synchronised to the DGC found at the anterior spiracles. These findings show that the subelytral spiracles play an active part in respiration at rest and suggest that the subelytral cavity has a role in respiration in accordance with its hypothesised function as a water-saving adaptation.

Materials and methods

Circellium bacchus (Fabricius) is a large, ball-rolling, flightless dung beetle endemic to the Eastern Cape of South Africa. The habitat is dry scrub on sandy soil ('Valley Bushveld'; Acocks, 1988) with a low (400 mm year^{-1}), unpredictable rainfall that will expose beetles to desiccating conditions during their 2–3-year life span (Coles, 1993). *C. bacchus* adults were collected from the Addo Elephant National Park (33°30′ S, 25°41′ E) in the Cape Province, South Africa, with permission from the National Parks Board, due to their conservation status. The beetles were housed in 10-litre bins, half filled with soil, in an insectary at 25°C with a 14 h:10 h light:dark cycle. They were fed fresh cow and horse dung twice weekly and survived for up to a year under laboratory conditions.

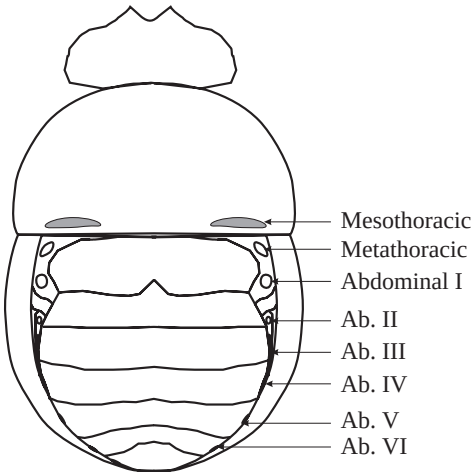

Fig. 1. Position of the spiracles in *Circellium bacchus*, dorsal view, elytra removed. Note that the anterior mesothoracic spiracles are actually between the prothorax and abdomen and are not visible in this view.

Volume of the subelytral cavity

To gain access to the subelytral cavity, one hole was drilled into each elytron, near the anterior lateral margin, of five beetles using a 3 mm dental drill. A short (5 mm) length of 3 mm-diameter tube (flexible nylon; Portex Ltd, Hythe, UK) was sealed into each hole using a combination of bee's wax and superglue. Between experiments, the tubes were closed off with dental wax plugs, which allowed the beetles to survive for several months in the laboratory. Therefore, we assume that the beetles were not incapacitated by this procedure.

To estimate the volume of the subelytral cavity, the wax plugs were removed, and distilled water was gently pumped into one elytral tube using a 10 ml syringe until it appeared at the second tube. After approximately 5 s, the beetle was tipped on its side and air was pumped through the upper tube while the water was collected as it came out of the lower tube. This water was weighed to ±0.1 mg (Precisa 160A balance) and used to estimate the volume of the subelytral cavity.

Continuous measurement of CO_2 emissions from the subelytral spiracles

A flow-through respirometry system was used to measure CO_2 emission in inactive beetles at room temperature (23±1°C). Simultaneous sampling of gas emissions from the

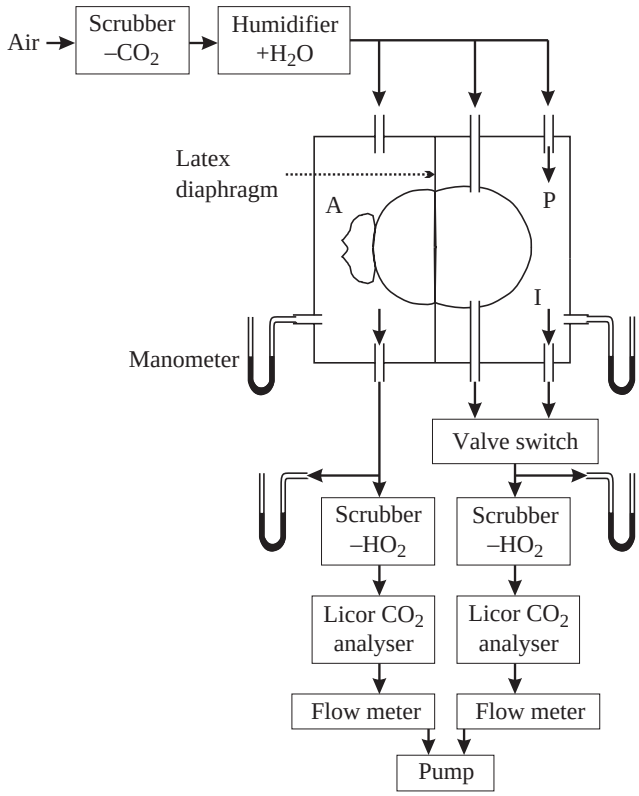

Fig. 2. Experimental set-up used in flow-through respirometry. A, anterior chamber; P, posterior chamber; I, route for gases to sample the contents of the posterior chamber during intermittent sampling; see text for details.

anterior (mesothoracic) and posterior (subelytral) spiracles was performed in a set-up with two separate compartments, similar to that described by Duncan and Byrne (2002), where the head and thorax are separated from the abdomen by a sheet of latex (Fig. 2). Each compartment had its own manometer, flow controller (Supelco flow meter) and gas analyser. Room air, scrubbed of CO_2 by a soda lime column, then humidified in a water column, was drawn through each compartment of the respirometer either at 50 ml min^{-1} or at a rate that generated equal pressures in all parts of the system. Initially, respiratory gases were sampled from the air spaces in each chamber. To measure the CO_2 output directly from the spiracles in the subelytral cavity, the saturated air was drawn in through one of the tubes inserted into an eltron and out through the other. Thus, the CO_2 emission from the subelytral spiracles was sampled in humid air, as we assumed that passing dry air through the subelytral cavity during the long periods required to make measurements would unduly stress the beetles and alter their respiratory patterns. The air withdrawn from both sides of the respirometer was dried using a magnesium perchlorate scrubber before measurement in a gas analyser. Either a Licor CO_2 analyser (LI-6251) or Licor CO_2/H_2O analyser (LI-6262), both with a resolution of 0.1 p.p.m., was used to measure the separate CO_2 emissions. The length of the tubes connecting each compartment to its respective analyser was kept to a minimum and was identical for each tube. Readings of the volume of CO_2 emitted were taken every 5 s and recorded using computerised data acquisition software (Datacan V, Sable Systems, Las Vegas, USA).

Measurements were made on individual beetles that were first weighed to ±0.1 mg (Precisa 160A balance). The beetles were measured for a minimum of 6 h in the dark, with the same conditions being used for all of the beetles. Separate, simultaneous measurements of the CO_2 emissions from the mesothoracic and subelytral spiracles allowed us to compare our results with previous work and to verify that the beetles remained inactive during sampling (Duncan and Byrne, 2002). Sealing the elytral holes with dental wax enabled us to use the same individuals for comparison of CO_2 emissions from the subelytral cavity with those from the anterior body half and those from outside the elytral case.

Baseline drift of the gas analysers was corrected during analysis from measurements taken at the beginning and end of each trial with the respirometer chamber empty. All measurements were corrected to standard temperature and pressure (STP). The CO_2 recordings were converted to rate of CO_2 production in ml h^{-1}. The discontinuous gas exchange cycle (DGC) characteristics were calculated as follows: the DGC frequency (= burst frequency) was calculated by determining the number of peaks of CO_2 per second, and the DGC duration was taken as one complete cycle. The mean rate of CO_2 emission was measured as the mean value over several complete DGC cycles, and integration of the area under the curve against time (in hours) was performed to obtain the emission volume.

Intermittent measurement of CO_2 emissions from the subelytral spiracles

To determine the gas composition within the subelytral cavity during the different periods of the respiratory cycle, we alternated sampling between the air inside the subelytral cavity and the air outside the elytral case, while continuously measuring CO_2 emission from the anterior spiracles. The subelytral cavity was sampled for short intervals on an intermittent basis. Air was withdrawn from the posterior chamber of the respirometer from either the space around the posterior end of the beetle or from within the subelytral cavity. At the same time, air was continuously sampled from the anterior chamber around the mesothoracic spiracles, which provided a benchmark for the stage of the normal DGC at which we sampled air from the subelytral cavity. By measuring the CO_2 emission from the anterior spiracles at the same time as intermittent sampling from within the subelytral cavity, we were able to determine the gas composition within the subelytral cavity at different periods of the normal DGC. The set-up was similar to that described above, except that the humidifier was removed from the intake side and the air was dried and scrubbed of CO_2 before entering the respirometer. In the posterior chamber, the air stream was periodically switched from passing over the elytral case to passing through the subelytral cavity using a solenoid valve switching mechanism

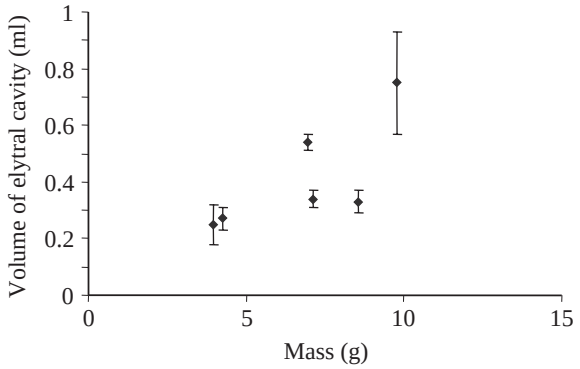

Fig. 3. The relationship between body mass and volume of the subelytral cavity of *Circellium bacchus* (regression equation: y=0.063x–0.013; r^2=0.56, P=0.08, N=7). Error bars represent S.D.

(Sable Systems). The subelytral cavity was sampled for 8-min periods, with the intervals between measurements ranging from 20 min to 1 h. A Licor CO_2/H_2O analyser (LI-6262) and O_2 sensor (Qubit Systems, Ontario, Canada; resolution of 0.1%) were used for the posterior chamber. Other parameters were treated as above. We were unable to synchronise the intermittent samples periods with the DGC of individual beetles as they only settle into their normal DGC after some time in the respirometer, when they become inactive. Therefore, we relied on fortuitous overlaps of the burst and closed period of the DGC from the anterior spiracles with the sampling periods of the subelytral cavity contents.

Statistics

Data are represented as means ± S.D. Sample size (N) is indicated as either representing individual beetles or, in the case of gas exchange characteristics, 3–10 discontinuous gas exchange cycles per beetle. Unless otherwise noted, statistical comparisons were made either with the Student's t-test or with analysis of variance (ANOVA). Significant ANOVAs were followed with the Newman–Keuls multiple range test. Regression analysis was done by the least-squares method, and the regression lines were compared using analysis of covariance (ANCOVA).

Results

Volume of the subelytral cavity

The volume of the subelytral cavity was estimated for seven beetles (mean mass, 6.76±2.32 g). The mean volume of the cavity was 0.413±0.19 ml. There was no significant correlation between the mass of the beetle and volume of the elytral cavity (Fig. 3).

Continuous measurement of CO_2 emissions from the subelytral spiracles

Carbon dioxide emissions from the anterior and posterior chambers of the respirometer showed what we consider to be a typical pattern for *C. bacchus* at rest. There is a marked DGC, with the majority of CO_2 being expelled at the anterior mesothoracic spiracles, and the absence of a flutter period (Fig. 4A). However, continual sampling from the subelytral cavity revealed a complete reversal of this pattern (Fig. 4B). The majority of CO_2 emitted was now detected from the subelytral spiracles, which clearly exhibited a flutter period before the burst period. Measurements from six beetles showed a similar pattern and are summarised in Table 1. By combining the amounts of CO_2 emitted from the subelytral space and the mesothoracic spiracles, the total recorded from two halves of a beetle is similar to that recorded from one beetle measured as a single entity; mean CO_2 partial pressure ($\dot{V}_{CO_2}$) of 295.7±195.2 μl h^{-1} from six beetles of mean mass 6.94±2.37 g (this study) compared with mean $\dot{V}_{CO_2}$ of 407±204 μl h^{-1} from seven beetles of mean mass 7.285±2.93 g (Duncan and Byrne, 2000), respectively; $t_{0.05,11}$=1.0, P>0.05.

During the continuous removal of air from the subelytral cavity, almost no CO_2 left *via* the mesothoracic spiracle

Table 1. *Comparison of rates of CO_2 emission from the anterior mesothoracic spiracle and the posterior subelytral spiracles of* Circellium bacchus *during continuous sampling from either outside or inside the subelytral cavity*

		Absolute rate of CO_2 emission (μl h^{-1})					
		From inside the SEC			From outside the SEC		
Beetle number	Mass (g)	Mesothoracic spiracles	Subelytral spiracles	A:P ratio	Mesothoracic spiracles	Elytral case	A:P ratio
1	8.012	56.29	187.98	1:3.3	–	–	
2	7.122	3.94	288.92	1:73.3	289.31	52.29	5.5:1
3	8.539	1.83	224.0	1:122.4	428.06	60.05	7.1:1
4	3.94	0.82	211.63	1:257.3	116.88	39.65	3.0:1
5	4.238	6.17	115.19	1:18.6	106.5	28.06	3.8:1
6	9.79	7.9	669.36	1:84.7	195.39	46.68	4.0:1

A, anterior mesothoracic spiracle; P, posterior subelytral spiracle; SEC, subelytral cavity; –, no measurement taken.

Readings from six individuals are shown to illustrate that CO_2 is normally expired from the mesothoracic spiracle unless it is experimentally removed from the subelytral cavity. Readings from outside the elytral case were taken on individuals that had elytral holes resealed with dental wax. Slight leakage from these holes might account for the relatively high levels of CO_2 measured here.

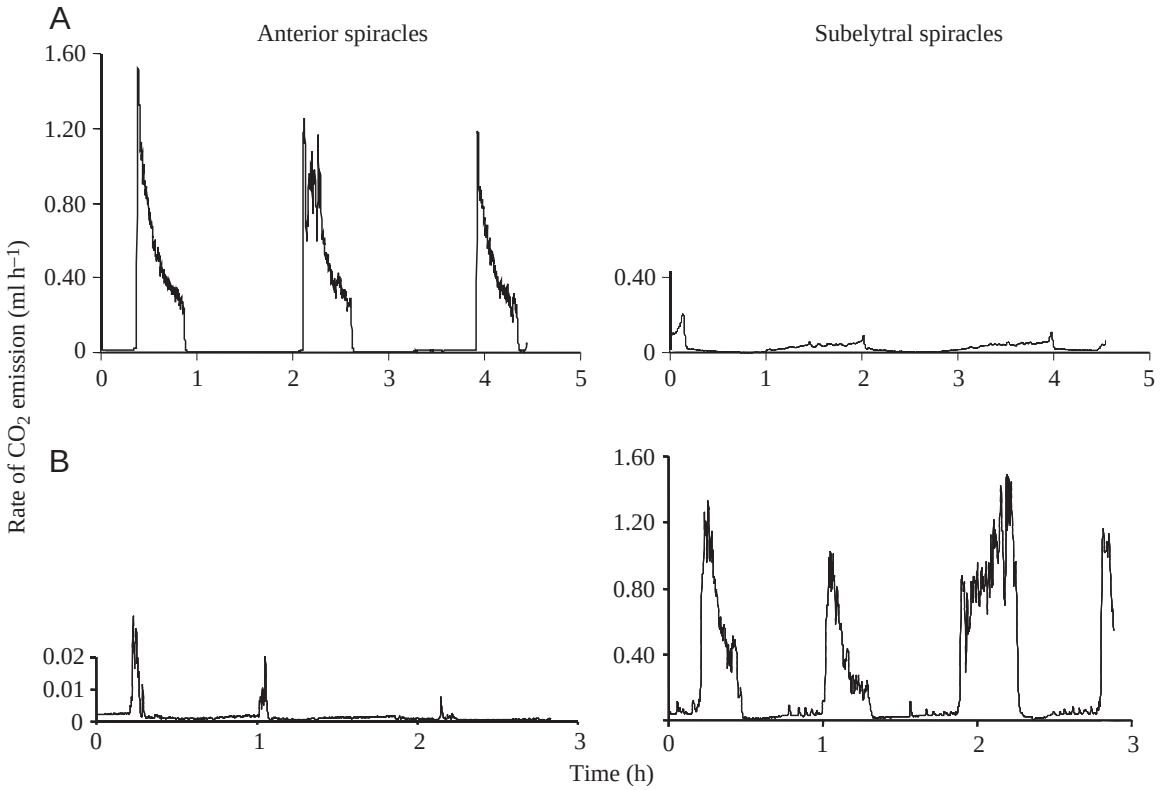

Fig. 4. Recordings of CO_2 emissions of *Circellium bacchus* (mass, 7.122 g) from (A) the posterior and anterior spiracles when air was drawn through the respirometer chamber and over the elytral case (route I; see Fig. 2) and (B) the posterior and anterior spiracles when air was drawn through the subelytral cavity, i.e. over the subelytral spiracles.

Table 2. *Characteristics of the discontinuous gas exchange cycle of* Circellium bacchus *during continuous sampling from either outside or inside the subelytral cavity*

	DGC characteristics			
	From the mesothoracic spiracle		From the subelytral spiracles	
Variable	Mean	S.D.	Mean	S.D.
Body Mass (g)	6.93	2.83	6.94	2.37
N	5		6	
DGC:				
Frequency (mHz)	0.26	0.10	0.25	0.07
Duration (min)	77.35	23.1	72.4	20.6
Burst period:				
CO_2 volume (µl)	318.5	268.9	272.6	134.2
Duration (min)	33.7	9.1	32.1	10.6
Flutter period:				
CO_2 volume (µl)	–	–	25.2	17.5
Duration (min)	–	–	25.4	7.0

DGC, discontinuous gas exchange cycle; SEC, subelytral cavity; –, no measurement taken.

Total DGCs = 51, approximately eight per beetle. Readings from the mesothoracic spiracles were taken on individuals that had elytral holes resealed with dental wax.

(Table 1). The rate at which CO_2 emission occurred into the subelytral cavity was, on average, 22 times greater than that emitted through the mesothoracic spiracle at that time. The mean rate of CO_2 emission and mass-specific rate of CO_2 emission from the subelytral spiracles was significantly greater than that from the mesothoracic spiracle (t=3.33, P<0.001; t=5.14, P<0.01, respectively).

The characteristics of the DGC obtained from the subelytral spiracles are given in Table 2. There is a significant positive relationship between the length of the periods (closed, burst and flutter) and the volume of CO_2 emitted during those periods. The slopes of these curves are identical but the intercepts are not (ANCOVA; flutter period, $F_{0.05,4,40}$=1.48; burst period, $F_{0.05,4,40}$=1.67, closed period, $F_{0.05,3,24}$=0.55). This indicates that the beetles adjust their CO_2 output by increasing the lengths of the respective respiratory periods rather than increasing their frequency. Comparing the values obtained for the DGC characteristics for the subelytral spiracles with those reported for the mesothoracic spiracle (Duncan and Byrne, 2002), there are no significant differences in the frequency and duration of the cycle, volume of CO_2 emitted during the burst period and duration of the burst period (P>0.05 in all cases).

Maintaining the same flow rate (50 ml min^{-1}) at the anterior and posterior chambers during continuous sampling of the

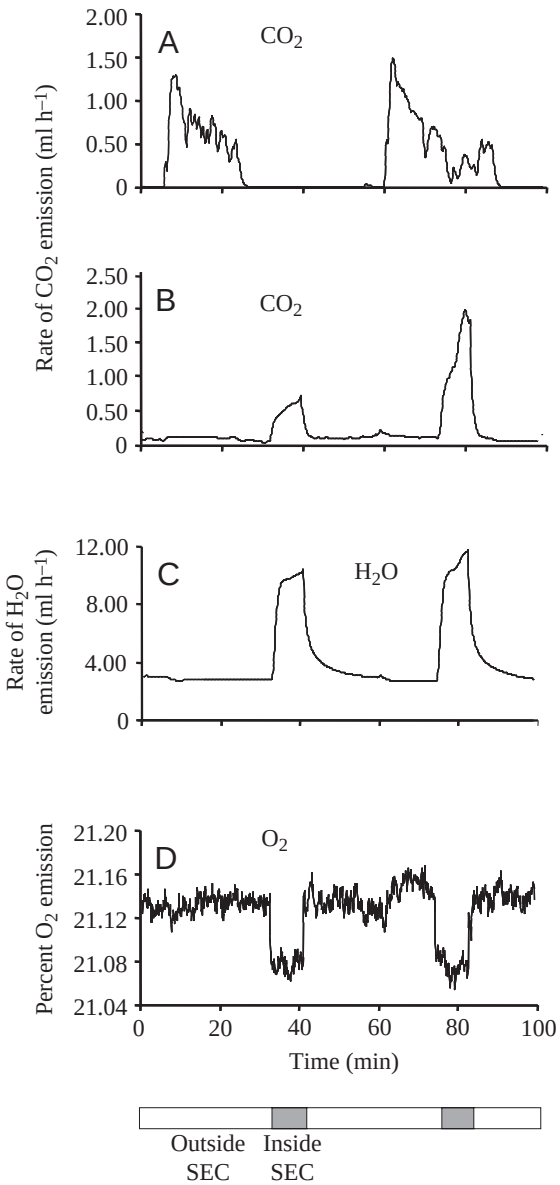

Fig. 5. Results of intermittent sampling of CO_2, O_2 and water emissions from the posterior and anterior spiracles of *Circellium bacchus* (mass, 8.539 g) from outside the elytral case and inside the subelytral cavity (SEC). (A) CO_2 emission from the anterior respirometer chamber. (B–D) Gas emissions from the posterior respirometer chamber.

subelytral cavity flushed almost all of the CO_2 out of the cavity (Fig. 4B), whereas equal pressures in both respirometer chambers and a lower flow rate through the subelytral cavity allowed a build up of CO_2 in the cavity, and more CO_2 left from the mesothoracic spiracle. Nevertheless, the pattern of CO_2 emission was the same in both cases.

Intermittent measurement of gas composition in the subelytral cavity

Intermittent sampling from the subelytral cavity did not disturb the normal DGC seen in *C. bacchus* (Fig. 5) and allowed us to gather a more complete picture of events during the respiratory cycle. At any time, the air inside the subelytral cavity is high in CO_2 and water vapour, while the concentration of O_2 is lower than atmospheric. The absolute concentration of CO_2 sampled changed according to whether or not air had recently been expelled through the mesothoracic spiracle (Fig. 5; Table 3). The amount of CO_2 in the subelytral cavity was significantly lower when sampled after CO_2 had been expelled from the mesothoracic spiracle (i.e. during the closed period of the mesothoracic spiracle; Fig. 5A) than when measured immediately before or during the burst period of the mesothoracic spiracle's DGC (Fig. 5B) ($F_{0.05,2,35}$=7.754, P=0.0016, N=5 beetles). In one specific individual in which several measurements were obtained from all three stages of the mesothoracic DGC, the same result was obtained ($F_{0.05,2,11}$=5.26, P=0.025). Thus, the concentration of CO_2 within the subelytral cavity is lowered after CO_2 has been emitted through the mesothoracic spiracle.

Removal of CO_2 from the subelytral cavity immediately before or during the burst period of the mesothoracic DGC influenced the amount of CO_2 emitted through the mesothoracic spiracle (Table 4; Fig. 5). If a large amount of CO_2 was removed from the subelytral cavity, less was emitted through the mesothoracic spiracle. The total amount of CO_2 lost from the beetle was calculated and, in some cases, it compares well with the total amount of CO_2 emitted from the mesothoracic spiracle (Table 4). Therefore, the total amount of CO_2 released is similar by whichever route CO_2 is emitted or sampled from the beetles.

The method for sampling water concentration used in this study did not enable the water content of the air in the subelytral cavity to be determined accurately. However, the data in Table 3 show that this air was kept at a high humidity, regardless of the stage of the DGC cycle at the mesothoracic spiracle. These data indicate that, should the elytra be opened, the proportional increase in water loss will be approximately 74% (Table 3).

The O_2 concentration of the air within the subelytral cavity was lower than the air around the elytral case, irrespective of the time of sampling (Fig. 5). Because small differences of O_2 concentration could be due to baseline fluctuations, we could not compare the differences in O_2 concentration within the subelytral cavity at the different DGC periods of the mesothoracic spiracle. Substantial drift in the baseline readings of the O_2 measurements occurred during the sampling period of several hours, which is typical for these analysers; nevertheless, a qualitative assessment of O_2 consumption can still be made. The noticeable drop in O_2 concentration when air was sampled from within the subelytral cavity was not due to dilution by CO_2 because an H_2O/CO_2 scrubber was placed before the O_2 analyser in the experimental set-up (Withers, 1977).

Discussion

From these results, we conclude that the spiracles that open into the subelytral cavity are used in respiration during the DGC in *C. bacchus*. Continuous measurements showed that

Table 3. *Composition of air within the subelytral cavity, when sampled at different times during the discontinuous gas exchange cycle of CO_2 emission from the mesothoracic spiracle*

Period of DGC cycle	*N*	Volume of CO_2 (μl g^{-1})	Maximum $\dot{V}_{CO_2}$ (μl g^{-1})	Percentage increase in water loss rate* (%)
Before burst period	9	16.19±6.98[a]	211.46±72.96[a]	75.6±8.4[a]
During burst period	11	18.71±10.27[a]	269.19±141.86[a]	75.9±4.4[a]
After burst period	18	8.24±5.38[b]	97.5±34.01[b]	71.2±1.8[a]

DGC, discontinuous gas exchange cycle; SEC, subelytral cavity; $\dot{V}_{CO_2}$, CO_2 partial pressure.

Values represent means ± S.D. *N* = number of samples taken during each period from recordings from five different beetles. Values in the same column followed by the same letter are not significantly different at *P*=0.05.

*Percentage increase = (highest water loss rate sampled from subelytral cavity – water loss rate sampled from outer elytral case)/(highest water loss rate sampled from subelytral cavity) × 100.

Table 4. *Contribution of the mesothoracic spiracle to burst period CO_2 volume during the DGC in* Circellium bacchus, *when CO_2 has been removed from the subelytral cavity immediately before or during the burst period of the DGC*

	Volume of CO_2 in burst period (μl)			
	Before sampling from the SEC	During sampling from the SEC		
Beetle number	Mesothoracic spiracles	Mesothoracic spiracles	SEC spiracles	Totals
2	335	–	–	335
		63	266	329
		170	243	413
		262	78	340
		160	241	401
3	351	–	–	351
		326	139	465
		209	98	307
		356	80	436
4	332	–	–	332
		119	201	320
		133	234	367
6	566	–	–	566
		540	68	608
		504	74	578

DGC, discontinuous gas exchange cycle; SEC, subelytral cavity; –, no measurement taken.

For comparison, the total amount removed during the burst period and the burst period volume before sampling from the subelytral cavity are given.

the subelytral spiracles are synchronised to the normal DGC (Fig. 4), and all three periods (including the flutter period) of the DGC are present, which is a notable difference to the DGC recorded only from the mesothoracic spiracle (Fig. 4A). As a result of the action of the subelytral spiracles, the air inside the subelytral cavity has a high CO_2 concentration, high water vapour concentration and lower than atmospheric O_2 concentration. The volume of CO_2 within the subelytral cavity decreases after CO_2 has been expelled through the mesothoracic spiracle, which is in accordance with our previous findings that CO_2 does not exit from the subelytral cavity by lifting of the elytra (Duncan and Byrne, 2002). These results support the hypothesis that ventilation in *C. bacchus* involves tidal, or anterograde, respiratory airflow. More importantly, they suggest a role for the subelytral cavity as a storage site for respiratory CO_2 in flightless beetles, which in turn could assist in diffusive uptake of O_2.

Spiracular coordination

Distinct functions and capabilities of thoracic and abdominal spiracular groups, working in coordination, were found in the desert ant *Cataglyphus bicolor* (Lighton et al., 1993). The authors concluded that the thoracic spiracles act as a high-capacity entrance to the tracheal system, whereas the abdominal spiracles are more specialised and play a major role during the early flutter period of the DGC, which we also propose for *C. bacchus*. The same pattern of spiracular use is employed by both insects for the same purpose of water retention, but we would expect that the effectiveness would be enhanced by the presence of the subelytral cavity in *C. bacchus*, which will allow for the maintenance of a high relative humidity over the posterior spiracles.

Sampling air from inside the subelytral cavity revealed a flutter period that was not apparent from outside the subelytral cavity. The flutter period in *Cecropia* pupae starts when the endotracheal O_2 concentration drops below about 3% (Levy and Schneiderman, 1966) and enables the bulk inflow of O_2 into the trachea along its concentration gradient while minimising the loss of H_2O. In arid-dwelling beetles, a large proportion of the CO_2 expelled exits during the flutter period. Lighton (1991) found that 24% of the total CO_2 release occurred during the flutter period for nine species of Namib Desert tenebrionid beetles, with one species reaching 47%. Duncan et al. (2002) reported values of 48% and 29% for two species of Negev Desert tenebrionid beetles. By contrast, *C. bacchus* released only 10% of the total CO_2 measured from the

subelytral spiracles during the flutter period. In flighted dung beetles, the flutter period could also be an important means of eliminating CO_2 whilst enabling inflow of O_2 and reducing water loss (Lighton and Garrigan, 1995). Our unpublished results on flighted dung beetles (without sealed elytra) indicate that the subelytral spiracles are the main site of gas exchange in these beetles at rest (M. J. Byrne and F. D. Duncan, unpublished data). We have measured a distinct flutter period that persists for 30% of the duration of the DGC in *C. bacchus*, which compares well with the above species.

Continuous sampling from the subelytral cavity did not disturb the normal pattern of the DGC seen in *C. bacchus*, just as holes into the elytra of the desert tenebrionid *Onymacris plana* caused no change in its cyclic respiratory pattern (Bartholomew et al., 1985). This suggests that the DGC is driven from CO_2 concentration within the trachea rather than from CO_2 or H_2O levels outside the spiracles (Lighton, 1996). The total amount of CO_2 removed from the beetle during the gas exchange cycles remained fairly constant (Table 4) regardless of whether it was removed from the subelytral cavity directly or exited naturally *via* the mesothoracic spiracle. Continuous sampling did, however, shift the bulk of gas exchange from the anterior to the posterior spiracles when a high flow rate was maintained through the subelytral cavity. Intermittent sampling or reduced flow rates through the subelytral cavity allowed water vapour and CO_2 to build up in the cavity; therefore, we assume that these are maintained at elevated levels throughout the normal DGC. Zachariassen (1991) showed that experimental manipulation of the relative humidity of the air inside the subelytral cavity of a tenebrionid beetle had a strong effect on the rate of water loss. The volume and pattern of CO_2 accumulation within the subelytral cavity of *C. bacchus* suggest that it is expelled from the subelytral spiracles rather than diffused through the cuticle, as in termites (Shelton and Appel, 2000).

Role of subelytral cavity in water conservation

The air within the subelytral cavity of *C. bacchus* has a high water vapour content, which was kept relatively constant during the course of the DGC. Ahearn and Hadley (1969) showed that removal of sections of the elytra increased water loss from desert tenebrionids. We calculated that if the subelytral cavity of *C. bacchus* was open, there would be an approximately 74% increase in the rate at which water is lost from the posterior body. Thus, lifting the elytra to create an aperture above the anus through which CO_2 could be expelled, as previously suggested for tenebrionids by Nicolson et al. (1984), would cause a substantial increase in the rate of water loss. Depending on the timing of this opening, water would be lost from the respiratory passages, as well as from the subelytral cavity. By restricting CO_2 exchange with the atmosphere to only one mesothoracic spiracle, water loss is confined to a small area of the total respiratory system (Lehman, 2001). In addition, these respiratory passages are only open to the atmosphere for short periods during the mesothoracic spiracle's burst period. Water loss rates increase when the mesothoracic spiracle opens but this only contributes 4% to the total water loss rates (Duncan, 2002). Thus, the evolution of a tidal airflow and a subelytral cavity could be important in reducing water loss in these arid-dwelling beetles.

The subelytral cavity as a CO_2 sink and O_2 sponge

Differential control of the mesothoracic and subelytral spiracles in a coordinated DGC has been shown in these results; with respect to other insects such as dragonflies (Miller 1961), ants (Lighton et al., 1993) and moth pupae (Slama, 1999), this is not remarkable. However, combined with the discovery of anterograde airflow and the use of a single spiracle for CO_2 emission in *C. bacchus*, these findings allow us to extend the hypothesis of the subelytral cavity as a water-saving device as follows.

The anterior mesothoracic spiracles are shut during the closed period, while the subelytral spiracles progress from being closed to rapid fluttering. This allows oxygen to enter the tracheal system from the subelytral cavity down its diffusion gradient. However, for O_2 to continue diffusing into the trachea *via* the subelytral spiracles during the 20 min flutter period, it will have to be replenished from the atmosphere, leading to a paradoxical role for the subelytral cavity where it will have to allow entrance of O_2 from the atmosphere while retaining water vapour and CO_2. This mirrors the proposed action of the tracheae and spiracles during the DGC, where CO_2 will theoretically unload from the respiratory system 15 times slower than O_2 will load, despite similar diffusion coefficients (Snyder et al., 1995; but see Lighton and Garrigan, 1995). Gorb (1998) has shown the subelytral cavity of an arid-adapted tenebrionid to be tightly sealed by microtrichia, and similar structures have been found in *C. bacchus* (M. J. Byrne and F. D. Duncan, unpublished data). Our results from this and a previous study (Duncan and Byrne, 2002) demonstrated differential movement of O_2 into the subelytral cavity and consumption from the subelytral cavity without a corresponding emission of CO_2.

Retention of CO_2 as a consequence of water retention may then actually be advantageous, whereby elevated levels of CO_2 in the subelytral cavity serve a dual function. Carbon dioxide can be sequestered in the subelytral cavity, increasing the overall CO_2 capacity of the body and, in that way, lengthening the DGC period, thereby reducing respiratory water loss rates (Kestler, 1985; Lighton et al., 1993) and contributing to a diffusion gradient that will draw atmospheric O_2 into the subelytral cavity. Water vapour in the subelytral cavity will also contribute to water retention in the flutter period by reducing its concentration gradient between the tracheae and ambient air (Kestler, 1985).

This state will persist until, finally, O_2 diffusion and replenishment from the atmosphere will lead to a build up of N_2 and CO_2 in the subelytral cavity and in the tracheae (Schneiderman, 1960) and the hypoxic trigger point is reached at the mesothoracic spiracle, which opens. These are the largest spiracles and appear to have the largest diffusive

capacity and, therefore, are best suited for CO_2 exchange *en masse* (Lighton et al., 1993). Consequently, large amounts of CO_2 are expelled from the anterior spiracle in its burst period. Whether or not this is achieved by active ventilation is unknown to us, and the theoretical arguments in favour (Kestler, 1985) or against (Lighton et al., 1993) are still unresolved.

The burst period of the anterior mesothoracic spiracle is probably preceded by the burst period of the subelytral spiracles but with considerable overlap (Figs 4, 5). However, very little CO_2 was detected outside the subelytral cavity to indicate a burst period from the posterior spiracles, providing further evidence of the subelytral cavity being CO_2 tight (Fig. 4A). Therefore, CO_2 from the subelytral cavity must be moved forward through the body, as we have shown O_2 to do (Duncan and Byrne 2002), and be expelled at the mesothoracic spiracle during its burst period. We do not know if active ventilation is responsible for this movement of gas. However, observations of dorsal abdominal pumping through holes drilled in the elytra, and the lack of correlation between body size and the volume of the subelytral cavity (Fig. 3), suggesting that it can be voluntarily altered by the beetle, both indicate that CO_2-laden air could be pumped forwards out of the subelytral cavity. Small CO_2 volleys, overlaying the burst period from both the anterior and posterior spiracles, can be seen in Fig. 4A,B and are indicative of active ventilation. The anatomical route of CO_2 from the subelytral cavity to the mesothoracic spiracle still needs to be resolved, but the system of large air sacs attached to the trachea coupled with abdominal pumping could be involved in moving CO_2 through the body through longitudinal tracheae in *C. bacchus* (Wasserthal, 1996).

C. bacchus is a monotypic, canthonine dung beetle, which is rare and restricted to fragmented patches of dense, arid bush in South Africa (Scholtz, 2000). It is assumed that competition from large, flighted dung beetles has confined it to these areas, where its apterous state and large size are assumed to be advantageous in minimising water loss (Chown et al., 1995). Klok (1994) found individuals of *C. bacchus* to be the most resistant to desiccation of the 12 species of dung beetles he tested. While these findings only correlate with our assumptions about the role of the subelytral cavity in reducing water loss, we can assume that, if our hypothesis is correct, this mechanism will be widespread among related species. Thirty percent of the Canthonini are flightless, and most are found in tropical or subtropical stable habitats (Scholtz, 2000). Secondary loss of flight is widespread in the Scarabaeidae and is polyphyletic within the Scarabaeini (Harrison, 1999). Flightless members of the subfamily, from the genus *Pachysoma*, occur in the West Coast deserts of southern Africa, and look superficially similar to *C. bacchus*. Convergent evolution of a large, rounded body shape is assumed to improve water saving in these species (Chown et al., 1998), and discovery of a shared respiratory mechanism would provide strong support for its role in water retention.

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