Gas exchange patterns for a small, stored-grain insect pest, *Tribolium castaneum*

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Abstract

Insects breathe using one or a combination of three gas exchange patterns; continuous, cyclic and discontinuous, which vary in their rates of exchange of oxygen, carbon dioxide and water. In general, there is a trade-off between lowering gas exchange using discontinuous exchange that limits water loss at the cost of lower metabolic rate. These patterns and hypotheses for the evolution of discontinuous exchange have been examined for relatively large insects (>20 mg) over relatively short periods (<4 h), but smaller insects and longer time periods have yet to be examined. We measured gas exchange patterns and metabolic rates for adults of a small insect pest of grain, the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae), using flow-through respirometry in dry air for 48 h. All adults survived the desiccating measurement period; initially they used continuous gas exchange, then after 24 h switched to cyclic gas exchange with a 27% decrease in metabolic rate, and then after 48 h switched to discontinuous gas exchange with increased interburst duration and further decrease in metabolic rate. The successful use of the Qubit, a lower cost and so more common gas analyser, to measure respiration in the very small *T. castaneum*, may prompt more flow-through respirometry studies of small insects. Running such studies over long durations may help to better understand the evolution of respiration physiology and thus suggest new methods of pest management.

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Introduction

Insects breathe using one or a combination of three distinct forms of gas exchange based on spiracular control patterns: continuous, cyclic and discontinuous (Marais *et al.*, 2005; Chown *et al.*, 2006; Terblanche and Woods, 2018). Continuous gas exchange (CGE), the ancestral condition (Marais *et al.*, 2005), occurs when the spiracles remain open to support continuous exchange of gases (oxygen into and carbon dioxide out of the body). Cyclic gas exchange consists of two phases, burst (spiracles open) and interburst (spiracles partially closed temporally and spatially), which produce periodic and often erratic cycles of gas exchange, but the spiracles never close completely in the interburst (Nespolo *et al.*, 2007). Discontinuous gas exchange (DGE; Lighton, 1996; Quinlan and Gibbs, 2006; Matthews, 2018) has three distinct phases, with spiracles completely closed (C), fluttering (F) and open (O); the interburst phase includes (C) and (F).

The evolutionary origins of DGE are unclear, and the factors promoting it are highly debated. Eight adaptive and two non-adaptive hypotheses have been proposed to explain the role and evolution of DGE, which vary between taxonomic groups and different habitats. Although none of these hypotheses has unequivocal support (reviewed by Terblanche and Woods, 2018), three adaptive hypotheses have more support than others. The hygric hypothesis suggests that DGE reduces respiratory water loss (during the closed and flutter phase) for insects experiencing water stress, such as in arid habitats (Buck *et al.*, 1953; Lighton, 1996). The chthonic hypothesis proposes that DGE improves O_2 and CO_2 exchange in hypoxic and/or hypercapnic habitats, such as underground (Lighton and Berrigan, 1995). The oxidative damage hypothesis argues that DGE reduces tissue damage during periods of low metabolic demand by hyperoxia in tissues of insects with a respiratory system adapted for high metabolic rates (Hetz and Bradley, 2005).

These gas exchange patterns and metabolic rate have been measured for more than 150 insect species (Marais *et al.*, 2005; Terblanche and Woods, 2018). Most studies of insect gas exchange patterns and metabolic rates use flow-through respirometry (Withers, 2001; Lighton, 2018), with metabolic rate measured as the rate of CO_2 emission ($\dot{V}_{CO_2}$) rather than O_2 consumption ($\dot{V}_{O_2}$) because it is technically more precise to measure small changes in CO_2 emission compared to O_2 consumption (e.g. Lighton, 1996, 2018). Metabolic rates measured as $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ differ depending on the metabolic substrate, which can be expressed in energy units (e.g. joules per hour) using appropriate conversion coefficients (e.g. Withers, 1992). Generally, these studies have examined the effects of a variety of factors, such as age/instar, sex, activity, absorptive state, temperature, nutrition level and hydration

status, on gas exchange patterns and metabolic rate (Contreras and Bradley, 2009, 2010; Schimpf *et al.*, 2012; Rolandi *et al.*, 2014).

There are nevertheless gaps in our knowledge of respiration for insects. First, more than 150 insect species with information are from seven orders, thus there are another 23 insect orders without information (Marais *et al.*, 2005; Terblanche and Woods, 2018). Second, almost all insects measured for gas exchange patterns are large, from 20 to 26,000 mg, and there is little to no information for small insects (Marais *et al.*, 2005; Woodman *et al.*, 2007). Third, the duration of experiments, in general, is short (2–10 h; Terblanche *et al.*, 2010; Huang *et al.*, 2014). There are a few studies where insects have been measured for periods longer than 10 h; all species are from relatively damp habitats. Three are small dipterans: one vinegar fly, *Drosophila melanogaster* (up to 36 h; Williams *et al.*, 1997; Williams and Bradley, 1998), and two mosquitoes, *Anopheles gambiae* and *A. arabiensis* (up to 20 h; Gray and Bradley, 2005). A recent example had Madagascan hissing cockroaches (*Gromphadorhina portentosa*) being recorded for 23 h in each of two treatments for a total of 46 h (Rowe *et al.*, 2022). Finally, besides metabolic rate measurements of insect grain pest species (Emekci *et al.*, 2002; Pimentel *et al.*, 2007; Arnold *et al.*, 2016), not a single species has been measured for gas exchange patterns by flow-through respirometry. Consequently the current knowledge of insect breathing patterns and metabolic rates does not represent the majority of insect species, and may not be particularly relevant to small grain-pest species.

The objective of our study was to measure patterns of gas exchange and metabolic rate for a small grain-pest insect. Small insects have a high metabolic rate and mass-specific evaporative water loss (Hadley, 1994; Fields and White, 2002) and stored grain is generally a dry environment (Jian and Jayas, 2012), which may constrain their pattern of gas exchange, and so provide evidence for the hygric hypothesis for DGE. We studied adults of the red flour beetle *Tribolium castaneum* (Coleoptera, Tenebrionidae – darkling beetles) as they are small and inhabit stored grain, thus results should inform possible function of DGE and its implications for CO_2 and H_2O loss. We used flow-through respirometry over an extended duration (48 h) and hypothesised that if the hygric hypothesis was relevant then we would observe increased reliance on DGE and reduction of ($\dot{V}_{CO_2}$) as these beetles desiccated over time. The results of our study will improve our understanding of small insect ecophysiology by adding to previous closed-system studies of metabolism of these pests (Emekci *et al.*, 2002; Pimentel *et al.*, 2007; Arnold *et al.*, 2016), and understanding their patterns of gas exchange may suggest methods for pest management.

Materials and methods

Insects

A 'wild type' phosphine-susceptible strain of the red flour beetle (*T. castaneum*, MUWTC-5000) was obtained from Murdoch University (Professor Yonglin Ren). Beetles were provided with flour and yeast (12:1) in a plastic jar with vents in the lid for gas exchange (following Alnajim *et al.*, 2019). Beetles were maintained in a constant temperature room at standard conditions of 25 ± 1°C, 12L:12D photoperiod, ambient gaseous conditions (21% O_2, 0.03% CO_2) and ambient RH of 60–80% (Emekci *et al.*, 2002; Lu *et al.*, 2009). Adult *T. castaneum* are a long-lived life stage and

survive for months in dry conditions (grain storage), hence they presumably can experience desiccation for extended durations. The adults are easy to handle because they become immobile when touched.

Respirometry

Carbon dioxide emission was measured using standard flow-through respirometry at an ambient temperature of 25°C (following Abbas *et al.*, 2020). Each adult beetle was selected randomly and irrespective of sex for respirometry. Air from a compressed air cylinder (BOC Gases, Canning Vale, WA, Australia) was used as a stable air source, with low and constant CO_2 and H_2O concentration; it was not scrubbed of these gases for experiments. Relative humidity (RH) of the compressed air was verified using a humidity probe (HMP113 Vaisala Corporation, Helsinki, Finland) to be 5–6%. Limitations of respirometric analysis precluded us from making measurements at high RH, equivalent to the maintenance conditions. A glass syringe barrel (1 ml volume) was used as a respirometry chamber for individual beetles. Two respirometry chambers, with stopcocks to switch air flow between them, were used. One contained the beetle (insect system, hereafter) whereas the other (baseline system, hereafter) was empty to periodically record baseline CO_2. Air flow was regulated at 25 ml min^{-1} Standard temperature and pressure, dry (STPD) using a mass flow controller (AFC 2600 Aalborg, Orangeburg, NY, USA). A bubble flow meter (Gilian Gilibrator 2, Sensidyne, St. Petersburg, FL, USA) was used to calibrate the mass flow controller. A Vaisala HMP113 probe was used to measure the RH and temperature of the air leaving each chamber; the probe was RH calibrated using a DewPoint Generator DG-4 (Sable Systems International, Las Vegas, NV, USA) and temperature calibrated using a traceable mercury in glass thermometer (Australian Calibrating Services, Melbourne, Vic, Australia). A CO_2 analyser (S151 Qubit systems, Kingston, Ontario, CA, USA) measured the CO_2 concentration of the excurrent air. It was calibrated using CO_2 free air (using Sodasorb CO_2 absorbent, W. R. Grace & Co., Chicago, IL, USA) and a certified span gas (0.153% CO_2, BOC Gases). We have used this system to measure two insects of 500–800 mg (the speckled cockroach *Nauphoeta cinerea* and the darkling beetle *Zophobas morio*; Abbas *et al.*, 2020); here we attempt to extend the Qubit analyser to insects of 2 mg.

We measured only adult, reproductive flour beetles (completely melanised cuticle) and expressed metabolic rate in mass-specific units (ul CO_2 g^{-1} h^{-1}), as reproductive status and body mass significantly affect metabolic rate, but sex *per se* does not (Arnold *et al.*, 2016). A single respirometry trial lasted for 48 h, during which the airflow was primarily through the insect channel. Each beetle was weighed to ±0.01 mg with a digital balance (ER-182A A&D Company Limited, Toshima-Ku, Tokyo, Japan) immediately before and after each respirometry trial. Each individual insect was placed in the respirometry system immediately after the initial weighing; the insect chamber was darkened throughout the respirometry trial to reduce activity. After an acclimation period of 5 h (following Emekci *et al.*, 2002; Pimentel *et al.*, 2007), the airflow was switched to the baseline channel for 40 min to record a start baseline, then to the insect system. Air flow was switched to the baseline chamber for 40 min periodically during the 48 h experiment; the insect's CO_2 emission was recorded in between baselines. Rate of CO_2 release was analysed at 5, 23, 48 h; it was important to measure $\dot{V}_{CO_2}$ close to a baseline period as there was long-term erratic baseline

drift of the CO_2 analyser over time. Although we attempted to measure 30 beetles, baseline drift in the Qubit analyser prevented the data from 22 beetles from being useful. Here we present data for the eight adult flour beetles for which baseline drift was not problematic.

Data acquisition and analysis

Digital multimetres (Protek 506, Hung Chang, Seoul, Korea and Thurlby 1905a, Thurlby Electronics Ltd, Huntingdon Cambridgeshire, UK) were used to measure analogue voltage signals from the CO_2 analyser and Vaisala probe. These multimetres were connected via a USB port hub (UC2324, ATEN, North Ryde, Australia) to a desktop PC. Voltage signals were sampled every 0.2 s and converted to ppm for CO_2 concentration and °C for temperature using a custom-written Visual Basic program (VB6, written by PC Withers). Data were stored continuously in an Excel file during the respirometry trial. Acquired data were analysed with a laptop using an Excel spreadsheet (written by PC Withers and W Abbas). Raw values of CO_2 and temperature were first calibration corrected, then the rate of CO_2 emission $\dot{V}_{CO_2}$ (ml STPD g^{-1} h^{-1}) was calculated based on the following equation from Withers (2001).

$$\dot{V}_{CO_2} = [(\dot{V}_e \times F_eCO_2) - (\dot{V}_i \times F_iCO_2)] \times 60/M_b$$

where $\dot{V}_i$ is the incurrent flow rate (STPD ml min^{-1}), $\dot{V}_e$ is the excurrent flow rate of air (STPD ml min^{-1}), F_iCO_2 is the incurrent fraction of CO_2, F_eCO_2 is the excurrent fraction of CO_2 and M_b is the body mass (g). $\dot{V}_e$ was calculated from $\dot{V}_i$, F_iCO_2 and F_eCO_2 (Withers, 2001); see Abbas et al. (2020) for detailed equations.

Mass loss was determined gravimetrically by difference between the initial and final mass, divided by average mass and the total experimental time (48 h). This mass loss approximates average evaporative water loss ($\dot{V}_{H_2O}$, mg g^{-1} h^{-1}) over the experimental period (ignoring mass loss as excretion and CO_2). Unfortunately, we were unable to measure total evaporative water loss (EWL) separately for flour beetles as the Vaisala RH probe was not sufficiently sensitive to measure the low EWL (in absolute terms) of these small beetles, so we could not partition total EWL into respiratory and cuticular water loss. However, we could estimate the partitioning of total EWL into respiratory and cuticular water loss from $\dot{V}_{CO_2}$ (see Discussion).

Gas exchange patterns

The CO_2 emission trace was examined for each individual beetle at different time intervals to characterise its respiratory pattern. Each beetle used either CGE, cyclic or DGE, at different time intervals. CGE was clearly identified from CO_2 emission traces, which were always above baseline, and lacked any periodicity, whereas cyclic gas exchange had more periodic CO_2 concentrations that approached baseline. The CGE and cyclic rates of CO_2 emission were calculated as the average $\dot{V}_{CO_2}$ over time. A beetle was considered to have DGE if its CO_2 emission trace regularly was at or close to baseline (C) and there was a discernable interburst period (Marais et al., 2005). For calculations of DGE $\dot{V}_{CO_2}$, both closed (C) and flutter (F) were combined as the inter burst phase (IB) as there was no clear distinction between them (Wobschall and Hetz, 2004). We analysed DGE cycles ($n = 2–8$) for each individual beetle to calculate $\dot{V}_{CO_2}$ and the

duration (sec) for each phase, burst (open phase, here after called burst or B for consistency with interburst or IB) and the entire cycle (IB + B).

Statistical analyses

Metabolic rate was compared at different time intervals for beetles by general linear models using the nlme package (v.3.1-140, Pinheiro et al., 2019) in R (v.3.6.1, R Development Core Team, 2020). A simple model (gls function) with time as a fixed factor, and a complex model (lme function) including individual as a random intercept factor (to account for individual variation for repeats) were compared by analysis of variance (ANOVA). For both models, assumptions of normal distributions of residuals and homogeneity of variance were tested using Shapiro–Wilk normality and Levene's tests respectively (car package; Fox and Weisberg, 2019). Individual variability was significant (likelihood ratio $\chi_5^2 = 13.05$, DF = 5, $P < 0.001$), so the complex model was used for metabolic rate comparison. The ANOVA model output used the metabolic rate for the middle time interval (24 h, coded as T1) as the reference level for comparison with metabolic rates at initial (5 h, coded as T2) and final time intervals (48 h, coded as T3) to compare cyclic and DGE $\dot{V}_{CO_2}$ at 24 and 48 h with CGE at 5 h. Pattern characteristics of cyclic and DGE (B and IB phase duration and $\dot{V}_{CO_2}$ as well as total cycle duration) were compared at 24 and 48 h time intervals by paired t-test. Means are provided with standard error. For comparison, we quantified the effects of body mass and temperature (10–30°C) on the IB phase duration for 21 species, using published data and that from this study (Supplementary table S1). We also compared the metabolic rates (CGE) of T. castaneum (at 5 h) with the allometric relationship for other tenebrionid beetles of varying sizes, and other similarly sized small dipterans and hymenopterans (Supplementary table S2) to determine the extent of variation in metabolic rate resulting from body mass.

Results

All beetles survived the 48 h respirometry experiments. They lost an average of $10.8 \pm 2.52\%$ of their initial body mass by the end of the respirometry trial, corresponding to an average evaporative water loss of 2.11 mg g^{-1} h^{-1} over 48 h.

The gas exchange pattern of beetles changed over time. Initially, they used CGE (at 5 h; fig. 1a) but switched to cyclic release of CO_2 at 24 h (interburst $\dot{V}_{CO_2}$ was low but did not reach zero; fig. 1b). Beetles used DGE at 48 h, with interburst $\dot{V}_{CO_2}$ even lower, for longer periods (fig. 1c).

The transition of gas exchange from continuous to cyclic at 24 h was coupled with a 27% reduction of metabolic rate ($t_{14} = 14.8$, $P < 0.001$, table 1). The DGE pattern at 48 h (fig. 1c) had a small but significant ($t_{14} = 2.17$, $P = 0.048$) reduction in metabolic rate (5.7%) compared to that at 24 h, and a slightly greater reduction of 31% compared to CGE at 5 h (table 1).

The cycle duration increased significantly from $54.5.7 \pm 3.06$ s at 24 h to 66.1 ± 4.53 s at 48 h ($t_7 = 3.52$, $P = 0.010$; table 1). The IB phase also increased significantly from 31.5 ± 3.21 s at 48 h compared to 21.8 ± 2.28 s at 24 h ($t_7 = 5.35$, $P = 0.001$), being almost 47% of the cycle duration at 48 h compared to 40% at 24 h. The IB phase $\dot{V}_{CO_2}$ was reduced as the IB phase duration increased with time, contributing 17.5% to metabolic rate at 48 h compared to 31% at 24 h. The B phase duration did not change significantly between 24 and 48 h ($t_7 = 2.05$, $P = 0.080$; table 1). The $\dot{V}_{CO_2}$ did
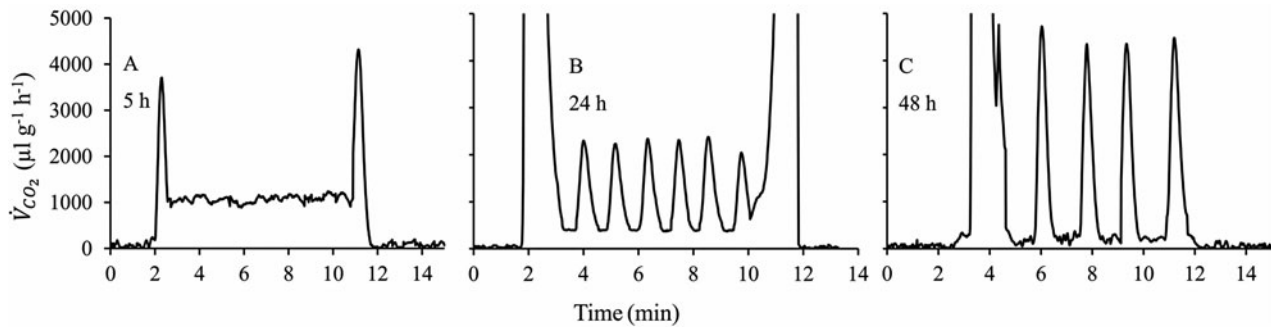
Figure 1. Example of gas exchange patterns identified from the CO_2 emission of a red flour beetle during its exposure to desiccation/starvation in the flow-through respirometry chamber for 48 h at 25°C. (a) At 5 h showing continuous gas exchange. (b) At 24 h showing cyclic release of CO_2. (c) At 48 h showing discontinuous gas exchange. The short sections of zero release at the beginning and end of each trace indicate baseline recordings from an empty chamber.

not differ significantly between 24 and 48 h for B ($t_7 = 1.79$, $P = 0.117$) or IB ($t_7 = 1.98$, $P = 0.088$).

Allometry of interburst duration and metabolic rate for beetles

The allometric relationship for log (interburst duration; min) with log (mass; g) and log (T_a; °C) was 2.98 (±0.30) + 0.288 (±0.054) log (M) – 0.052 (±0.013) log (T_a), which was highly significant ($F_{33,2} = 23.7$, $P < 0.001$). Both mass and T_a factors were significant, with higher mass increasing ($t_{33} = 5.34$, $P < 0.001$) and higher temperature decreasing ($t_{33} = 4.11$, $P = 0.002$) the IB duration. Q_{10}-adjusting the temperature for all the data to 25°C clearly shows the positive allometry of IB duration with mass (fig. 2).

After adjusting metabolic rate to an ambient temperature of 25°C (assuming a Q_{10} of 2; Lighton, 2018; Supplementary table S2), we found that the scaling relationship between metabolic rate and body mass of the compared species (fig. 3) was significant (log (metabolic rate, MR; µl CO_2 h^{-1}) = 0.79 (±0.026) log (M; g), $F_{17,1} = 860$, $P < 0.001$). The r^2 value of 0.98 indicates that only 2% variation in metabolic rate between species was not explained by differences in body mass.

Discussion

This is the first study to measure metabolic rate concurrently with gas exchange patterns for a small insect that is a pest of stored grain, in a flow-through respirometry system. The only other similar-sized insects measured for breathing patterns, water loss and/or metabolic rates are dipterans. *Drosophilia melanogaster* adults survived for just 10 h in similar desiccating conditions, with an apparent CGE gas exchange pattern (Williams *et al.*, 1997: fig. 2b). Two species of mosquito, *Anopheles arabiensis* and *A. gambiae*, survived 5–20 h (age dependent) in 10% RH still air (fig. 1 of Gray and Bradley, 2005) i.e. not in a respirometry chamber with air flow, which would increase EWL and so likely decrease survival time. These three species live in moist habitats and consume moist food, suggesting that the drier habitat and food of *T. castaneum* has been a factor in their evolution of a strong desiccation resistance.

Gas exchange patterns

Tribolium beetles transitioned from CGE to cyclic and DGE over time, with a significant reduction in metabolic rate. Beetles experienced both starvation and desiccation during measurement,

Table 1. Body mass (mg), gas exchange patterns changing from continuous (CGE) to cyclic and discontinuous (DGE) over time, characteristics of interburst (IB) and burst (B) phases, and the cycle duration at 24 and 48 h, and average $\dot{V}_{CO_2}$ (metabolic rate, µl g^{-1} h^{-1}) and evaporative water loss (EWL, mg g^{-1} h^{-1}) of red flour beetles ($n = 8$) measured for 48 h at 25°C

Variables	Measurement time (h)				
	0	5	24	48	P
Body mass (mg)	1.78 ± 0.074*			1.57 ± 0.030*	$P = 0.011$
Gas exchange pattern		CGE	Cyclic	DGE	
B phase duration (s)		–	31.7 ± 0.90	34.5 ± 1.6	$P = 0.080$
IB phase duration (s)		–	21.8 ± 2.28*	31.5 ± 3.21*	$P = 0.001$
Cycle duration (s)		–	54.5 ± 3.06*	66.1 ± 4.53*	$P = 0.010$
B phase $\dot{V}_{CO_2}$ (µl g^{-1} h^{-1})			1319 ± 48.9	1457 ± 63.7	$P = 0.117$
IB phase $\dot{V}_{CO_2}$ (µl g^{-1} h^{-1})			276 ± 37.6	147 ± 29.3	$P = 0.088$
Average $\dot{V}_{CO_2}$ (µl g^{-1} h^{-1})		1209 ± 32.3*	886 ± 19.6*#	839 ± 27.7*#	*$P < 0.001$ #$P = 0.048$
EWL (mg g^{-1} h^{-1})	2.26 ± 0.525				

Values are mean ± SEM. Significant differences for interburst (IB) and cycle duration at 24 and 48 h are indicated by a superscript * or #, with *P* value. Average $\dot{V}_{CO_2}$ of the beetles at 24 h was significantly different from 5 (*) and 48 h (#).
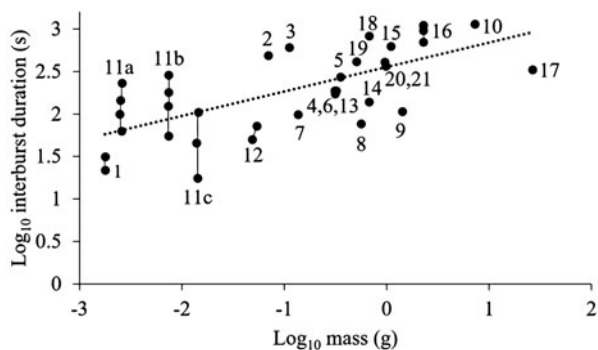
Figure 2. Allometry of DGE interburst duration with body mass for *Tribolium castaneum* from our study and other species from literature data (species are labelled from 1–21, with *Tribolium castaneum* from our study labelled as 1 with other Coleoptera from literature as 2–10, Hymenoptera as 11, Blattodea as 12–17 and Orthoptera as 18–21; see Supplementary table S1 for details) after Q_{10}-adjusting the temperature for all the data to 25°C.

which makes it difficult to separate the relative roles of each potential stress to the transition of the gas exchange pattern from CGE to DGE. However, *Tribolium* beetles are resistant to starvation, capable of surviving at least ~11 days when starved even at 34°C and 60% RH (Scharf *et al.*, 2015), so the effect of desiccation may be stronger. Unfortunately, as we could not weigh beetles throughout the experiment (which would require stopping the respirometry experiment at each time point), we were not able to measure EWL over time to see if there was reduction in EWL with or without DGE, to provide a test of hygric hypothesis (Buck *et al.*, 1953; Lighton, 1996). This approach would be useful for future work.

DGE has been observed for adults of many insect species, ranging in body mass from 2.5 mg for worker fire ants (*Solenopsos invicta*, Vogt and Appel, 2000) that are around 40% heavier than *T. castaneum*, to 26 g for the giant burrowing cockroach (*Macropanesthia rhinoceros*, Woodman *et al.*, 2007) that is four orders of magnitude heavier than *T. castaneum* (1.78 mg). The IB duration increases with body mass increase, presumably reflecting the lower mass-specific metabolic rate of larger species (fig. 2). For example, the IB duration of *M. rhinoceros* (10 min) is 2.28-fold higher than for *S. invicta* (4.4 min) at the same temperature (20°C). Increased ambient temperature has a negative effect on IB duration, reflecting the higher metabolic rate at higher ambient temperature. For example, the IB duration of the

Table Mountain cockroach (*Aptera fusca*; body mass 2.29 g) is 9.5-fold higher at 10°C than 30°C (Groenewald *et al.*, 2013).

Resting metabolic rate

The metabolic rate of *T. castaneum* after the initial 5 h acclimation period (see Emekci *et al.*, 2002; Pimentel *et al.*, 2007) of 1209 µl $g^{-1} h^{-1}$ measured in the current study was lower than previous measurements using closed-system respirometry of 1680 µl $g^{-1} h^{-1}$ for 200 adults with food at 70% RH averaged over 1 h (at the same temperature of 25°C; Emekci *et al.*, 2002). Pimentel *et al.* (2007) measured a slightly higher rate of 2.30 µl h^{-1} using closed-system respirometry for a group of 20 beetles, than our value of 2.15 µl h^{-1}. Our lower values for *T. castaneum* are likely attributable to our instantaneous measurement of food-deprived individual adults by flow-through respirometry, which would be expected to result in lower values than measurements of groups of individuals or closed-system measurement (Lighton, 2018). *Tribolium castaneum* is the smallest of all tenebrionid beetles measured and has the lowest absolute metabolic rate, which agrees well with its allometrically predicted metabolic rate (fig. 3, Supplementary table S2).

Metabolic rate varies in response to a range of factors other than body mass and temperature e.g. starvation and desiccation (Contreras and Bradley, 2009, 2010; Schimpf *et al.*, 2012; Rolandi *et al.*, 2014). The metabolic rate of *T. castaneum* was downregulated over time, presumably reflecting pattern transition from CGE to DGE, in addition to the combined stress of starvation and/or desiccation in our experiment as explained above. Although DGE might have a beneficial reduction of EWL, it has potentially a disadvantageous metabolic consequence.

Physiological responses to stress such as variation of metabolic rate, water loss and gas exchange patterns of insect pests, other than stored-grain pests, are well documented (see table 1 of Karise and Mänd, 2015). For example, the American cockroach (*Periplaneta americana*) has a decreased metabolic rate and water loss with the transition from DGE to CGE after exposure to phosphine (800 ppm; Woodman *et al.*, 2008). Less is known for stored-grain pests such as *T. castaneum*; metabolic rate varies in response to controlled atmosphere treatments i.e. increased CO_2 levels and/or decreased O_2 levels (Emekci *et al.*, 2002, 2004) but it is not known if these variations are linked with gas exchange patterns. However, we have found that *T. castaneum* beetles switch to DGE and down-regulate $\dot{V}_{CO_2}$ with desiccation/starvation stress.

Evaporative water loss

Red flour beetles survived 48 h at low RH with an average EWL of 2.11 mg $g^{-1} h^{-1}$ despite their small mass hence high surface-to-volume, which is associated with a high mass-specific evaporative water loss. Their switch from CGE (5 h) to cyclic (24 h) then DGE (48 h) may provide a fitness benefit of reduced EWL in the low RH environment of dry stored grain i.e. support the hygric hypothesis. Unfortunately, we were unable to measure respiratory EWL (REWL) separately for 5, 24 and 48 h for flour beetles, to determine if it decreased during DGE, but we can estimate REWL from average $\dot{V}_{CO_2}$ (table 1) using the average ratio of 1 mg respiratory H_2O ml O_2^{-1} (calculated for 30 insect species; Woods and Smith, 2010). Assuming a respiratory exchange ratio of 0.85 (midway between 0.7 for lipids and 1.0 for carbohydrate; calculated from Withers, 2001), REWL would have been
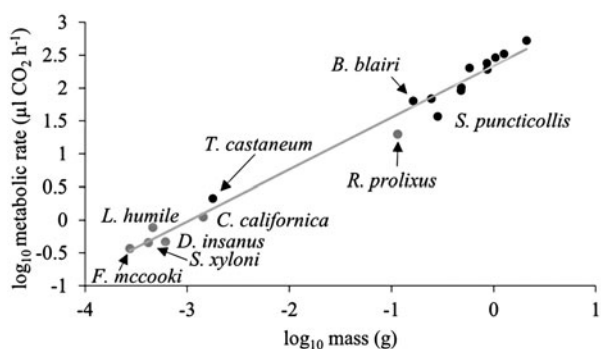
Figure 3. Allometry of CGE metabolic rate for *Tribolium castaneum* with tenebrionid beetles (black circles) and small species from several other insect orders (grey circles; see Supplementary table S2).

$1.42\,\mathrm{mg\,g^{-1}\,h^{-1}}$ at 5 h, $1.04\,\mathrm{mg\,g^{-1}\,h^{-1}}$ at 24 h and $0.99\,\mathrm{mg\,g^{-1}\,h^{-1}}$ at 48 h (DGE); these estimates correspond to a 27% reduction for cyclic gas exchange (24 h) and 31% decrease for DGE (48 h), which by way of our estimation method are necessarily the same as % reductions in $\dot{V}_{CO_2}$. However, haemolymph P_{CO_2} is relatively constant during CGE but is cyclic during DGE, so the P_{CO_2} gradient between haemolymph and ambient air during the open phase of DGE is variable. For the hissing cockroach (*G. portentosa*) the haemolymph P_{CO_2} during CGE of about 2.0 kPa is similar to the mean P_{CO_2} during DGE of about 1.9 kPa (varying from about 1.3 to 2.5 kPa; Rowe *et al.*, 2022) so the average EWL/$\dot{V}_{CO_2}$ could be similar for continuous and DGE. The lower REWL (reflecting changes in $\dot{V}_{CO_2}$) has a more modest effect on total EWL of 16% reduction at 24 h (cyclic) and 19% at 48 h (DGE). We can also estimate cutaneous EWL (CEWL) as $1.11\,\mathrm{mg\,g^{-1}\,h^{-1}}$, calculated as the difference between the total mass loss over the experimental duration of $2.26\,\mathrm{mg\,g^{-1}\,h^{-1}}$ and the average REWL estimated over the experimental duration of $1.15\,\mathrm{mg\,g^{-1}\,h^{-1}}$. Actual measurements of TEWL and partitioning of REWL and CEWL are required, to confirm upon these estimates of water loss.

Qubit S151 analyser for very small insects

High precision gas analysers are used for small animals, due to the low volumes of gas fluxes (Withers and Cooper, 2011). Consequently, respirometry studies on insects usually use high-precision gas analysers to characterise gas exchange patterns (Gray and Bradley, 2006). We have now shown that the low-cost Qubit analyser, normally used for larger vertebrates and insects heavier than 1 g (e.g. Robertson *et al.*, 2017; Lailvaux *et al.*, 2018) can be used successfully to characterise gas exchange patterns in *T. castaneum* with a mass of around 2 mg. There are issues with the system, most importantly baseline drift. Baseline drift can make around the data from 73% of replicates unusable for analysis. Even with this issue, using the lower cost, and more common Qubit analyser may promote more studies of insect respiration.

In conclusion, this is the first study to determine gas exchange patterns in a stored grain insect, with associated changes in metabolic rate. We believe that this information for *Tribolium* of a switch from CGE to cyclic then DGE with progressive stress from dehydration, and the consequent decrease in metabolic rate, may be of use in applied settings, such as pest management. Pest management of stored grain insects is currently almost entirely achieved by fumigation with toxic gases (e.g. phosphine), but there is considerable research into the potential use of unusual concentration of natural atmospheric gases (i.e. low oxygen, high carbon dioxide, etc.), known as controlled atmospheres (Navarro, 2012). We suggest that knowledge of the gas exchange patterns of pest insects coupled with their effects on metabolic rate and evaporative water loss may help to find new pest management methods.

References

Abbas W, Withers PC and Evans TA (2020) Water costs of gas exchange by a speckled cockroach and a darkling beetle. *Insects* **11**, 632.

Alnajim I, Du X, Lee B, Agarwal M, Liu T and Ren Y (2019) New method of analysis of lipids in *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (Fabricius) insects by direct immersion solid-phase microextraction (DI-SPME) coupled with GC–MS. *Insects* **10**, 363.

Arnold PA, Cassey P and White CR (2016) Maturity matters for movement and metabolic rate: trait dynamics across the early adult life of red flour beetles. *Animal Behaviour* **111**, 181–188.

Buck J, Keister M and Specht H (1953) Discontinuous respiration in diapausing *Agapema* pupae. *Anatomical Records* **117**, 541–541.

Chown SL, Gibbs AG, Hetz SK, Klok CJ, Lighton JRB and Marais E (2006) Discontinuous gas exchange in insects: a clarification of hypotheses and approaches. *Physiological Biochemical Zoology* **79**, 333–343.

Contreras HL and Bradley TJ (2009) Metabolic rate controls respiratory pattern in insects. *Journal of Experimental Biology* **212**, 424–428.

Contreras HL and Bradley TJ (2010) Transitions in insect respiratory patterns are controlled by changes in metabolic rate. *Journal of Insect Physiology* **56**, 522–528.

Emekci M, Navarro S, Donahaye E, Rindner M and Azrieli A (2002) Respiration of *Tribolium castaneum* (Herbst) at reduced oxygen concentrations. *Journal of Stored Products Research* **38**, 413–425.

Emekci M, Navarro S, Donahaye E, Rindner M and Azrieli A (2004) Respiration of *Rhyzopertha dominica* (F.) at reduced oxygen concentrations. *Journal of Stored Products Research* **40**, 27–38.

Fields PG and White NDG (2002) Alternatives to methyl bromide treatments for stored-product and quarantine insects. *Annual Review of Entomology* **47**, 331–359.

Fox J and Weisberg S (2019) *An R Companion to Applied Regression*. Thousand Oaks, CA: Sage.

Gray EM and Bradley TJ (2005) Physiology of desiccation resistance in *Anopheles gambiae* and *Anopheles arabiensis*. *American Journal of Tropical Medicine and Hygiene* **73**, 553–559.

Gray EM and Bradley TJ (2006) Evidence from mosquitoes suggests that cyclic gas exchange and discontinuous gas exchange are two manifestations of a single respiratory pattern. *Journal of Experimental Biology* **209**, 1603–1611.

Groenewald B, Bazelet CS, Potter CP and Terblanche JS (2013) Gas exchange patterns and water loss rates in the Table Mountain cockroach, *Aptera fusca* (Blattodea: Blaberidae). *Journal of Experimental Biology* **216**, 3844–3853.

Hadley NF (1994) Ventilatory patterns and respiratory transpiration in adult terrestrial insects. *Physiological Zoology* **67**, 175–189.

Hetz SK and Bradley TJ (2005) Insects breath discontinuously to avoid oxygen toxicity. *Nature* **433**, 513–516.

Huang S-P, Sender R and Gefen E (2014) Oxygen diffusion limitation triggers ventilatory movements during spiracle closure when insects breathe discontinuously. *Journal of Experimental Biology* **217**, 2229–2231.

Jian F and Jayas DS (2012) The ecosystem approach to grain storage. *Agricultural Research* **1**, 148–156.

Karise R and Mänd M (2015) Recent insights into sublethal effects of pesticides on insect respiratory physiology. *Insect Physiology* **5**, 31–39.

Lailvaux SP, Wang AZ and Husak JF (2018) Energetic costs of performance in trained and untrained *Anolis carolinensis* lizards. *Journal of Experimental Biology* **221**, jeb176867.

Lighton JRB (1996) Discontinuous gas exchange in insects. *Annual Review of Entomology* **41**, 309–324.

Lighton JRB (2018) *Measuring Metabolic Rates: A Manual for Scientists*. Oxford, UK: Oxford University Press.

Lighton JRB and Berrigan D (1995) Questioning paradigms: caste-specific ventilation in harvester ants, *Messor pergandei* and *M. julianus* (Hymenoptera: Formicidae). *Journal of Experimental Biology* **198**, 521–530.

Lu B, Ren Y, Du Y-Z, Fu Y and Gu J (2009) Effect of ozone on respiration of adult *Sitophilus oryzae* (L.), *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (F.). *Journal of Insect Physiology* **55**, 885–889.

Marais E, Klok CJ, Terblanche JS and Chown SL (2005) Insect gas exchange patterns: a phylogenetic perspective. *Journal of Experimental Biology* **208**, 4495–4507.

Matthews PGD (2018) The mechanisms underlying the production of discontinuous gas exchange cycles in insects. *Journal of Comparative Physiology B* **188**, 195–210.

Navarro S (2012) The use of modified and controlled atmospheres for the disinfestation of stored products. *Journal of Pest Science* **85**, 301–322.

Nespolo RF, Artacho P and Castañeda LE (2007) Cyclic gas-exchange in the Chilean red cricket: inter-individual variation and thermal dependence. *Journal of Experimental Biology* **210**, 668–675.

Pimentel MAG, Faroni LRDA, Tótola MR and Guedes RNC (2007) Phosphine resistance, respiration rate and fitness consequences in stored-product insects. *Pest Management Science* **63**, 876–881.

Pinheiro J, Bates D, DebRoy S and Sarkar D (2019) nlme: linear and nonlinear mixed mffects models.

Quinlan MC and Gibbs AG (2006) Discontinuous gas exchange in insects. *Respiratory Physiology & Neurobiology* **154**, 18–29.

R Development Core Team (2020) *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Robertson RM, Spong KE and Srithiphaphirom P (2017) Chill coma in the locust, *Locusta migratoria*, is initiated by spreading depolarization in the central nervous system. *Scientific Reports* **7**, 1–12.

Rolandi C, Iglesias MS and Schilman PE (2014) Metabolism and water loss rate of the haematophagous insect *Rhodnius prolixus*: effect of starvation and temperature. *Journal of Experimental Biology* **217**, 4414–4422.

Rowe TT, Gutbrod MS and Matthews PG (2022) Discontinuous gas exchange in Madagascar hissing cockroaches is not a consequence of hysteresis around a fixed P CO2 threshold. *Journal of Experimental Biology* **225**, jeb242860.

Scharf I, Galkin N and Halle S (2015) Disentangling the consequences of growth temperature and adult acclimation temperature on starvation and thermal tolerance in the red flour beetle. *Evolutionary Biology* **42**, 54–62.

Schimpf NG, Matthews PGD and White CR (2012) Cockroaches that exchange respiratory gases discontinuously survive food and water restriction. *Evolution* **66**, 597–604.

Terblanche JS and Woods HA (2018) Why do models of insect respiratory patterns fail? *Journal of Experimental Biology* **221**, jeb130039.

Terblanche JS, Clusella-Trullas S and Chown SL (2010) Phenotypic plasticity of gas exchange pattern and water loss in *Scarabaeus spretus* (Coleoptera: Scarabaeidae): deconstructing the basis for metabolic rate variation. *Journal of Experimental Biology* **213**, 2940–2949.

Vogt JT and Appel AG (2000) Discontinuous gas exchange in the fire ant, *Solenopsos invicta* Buren: caste differences and temperature effects. *Journal of Insect Physiology* **46**, 403–416.

Williams AE and Bradley TJ (1998) The effect of respiratory pattern on water loss in desiccation-resistant *Drosophila melanogaster*. *Journal of Experimental Biology* **201**, 2953–2959.

Williams AE, Rose MR and Bradley TJ (1997) CO_2 release patterns in *Drosophila melanogaster*: the effect of selection for desiccation resistance. *Journal of Experimental Biology* **200**, 615–624.

Withers PC (1992) *Comparative Animal Physiology*. Philadelphia: Saunders College Pub.

Withers PC (2001) Design, calibration and calculation for flow-through respirometry systems. *Australian Journal of Zoology* **49**, 445–461.

Withers PC and Cooper CE (2011) Using a priori contrasts for multivariate repeated-measures ANOVA to analyze thermoregulatory responses of the dibbler (*Parantechinus apicalis*; Marsupialia, Dasyuridae). *Physiological and Biochemical Zoology* **84**, 514–521.

Wobschall A and Hetz SK (2004) Oxygen uptake by convection and diffusion in diapausing moth pupae (*Attacus atlas*). *International Congress Series* **1275**, 157–164.

Woodman JD, Cooper PD and Haritos VS (2007) Cyclic gas exchange in the giant burrowing cockroach, *Macropanesthia rhinoceros*: effect of oxygen tension and temperature. *Journal of Insect Physiology* **53**, 497–504.

Woodman JD, Haritos VS and Cooper PD (2008) Effects of phosphine on the neural regulation of gas exchange in *Periplaneta americana*. *Comparative Biochemistry and Physiology C* **147**, 271–277.

Woods HA and Smith JN (2010) Universal model for water costs of gas exchange by animals and plants. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 8469–8474.