



# Hold your breath beetle—Mites!

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Received March 25, 2015

Accepted November 11, 2015

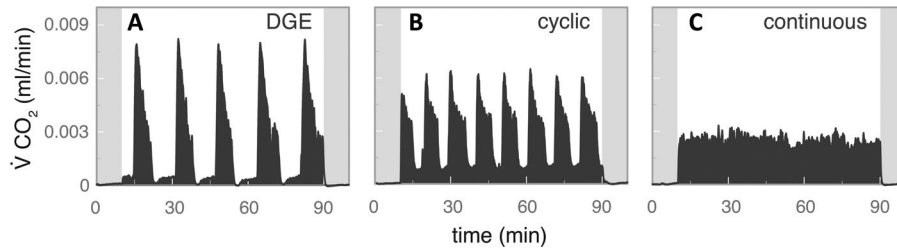
Respiratory gas exchange in insects occurs via a branching tracheal system. The entrances to the air-filled tracheae are the spiracles, which are gate-like structures in the exoskeleton. The open or closed state of spiracles defines the three possible gas exchange patterns of insects. In resting insects, spiracles may open and close over time in a repeatable fashion that results in a discontinuous gas exchange (DGE) pattern characterized by periods of zero organism-to-environment gas exchange. Several adaptive hypotheses have been proposed to explain why insects engage in DGE, but none have attracted overwhelming support. We provide support for a previously untested hypothesis that posits that DGE minimizes the risk of infestation of the tracheal system by mites and other agents. Here, we analyze the respiratory patterns of 15 species of ground beetle (Carabidae), of which more than 40% of individuals harbored external mites. Compared with mite-free individuals, infested one's engaged significantly more often in DGE. Mite-free individuals predominantly employed a cyclic or continuous gas exchange pattern, which did not include complete spiracle closure. Complete spiracle closure may prevent parasites from invading, clogging, or transferring pathogens to the tracheal system or from foraging on tissue not protected by thick chitinous layers.

**KEY WORDS:** Adaptive response, DGC, DGE, insect evolution, mite–host interaction, respiration pattern.

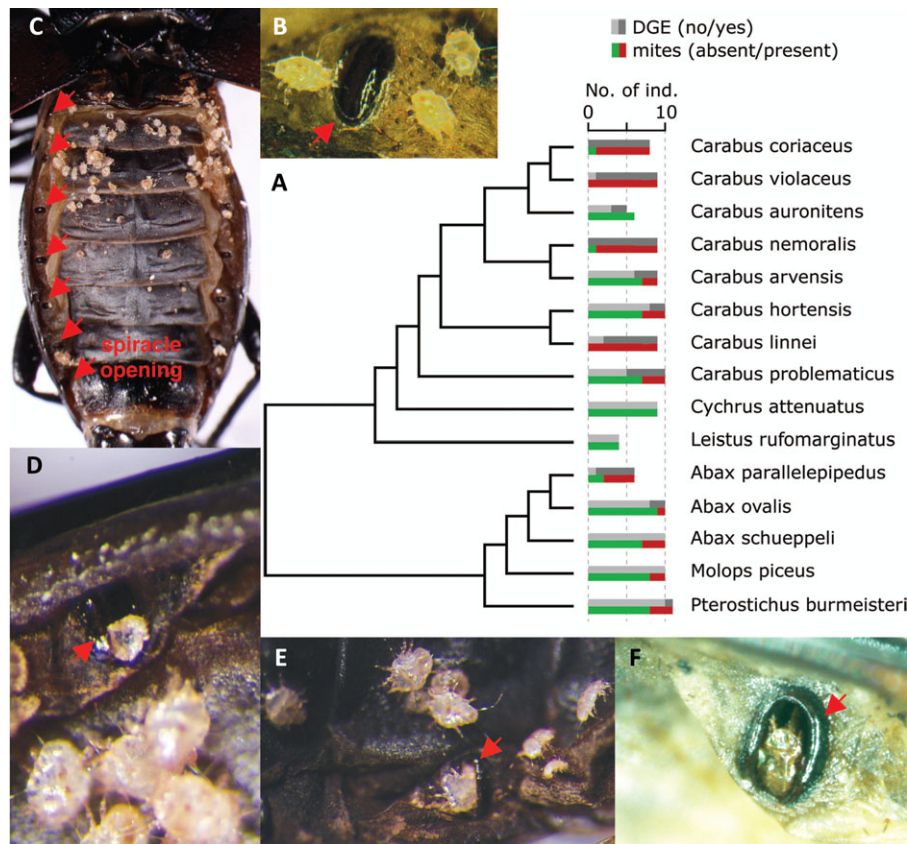
Discontinuous gas exchange (DGE) is a unique and enigmatic breathing pattern characterized by periods of zero gas exchange (Fig. 1A) that is observed principally in resting or quiescent arthropods (Lighton 1996; Marais et al. 2005; Quinlan and Gibbs 2006; Chown 2011; Contreras et al. 2014). This gas exchange pattern is likely to have evolved by natural selection as it is heritable (Schimpf et al. 2013) and has evolved independently several times in arthropods (Marais et al. 2005). Many animals rely on metalloproteins, such as hemocyanin or hemoglobin, to transport O<sub>2</sub> and CO<sub>2</sub> between the environment and cells, but the majority of adult insects use a branching tracheal system and therefore have more direct gas exchange between the air and their cells (Burmester 2015). The opening and closing of spiracles defines the three phases associated with DGE: open, closed, and flutter (Miller 1974; Mill 1985; Hetz 2007; Contreras et al. 2014). In animals capable of DGE, gas exchange with the environment decreases to values close to zero during the closed phase, indicating complete closure of the spiracles (Fig. 1A) with only cuticular gas exchange remaining (Quinlan and Gibbs 2006; Terblanche

et al. 2008; Chown 2011). Rhythmic repetition of CO<sub>2</sub> bursts and interburst intervals with incomplete closure of the spiracles are typical of cyclic gas exchange (Fig. 1B). The continuous pattern (Fig. 1C) lacks any rhythmicity. Species and individuals reveal substantial variation, and certain species or individuals can even engage in all three modes of gas exchange (Marais and Chown 2003; Marais et al. 2005; Bradley 2007).

Why individuals employ these different respiratory strategies is not known. One of the hypotheses proposed to explain DGE, summarized by Chown et al. (2006), is called “the strolling arthropod hypothesis.” This hypothesis was originally proposed by insect respiratory specialist Peter Miller in the 1970s (Miller 1974) and is based on the idea that the complete closure of spiracles may be a mechanism to prevent other arthropods, particles, bacteria, and fungal spores from entering the tracheal system (Miller 1974; Mill 1985; Chown et al. 2006; Quinlan and Gibbs 2006). However, this hypothesis has never been tested (Chown et al. 2006; Chown 2011; Woods 2011; Contreras et al. 2014). Here, we provide a much-needed assessment of this hypothesis.



**Figure 1.** The gas exchange patterns of ground beetles. Three gas exchange patterns of ground beetles, (A) discontinuous gas exchange (DGE), (B) cyclic gas exchange, and (C) continuous gas exchange were measured with open-flow respirometry. The measurement of each gas exchange pattern was preceded and followed by baseline measurements (in gray).



**Figure 2.** Ground beetles infested with external mites. (A) The phylogenetic tree used to create a phylogenetically adjusted generalized linear mixed model to test whether beetles infested with mites employed DGE more often than did mite-free individuals. For each species bars show the number of individuals that employed DGE and the number of individuals infested with mites. (B) Three subadult mites (deutonymphs, *Photia hejriana*) are located near a spiracle (red arrow), a tracheal system opening in the exoskeleton of *Carabus nemoralis*. (C) The abdomen of *C. nemoralis* after removal of the elytra reveals mite infestation. A pair of spiracles is found on each side of the abdominal segments. Red arrows indicate spiracles on the left abdominal side, while the mirror-sided spiracles on the right abdominal side are not indicated. *Carabus auronitens* (D) and *C. nemoralis* (E) with a mite partially located in the spiracle opening. (F) A mite inside the spiracle opening of *C. linnei*.

## Materials and Methods

We recorded gas exchange using open-flow respirometry (Lighton and Halsey 2011) for a total of 129 ground beetles of 15 species in the family Carabidae (Figs. 2A and S2). This family is a species-rich taxon within the Coleoptera. Immediately after respiratory measurements, the beetles were euthanized and examined under a

stereo microscope for the presence of external mites, either clearly visible on the exoskeleton or below the elytra (chitinized first wing pair), a common location of mites on beetles (Fig. 2B–F).

## EXPERIMENTAL ANIMALS

Carabid beetles were collected in September and October 2013 in southern Poland (Myślenice Forestry District, 49°48′19.9″N,

19°56'01.8"E to 49°43'50.1"N, 19°52'46.0"E) with permission from the General Directorate of Environmental Conservation in Warsaw, Poland (no. DOP-oz. 6401.01.12.2013 JRO). Fifteen species were identified (Freude et al. 2004): *Carabus coriaceus* L. 1758, *C. violaceus* L. 1758, *C. auronitens* F. 1792, *C. nemoralis* O. F. Müll. 1764, *C. arvensis* Hbst. 1784, *C. hortensis* L. 1758, *C. linnei* Duft. 1812, *C. problematicus* Hbst. 1786, *Cychrus attenuatus* (F. 1792), *Leistus rufomarginatus* (Duft. 1812), *Abax parallelepipedus* (Pill. and Mitt. 1783), *A. ovalis* (Duft. 1812), *A. schueppeli* Pall. 1825, *Molops piceus* (Pz. 1793), and *Pterostichus burmeisteri* Heer 1841. Beetles were maintained in a climate chamber under a 12L:12D light regime, 20°C, and 70% relative humidity for one to four weeks before metabolic rate (MR) was measured. Within the climate chamber, the beetles were kept in plastic containers with sand and clay pots as shelters.

We determined presence or absence of external mites of all individual beetles. Immediately after the MR measurement, beetles were euthanized with ethyl acetate (Avantor, Poland) and dissected under a stereo microscope (SZ40, Olympus, Japan) to detect the presence of external and subelytral mites. Based on published material, the 15 ground beetle species of this study are reported to be associated with more than 25 mite species from more than 10 families and at least three orders (Cooreman 1975; Haitlinger 1988; Gwiazdowicz 2000; Szczepanska and Magowski 2006; Salmane and Telnov 2009; Gwiazdowicz and Gutowski 2012; Haitlinger and Łupicki 2012). For eight of the carabid beetle species associations with several (maximum of 13) mite species have been reported. Even two or three parasitic mite species have been recorded for the same host ground beetle individual (Regenfuss 1968; Husband and Husband 2007). Feeding ecology is unknown for many of the larval stages of mites and even for several species, while some of the 25 mite species known to be associated with carabid beetles are considered commensals or parasitic (Krantz and Walter 2009).

### MR MEASUREMENTS

A total of 129 beetles were subjected to MR measurements. Beetles were kept in individual boxes and provided the same number of mealworms daily (at the sixth hour of lights-on). For beetles confirmed to have fed on at least one mealworm (*Tenebrio molitor*) from 1500h one day to 1500h the next day any remaining food was removed from their enclosure to ensure accurate postabsorptive MR measurements starting at one hour after lights-on the following morning. Thus, MR was measured in individuals at least 17 h following the last feeding event. Individual mass was recorded to the nearest 0.01 mg (Toledo XP26, Mettler, Switzerland) immediately before the MR measurements. To constrain movement before being placed in a 20 cm<sup>3</sup> experimental chamber (Sable Systems) each beetle was carefully wrapped in synthetic gauze by rolling the beetle tightly into the gauze and subsequently

folding closed the two terminal sides. MR measurements were therefore not affected by locomotor activity.

All respirometry measurements were performed in an environmental chamber at 20°C. Flow-through respirometry was used to measure CO<sub>2</sub> release (Sable System) in eight chambers (one baseline measurement and seven beetle measurements) controlled by a multiplexer (Sable System). A push flow system was used, with a flow rate of 50 ml min<sup>-1</sup> controlled by a mass flow controller (Sidetrack). Incoming air drawn from the outside of the building was maintained at 60% relative humidity (DG-4 Dew-point generator, Sable System), and animal chambers that were not in use were flushed with an air flow with relative humidity that was similar to the sample air stream. Air leaving the experimental chamber was dried with magnesium perchlorate (Merck KGaA, Germany) before CO<sub>2</sub> (Li-Cor model 7000 infrared;) and oxygen (Oxzilla, Sable System) were measured. Each individual was measured for 80 continuous minutes once during the day and once during the night whereby starting time of measurements was randomized for each species. Baseline measurements were collected for 10 min between each individual measurement and the next. Acquired baseline was used to provide accurate zero values and to correct for instrumental drift. Immediately after the MR measurement we determined the presence of external and subelytral mites. The MR of *Poecilochirus carabi* mites was examined under the same conditions as those of beetles. Up to 70 mites of this species were collected from multiple beetles and measured in a single respirometry chamber. However, the mites did not produce a detectable CO<sub>2</sub> deflection, which excludes the possibility that the beetle MR readings were biased by mite respiration.

### DATA ANALYSIS

MR measurements were recorded every 1 sec and analyzed using ExpeData software (Sable Systems). For every recording, data analysis was a process that began with transformations of the original data. During transformation, the CO<sub>2</sub> trace was baseline corrected and converted to ml min<sup>-1</sup> of  $\dot{V}CO_2$  by using the following formula (Lighton 2008):  $\dot{V}CO_2 = FR_i [(F'_e CO_2 - F'_i CO_2) - F'_e CO_2 (F_i O_2 - F'_e O_2)] / (1 - F'_e CO_2)$ , where  $\dot{V}CO_2$  is the CO<sub>2</sub> emission rate, FR is the flow rate of air through a chamber (incurrent; FR<sub>i</sub>), and F is the fractional concentration of a given gas (measured upon entering [incurrent; F<sub>i</sub>] or leaving [excurrent; F<sub>e</sub>] a respirometer chamber) without (F<sub>e</sub>) or with H<sub>2</sub>O scrubbing (F'<sub>e</sub>). Flow rate was measured in a wet air stream and corrected for 60% relative humidity. Three different gas exchange patterns were determined: two periodic (DGE and cyclic) and one continuous, as defined by Marais et al. (2005) and Chown (2011). Continuous gas exchange is defined as gas exchange lacking any zero-value period and having a majority of datapoints above an average  $\dot{V}CO_2$  value of 30%. If the percentage was <30% of the average  $\dot{V}CO_2$ , the trace is more likely to be considered periodic

(Marais et al. 2005). Discontinuous and cyclic gas exchange were distinguished according to Chown (2011). During DGE, gas exchange with the environment drops to values close to zero due to completely closed spiracles, while cuticular gas exchange may still occur (Terblanche et al. 2008; Berman et al. 2013). The flutter phase is characterized by rapid and repeated limited openings of the spiracles, and the open phase is usually characterized by a burst of CO<sub>2</sub> release (Hetz 2007; Chown 2011) (Fig. 1A). Cyclic gas exchange is characterized by regular peaks and troughs of gas exchange, but the  $\dot{V}CO_2$  trace never achieves values close to zero value (Fig. 1B). For separation of DGE from cyclic gas exchange pattern, we estimated the minimum traces of CO<sub>2</sub> release relative to the mean CO<sub>2</sub> release for the entire cycle. Minimum  $\dot{V}CO_2$  was either below 6% of the mean  $\dot{V}CO_2$  or above 10% of the mean  $\dot{V}CO_2$  (Fig. S1). Therefore, we employed a 6% threshold value for our analysis and considered beetles with CO<sub>2</sub> release below this threshold to represent DGE and beetles above this threshold to represent cyclic gas exchange pattern. Thus, we use minimum  $\dot{V}CO_2$  for the separation between DGE and cyclic gas exchange pattern, which is in agreement with Wobschall and Hetz (2005) who reported zero CO<sub>2</sub> release during the flutter phase and therefore use the flutter phase as a determining characteristic for DGE pattern.

Day and night measurements of each individual were classified as either representing a DGE, cyclic, or continuous pattern. An individual was considered to show DGE if it used this pattern during the day, night, or both. If a DGE pattern was never observed, the individual's recordings were considered to be cyclic. If no DGE and no cyclic pattern were observed, the individual was classified as having a continuous gas exchange pattern. If day and night gas exchange patterns were similar, the measurement with the lower MR was used in the analysis. For 10 individuals, only one recording (day or night) was available. For most of the beetle individuals that showed a rhythmic gas exchange pattern, we analyzed three consecutive cycles or more, but for 16 individuals analysis was performed on less than three cycles. We removed measurements (two of 129 total measurements) from our analysis that contained chaotic and indeterminate  $\dot{V}CO_2$  traces and had MRs that were two- or threefold higher than the mean standard MR for the species; it was assumed that individuals with these readings moved during the test, despite the constraints.

## STATISTICAL ANALYSIS

All analyses were performed in the R computational environment (version 3.2.1) (R Core Team 2014). Presence or absence of DGE was tested using a generalized linear mixed model with a binomial error distribution and a logit link function (Butler 2009). The response variable was presence/absence of DGE, that is, the coefficients quantified trends in the probability of observing DGE. The model included one categorical fixed effect (presence/absence of mites), the covariate (centered) body mass, and two random

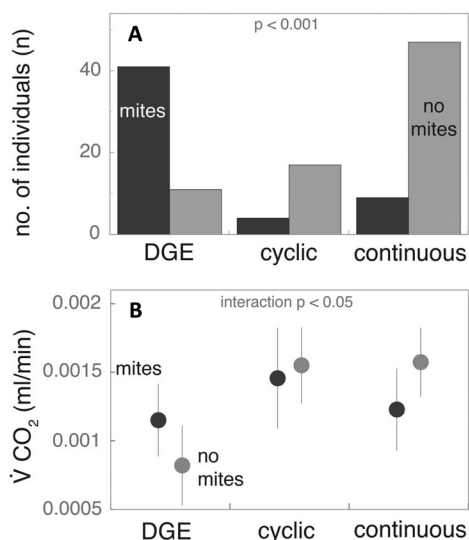
effects (a species effect not related to phylogeny and a phylogenetic effect included as a phylogenetic relationship matrix between all species). Including species and phylogenetic effects accounts for variation observed between species and thus enables comparisons of between species patterns without confounding them by within-species specific patterns. The model was run with **S** parametrization of the phylogenetic effect, which means that the phylogenetic relationship matrix included all nodes of the tree rather than only the terminal tips of the tree. For each pair of tips/nodes, elements of the **S** matrix contain the summed distances from the root of the tree to the most recent common ancestor of these nodes/tips. For ultrametric trees scaled to unity length, the **S** matrix becomes a correlation matrix. Body mass was not significantly related to occurrence of DGE (larger beetles tended to exhibit DGE more often;  $F_{df,1,30} = 3.57$ ,  $P = 0.06$ , regression coefficient  $b = 0.002 \pm 0.002$ ).

Fixed effects were tested using an approximate conditional Wald statistic (*wald.asreml* function), and random effects were tested using a likelihood-ratio test that compared models with and without particular random effects. The resulting likelihood ratios were tested against a chi-square distribution with  $df = 1$  (Lynch and Walsh 1998).

MR was analyzed using a linear mixed model with a Gaussian error structure. All analyzed models conformed to the assumption of i.i.d.: they were normally distributed with homogenous variances. The response variable was the MR (mean  $\dot{V}CO_2$ ) with the fixed effects of respiration pattern (categorical: DGE, cyclic, continuous), presence of mites (categorical: present/absent), and the interaction of mites and respiration pattern, with (centered) body mass used as a covariate. Species and phylogenetic effect were random effects in this model. After confirming that the interaction was significant, we split the data into two groups (mite-free and mite-infested) and reanalyzed the data to examine the differences between respiration patterns between groups.

All linear models were constructed using *asreml-R* (Butler 2009). Because a complete phylogeny was not available, we manually assembled a phylogeny based on the available phylogenetic relationships (Maddison et al. 1999; Raupach et al. 2010; Deuve et al. 2012). Branch lengths were automatically generated using the *inverseA* function from *MCMCglmm* package (Hadfield 2010). To account for possible dependence of our results on the branch lengths, we generated 100 trees based on identical topology, with branch length sampled randomly from a normal distribution and allowing up to a twofold difference in branch length within a tree (while maintaining the tree ultrametric). The fraction of models with a significant parasite effect on the occurrence of DGE was  $f = 1$ , and the analogous proportion of models with significant parasite  $\times$  breathing pattern interactions on MR was  $f = 0.98$ . Thus, the unavailability of branch lengths did not significantly bias our results.





**Figure 3.** Ground beetles infested with external mites employ DGE significantly more often than mite-free beetles. (A) The number of infested and noninfested beetles using each of the three gas-exchange patterns, with adjustments for phylogeny and body mass. (B) Metabolic rates, measured as the  $\dot{V}CO_2$  in three gas-exchange patterns, in beetles with and without mites. Whiskers indicate 95% confidence intervals. The average metabolic rate is not affected by infestation, but the interaction of mite presence and respiration is significant ( $P = 0.04$ ) after adjusting for the effects of phylogeny and body mass.

## Results and Discussion

In agreement with the strolling arthropod hypothesis, beetles employed DGE significantly more often when harboring external mites in comparison with individuals not carrying mites (Fig. 3A, phylogenetic comparative mixed model:  $F_{df: 1, 126} = 12.35$ ;  $P < 0.001$ ). Phylogenetic signal in this trait was nonsignificant (likelihood-ratio test;  $\chi^2_1 = 0.19$ ,  $P = 0.53$ ; phylogenetic heritability  $\pm$  SE:  $h^2 = 0.21 \pm 0.27$ ), such that different species were equally likely to exhibit DGE when harboring mites. More than 40% of all individuals that we examined carried at least one of several species of carabid-infesting mites detected in this study. Eleven individuals from eight species employed DGE without being infested with mites (Figs. 2A and S2), indicating that the physical presence of mites is not the only trigger of DGE; other, nonmutually exclusive hypotheses may explain DGE in beetles that do not harbor mites.

DGE typically ceases when the demand for oxygen increases (Contreras and Bradley 2009). To test the hypothesis that DGE in infested beetles is the result of a poor physiological state and, thus, low oxygen demand, we compared MR (expressed as  $CO_2$  emission) between beetles with and without mites. Overall,  $CO_2$  production was related to the gas exchange pattern ( $P = 0.008$ ). However, this effect depended on the presence of mites (Fig. 3B;

mites  $\times$  pattern interaction:  $P = 0.04$ ). Infested beetles showed no relationship between gas exchange pattern and MR ( $P = 0.40$ ), whereas a significant relationship ( $P < 0.001$ ) was observed for noninfested beetles such that lower  $CO_2$  production corresponded to increased DGE. Interestingly, infested beetles did not conserve energy when employing DGE as did the noninfested beetles (Fig. 3B). Instead, infested beetles appear to employ DGE for reasons other than reducing their MR, as hypothesized by the energy savings hypothesis (Chown 2011).

Mites living within insect respiratory systems can undoubtedly reduce host evolutionary fitness. Whether mites alone or mites in concert with mite-associated viruses, bacteria, fungi, protozoan, or nematodes (Poinar and Poinar 1998) contribute to the negative effects on the host is not known. Tracheal clogging is known to occur in honeybees, hampers oxygen delivery to cells having high metabolic demands (Harrison et al. 2001), and also reduces survival by limiting thermoregulatory capacity (McMullan and Brown 2009; McMullan 2010). The presence of mites in the tracheal systems of bumblebees impairs their foraging behavior (Otterstatter et al. 2005) and shortens their life span under laboratory conditions (Otterstatter and Whidden 2004). Ground beetles are generally fast runners (with some species like the tiger beetle running exceptionally fast up to 170 body lengths per second; Kamoun and Hogenhout 1996), an activity that requires very rapid oxygen delivery. Tracheal system clogging by mites may not constrain gas exchange when oxygen demand is moderate, but clogging most likely impairs performance during activities that require intense aerobic metabolism (Harrison et al. 2001), such as hunting or predator escape. Thus, the adaptive value of avoiding respiratory system infestation by mites appears indisputable. Because of the low metabolic demand when insects are at rest, DGE provides an adequate oxygen supply for cellular metabolism. Switching to other gas exchange patterns is unavoidable when insects are active (Contreras and Bradley 2009); however, mite invasion may be more difficult when beetles are active at high speeds.

Alternative hypotheses have been proposed to explain the function of DGE. These hypotheses invoke the beneficial role of closed spiracles in maintaining an insect's water budget and coping with subterranean conditions (chthonic) by generating greater partial pressure gradients between the animal and its environment; spiracles may optimize subterranean water maintenance and minimize oxidative stress or energy use (Hetz and Bradley 2005; Chown et al. 2006; Quinlan and Gibbs 2006; Chown 2011; Groenewald et al. 2014). Possibly because there are diverse advantages to DGE, none of these adaptive hypotheses has received overwhelming support. For this reason, a recent review on the adaptive functions of DGE (Contreras et al. 2014) has considered these hypotheses nonmutually exclusive. Cyclic gas exchange (Fig. 1B) is considered to be the ancestral respiratory

mode, and DGE has subsequently evolved independently at least five times in insects (Marais et al. 2005; Bradley et al. 2009). The selective regimes under which DGE evolved are not known, and the current dependence of DGE on particular environmental factors (namely, mite presence) is not necessarily indicative of the conditions under which DGE evolved (Chown et al. 2006; Quinlan and Gibbs 2006; Woods 2011; Contreras et al. 2014). Still, the current relationships of DGE with certain environmental factors provide potential explanations for DGE evolution. Recently, DGE has even been proposed to be an exaptation, a trait that provides additional adaptive functions outside of its original adaptive purpose (Contreras et al. 2014).

## CONCLUSION

The present study cannot unquestionably answer if the strolling arthropod hypothesis should be accepted or rejected, and cannot distinguish whether DGE evolved for the purpose of avoiding mite invasion or if it evolved under other selective pressures and was later adopted to protect against mites. However, this study provides to our best knowledge the first data on how the presence or absence of external mites is related to the employed gas exchange pattern and the associated MR. Presence of mites likely increases the presence of mite-associated particles, bacteria, spores, and pathogens in close proximity to tracheal openings, exclusion of which could be as adaptive as mite exclusion itself. The associations of mites with the ancestors of extant Hymenoptera and Coleoptera date at least to the Eocene (almost 50 myr) (Dunlop et al. 2013, 2014), so sufficient time would have elapsed for DGE to evolve or to be adopted as an antimite defensive mechanism.

## ACKNOWLEDGMENTS

We thank M. Czarnoński for input on study design, W. Witaliński for mites identification, K. Kapela, L. Kuriańska-Piątek, A. Filiczowska, and P. Gumienny for help with beetle maintenance; N. Derus for support with dissections; F. Kapustka for help with creating phylogenetic tree; M. Gudowski and C. Hartmann for photographs; M. Bauchinger for collection specimens; and M. Cichoń, E. Sadowska, S. McWilliams, and J. S. Terblanche for comments on the manuscript. This research was supported by the National Science Centre in Poland, MAESTRO grant to JK (grant no. 2011/02/A/NZ8/00064) and Jagiellonian University (DS K/2DS/004151) and Institute of Environmental Sciences DSC (K/DSC/001714 and K/DSC/001697). We are grateful for constructive editorial and reviewer comments that helped to improve the manuscript. Beetle collection and maintenance were done by BWS, AG, and UB. Preparation of animals for measurements was done by BWS and AG. Respirometric measurements and analysis were done by AG and UB. Beetle dissection and mite presence determination were planned and performed by AML. Statistical analysis and figure preparations were done by AG, SD, and UB. Manuscript writing was done by AG, SD, JK, and UB. All authors commented on final manuscript.

## DATA ARCHIVING

The doi for our data is <http://dx.doi.org/10.5061/dryad.d0753>.

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Associate Editor: M. Forister  
Handling Editor: J. Conner

## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** The number of individuals that showed a minimum of CO<sub>2</sub> release expressed as the percentage of the mean CO<sub>2</sub> release of the measured cycles (in steps of 2%).

**Figure S2.** Examples of gas exchange patterns (measured as rate of CO<sub>2</sub> emission) of beetles not harboring mites (left column, DGE, cyclic, and continuous pattern graphs with white backgrounds) and beetles that were harbouring mites (right column, DGE, cyclic, and continuous pattern graphs with gray backgrounds).

**Table S1.**