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**SHORT COMMUNICATION**

# The closed spiracle phase of discontinuous gas exchange predicts diving duration in the grasshopper *Paracinema tricolor*

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## ABSTRACT

The discontinuous gas exchange (DGE) pattern of respiration shown by many arthropods includes periods of spiracle closure (C-phase) and is largely thought to serve as a physiological adaptation to restrict water loss in terrestrial environments. One major challenge to this hypothesis is to explain the presence of DGE in insects in moist environments. Here, we show a novel ecological correlate of the C-phase, namely, diving behaviour in mesic *Paracinema tricolor* grasshoppers. Notably, maximal dive duration is positively correlated with C-phase length, even after accounting for mass scaling and absolute metabolic rate. Here, we propose that an additional advantage of DGE may be conferred by allowing the tracheal system to act as a sealed underwater oxygen reservoir. Spiracle closure may facilitate underwater submersion, which, in turn, may contribute to predator avoidance, the survival of accidental immersion or periodic flooding and the exploitation of underwater resources.

KEY WORDS: Respiratory gas exchange, Submersion, CT scan, Scaling, Orthoptera

## INTRODUCTION

Several air-breathing terrestrial animals use an aquatic environment opportunistically (Seymour and Matthews, 2013). Behavioural responses to water, such as diving and underwater locomotion, can be an effective means of crossing water bodies during migration or accessing a range of resources otherwise unavailable on land (Vinnersten et al., 2009). Entering the water can also be a strategy to avoid predation from terrestrial or aerial predators (Gibbs, 2002). However, prolonged submersion can have significant physiological effects and requires specific respiratory strategies to achieve cellular respiration (Heitler et al., 2005).

Many insects display discontinuous gas exchange (DGE) cycles in the resting state, characterised by a periodic sustained spiracle closure phase (C-phase) and the suspension of external gas exchange alternating with bursts of gas exchange (Fig. 1A). Although DGE is widely documented (Marais et al., 2005), its physiological and evolutionary costs and benefits remain unresolved. Several alternative adaptive hypotheses have been proposed, and the oldest, most widely discussed idea suggests that DGE is an adaptation to reduce respiratory water loss (Buck et al., 1953; Terblanche et al., 2010; reviewed recently in Matthews and Terblanche, 2015). Several observations challenge this hypothesis,

not least is that some species show DGE in moist environments or when they are not desiccation stressed (Contreras and Bradley, 2011). Consequently, there may be other ecological or evolutionary advantages to having closed spiracles that have not yet been well explored.

When an animal maintains DGE in moist environments, what could the reason(s) be? There is a well-established suite of factors determining insect respiratory patterns: (1) primarily maintaining oxygen supply, then (2) pH regulation (and $CO_2$ excretion), and, finally, (3) water saving and/or minimising oxidative damage (Groenewald et al., 2014). It is reasonable that spiracle closure during DGE for water saving could also restrict unwanted foreign bodies from entering the tracheal system, as recently demonstrated in carabid beetles (Gudowska et al., 2015). An alternative novel hypothesis that we propose here is that spiracle closure may provide an advantage by restricting respiratory water loss, but at the same time by keeping water from entering the tracheal system during immersion. A diverse suite of factors can influence whether water will enter the tracheal system, including water tension, hydrophobicity of the cuticle, intratracheal gas pressure and morphological structures forming plastrons or bubbles (e.g. hairs). Indeed, spiracle water-tightness is critical to the survival of submersion in *Drosophila* larvae (Parvy et al., 2012), despite their low metabolic requirements and small size, which theoretically should enable sufficient diffusion of respiratory gases from their respiratory system.

Here, in *Paracinema tricolor*, a grasshopper associated with mesic habitats (wetlands) and that readily shows DGE (Fig. 1A), we examined (1) whether respiratory adaptations, such as sustained closed spiracle periods, may be correlated with immersion behaviours in an air-breathing insect, and (2) what physiological and morphological traits might contribute to their overall diving behaviour.

## MATERIALS AND METHODS
### $CO_2$ emission rate measurement as a proxy for metabolic rate

*Paracinema tricolor* (Thunberg 1815) (Orthoptera: Acrididae) were collected from wetlands in the JS Marais Park and Jonkershoek Nature Reserve (South Africa) and housed in the laboratory at 25°C (60–80% relative humidity, 14 h:10 h light:dark). All grasshoppers collected during field work were winged. They were fed with fresh lettuce and oatmeal, and given Restionaceae grasses from their natural environment. Grasshoppers were fasted for at least 8 h prior to metabolic rate measurements, and weighed (to 0.1 mg) before and after each trial. Respirometry measurements were carried out in a programmable refrigeration bath (Huber CC-410-WL, Peter Huber Kältemaschinenbau, Offenburg, Germany) at 15°C. Ambient air was scrubbed of $CO_2$ and water using a column containing soda lime (Merck, Gauteng, South Africa) and another containing silica gel and Drierite (ratio 1:1) (Merck; Sigma-Aldrich,
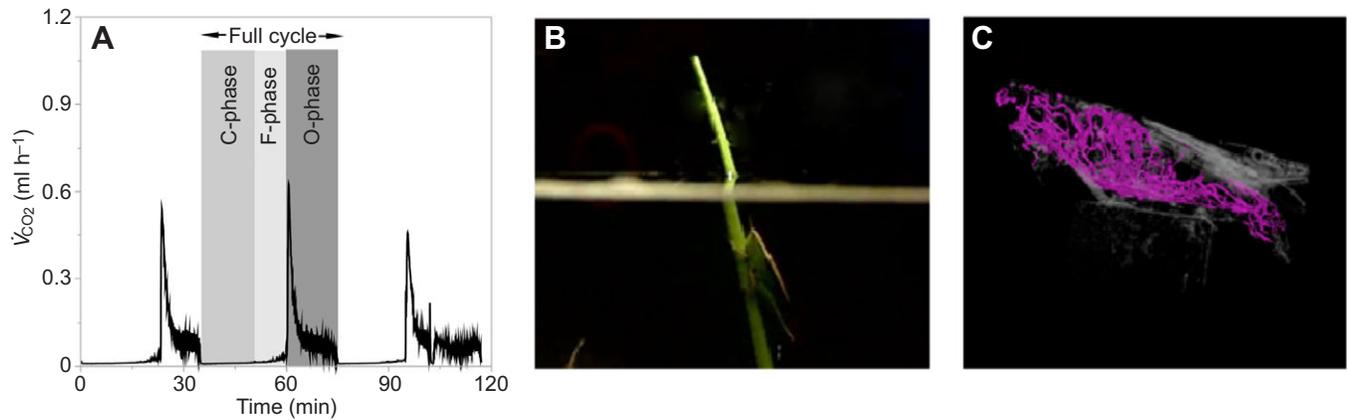
**Fig. 1. *Paracinema tricolor*.** (A) Typical $CO_2$ emission trace of a 0.268 g male. (B) *Paracinema tricolor* individual during underwater diving. (C) An example CT scan of the air stores in the respiratory system (pink) (see also Movie 1).

St Louis, MO, USA). Scrubbed air was pushed at a constant flow rate of 200 ml min$^{-1}$ and then passed through a 15 ml cuvette (for males) and a 20 ml cuvette (for females). Flow-through respirometry was undertaken with an infrared $CO_2$/$H_2O$ analyser (Li-7000, Li-Cor, Lincoln, NE, USA) to record the rate of $CO_2$ uptake ($\dot{V}_{CO_2}$). Each individual (total $n$=28) was measured once continuously for 3–12 h. Data were converted to ml h$^{-1}$, baselined and drift corrected in ExpeData software (Version 1.7.15, Sable Systems International, Las Vegas, NV, USA). For analyses, only periods where no activity was visible were used, based on recordings from an electronic infrared activity detector (AD2, Sable Systems International). Where individuals displayed DGE, mean $\dot{V}_{CO_2}$ release from two to five consecutive cycles per individual was extracted. The closed (C) phase was identified as a period with stable zero or close to zero $\dot{V}_{CO_2}$. The washout time of the system (time to 99% equilibration) is 22.5 and 30 s for males and females, respectively, indicating that possible error

in C-phase length estimation was 3–6% for males and 2–3% for females.

### Diving duration

After respirometry, diving experiments were conducted over the next few days in a glass aquarium tank filled with de-chlorinated tap water maintained at 15±1°C. At the start of each trial, the grasshopper's abdomen was gently moistened and after 2 s the entire individual was submerged under the water (20 cm depth) close to the longest restio grass, allowing individuals to grasp the restios. The diving duration of individuals was recorded. To force grasshoppers to maximal diving duration, every 10 s the water was disrupted by the hand of the observer, to serve as a cue to the animal to remain submerged (Fig. 1B). To check spiracular activity underwater, grasshoppers were submerged in Petri dishes filled with tap water and the state of the spiracles was assessed under a binocular microscope (Leica Microsystems, Germany).
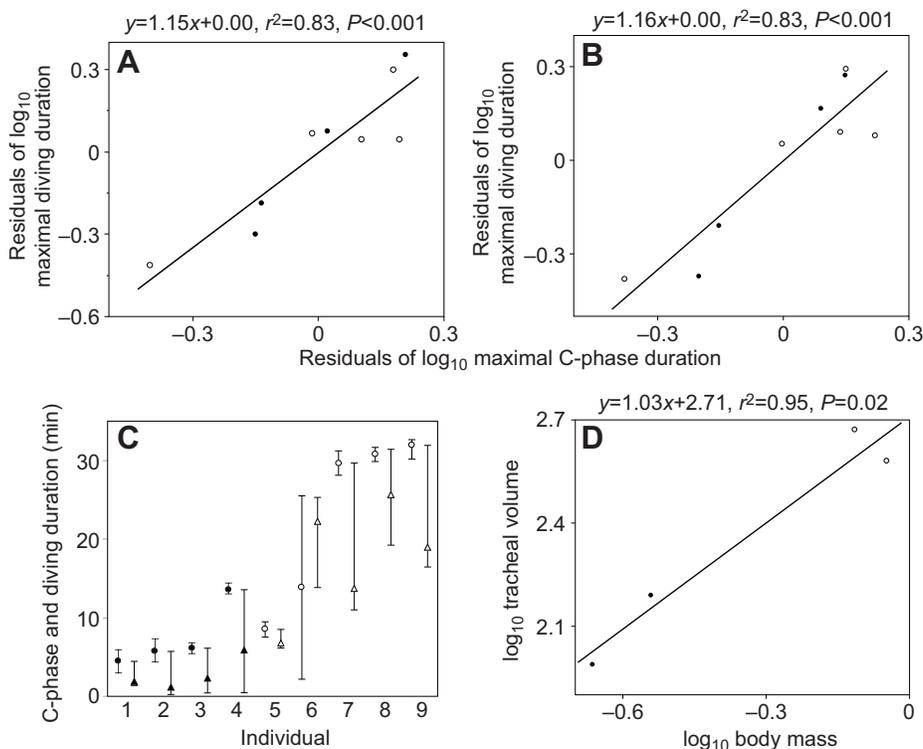


**Fig. 2. Scatterplots of the relationships between respiratory physiology, morphology and behaviour.** (A) Body mass residuals of log$_{10}$ maximal diving duration (min) and log$_{10}$ maximal C-phase length (min). (B) Metabolic rate residuals of log$_{10}$ maximal diving duration (min) and log$_{10}$ maximal C-phase length (min). (C) C-phase length (min) and diving duration (min); each point on the graphs represents the mean value of two to five C-phase length estimates (circles) and the mean value of two to seven diving trials (triangles) per individual (±minimum and maximum). (D) log$_{10}$ tracheal volume (mm$^3$) and log$_{10}$ body mass (g). Open symbols, females ($n$=5); filled symbols, males ($n$=4) (tracheal volume $n$=4).

### Tracheal volume measurement

Internal body air stores were imaged in a subset ($n$=4) of individuals using a computed tomography (CT) scanner (0.015–0.021 mm$^3$ resolution, Phoenix Nanotom S, General Electric) (Fig. 1C). Segmentation and analyses were performed using Volume Graphics VGStudio Max 2.2. The grasshopper was separated from the background (mounting) using the manual ellipse segmentation tool. Next, an automatic region grower tool was used to select all the external air surrounding the sample [region of interest (ROI)]. This ROI was then inverted to include all the internal air. Lastly, region-growing tools were manually applied to the ROI to select the internal air. The volume of internal air included estimates of tracheae and all visible air sacs (Movie 1).

## RESULTS AND DISCUSSION

While underwater, insects must obtain sufficient oxygen to sustain cellular aerobic metabolism while buffering the accumulation of $CO_2$. Orthoptera are not considered particularly specialized for freshwater aquatic life, but they are capable of intermittent diving and short-term survival in aquatic environments (Heitler et al., 2005). Does the length of the C-phase correlate with diving duration? As expected, heavier grasshoppers exhibited a longer C-phase and remained underwater for longer ($y$=1.62$x$+1.30, $r^2$=0.80, $P$=0.001). Maximal diving duration was correlated with C-phase length after adjusting for the effect of body mass ($y$=1.15$x$+0.00, $r^2$=0.83, $P$=0.0006; Fig. 2A) or metabolic rate ($y$=1.16$x$+0.00, $r^2$=0.83, $P$=0.0006; Fig. 2B, Table S1). Moreover, maximal diving duration never exceeded C-phase duration, and ranged from 52 to 99% of C-phase duration for females and from 38 to 77% for males (Fig. 2C). Upon submergence, ventilatory and spiracular activity ceased, contrary to findings reported from *Schistocerca gregaria* (Heitler et al., 2005). *Paracinema tricolor* could behave differently because of their high tracheal volume, and the study methods could also have contributed to the observed differences (*S. gregaria* were not forced to submerge). The present results suggest there is no gas exchange with the water during diving. *Paracinema tricolor* likely rely on internal oxygen supply accumulated in the respiratory system, which suggests that grasshoppers re-surfaced with some reserves of oxygen (or perhaps in the case of one female, may have entered anaerobic metabolism) (Table S2).

The upper limit to diving duration is likely set by a combination of factors including tracheal volume and/or $CO_2$ accumulation in haemolymph, but this depends on metabolic rate and the body-mass scaling of both of these relationships. In *P. tricolor*, tracheal volume scales isometrically with body mass ($y$=1.03$x$+2.71, $r^2$=0.95, $P$=0.02; Fig. 2D, Movie 1) and also isometrically for the metabolic rate–body mass scaling relationship [$y$=0.87$x$−0.69 (±0.34 CI), $r^2$=0.85, $P$<0.001]. Taking into account these two relationships, larger grasshoppers store similar air (oxygen) volumes in the tracheal system, metabolise that oxygen at similar rates and yet dive proportionally longer relative to smaller individuals. This suggests that another factor may determine the termination of diving, and the trigger for spiracle opening may be the accumulation of $p_{CO_2}$ (or a critical pH threshold) (Matthews and Terblanche, 2015); however, the body-mass scaling of $CO_2$ buffering is unknown for insect respiration. Moreover, *P. tricolor* has a relatively large tracheal volume compared with other grasshopper species (Huang et al., 2015), which suggests a possible advantage in the form of extended diving duration (Table S3).

The phenomenon of complete spiracle closure allows for suspended gas exchange with the external environment during DGE. Therefore, on the basis of the limited data at hand, we propose that the ecological and evolutionary benefits of DGE extend more broadly to novel aspects of organismal biology that have previously not been widely examined. DGE contributes to fitness by allowing predation avoidance via diving, exploiting underwater resources and the survival of accidental immersion or periodic flooding. Further work exploring the potential costs and benefits for immersion survival would be valuable.

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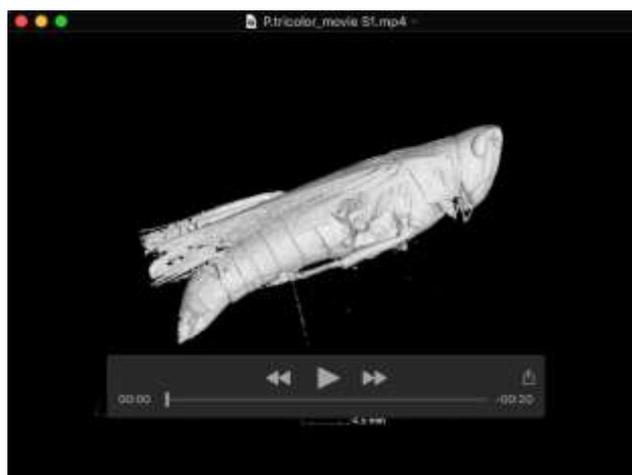
Movie S1. **CT scan of respiratory system in *P. tricolor*.**

Table S1. **Correlation matrix (*r*-values) between all estimated physiology, morphology and behaviour parameters (p<0.05 in bold, n=9 except tracheal volume n=4).**

| | $\log_{10}$ mass | $\log_{10}$ MR | $\log_{10}$ mean C-phase length | $\log_{10}$ mean O-phase length | $\log_{10}$ mean diving duration | $\log_{10}$ tracheal volume |
|---|---|---|---|---|---|---|
| $\log_{10}$ mass | 1.00 | | | | | |
| $\log_{10}$ MR | **0.92** | 1.00 | | | | |
| $\log_{10}$ mean C-phase length | **0.71** | **0.77** | 1.00 | | | |
| $\log_{10}$ mean O-phase length | 0.33 | 0.55 | 0.48 | 1.00 | | |
| $\log_{10}$ mean diving duration | **0.89** | **0.90** | **0.89** | 0.50 | 1.00 | |
| $\log_{10}$ tracheal volume | **0.97** | **0.99** | 0.67 | 0.59 | 0.89 | 1.00 |

Table S2. **Comparison between tracheal volume (cm$^3$) as an internal oxygen stores, oxygen consumption rates (mL h$^{-1}$) calculated using an RQ=0.85 and maximal diving time (min) and C-phase length (min); assuming 21% O$_2$ in tracheal gas.**

| ID | sex | tracheal volume (cm$^3$) | O$_2$ consumption rates (mL h$^{-1}$) | maximal diving time (min) | maximal C-phase length (min) | potential maximal time without open spiracles (min) |
|----|-----|------|------|-------|------|------|
| 5 | M | 0.097 | 0.049 | 4.21 | 7.4 | 24.9 |
| 14 | M | 0.155 | 0.089 | 11.19 | 14.5 | 21.9 |
| 11 | F | 0.470 | 0.308 | 31.47 | 31.7 | 19.2 |
| 12 | F | 0.382 | 0.213 | 7.34 | 9.5 | 22.6 |

Table S3. **Comparison between tracheal volume for *P.tricolor* and other grasshopper species reported in Huang et al. (2015) (after body mass correction).**

| species | tracheal volume ($mm^3$ $g^{-1}$) |
|---|---|
| *P. tricolor* (F) | 513.25 |
| *P. tricolor* (M) | 484.62 |
| *T. pulchripennis* | 350.98 |
| *O. bethlemita* | 241.67 |
| *O. lividipes* | 275.51 |