

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

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Summary statement

The ability to tolerate periods of spiracular closure in *Paracinema tricolor* grasshoppers can facilitate predation avoidance via diving or be used for exploiting underwater resources.

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 aid exploiting underwater resources.

Introduction

Several air-breathing terrestrial animals use an aquatic environment opportunistically (Seymour and Matthews, 2013). Behavioural responses to water, like diving and underwater locomotion, can be an effective means of crossing water bodies during migration or for 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 challenges 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 withstanding 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 are a well-established suite of factors determining insect respiratory patterns: 1) primarily maintaining oxygen supply, then 2) pH regulation (and CO₂ excretion), and finally, 3) water saving and/or minimizing 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 watertightness 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 which readily shows DGE (Fig. 1A), we examined (i) whether respiratory adaptations, such as sustained closed spiracle periods, may be correlated with immersion behaviours in an air-breathing insect, and (ii) what physiological and morphological traits might contribute to their overall diving behaviour.

Material and methods

a. CO₂ emission rate measurement as a proxy for metabolic rate

Paracinema tricolor (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% RH, 14:10 L:D). 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 (MR) measurements, and weighed (to 0.1 mg) before and after each trial. Respirometry measurements were carried out in programmable refrigeration bath (Huber CC-410-WL, Peter Huber Kältemaschinenbau, Offenburg, Germany) at 15°C. Ambient air was scrubbed of CO₂ and water using columns containing soda lime (Merck, Gauteng, RSA) and another containing silica gel and Drierite (ratio 1:1) (Merck, Gauteng, RSA/Sigma-Aldrich, St Louis, MO, USA). Scrubbed air was pushed at a constant flow rate of 200 mL min⁻¹ and then passed through the 15 mL cuvette (for males) and 20 mL (for females). Flow-through respirometry was undertaken with a infrared CO₂/H₂O analyser (Li-7000, Li-Cor, Lincoln, NE, USA) to record $\dot{V}\text{CO}_2$. Each individual (total n=28) was measured once continuously for 3-12 h. Data were converted to mLh⁻¹, baselined and drift corrected in ExpeData software (Version 1.7.15, Sable Systems International (SSI), 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, SSI). Where individuals displayed DGE, mean $\dot{V}\text{CO}_2$ release from 2-5 consecutive cycles per individual was extracted. The closed (C) phase was identified as a period with stable zero or close to zero $\dot{V}\text{CO}_2$. The washout time of the system (time to 99% equilibration) is 22.5 s 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.

b. 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

ten seconds 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 spiracles assessed under a binocular microscope (Leica Microsystems CMS GmbH, Germany).

c. tracheal volume measurement

Internal body air stores were imaged in a subset ($n=4$) of the individuals using a Computed Tomography (CT) Scanner (0.015–0.021 mm³ 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 (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 S1).

Results and Discussion

While underwater, insects must obtain sufficient oxygen to sustain cellular aerobic metabolism while buffering the accumulation of CO₂. 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 have a longer C-phase and remain underwater for longer ($y=1.62x+1.30$; $r^2=0.80$; $p=0.001$). Maximal diving duration correlate with C-phase length after adjusting for the effect of body mass ($y=1.15x+0.00$; $r^2=0.83$; $p=0.0006$) (Fig. 2A) or metabolic rate ($y=1.16x+0.00$; $r^2=0.83$; $p=0.0006$) (Fig. 2B, Table S1). Moreover, maximal diving duration never exceeded C-phase duration, and ranged from 52–99% of C-phase duration for females to 38–77% for males (Fig. 2C). Upon submergence ventilatory and spiracle activity ceased, contrary to findings reported from *S. gregaria* (Heitler et al., 2005). *Paracrinema tricolor* could behave differently due to their high tracheal volume as well as study methods (*S. gregaria* were not forced to submerge). These results suggest there is no gas exchange with the water during diving. *P. tricolor* likely rely on internal oxygen supply accumulated in the respiratory system, and suggests that grasshoppers resurfaced 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 CO₂ 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 ($y=1.03x+2.71$; $r^2=0.95$; $p=0.02$) (Fig. 2D; Movie S1) and also isometrically for the metabolic

rate-body mass scaling relationship ($y=0.87x-0.69$ (± 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, metabolize that oxygen at similar rates and yet dive proportionally longer relative to smaller individuals. This suggests another factor may determine the termination of diving, and the trigger for spiracle opening may be the accumulation of $p\text{CO}_2$ (or a critical pH threshold) (Matthews and Terblanche, 2015); the body mass-scaling of CO_2 buffering is however unknown for insect respiration. Moreover, *P. tricolor* has a relatively large tracheal volume compared to 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 contribute 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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The authors declare no competing interests.

Authors' contributions

AG, LB and JST designed the study, performed the measurements, analysed the data and drafted the manuscript.

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Figures

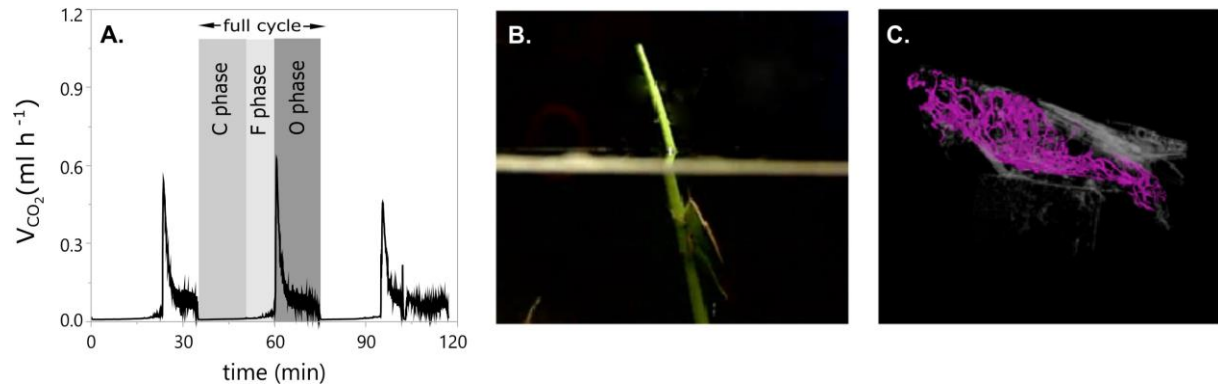


Figure 1. *Paracinema tricolor* A. typical CO₂ emission trace of a 0.268 g male, B. during underwater diving, and C. an example CT scan of the air stores in the respiratory system (pink) (see also Movie S1).

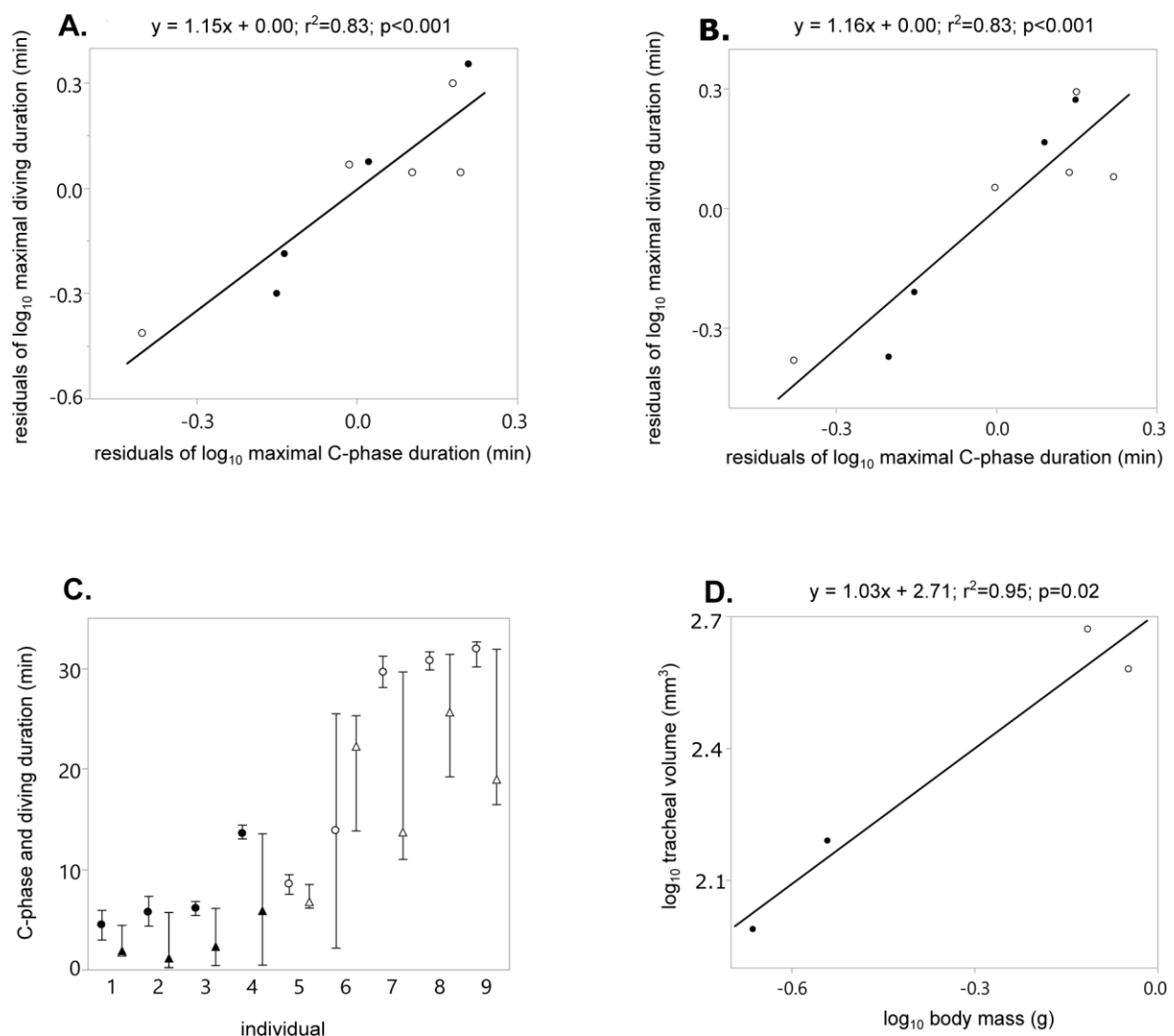


Figure 2. **Scatterplots of the relationships between respiratory physiology, morphology and behaviour.** A. body mass residuals of \log_{10} maximal diving duration (min) and \log_{10} maximal C-phase length (min), B. MR-residuals of \log_{10} maximal diving duration (min) and \log_{10} maximal C-phase length (min), C. C-phase length (min) and diving duration (min), each point on the graphs represents the mean value of 2–5 C-phase length estimates (circles) and the mean value of 2–7 diving trials (triangles) per individual (\pm minimum and maximum), D. \log_{10} tracheal volume and \log_{10} body mass (g). Open symbols – females ($n=5$), filled symbols – males ($n=4$); (tracheal volume $n=4$).