

## Research article

# Metabolic rate controls respiratory pattern in insects

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### Summary

The majority of scientific papers on the subject of respiratory patterns in insects have dealt with the discontinuous gas-exchange cycle (DGC). The DGC is characterized by the release of bursts of CO<sub>2</sub> from the insect, followed by extended periods of spiracular closure. Several hypotheses have been put forward to explain the evolutionary origin and physiological function of this unusual respiratory pattern. We expand upon one of these (the oxidative damage hypothesis) to explain not only the occurrence of the DGC but also the mechanistic basis for the transition to two other well-characterized respiratory patterns: the cyclic pattern and the continuous pattern. We propose that the specific pattern employed by the insect at any given time is a function of the amount of oxygen contained in the insect at the time of spiracular closure and the aerobic metabolic rate of the insect. Examples of each type of pattern are shown using the insect *Rhodnius prolixus*. In addition, contrary to the expectations deriving from the hygric hypothesis, it is demonstrated that the DGC does not cease in *Rhodnius* in humid air.

Key words: insect respiration, cyclic, continuous, DGC, *Rhodnius*, metabolic rate.

### Introduction

This special issue is in honor of Dr Simon H. P. Maddrell for his contributions to the field of insect physiology. In the spirit of this, we will describe some of our recent results in the area of insect respiration, and will attempt to illustrate these results using the insect *Rhodnius prolixus*, Dr Maddrell's favorite experimental subject. We hope that this paper serves not only to celebrate Dr Maddrell's many scientific contributions but also to emphasize the surprising diversity of fields in insect physiology that can and have been explored using this extraordinary insect.

By far the majority of scientific papers on the subject of insect respiration have dealt with the discontinuous gas-exchange cycle (DGC) (for reviews, see Slama, 1988; Lighton, 1996; Chown et al., 2006). The DGC is characterized by having three phases: a phase in which the spiracles are fully closed (the closed phase), one where the spiracles are fully open (the open phase) and one where the spiracles open and close rapidly (the flutter phase). This respiratory pattern is observed in a large number of insects in groups with very diverse taxonomic breadth (Marais et al., 2005).

A number of papers have examined the issue of the evolutionary origins of the DGC and the physiological significance of its occurrence. Several hypotheses for the occurrence of the DGC have been put forward (Levy and Schneiderman, 1966; Lighton, 1998; Hetz and Bradley, 2005; Chown et al., 2006; Quinlan and Gibbs, 2006; Slama et al., 2007).

Buck and colleagues (Buck et al., 1953) proposed a hypothesis, later expanded by Levy and Schneiderman (Levy and Schneiderman, 1966), that has dominated the textbooks for nearly 50 years. This hypothesis [recently named the hygric hypothesis (Chown et al., 2006)] proposes that the evolutionary force promoting discontinuous ventilation is the reduction of respiratory water loss occasioned by the closed phase and bulk inward flow of air during the flutter phase. Although this hypothesis still has adherents (Slama et al., 2007) recent results have demonstrated

directly that no reduction in water loss is achieved when insects respire using the DGC relative to other respiratory patterns (Williams and Bradley, 1998; Gibbs and Johnson, 2004; Lighton and Turner, 2008).

Lighton and Berrigan (Lighton and Berrigan, 1995) proposed the chthonic hypothesis for the occurrence of the DGC in insects. They pointed out that many of the insects exhibiting DGC have portions of their life cycles that are spent underground. The DGC can be beneficial in an environment in which  $P_{O_2}$  is low and  $P_{CO_2}$  is high. For example: (a) partial closing of the spiracles promotes a low  $P_{O_2}$  in the tracheal lumina, allowing inward diffusion in hypoxic environments; (b) partial closing of the spiracles promotes the accumulation of CO<sub>2</sub> in the tissues and tracheae, promoting the rapid release of CO<sub>2</sub> during the open phase in hypercapnic environments; and (c) the discontinuous nature of the respiratory exchange allows for the diffusion of gases surrounding the animal in an environment where convective gas exchange is limited.

Chown and Holter (Chown and Holter, 2000), in a study examining the respiratory pattern in a dung beetle, proposed the emergent properties hypothesis in which the DGC was the result of two competing and interacting sensory and regulatory systems, with one responding to oxygen levels and the other to carbon dioxide. Their model, which was not mechanistic, simply suggested that the two systems would lead to an oscillatory pattern.

Hetz and Bradley (Hetz and Bradley, 2005) proposed the oxidative damage hypothesis. They demonstrated that the partial pressure of oxygen in the tracheae reaches a high level during the open phase. They suggested that the extended period of spiracular closure, which follows the open phase, serves to lower the partial pressure of oxygen in the tracheae and surrounding tissues. This protects the tissues from oxidative damage. The flutter phase, which follows the closed phase, continues to regulate  $P_{O_2}$  at low levels. During this phase, CO<sub>2</sub> accumulates in the insect, eventually reaching a level that forces spiracular opening and initiates the next

open phase. It is this  $O_2$  regulation that produces the observed discontinuous release of  $CO_2$ .

In the current paper, we wish to address not only the issue of why insects occasionally exhibit DGC but also why they often do not. While the majority of papers deal with the DGC, it is probably accurate to say that most of the time most insects show different, non-DGC respiratory patterns. Two additional patterns, which occur frequently enough to have received specific names, are the cyclic pattern and the continuous pattern (Gibbs and Johnson, 2004). These authors observed a correlation between metabolic rate and respiratory pattern.

Our goal is to provide a mechanistic explanation for the variations in insect respiratory patterns. We propose that the closed phase is used to lower  $P_{O_2}$  in the insect. As metabolic rate increases, the closed phase shortens and disappears leading to a cyclic pattern. Further increases in metabolic rate shorten the flutter phase. Its elimination leads to a continuous respiratory pattern. This hypothesis, if true would explain the changes observed over time in a single insect, as well as the differences observed between species.

## Materials and methods

### Insects

Dr Michael Quinlan provided *Rhodnius prolixus* (Stal) from Midwestern University, Glendale, AZ, USA, to found a colony at the University of California, Irvine. The colony was maintained at 27°C and 80% relative humidity (RH) on a 12h:12h day/night cycle. Each adult male used for this study was kept in a separate 15 ml vial for easy identification. For unfed trials, insects were not fed for at least 3 weeks prior to the experiment. For fed trials, insects were blood fed on a rabbit 24 h before experimental trials commenced.

Initially the respiratory patterns of adult male *Rhodnius prolixus* were examined in fed and unfed conditions at 25°C and 35°C ( $N=10$ ). To examine the effect of humidity on respiratory pattern, the  $\dot{V}_{CO_2}$  of 10 adult male *Rhodnius* was measured in a random block design under one of four different treatments: dry fed, dry unfed, humid fed and humid unfed.

### Respirometry

All respirometry measurements were carried out at 25°C with the exception of one, which was carried out at 35°C (Fig. 1C). *Rhodnius* were placed in the experimental chamber and left undisturbed for 55 min before the measurement period commenced. Flow-through respirometry was used to measure  $CO_2$  release with Sable Systems (Henderson, NV, USA) data acquisition software controlling an 8-channel multiplexer and logging data from an infrared  $CO_2$  analyzer (Li-Cor model 6262 infrared; Lincoln, NE, USA). Two chambers were attached to the multiplexer: a baseline (empty) and an experimental (containing the insect) chamber. Measurements were conducted in 2 ml chambers with an airflow of  $200\text{ ml min}^{-1}$ . Air leaving the experimental chamber passed into the  $CO_2$  analyzer. When an insect was not being measured, its chamber was still perfused with air at a rate equal to the regulated flow entering the measured chamber. An experimental run lasted 55 min in total. During a run, three 5 min baselines were recorded (where an empty chamber was read) at the beginning, middle and end of the total run. Baseline values were used to provide accurate zero values and to correct for instrumental drift.

During a dry experimental trial, room air was pumped through two silica and one Ascarite/Drierite column to be scrubbed of water and  $CO_2$ . In high humidity trials, room air was first scrubbed of

$CO_2$  and water, and was then bubbled with a diffuser through two flasks containing a dilute sodium hydroxide solution before entering the experimental chamber. The humidity of air entering the chambers was measured using a hygrometer (Sperry STK-3026, Hauppauge, NY, USA) and found to exceed 85% RH.

### Data analysis

Sable Systems Expedata analysis software was used to process  $\dot{V}_{CO_2}$  measurements.  $CO_2$  levels were recorded in parts per million and, after data were zeroed using baseline values, converted to microliters per minute. Data were then transported into Excel.

The following protocol was used to identify a burst of  $CO_2$  release in the respirometry data. The average  $CO_2$  release was

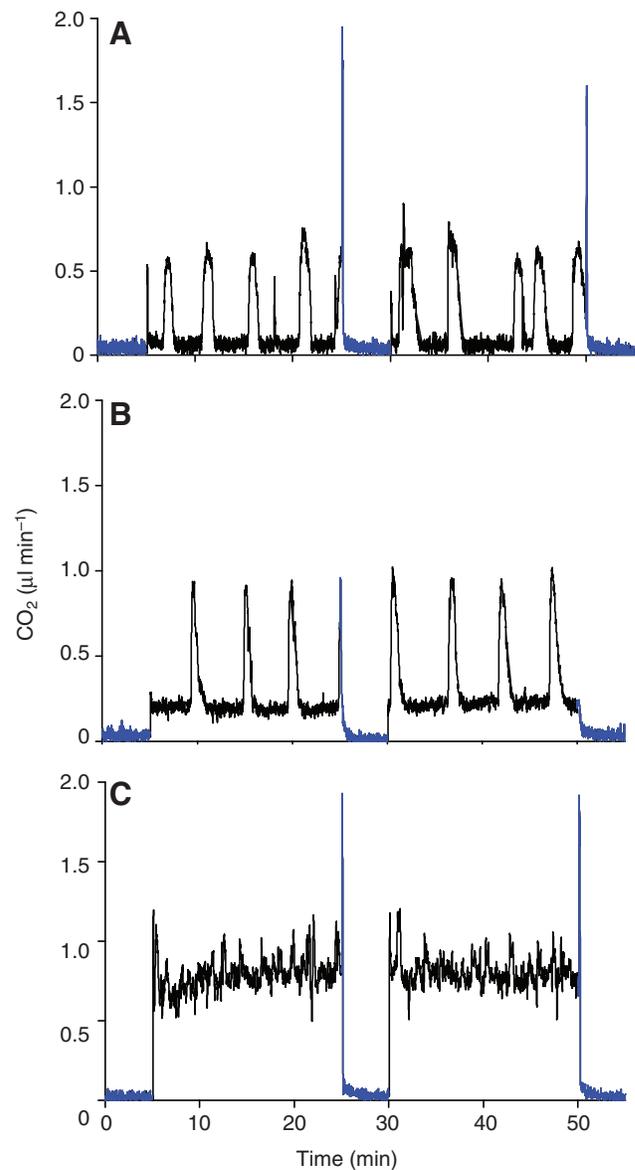


Fig. 1. Examples of (A) discontinuous, (B) cyclic and (C) continuous respiration in male *Rhodnius prolixus*. The experimental trial lasted 55 min with three 5 min baselines (where an empty chamber was read) at 0–5 min, 25–30 min and 50–55 min (blue line) and two 20 min experimental readings at 5–25 min and 30–50 min (black line).  $CO_2$  bursts were observed in all treatments although the overall pattern seemed to differ as metabolic rate increased.

determined for each run. CO<sub>2</sub> was considered to be in a burst if: (1) the ratio of release exceeded twice this average and (2) this period of CO<sub>2</sub> release was preceded or followed by a period in which CO<sub>2</sub> values fell below the average release for that run. Using this definition, the number of bursts for each individual, under each treatment, was recorded and averaged.

In order to obtain an average rate of CO<sub>2</sub> release for the four experimental conditions, the Boolean:data application in Expedata software was used to format each experimental trace. Following the above guidelines, CO<sub>2</sub> values that were not part of a burst were deleted. After this deletion, the experimental trace was corrected so that values were re-zeroed and the average release of CO<sub>2</sub> during bursts was calculated.

The average number of bursts and the rate of CO<sub>2</sub> release per burst under the four treatments were analyzed using a repeated measures analysis of variance (ANOVA).

### Results

Fig. 1 shows the rate of CO<sub>2</sub> release from insects as revealed by flow-through respirometry. All three traces are derived from adult males of *Rhodnius prolixus* and illustrate the three insect respiratory patterns described in the Introduction.

In Fig. 1A, the insect is employing the DGC. Large bursts are interspersed with periods of near-zero CO<sub>2</sub> release. In Fig. 1B the insect is employing the cyclic pattern. In this pattern, bursts of CO<sub>2</sub> release still occur with a certain degree of regularity. Between bursts, however, the release of CO<sub>2</sub> rarely if ever goes completely to zero. This is generally interpreted as an oscillation between an open phase and a phase of relatively reduced release. In Fig. 1C the insect is employing the continuous pattern in which no obvious

rhythmic bursts of CO<sub>2</sub> release are observed, and no extended periods of spiracular closure are seen.

As discussed in the Introduction, one of the leading hypotheses regarding the function of the DGC is that it serves to reduce respiratory water loss. If this is true, one might expect insects to abandon the DGC when not under water stress or when in humid air. Slama and colleagues have reported that the termite *Prorethinius simplex* uses the DGC in dry air but not humid air (Slama et al., 2007). We explored this issue as well using adult male *Rhodnius*. When the insects had a low metabolic expenditure (i.e. when non-locomotory and non-fed) they always used the DGC in both dry and humid air (Fig. 2A,C). When fed a bloodmeal, the insects transitioned to a cyclic pattern (i.e. with an absence of closed periods) due to the increase in metabolic rate associated with specific dynamic action (Bradley et al., 2003), but burst releases of CO<sub>2</sub> continued in both dry and humid air (Fig. 2B,D).

The types of measurements shown in Fig. 2 were replicated on 10 adult male *Rhodnius*. These data were analyzed using a repeated measures ANOVA (Table 1). It can be seen that neither feeding nor increases in external humidity had a statistically significant effect on the number of bursts measured over the 40 min experimental period. Similarly, the average of the maximum rate of CO<sub>2</sub> release from these bursts was unaffected by these treatments.

### Discussion

To understand the diversity of the respiratory patterns observed in insects, we should begin with a fuller understanding of the DGC. The DGC is characterized by a period in which the spiracles open (the open phase) and gases are free to diffuse and/or exchange between the tracheal space and the external atmosphere. It has been

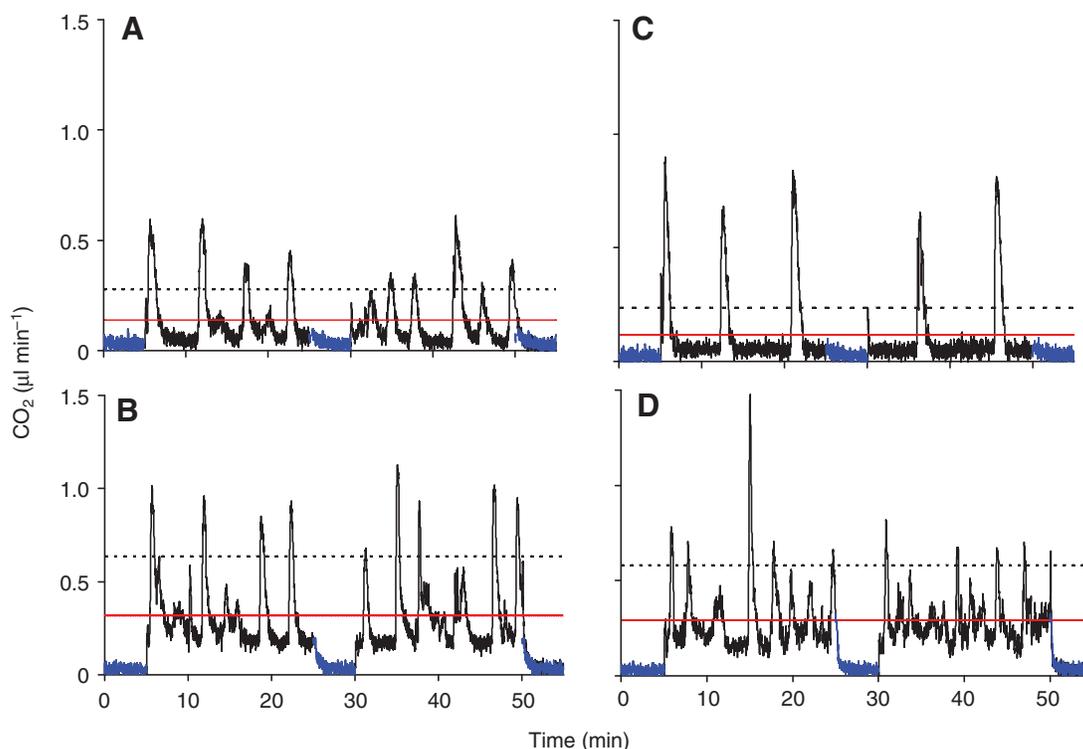


Fig. 2. Four experimental traces for a single insect under (A) dry unfed, (B) dry fed, (C) humid unfed and (D) humid fed conditions. The experimental trial lasted 55 min with three 5 min baselines (where an empty chamber was read) at 0–5 min, 25–30 min and 50–55 min. CO<sub>2</sub> bursts were observed in all treatments although the overall pattern seemed to differ between fed and unfed individuals. Bursts were defined as values above twice the  $\dot{V}_{CO_2}$  average (dashed line) for that trial, which were preceded or followed by at least a minute of values falling below the  $\dot{V}_{CO_2}$  average (red solid line).

Table 1. Analysis of the effects of feeding and environmental humidity on the CO<sub>2</sub> bursts in *Rhodnius prolixus*

	Dry, unfed	Dry, fed	Humid, unfed	Humid, fed	<i>F</i>	d.f.	<i>P</i>
Number of bursts	7.2±0.79	7±0.77	8.1±1.06	7.9±0.66	0.38	3	0.69
Burst $\dot{V}_{CO_2}$ ( $\mu\text{l min}^{-1}$ )	0.022±2.69E-3	0.016±3.69E-3	0.021±3.32E-3	0.012±3.35E-3	1.29	3	0.30

Top line, mean number ( $\pm$ s.e.m.) of CO<sub>2</sub> bursts in *Rhodnius* over the 40 min experimental period.

Bottom line, mean ( $\pm$ s.e.m.) maximal rate of CO<sub>2</sub> release ( $\mu\text{l min}^{-1}$ ) achieved in the CO<sub>2</sub> bursts produced under each of the conditions examined.

d.f., degrees of freedom.

demonstrated (Hetz and Bradley, 2005) that the partial pressure of oxygen in the tracheae during the open phase approaches a value very similar to the external  $P_{O_2}$ . Fig. 3 is a diagrammatic representation of the  $P_{O_2}$  in the tracheae during the open phase and closed phase of the DGC. In this figure a peak  $P_{O_2}$  of about 18 kPa is reached in the tracheae. The open phase is followed by the closed phase. During this period of spiracular closure, the  $P_{O_2}$  drops substantially due to the consumption of oxygen through aerobic metabolism. Eventually, the  $P_{O_2}$  reaches a critical low level (shown as being about 3.5% in Fig. 3) and the spiracles begin to flutter. During the flutter phase the level of oxygen is closely regulated (Hetz and Bradley, 2005).

Following the above logic, the length of the closed phase should be negatively correlated with the metabolic rate of the insect. The effect of an increase in metabolic rate can be seen by comparing Fig. 3A with Fig. 3B. In the latter, the increased metabolic rate of the insect leads to a shortening of the closed phase because the  $P_{O_2}$  inside the insect drops more rapidly.

We propose that it is the insect's metabolic rate that determines the pattern of respiration observed. In an insect employing the DGC, as the insect's metabolic rate begins to increase, the closed

phase will get shorter and shorter until it can no longer be discerned (Levy and Schneiderman, 1966; Lighton, 1996). At this point the insect will have transitioned to the cyclic pattern, in which bursts of CO<sub>2</sub> release continue to be observed but the closed phase is missing. Further increases in metabolic rate will require the spiracles to open more fully in the flutter phase, allowing more CO<sub>2</sub> to escape in this phase. Finally, when the metabolic rate is sufficiently high, oxygen entry will match CO<sub>2</sub> release, resulting in a continuous pattern of respiration in which no large, rhythmic bursts of CO<sub>2</sub> are observed.

The transitions described above are consistent with our observations in *Rhodnius* (Fig. 1). The insect in Fig. 1A was measured unfed at 25°C in the absence of locomotory activity. These factors led to a low metabolic rate (0.194  $\mu\text{l CO}_2 \text{ min}^{-1}$ ) and the insect employed the DGC as a respiratory pattern. The insect shown in Fig. 1B was held at 25°C but was fed. It therefore had a higher metabolic rate (0.301  $\mu\text{l CO}_2 \text{ min}^{-1}$ ), resulting in the use of the cyclic pattern of respiration. The insect in Fig. 1C was measured at 35°C and was also actively moving during the period shown. At the resulting high metabolic rate (0.802  $\mu\text{l CO}_2 \text{ min}^{-1}$ ) the insect exhibited continuous respiration.

In summary, the respiratory pattern employed by an insect is affected by three factors. The length of the closed phase is (1) positively correlated with the partial pressure of oxygen in the atmosphere surrounding the insect, (2) positively correlated with the amount of oxygen stored in the insect at the end of the open phase (presumably the sum of the gaseous oxygen in the tracheae and the dissolved oxygen in the tissues and hemolymph), and (3) negatively correlated with the metabolic rate of the insect. The external oxygen concentration in the atmosphere varies very little in most habitats, but it can be substantially lower for insects underground and it certainly can be manipulated in the laboratory. The amount of oxygen stored in the insect at the end of the open phase is probably most significantly affected by the volume of air contained in the trachea upon spiracular closure. This will vary from one insect species to another and may be a major factor in explaining species-specific respiratory patterns. It has been shown, however, that growth within an instar and feeding can reduce tracheal volume (Greenlee and Harrison, 2003). Despite the importance of the above factors influencing oxygen delivery, we submit that the major variable affecting respiratory patterns in insects is the metabolic rate of the insect. This can vary over more than an order of magnitude in many insects and therefore is the major variable affecting respiratory pattern.

The hygric hypothesis, which has been the leading explanation for the DGC in textbooks of insect physiology for the past 50 years, has now been questioned by several researchers. Many insects do not show DGC under conditions where the hygric hypothesis would suggest they should (Hadley and Quinlan, 1993; Chappell and Rogowitz, 2000; Chown and Holter, 2000), while other studies indicate that the DGC does not reduce respiratory water loss compared with other respiratory patterns (Williams and Bradley, 1998; Gibbs and Johnson, 2004; Lighton and Turner, 2008).

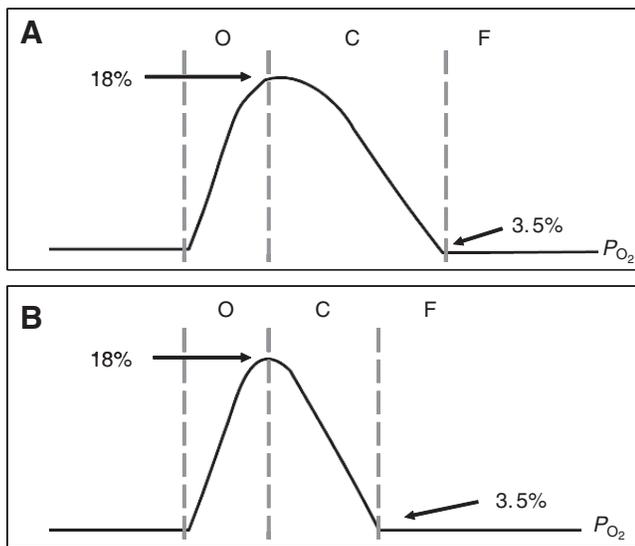


Fig. 3. A diagrammatic representation of the  $P_{O_2}$  in the tracheal lumen of an insect during the open (O), closed (C) and flutter (F) phases of the discontinuous gas-exchange cycle (DGC). The partial pressure of O<sub>2</sub> increases during the open phase and decreases during the closed phase. The closed phase is terminated when the partial pressure of oxygen reaches a critically low level (3.5% in this example). During the flutter phase the insect regulates the spiracles to maintain a steady but low  $P_{O_2}$ . When the insect is experiencing a low metabolic rate (A) the closed phase is relative long. At higher metabolic rates (B) the closed phase is shorter because the oxygen present in the tracheae and tissues is more rapidly depleted.

The oxidative damage hypothesis not only explains the presence of the DGC in insects at low rates of metabolism but, in the extension we offer here, now also provides a rationale for the other respiratory patterns observed in insects. The hypothesis is testable through the manipulation of external  $P_{O_2}$ , humidity and the metabolic rate of the insect. We look forward to further tests of this hypothesis and the insights these experiments will provide into the control of respiration and metabolism in insects.

### Epilogue

One of us (T.J.B.) had the pleasure of working with Simon Maddrell in British Columbia, Cambridge and California. I came away impressed with Simon's experimental expertise, theoretical insights and, not least, the speed with which he could dissect the Malpighian tubules out of *Rhodnius prolixus*. I am left with the feeling that the next generation of insect physiologists will have a hard time matching Simon's expertise. I offer therefore this:

### Ode to Simon

O Simon, who shall forthwith tease the tube?  
 Can yet any man or maid so rapidly divide  
 And pin and pluck the tissue from the chaff?  
 I swear, the youngsters strive and yet they seem the rube.  
 They lack the touch, the techniques true and tried.  
 They strive to match the master but alas, they come up half.

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