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RESEARCH ARTICLE

Temperature-dependent variation in gas exchange patterns and spiracular control in *Rhodnius prolixus*

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ABSTRACT

Insects display an array of respiratory behaviors, including the use of discontinuous gas exchange. This pattern is characterized by periods of spiracular closure, micro-openings (flutter), and complete openings during which the majority of gas exchange takes place. A current model of insect spiracular control suggests that spiracles are controlled by two interacting feedback loops, which produce the discontinuous pattern. The flutter period is thought to be initiated by a critically low partial pressure of oxygen, while the open period is initiated by a critically high CO_2 threshold. The goal of our study was to test this control model under conditions of feeding-induced or temperature-induced changes in metabolic rate. We manipulated the metabolic rate of the insect *Rhodnius prolixus* using two discrete mechanisms: (1) feeding the insects a bloodmeal or (2) exposing them to a range of temperatures (18–38°C). Examining the variation in the gas exchange patterns produced by insects in each of these treatments allowed us to determine whether spiracular control is sensitive to metabolic rate and/or temperature. We found that increases in temperature caused significant decreases in open phase burst volumes and premature abandonment of discontinuous gas exchange cycles. These effects were not observed in fed individuals maintained at a single temperature despite their higher metabolic rates. Our results indicate that some part of the spiracular control mechanism is temperature sensitive, suggesting a possible role for pH in CO_2 sensing.

KEY WORDS: DGC, Insect, Metabolism, Respiration, Spiracles, Temperature

INTRODUCTION

The insect respiratory system provides tightly controlled and highly efficient mechanisms for oxygen uptake and carbon dioxide release. It is composed of a network of air-filled tracheal tubes that open to the atmosphere along the lateral sides of the body and branch throughout the insect to deliver oxygen directly to metabolically active tissues. Spiracular valves, located at each tracheal opening, control the exchange of gases between the atmosphere and the tracheal system. Although much is known about the responses of the spiracles to different stimuli, it remains unclear how and where respiratory gases are sensed. Understanding these aspects of spiracular control is a challenging problem because gas exchange in insects is uncoupled from hemolymph circulation. Insects face additional challenges in that they must control their respiratory behavior to account for changes in metabolic rate due to activity as well as temperature.

Insects utilize different patterns of respiratory gas exchange depending on their metabolic demands (Contreras and Bradley, 2009). These demands may vary as a result of fluctuations in ambient temperature, activity, or other costly processes such as digestion and reproduction. The most extensively studied gas exchange pattern is the discontinuous gas exchange cycle, which has been described in at least seven different insect orders (Lighton, 1996; Marais et al., 2005; Gray and Bradley, 2006; Chown et al., 2006; Contreras and Bradley, 2009). This behavior consists of three phases: an open phase in which the spiracles open to allow gas exchange to occur, a closed phase in which the spiracular valves remain tightly sealed for a prolonged period of time, and a flutter phase in which micro-openings of the spiracles allow oxygen to enter the body while a low oxygen partial pressure is maintained within the insect.

In the present study we used *Rhodnius prolixus* (Stål 1859) to test two current models of insect respiratory control, one concerning the effects of metabolic rate and one addressing spiracular control. The first is the model proposed by Contreras and Bradley (Contreras and Bradley, 2009), which suggests that insect gas exchange patterns are determined largely by metabolic rate. They propose that the pattern changes from discontinuous to continuous as metabolic rate is increased. The second model we have tested is Förster and Hetz's dual feedback model of spiracular control. This model indicates that spiracle activity is regulated by internal partial pressures of carbon dioxide and oxygen (Burkett and Schneiderman, 1974; Förster and Hetz, 2010). This model proposes that at the end of a spiracular closed phase, critically low oxygen content within the insect triggers the spiracles to begin fluttering, allowing oxygen to enter the tracheal system. As very little CO_2 is released by the insect during the flutter period, eventually the increasing internal CO_2 content triggers a second feedback mechanism to initiate spiracular opening when a critically high P_{CO_2} is reached. Chown and Holter (Chown and Holter, 2000) referred to this mechanism via their emergent properties hypothesis and argue that discontinuous gas exchange emerged as a function of this physiological feedback rather than serving any adaptive function (Chown et al., 2006). This control system consists of two interacting feedback loops that monitor internal P_{O_2} and P_{CO_2} and trigger spiracular opening events when critical thresholds are reached.

The precise mechanism by which the partial pressures of these gases are detected is unknown. It is also unclear what triggers the start of the closed phase. It is conceivable that spiracular closure may be initiated when sufficient CO_2 has been released to reach a critically low value, or when diffusion of oxygen to the tracheal system produces a critically high oxygen value. Conversely, spiracular openings may be of a constant duration resulting from the timing of neural inputs or spiracle-opening muscle contractile properties.

Based on the Förster and Hetz dual feedback loop model of the discontinuous gas exchange pattern, an increase in metabolic rate should lead to an increase in the frequency of open phases, as at higher metabolic rates CO_2 accumulates in the insect faster during

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the closed and flutter phases. Conversely, the volume of CO_2 released per respiratory burst should remain unchanged as it reflects the amount of accumulated CO_2 required to reach the threshold partial pressure that triggers spiracular opening. In contrast to this expectation, it has been demonstrated that increasing the metabolic rate by increasing temperature leads to a decreased volume of CO_2 released by both a silkworm and an ant during an open phase (Schneiderman and Williams, 1955; Lighton, 1988). A decreased volume of CO_2 release during a burst might indicate a reduction in critical P_{CO_2} required to open the spiracles. Consequently, these results might indicate that either the CO_2 sensor is itself sensitive to temperature or there is an effect of temperature on the P_{CO_2} detected by the sensor.

Rhodnius prolixus provides certain valuable features as a model insect for studying respiratory control models. *Rhodnius prolixus* is a blood-feeding insect. Before each molt, the insect ingests and processes a blood meal, which leads to an increase in metabolic rate up to 10 times that of the resting individual over 15 days (Zwicky and Wigglesworth, 1956; Bradley et al., 2003). However, in the absence of an appropriate host in the vicinity, the insects remain quite still. Therefore, after a period of acclimation, the insects remain very still in a respirometer. Additionally, although *Rhodnius* breathe discontinuously, they show no external signs of active ventilation. Finally, the metabolic rate of *Rhodnius* can be modified in two distinct ways, namely through changes in temperature or by feeding. This allows us to test models of respiratory control using two means of adjusting metabolic rate, and thus respiratory demand.

Here, we tested the Contreras and Bradley model (Contreras and Bradley, 2009) by exposing *R. prolixus* to two different metabolic stimuli: feeding and temperature manipulation. We then characterized and measured different aspects of the gas exchange pattern to determine whether metabolic rate alone predicts the gas exchange pattern utilized by the insect or whether there are additional factors that contribute to respiratory behavior. The Förster and Hetz spiracular control model was tested by comparing measurements of burst frequency and volume at different temperatures and metabolic rates. This model, as described above, indicates that the volume of CO_2 released in a respiratory burst should remain constant regardless of metabolic rate as an internal P_{CO_2} threshold controls spiracular opening. Elevated metabolic rates should therefore be compensated for exclusively by increased burst frequency.

To further examine the spiracular control model, we recorded CO_2 release patterns from insects exposed to hyperoxia to determine whether elevated atmospheric oxygen affected respiratory control. While the trigger for spiracular opening events appears to be tightly linked to intratracheal P_{CO_2}, the mechanism responsible for triggering the end of the open phase remains unknown. We hypothesized that if a critically high oxygen threshold triggers the end of the spiracular open phase, then insects exposed to atmospheric hyperoxia would produce decreased CO_2 burst volumes compared with insects in normoxic environments. An increased partial pressure gradient from the atmosphere to an internal oxygen sensor would decrease the time required to reach the oxygen threshold and trigger spiracular closure. Combined, these experiments test two models of spiracular control, and may provide insight into the molecular mechanism by which insects detect metabolic gases.

RESULTS

Metabolic variation in feeding and temperature manipulation trials

Fed individuals displayed a progressive increase in metabolic rate after feeding until about day 10, when metabolic rate began to decrease (Fig. 1A). The mean (±s.d.) peak metabolic rate for fed individuals (37.938±5.098 µl h^{-1}) was 13.9 times higher than the mean resting metabolic rate (2.732±0.597 µl CO_2 h^{-1}) for these insects at 26°C, the normal rearing temperature. When unfed individuals were exposed to a range of temperatures from 18 to 38°C, mean metabolic rate increased with temperature (ANOVA, $F_{10,108}$=24.05, P<0.001; Fig. 1B) and peak metabolic rates at 38°C were on average 3.5 times higher than the resting rate. There was no significant difference between the mean metabolic rate at 26°C across the two temperature trials ($t_{9.841}$=–0.5574, P=0.5897). Near the rearing temperature for these individuals, metabolic rate remained fairly constant, but decreased at temperatures below 26°C and increased at temperatures greater than 32°C. Metabolic rates of individuals in the temperature treatment and feeding treatment overlapped only during the first measurements made immediately after feeding, after which, metabolic rates of fed individuals surpassed the highest metabolic rates observed in temperature trials.

Unfed *Rhodnius* exhibit discontinuous gas exchange with discrete bursts of CO_2 release and intervening periods of very low CO_2 release, often indistinguishable from zero. Traditionally, the discontinuous gas exchange cycle has been characterized by the presence of three periods, the closed period, followed by the flutter period, followed by the open period. During flutter, the spiracles open briefly to allow oxygen into the tracheal system and to regulate the tracheal P_{O_2}. During this phase, the volume of CO_2 released is much smaller than the volume of oxygen taken up because of the larger partial pressure gradient for oxygen. As a result, only a very limited release of CO_2 occurs during spiracular flutter. CO_2 release during flutter is further limited in unfed *Rhodnius*, as a result of the notably low oxygen demand (Bradley et al., 2003). The difficulty in distinguishing the closed from the flutter phase on the basis of the

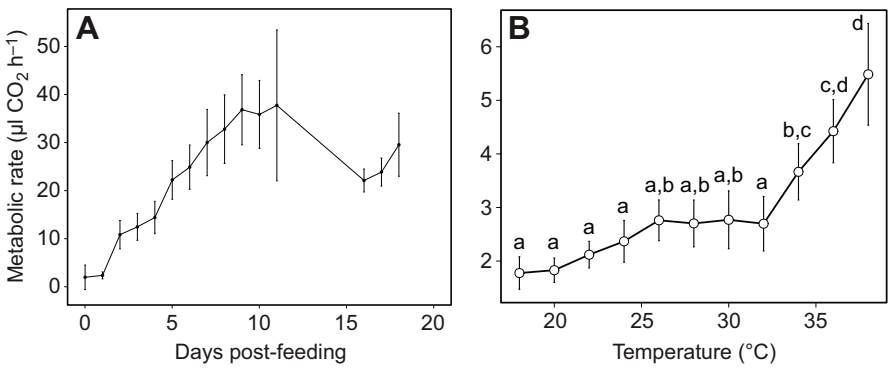

Fig. 1. Metabolic variation in temperature and feeding trials. (A) The rate of CO_2 release over time (days post-feeding) in seven, fifth instar *Rhodnius prolixus*. (B) The rate of CO_2 release as a function of ambient temperature in 14 unfed, fifth instar *R. prolixus*. Error bars represent 95% confidence intervals. Different letters indicate a significant difference between values.

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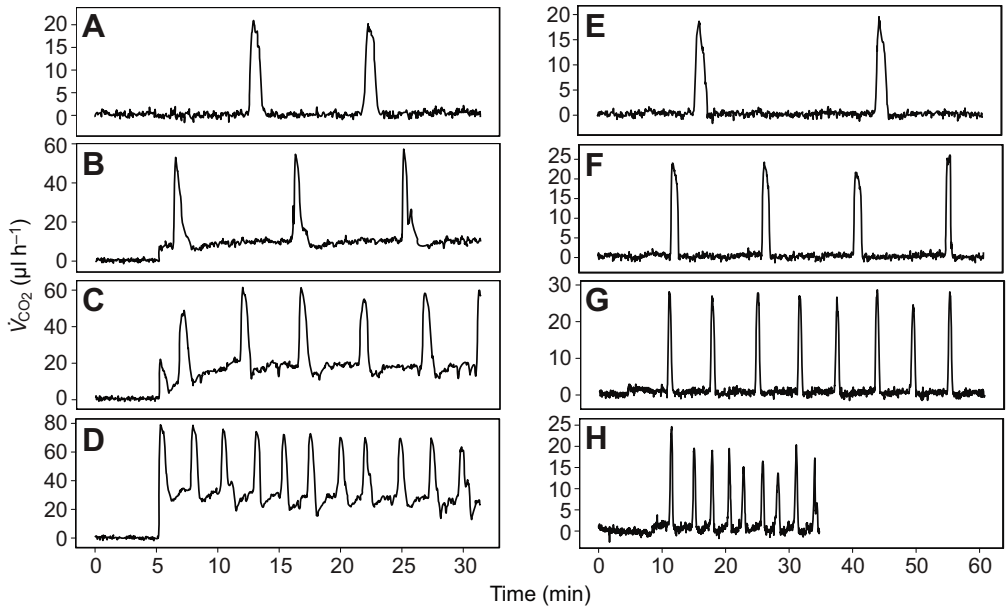

Fig. 2. Examples of respiratory patterns observed in fed and unfed *R. prolixus*. Each set of graphs displays the rate of CO_2 release over time from a single individual from each treatment. Shaded regions indicate baseline recordings. (A–D) Sample respiratory patterns for fed *R. prolixus* as metabolic rate increases at 0, 2, 6 and 10 days post-feeding, respectively. (E–H) Sample respiratory patterns for unfed *R. prolixus* at 18, 24, 30 and 36°C, respectively. Recordings were shortened at very high temperatures (H) as the high burst frequency allowed the appropriate measurements to be made over a shorter time period.

amount of CO_2 released has caused many investigators to treat these two phases as a single interburst phase (Chappell and Rogowitz, 2000; Shelton and Appel, 2001; Duncan et al., 2010). However, in our experiments, particularly at higher temperatures, a flutter phase is discernible, in that the rate of CO_2 release in the closed phase was distinctly lower than that in the flutter phase (Fig. 2H; supplementary material Fig. S1).

When insects were equilibrated to higher temperatures, the time interval between bursts was shortened, reflecting the higher metabolic rate of the insects. When the time between bursts becomes shorter than the time constant of the chamber, observed CO_2 release appears continuous (Gray and Bradley, 2006). As such, one out of six individuals in the first temperature treatment group and three out of six individuals in the second treatment group ceased to produce detectable discontinuous bursting patterns at 38°C with a flow rate of 200 ml min^{-1}. As a result, the recorded CO_2 release was continuous and characteristics of the gas exchange pattern were no longer examined at this temperature and above.

When *Rhodnius* were allowed to take blood meals to repletion, metabolic rate increased substantially over a period of several days (Fig. 1A), resulting in a CO_2 release pattern with more frequent bursts and an elevated rate of interburst CO_2 release (Fig. 2A–D). More O_2 is required between bursts in order to support the higher aerobic metabolic rate. This oxygen is supplied by increasing the duration and/or frequency of spiracular micro-openings during the interburst period. The increased uptake of oxygen therefore results in an increased rate of CO_2 release during flutter (Fig. 2D). In some traces, the rate of CO_2 release immediately after an open phase is lower than later in the interburst period (Fig. 2C,D). We interpret this to mean that there is a short closed phase after the open phase, but the high metabolic rate dictates a rapid transition to the flutter phase in fed individuals.

Respiratory characteristics in feeding trials

When fed a bloodmeal, *R. prolixus* maintained a discontinuous pattern of CO_2 release even as the metabolic rate increased. However, variations in the characteristics of the discontinuous pattern were observed. When burst volume (BV) was measured as the increase over the interburst release rate, the volume increased with an increase in metabolic rate (MR) (Fig. 3A; BV=0.003MR+0.266, R^2=0.217, $F_{1,75}$=22.03, P<0.001). When the burst volume was measured as the increase relative to a zero baseline (see Fig. 4, volume measurement 2), this relationship became more pronounced (BV=0.015MR+0.261, R^2=0.838). Respiratory bursts became shorter in duration as metabolic rate increased (Fig. 3B; duration=–0.549MR+76.132, R^2=0.401, $F_{1,75}$=51.82, P<0.001). The average cycle period displayed an initial decrease with increasing metabolic rate but began to reach a plateau at an intermediate metabolic state (Fig. 3C; period=–138.5lnMR+649.27, R^2=0.75, $F_{1,75}$=225.27, P<0.001). A decrease in burst duration and an increase in the volume of CO_2 released in a burst resulted in a significant increase in the calculated CO_2 release rate (burst volume/burst duration) (Fig. 3D; rate=0.00009MR+0.003, R^2=0.673, $F_{1,75}$=157.2, P<0.001).

Respiratory characteristics in temperature trials

Unlike the increase in burst volume that occurred with increased metabolic rate following feeding, burst volume decreased with an increase in temperature (T) (Fig. 5A, ANOVA, $F_{10,108}$=24.51, P<0.001; BV=–0.024T+0.980 above 27°C). Burst duration and cycle period also decreased with increasing temperature (Fig. 5B, ANOVA, $F_{10,108}$=122.4, P<0.001; Fig. 5C, $F_{10,108}$=51.63, P<0.001). A one-way ANOVA also indicated a significant relationship between the burst phase CO_2 release rate and temperature (Fig. 5D, $F_{10,108}$=4.694, P<0.001), with a Tukey HSD test demonstrating that all rates were similar with the exception of the rate measured at 38°C, which was significantly lower than rates at all temperatures except for 18 and 22°C.

Effects of hyperoxia on discontinuous gas exchange pattern characteristics

Table 1 provides results of two-way ANOVA tests performed on fed and unfed *R. prolixus* in 21% and 40% oxygen treatments. There was no significant effect of oxygen concentration on the metabolic rates of the unfed or fed groups. The volume of CO_2 released in a respiratory burst was not affected by feeding, but increased significantly in fed individuals exposed to hyperoxia (Fig. 6A). There was no effect of oxygen concentration on the burst duration (Fig. 6B). However, feeding had a significant effect on burst duration, with fed individuals displaying significantly longer bursts.

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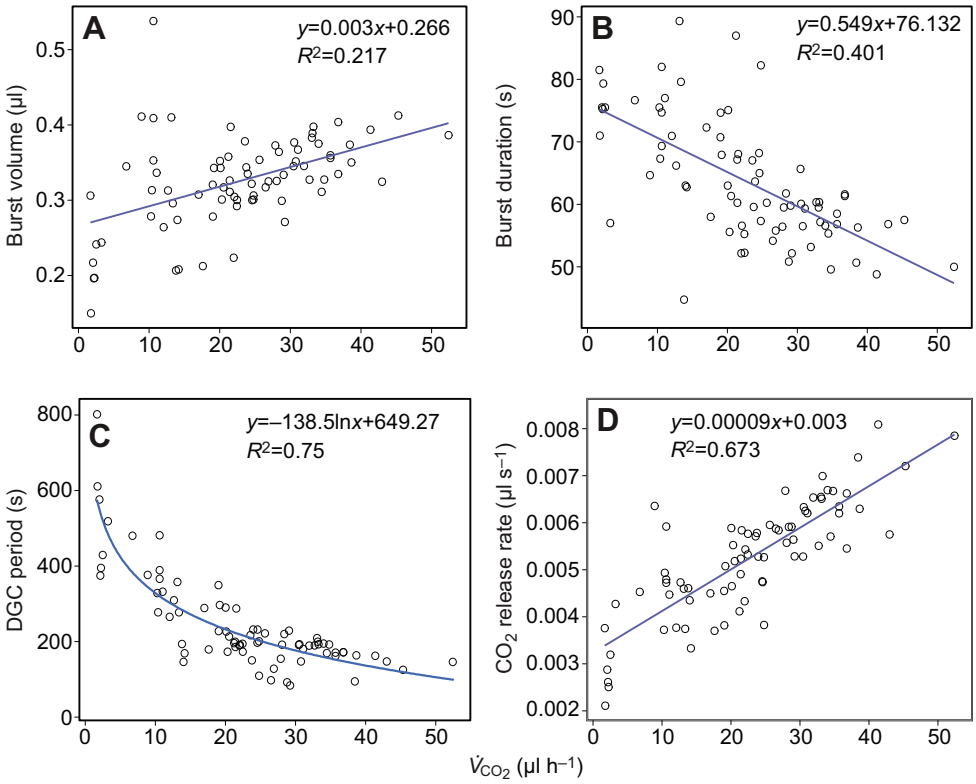

Fig. 3. Effects of feeding-induced changes in metabolic rate on measured respiratory characteristics. The change in burst volume (A), burst duration (B), discontinuous gas exchange cycle (DGC) period (C) and open phase CO_2 release rate (D) as a function of metabolic rate in fed *R. prolixus*. A slight increase in burst volume was observed as metabolic rate increased. This increase was more pronounced if burst volume measurements included the steady-state CO_2 release rate during interburst periods. An increase in burst volume and a decrease in burst duration resulted in a significant increase in the rate of CO_2 release during a spiracular opening period. The cycle period decreased initially and leveled off near 150 s per cycle. Blue lines are linear regressions in A, B and D and a logarithmic regression in C. For A–D, $P<0.001$.

The cycle period was significantly increased in fed compared with unfed individuals at 21% and 40% oxygen (Table 1). There was a significant effect of oxygen concentration on the rate of CO_2 release during a burst in fed individuals (Table 1), with the rate being higher in 40% oxygen. There was also a higher rate of CO_2 release during a burst in unfed individuals compared with fed individuals at 21% oxygen.

DISCUSSION

The dual feedback model of insect respiratory control (Levy and Schneiderman, 1966; Chown and Holter, 2000; Förster and Hetz, 2010) proposes that spiracular activity is controlled via two feedback mechanisms that regulate internal partial pressures of oxygen and CO_2. Spiracles initiate a fluttering response when tracheal P_{O_2} reaches a critically low threshold and spiracles open when tracheal P_{CO_2} reaches a critically high threshold. The interaction between these two sensory systems produces a pattern of respiratory CO_2 release in which large bursts of CO_2 are separated by prolonged periods of spiracular closure or low CO_2 release rates.

The presence of a critical P_{CO_2} threshold dictates that the amount of CO_2 released in a single respiratory burst, or spiracular open period, would remain constant as long as the threshold value does not change. However, many studies have demonstrated that the respiratory burst volume decreases with increased temperature in ants (Lighton, 1988; Quinlan and Lighton, 1999; Vogt and Appel, 2000), beetles (Duncan and Dickman, 2001) and the cecropia silkworm (Schneiderman and Williams, 1955). Others have found no change in burst volume with increasing temperature (Davis et al., 1999; Klok and Chown, 2005), or even an increase in burst volume (Shelton and Appel, 2001; Basson and Terblanche, 2011). Our results from temperature manipulation experiments in *R. prolixus* show a decrease in burst volume with increasing temperature (Fig. 5). Above the rearing temperature of 27°C, burst volume decreased linearly with temperature (BV=–0.024*T*+0.980). These

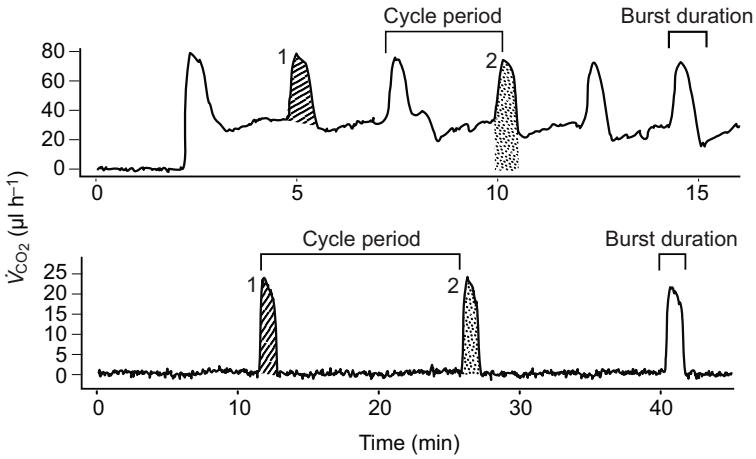

Fig. 4. Methods of data collection during different metabolic states. Two examples are shown. Burst volumes were collected as the volume of CO_2 released above the steady-state release during flutter (1), or as the total CO_2 release during an opening event (2). Twelve bursts were sampled from each individual to find an average burst volume for each treatment. Cycle periods were defined as the time between the start of one burst and the start of the next consecutive burst. Burst durations were defined as the time from the start of the burst to the point at which CO_2 release returns to the steady-state value.

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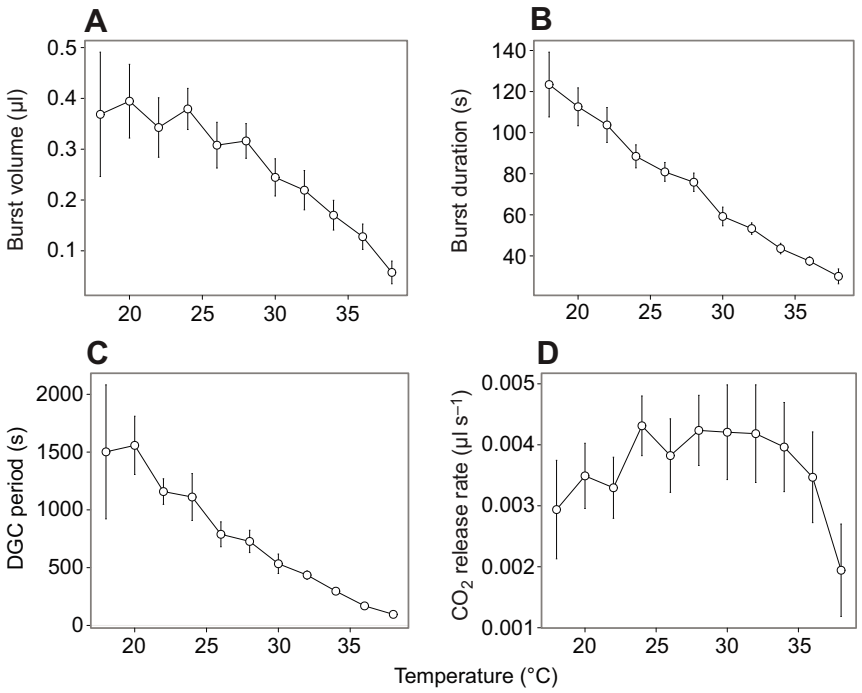

Fig. 5. Effects of temperature-induced changes in metabolic rate on measured respiratory characteristics. The change in burst volume (A), burst duration (B), discontinuous gas exchange cycle (DGC) period (C) and open phase CO_2 release rate (D) as a function of metabolic rate in unfed *R. prolixus* at temperatures ranging from 18 to 38°C. Increases in temperature led to decreases in burst volume, burst duration and cycle period. There was no significant change in the rate of CO_2 release during a burst over the range of 18 to 34°C. Error bars represent 95% confidence intervals.

results are inconsistent with expectations based on the dual feedback model, unless the critical CO_2 threshold changes with temperature.

To determine whether these changes in gas exchange pattern were due to a direct effect of temperature or an indirect effect on metabolic rate, we also measured these characteristics in gas exchange patterns obtained from *R. prolixus* during digestion of a blood meal. Throughout the duration of meal processing, *R. prolixus* experiences a range of metabolic rates up to 13 times their resting metabolic rate. In contrast to our observations from temperature trials, we found that burst volumes within the population did not decrease with increased metabolic rate but actually increased slightly (Fig. 3), regardless of whether burst volume was measured as the increase over flutter release rates or baseline zero values. However, these volume increases are much smaller in scale than the decreases observed in temperature trials. As a result, we conclude that the dual feedback model of spiracular control adequately describes respiratory behavior and can accommodate metabolic variation at a single temperature. The conundrum remains, however, that some aspect of the respiratory control mechanism is temperature sensitive and that the respiratory control model becomes more complex if changes in temperature are considered.

Effects of temperature on gas exchange patterns

There was a 3.5-fold increase in the average metabolic rate of *R. prolixus* over the temperature range of 18 to 38°C (Fig. 1). In addition to these changes in metabolism, the increase in temperature also induced changes in the gas exchange pattern. As temperature increased there was a significant decrease in the volume of CO_2

Table 1. Two-way ANOVA results for all respiratory variables in oxygen treatment trials

Measurement	Source	SS	d.f.	MS	*F*	*P*
Metabolic rate (µl CO_2 h^{-1})	Feeding	3277	1	3277	289.992	**<0.001**
	Oxygen	1	1	1	0.058	0.812
	Feeding:oxygen	3	1	3	0.231	0.635
	Error	271	24	11		
Burst volume (µl)	Feeding	0.002	1	0.002	1.067	0.312
	Oxygen	0.057	1	0.057	24.861	**<0.001**
	Feeding:oxygen	0.014	1	0.014	6.096	**0.021**
	Error	0.055	24	0.002		
Cycle period (s)	Feeding	2.3×10^{6}	1	2.3×10^{6}	181.584	**<0.001**
	Oxygen	43,785	1	43,785	3.445	0.0758
	Feeding:oxygen	58,103	1	58,103	4.572	**0.043**
	Error	3.1×10^{5}	24	12,709		
Burst duration (s)	Feeding	3412	1	3412	90.13	**<0.001**
	Oxygen	94	1	94	2.491	0.128
	Feeding:oxygen	90	1	90	2.384	0.136
	Error	909	24	38		
Open phase CO_2 release rate (µl s^{-1})	Feeding	1.1×10^{-5}	1	1.1×10^{-5}	17.264	**<0.001**
	Oxygen	5.5×10^{-6}	1	5.5×10^{-6}	8.942	**0.006**
	Feeding:oxygen	3.9×10^{-6}	1	3.9×10^{-6}	6.276	**0.019**
	Error	1.5×10^{-5}	24	6.2×10^{-7}		

Bold indicates significance.

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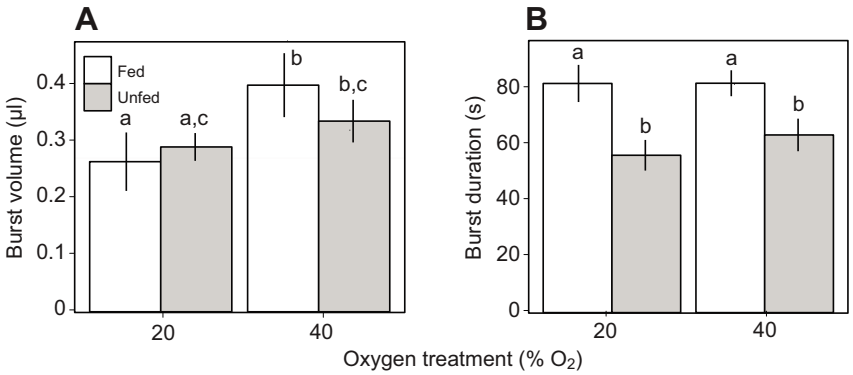

Fig. 6. Effects of oxygen content and feeding status on respiratory pattern. Respiratory burst volumes (A) and burst durations (B) for fifth instar *R. prolixus* under two treatments (unfed or fed a complete bloodmeal) following exposure to normoxic air (21% O_2) or acute exposure to hyperoxic (40% O_2) air. Error bars indicate 95% confidence intervals. See Table 1 for ANOVA results on all measured variables.

released during a burst as well as a decrease in burst duration (Fig. 5). We found no change in the rate of release of CO_2 during a burst with the exception of a decrease in rate at 38°C (Fig. 5). Cycle period decreased with increased temperature. In some insects, bursts became so frequent that they were no longer distinguishable via flow-through respirometry, and the pattern therefore appeared as continuous gas exchange. Overall, the results of the temperature experiments are consistent with the findings of Contreras and Bradley (Contreras and Bradley, 2009), in that CO_2 release patterns transitioned from discontinuous to continuous as metabolic rate increased with temperature.

Effects of feeding on gas exchange patterns

Fed insects experienced a much larger range of metabolic rates compared with temperature trial rates. Throughout the duration of meal processing, average peak metabolic rates increased up to 13.9 times the average resting value. However, despite the larger increase in metabolism, fed insects continued to produce discontinuous patterns of CO_2 release throughout the experiment. In contrast to the gas exchange pattern changes observed in temperature trials, burst volumes increased slightly and burst durations decreased with metabolic rate in fed insects (Fig. 3). As a result, CO_2 release rates (volume/duration) during a burst increased with metabolic rate. Fed insects produced more frequent bursts as their metabolic rate increased, but cycle period leveled off at about 150 s per cycle, allowing the maintenance of discontinuous CO_2 release patterns.

Contreras and Bradley (Contreras and Bradley, 2009) proposed that an insect's metabolic rate determines its gas exchange pattern. In the present study, the differences in gas exchange patterns between insects exposed to temperature or feeding treatments indicate that metabolic rate is not the only factor determining respiratory behavior in insects. At a constant temperature, gas exchange patterns can be predicted by metabolic rate and are explained by the dual feedback loop model of spiracular control (Levy and Schneiderman, 1966; Hetz, 2010). We found that temperature also has strong effects on gas exchange pattern independent of its effect on metabolic rate. These effects are not accommodated by the model put forward by Contreras and Bradley (Contreras and Bradley, 2009) or by the dual feedback loop model in its simplest form.

Analysis of temperature effects on gas exchange pattern characteristics

We found in *R. prolixus* that increased temperature led to decreased burst volume during spiracular openings. As the presence of a constant CO_2 trigger threshold for spiracular opening at a given temperature has been well described (Levy and Schneiderman, 1966; Harrison et al., 1995; Förster and Hetz, 2010), and seems to be verified in this insect (present study), a decrease in the amount of CO_2 released in a respiratory burst could be the result of premature initiation of the open phase or premature initiation of the closed phase.

It may be informative to consider the results presented here in the context of the process of CO_2 sensing. In vertebrates, CO_2 is sensed as pH in the brain, a site separated from the blood, which is highly buffered, by the blood–brain barrier (Guyton and Hall, 2011). The sensors in the brain are sensitive to pH and in this location the pH of the solution is directly proportional to P_{CO_2} in the blood. Premature spiracular opening in insects might also be explained by the physical properties of CO_2 or an effect of temperature on the respiratory control system. As temperature increases from 18 to 38°C, the solubility coefficient of CO_2 in water decreases from 4.149×10^{-2} to 2.474×10^{-2} mol l^{-1} atm^{-1} (Weiss, 1974). As a result, for any given quantity of CO_2 produced, less will become dissolved in the aqueous portions of the body, resulting in a higher P_{CO_2} in the tracheal system. As P_{CO_2} is in equilibrium throughout the body, this results in a higher P_{CO_2} at the sensor location, wherever it may be. As such, a smaller quantity of CO_2 is required to initiate spiracular opening, resulting in smaller volumes of CO_2 released per burst. Congruently, Levy and Schneiderman (Levy and Schneiderman, 1966) and Förster and Hetz (Förster and Hetz, 2010) note a positive relationship between temperature and the tracheal P_{CO_2} at the threshold for spiracle opening.

However, the observation that burst volume decreases with increasing temperature is also compatible with an alternative explanation. P_{CO_2} may be detected via pH in an isolated sensory organ, as in vertebrates. If so, then as temperature increases, an additional pH reduction is experienced in the sensory organ as a result of increased water ionization, causing the pH triggering threshold to be reached prematurely and the open phase to be initiated at a lower P_{CO_2} than expected. Hemolymph pH in grasshoppers decreases above 25°C (Harrison, 1988; Harrison, 1988), but does not change significantly throughout the respiratory cycle in grasshoppers (Harrison et al., 1990; Harrison et al., 1995) or cockroaches (Matthews and White, 2011). However, in vertebrates the CO_2-sensing neurons are separated from the blood by the blood–brain barrier. This allows the neurons to respond to P_{CO_2} through a change in pH of the cerebrospinal fluid while being insensitive to blood pH. Perhaps in insects, neurons are also sensing the pH of the perineural fluid, which is similarly isolated from the hemolymph (Treherne and Pichon, 1972). A two-layer barrier protects the brain, ganglia and major nerves from direct contact with the hemolymph, which is well buffered (Nation, 2002). This barrier consists of an outer acellular layer, which functions as a barrier to ions and osmolites in the hemolymph, and a second layer composed of perineural cells. The perineural cell layer is largely impermeable

as a result of an abundance of tight and gap junctions (Lane and Skaer, 1980).

The effects of CO_2 on spiracular function have been described (Hazelhoff, 1926; Wigglesworth, 1935; Hoyle, 1960), but the mechanism by which this signal is detected remains unknown. Case (Case, 1957) argued that spiracles open either in response to CO_2-induced acidity or as a result of the release of bound CO_2 by other acids. In other studies, Case found that hypoxia increased the sensitivity of the spiracles to CO_2 (Case, 1954; Case, 1955). He (Case, 1957) concluded that spiracular opening is therefore produced directly through the action of CO_2 on the spiracle muscle or another receptor cell that is permeable to CO_2 but not to hydrogen ions. Similarly, Hoyle (Hoyle, 1960) found that CO_2 plays a direct role in the relaxation of the spiracular closure muscle, even under continued nervous excitation, through interference with the transmission of action potentials across the neuromuscular junction. Badre et al. (Badre et al., 2005) provide evidence for direct inhibition of glutamate receptors of insect skeletal muscle by CO_2.

Based on this evidence, Förster (Förster, 2010) proposed two possible mechanisms by which this direct action of CO_2 may take place: (1) direct inhibition of glutamate receptors by CO_2 or (2) CO_2 diffusion into the post-synaptic cell causing a pH disturbance that inactivates the receptor. Our results support the second of these proposed models. Increased temperatures would reduce intracellular pH in addition to the pH disturbance caused by accumulated dissolved CO_2, causing premature receptor inactivation and spiracular opening events, which occur before the CO_2 threshold would have been reached based on CO_2-induced pH changes alone. These premature openings would explain the reduced CO_2 emission volumes during the spiracular open phase detected at high temperatures.

Effects of hyperoxia

The dual feedback model of insect respiratory control attributes the initiation of the flutter phase to a critically low P_{O_2} threshold, and the initiation of the open phase to a threshold high CO_2 concentration. It is silent with regard to the factors that cause the cessation of the open phase, i.e. the initiation of the closed phase.

One can hypothesize that the cessation of the open phase could be controlled by the oxygen concentration in the insects reaching a threshold high level, or by the CO_2 concentration in the insect reaching a threshold low level. Alternatively, cessation of the open phase could be simply a timing event such that the open phase had a specific temporal duration. We can rule out this last explanation based on the data in Figs 3 and 5. Regardless of how metabolic rate is increased, the duration of the open phase decreases with increasing metabolic rate.

We chose to test the oxygen threshold hypothesis by examining the duration of the open phase in 21% and 40% oxygen environments. We found that the duration of the open phase did not change significantly under these two oxygen regimes. We are left with the conclusion that the rate at which oxygen enters the insect during the open phase does not control the temporal length of the open phase.

The experiments we have described here are unable to either confirm or falsify the hypothesis that the open phase is terminated when P_{CO_2} reaches a threshold low level. It is clear that the amount of CO_2 released in a burst declines with increasing temperature. However, we are unable to determine on the basis of our results whether this is due to decreased solubility of CO_2 in water at elevated temperatures or to a shift in pH in cells responsible for spiracular control. Either process could explain the observed changes in burst volume.

Physiological and ecological implications

As small ectotherms, insects are subject to substantial temperature fluctuations on both a daily and a seasonal basis. The respiratory control system of *R. prolixus* is sensitive to temperature, resulting in different gas exchange patterns in response to both metabolic rate and ambient temperature. Increased temperature results in reduced CO_2 release during the open phase of the discontinuous gas exchange cycle and premature abandonment of the discontinuous pattern at metabolic rates lower than those reached by fed individuals who maintained this pattern.

Many researchers have argued that the discontinuous gas exchange cycle provides adaptive advantages to insects. Unmatched metabolic rate and oxygen supply may lead to excess oxidative damage (Hetz and Bradley, 2005), and an inability to produce prolonged spiracular closures may have detrimental effects on water balance (Chown and Davis, 2003). Oxidative damage and water loss would both increase with increasing temperature. The maintenance of the discontinuous respiratory cycle even at higher metabolic rates may have important physiological implications for the fed insects, which are experiencing high metabolic rates and rapid tissue growth. Alternatively, perhaps the discontinuous gas exchange cycle has no adaptive value and is simply a result of cycling partial pressures of respiratory gases (Chown and Holter, 2000).

Furthermore, researchers have often utilized temperature as a means of inducing changes in metabolic rate to examine the effects of different metabolic states on various physiological parameters. We have shown that the effects of temperature on respiratory behavior are significant and can overshadow the effects of metabolic rate alone. As a result, caution should be exercised when investigating physiological responses to metabolic rates induced via temperature. Interactions between effects of temperature and metabolism must be considered. When possible, researchers should attempt to include multiple sources of metabolic manipulation, such as temperature, feeding or activity, to gain a better idea of the variation in physiological responses to metabolic changes.

The fact that respiratory controls are temperature sensitive may provide new techniques for determining the sites and mechanisms by which insects sense the partial pressures of respiratory gases. Localized temperature manipulation, for example of the head or of a specific spiracle, could allow us to make predictions about the molecular mechanisms and specific tissues with which an insect senses respiratory gases. Additionally, localized tests of pH sensitivity, for instance in spiracular motor neurons, may provide supporting evidence for Hoyle (Hoyle, 1960) and Case's (Case, 1957) ideas about CO_2 sensing via pH changes at the neuromuscular junction. Future studies may also look more closely at the intratracheal P_{O_2} and P_{CO_2} threshold values for spiracular activity across a range of different temperatures to verify how tracheal gas partial pressure thresholds change with temperature.

Understanding the details of this control system will provide insight into how animals with vastly different respiratory physiologies – that is, vertebrates and insects – evolved strategies to solve the same basic problem of controlling the exchange of respiratory gases. Furthermore, these results display the importance of understanding the interactive effects of different metabolic stressors in ectothermic animals, and their effects on respiration.

MATERIALS AND METHODS

Animals

Fifth instar *R. prolixus* were obtained from a colony at the University of California, Irvine. The colony was maintained at 27°C and 80% relative humidity (RH) with a 12 h:12 h day:night cycle. Individuals were separated

into two treatment groups of six individuals each. The mean (±s.d.) mass of individuals was 41.53±13.07 mg in the first group and 43.08±6.69 mg in the second group. The first group was fed a blood meal to increase the metabolic rate, and the second group remained unfed and experienced a range of temperatures to induce changes in metabolic rate. Insects in both treatment groups were fasted for 3 weeks prior to the start of the experiment. Resting metabolic rates for insects in the fed treatment group were recorded 1 day prior to feeding. Insects in the fed treatment group then took a blood meal from a rabbit immediately prior to the first recordings.

After the completion of the above experiments, two additional groups of individuals were collected. One group of six individuals was used in a second trial of temperature experiments and another group of seven individuals was used for oxygen manipulation tests (mass 43.15±5.97 mg).

Procedure for temperature and feeding trials

CO_2 release patterns of individuals in the temperature treatment group were recorded at temperatures ranging from 18 to 38°C. To minimize the effects of acclimation or heat stress, the order of temperature exposure was determined by creating a semi-random sequence in which most temperature transitions did not exceed a change of more than 6°C and the highest temperature was experienced either first or last. Temperatures were encountered in the following order for the first temperature treatment group, and in the reverse order for the second temperature treatment group: 38, 24, 30, 34, 36, 32, 28, 24, 20, 18, 22, 26°C. The insects experienced each temperature for a total of 48 h, which included a 24 h equilibration period followed by another 24 h period in which two 30 min recordings were taken from each individual. Experiments were conducted in a temperature-controlled room that maintained a programmed temperature within ±0.5°C. The insects in the fed treatment group were maintained at 26±0.5°C for the duration of the study. Two 30 min recordings were taken daily from each individual for 17 days after feeding.

In both the feeding and temperature treatment groups, insects remained in their chambers throughout the duration of the study to reduce handling stress. Each insect was placed into a 2 ml cylindrical plastic chamber and provided with a 1 cm^2 piece of filter paper. Insects were perfused with a slow flow of hydrated room air (80% RH, ~25 ml min^{-1}) during periods when measurements were not being taken to prevent desiccation and CO_2 build-up. Room air was humidified by bubbling through a saturated ammonium sulfate solution.

During flow-through respirometry, a mass flow controller pushed air at a constant flow rate of 200 ml min^{-1} (Sierra Instruments Inc., Monterey, CA, USA) through columns of Drierite (W. A. Hammond Drierite Co. Ltd, Xenia, OH, USA) and Ascarite (Thomas Scientific, Swedesboro, NJ, USA) to remove water and CO_2. Air then passed through an 8-channel multiplexer (Sable Systems International, Las Vegas, NV, USA) which was programmed to direct the airstream through six channels leading to individual insects for 30 min intervals. Between each recording from an insect, the multiplexer directed air through a seventh channel containing an empty chamber to obtain a baseline recording. A 90 s pause in data recording was issued at the beginning of each channel switch while dry air began flowing through the new channel. After the chamber, air passed through a CO_2 gas analyzer (model 6262, Li-Cor Inc., Lincoln, NE, USA).

CO_2 readings were converted from analog to digital signals with a Sable Systems Universal Interface II and recorded with Expedata data acquisition software (Sable Systems). Data were zeroed in Expedata to account for any baseline drift and values for average metabolic rate, burst volume, burst duration and cycle period were recorded. These values were obtained from baseline-corrected recordings in Expedata and tabulated in Excel, where CO_2 release rates were converted from ppm to µl h^{-1} (Microsoft, Redmond, WA, USA).

As indicated in the Introduction, *Rhodnius* remain quiescent between molts unless a blood host is available. Our data collection occurred after 24 h of undisturbed acclimation at each temperature. Visual observation of the insects in the chambers during the discontinuous gas exchange cycle gave no indication of ventilatory movements of the abdomen or locomotor activity. To further verify these observations, we conducted simultaneous measurements of CO_2 release and activity, and video recordings under low (25°C) and high (35°C) temperature conditions to determine whether activity affected the gas exchange pattern. The insect was placed in a clear, cylindrical glass chamber housed inside a temperature-controlled cabinet (Pelt-5 Temperature Controller, Sable Systems). Respirometry plumbing was arranged as previously described. Supplementary material Fig. S1 shows data from an infrared AD2 activity detector (Sable Systems) recording movement of the insect. The results from these trials indicate that the insects breathe discontinuously while quiescent and that no visual evidence of ventilatory movements can be seen. Locomotory movements, such as those that occurred immediately after a change in temperature, clearly disturb the respiratory rhythms (supplementary material Fig. S1). The absence of such disturbed periods in our experimental recordings is evidence that activity was not a confounding variable during our measurement of gas exchange patterns.

Oxygen manipulation trials

Rhodnius prolixus were tested for changes in gas exchange patterns in 21% and 40% oxygen environments before and after feeding to determine whether a critically high oxygen threshold played a role in triggering spiracular closure. The temperature was maintained at 26°C for all oxygen tests. CO_2 release patterns were recorded from seven, fifth instar individuals using the same flow-through respirometry setup previously described excluding the multiplexer, as individuals were tested one at a time. Recordings were first taken in 21% oxygen for 1 h, followed by 1 h of recordings at 40% oxygen. A reservoir of hyperoxic air was produced by mixing dry room air and 100% oxygen in a large Mylar balloon. During the 40% oxygen trials, air was pulled from this reservoir by the mass flow meter and re-dried and scrubbed of CO_2 prior to entering the experimental chamber. Oxygen concentrations were verified by flowing air from the balloon through an Oxzilla Differential Oxygen Analyzer (Sable Systems). Oxygen percentages for all hyperoxic trials were maintained at 40±1%.

Data collection and analysis

One goal of the present study was to analyze the volume of CO_2 released per burst, or spiracular open phase, as a function of metabolic rate. The burst volume in unfed *R. prolixus* is fairly straightforward to measure from a flow-through respirometry recording. It represents the amount of CO_2 released during an open phase relative to the zero baseline (see Fig. 6). We used Expedata to measure these volumes (volume/s × s=volume). However, the burst volume in fed *R. prolixus* can be considered in two different ways. As in the unfed insects, one can consider the volume of a burst to be the amount of CO_2 released during the open phase over and above a zero rate of release. Alternatively, as fed individuals display an elevated rate of release in the interburst periods as a result of greater spiracular activity during the flutter phase, the volume of a burst can also be interpreted as the CO_2 release volume over and above the interburst release rate. CO_2 accumulates during the interburst periods until it reaches the critical partial pressure required to initiate a burst. Following the burst, the closed phase is very short in fed individuals as a result of the elevated metabolic rate, and therefore the rate of CO_2 release rapidly returns to the elevated flutter rate. The burst volume consequently can be viewed as the volume of CO_2 released at intervals, over and above this steady-state release rate (Fig. 4). We quantified burst volume in fed *R. prolixus* using both approaches, i.e. by measuring the volume released relative to zero rates of release, and relative to the rates observed during the interburst flutter phase.

To determine the average burst volume for an individual, a maximum of six individual bursts were measured per 30 min recording at each temperature or each day post-feeding. At the lowest metabolic rates, at least two bursts per recording were available for measurement. The beginning of a peak was defined as the point at which the CO_2 recording became greater than the average CO_2 value in the interburst period. The end of a burst was determined as the point at which the CO_2 recording returned to the average interburst period level. The rate of CO_2 release during a respiratory burst was calculated as the burst volume divided by the burst duration. Cycle periods were defined as being from the start of one open phase to the start of the next open phase. An average cycle period for each 30 min recording was found by measuring the time between the start of the first peak and the start of the last peak in the recording, and dividing this time by the number of peaks that occurred during that time period.

Statistical tests were performed in RStudio (rstudio.com). Averages were taken of all measurements for one individual at a given temperature,

or day after feeding, for use in analyses. Data from temperature trials were combined to produce a sample size of 12 individuals per temperature. Data from feeding trials were evaluated via regression analyses with metabolic rate as a continuous variable. Temperature trial data were analyzed via one-way ANOVA. Oxygen manipulation trials were analyzed using two-way ANOVA with feeding status and oxygen treatment as grouping variables.

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Competing interests
The authors declare no competing financial interests.

Author contributions
E.C.H. and T.J.B. designed the study, interpreted the results, and wrote the manuscript. Experiments and data analyses were conducted by E.C.H.

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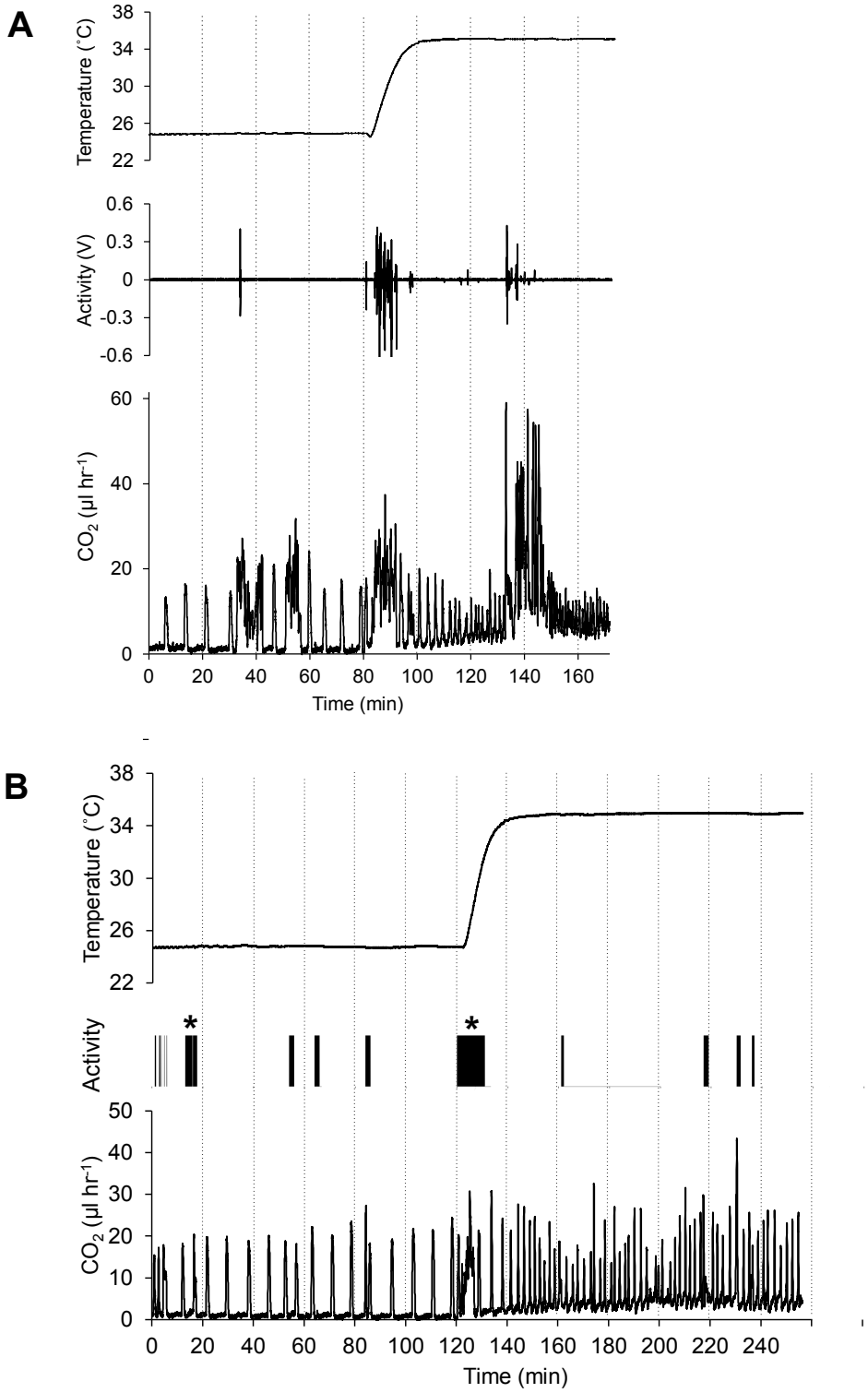

Fig. S1. Activity and CO_2 release in an unacclimated *Rhodnius* over time at a low (25 ˚C) and high (35 ˚C) temperature. Overall activity patterns did not differ across the two temperatures. (A) Periods of activity detected by an infrared activity detector result in a disruption of the discontinuous gas exchange pattern and an elevated rate of CO_2 release at both temperatures. One disruption of the pattern was not associated with activity. (B) Periods of intense movement observed visually from video recordings (e.g. escape behavior, indicated with asterisks) resulted in a disruption of the discontinuous pattern in one case, but the pattern was maintained during another. Other periods of minimal movement (grooming, adjusting body position) had similar effects in that they only occasionally disrupted the discontinuous pattern. After the 24 h acclimation period utilized in our study, lengthy periods of discontinuous gas exchange were observed with no apparent effects of locomotion.