

## RESEARCH ARTICLE

# The effect of ambient humidity and metabolic rate on the gas-exchange pattern of the semi-aquatic insect *Aquarius remigis*

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### SUMMARY

We have examined the effects of temperature on metabolic rate and respiratory pattern in the water strider *Aquarius remigis*. As temperature was increased from 10 to 30°C, the metabolic rate of the insects increased and the respiratory pattern transitioned from discontinuous, to cyclic, to continuous. The discontinuous gas-exchange cycle (DGC) was observed even in insects standing on water when the respirometry chamber was being perfused with humid (>95% relative humidity) air. Comparisons of insects at 20°C in humid and dry air showed no statistically significant differences in metabolic rate or respiratory pattern ( $P>0.05$ ). The proportion of time that the spiracles were closed was greater at 10°C than at 20°C ( $P<0.01$ ), and greater at 20°C than at 30°C ( $P<0.05$ ). These results are compatible with the hypothesis that the respiratory patterns of insects are determined by the relationship between oxygen supply and oxygen demand. There was no evidence in this insect that humidity had any effect on the respiratory pattern. The results are discussed in the context of the ongoing discussion in the literature of the origin, maintenance and adaptive significance of the DGC in insects.

Key words: discontinuous, insect, pattern, respiratory, waterstrider.

### INTRODUCTION

Insects are known to employ three different gas exchange patterns: discontinuous, cyclic and continuous (Gibbs and Johnson, 2004; Marais et al., 2005; Bradley, 2008; Contreras and Bradley, 2009). The discontinuous gas-exchange cycle (DGC) is distinguished from the other two patterns by the presence of clear closed periods in which the spiracles are sealed and no gas exchange occurs (Lighton, 1994). The cyclic pattern lacks clear closed periods but retains bursts that impart rhythmicity to the CO<sub>2</sub>-release traces. The continuous pattern lacks both closed periods and any evidence of rhythmicity (Marais et al., 2005; Bradley, 2008).

The DGC is by far the pattern most thoroughly studied by insect physiologists (Lighton, 1994; Lighton, 1996; Lighton, 1998; Hadley, 1994; Slama, 1999; Chown and Nicolson, 2004), largely because of the long-standing debate regarding the adaptive significance and evolutionary origins of DGC in insects (Chown et al., 2006). Early work on insect gas-exchange patterns suggested that the DGC served to conserve water (Hazelhoff, 1927; Buck et al., 1953; Buck and Keister, 1955; Burkett and Schneiderman, 1974a; Burkett and Schneiderman, 1974b; Kestler, 1985). Under this hygric hypothesis, the DGC conserves water not only by reducing spiracular opening (Burkett and Schneidermann, 1974a; Lighton et al., 1993) but also by restricting water loss during the flutter phase when there can be a bulk inflow of air (Kestler, 1985; Wobschall and Hetz, 2004). Although convective inflow of air has been shown in many insects (Levy and Schneidermann, 1966b; Kestler, 1985; Slama and Coquillaud, 1992; Snyder et al., 1995; Slama, 1999), it is not employed consistently by all species (Lighton, 1988; Lighton and Garrigan,

1995; Chappell and Rogowitz, 2000) and may actually be uncommon (Lighton, 1996; Lighton, 1998).

The hygric hypothesis has been called into question by some investigators, however. It has been shown that insects that are dehydrated do not necessarily show the DGC when it would seem beneficial and that the DGC may be employed even when ambient humidity levels are high (Hadley and Quinlan, 1993; Contreras and Bradley, 2009). The oxidative damage hypothesis was presented as an alternative explanation for the presence of the DGC in insects. This hypothesis proposes that the DGC did not evolve to conserve water but rather to reduce the amount of oxygen entering the respiratory system of resting insects because high levels of O<sub>2</sub> are toxic to tissues (Hetz and Bradley, 2005). The open phase, which serves to rid the system of accumulated CO<sub>2</sub>, also exposes tissues to high levels of O<sub>2</sub>. By closing the spiracles once CO<sub>2</sub> has been adequately released, O<sub>2</sub> levels near the tissues are rapidly reduced. Furthermore, as was previously suggested by Levy and Schneidermann (Levy and Schneidermann, 1966a), the flutter phase serves to maintain O<sub>2</sub> levels at a low constant level (~4 kPa) (Hetz and Bradley, 2005). Therefore the closed and flutter phases serve to protect the tissues from oxidative damage.

Independently, Hetz (Hetz, 2007) and Bradley (Bradley, 2008) expanded upon this idea by suggesting that the type of gas exchange employed by an insect, at any point in time, is dictated by an interaction between an insect's metabolic rate and the capacity of the respiratory system to deliver oxygen to this metabolically active tissue. Therefore, insects with a low metabolic rate may show a long period of spiracular closure (C phase). As metabolic rates increase, the C phase becomes shorter and shorter until eventually,

if metabolic rates are high enough, this phase completely disappears. In this manner, insects transition from the DGC to a cyclic gas exchange pattern. If metabolic rates are increased further, the period of spiracular flutter (F phase) may begin to shorten. Eventually, this phase may also disappear if metabolic rates are high, and the insect will maintain the spiracles in an open state. Insects, therefore, will transition from a cyclic to a more continuous gas exchange pattern. Studies on the effects of temperature on the gas-exchange patterns of *Rhodnius prolixus* (Contreras and Bradley, 2009; Contreras and Bradley, 2010) and *Gromphadorhina portentosa* (Contreras and Bradley, 2010) show that as metabolic rates increase, insects transition from the DGC to a cyclic and finally to a continuous gas pattern. Further studies on ants (Lighton and Wehner, 1993) and beetles (Davis et al., 1999; Chappel and Rogowitz, 2000; Kaiser et al., 2009) have shown that as metabolic rates increase (in response to temperature), the C phase of the DGC becomes shorter and shorter.

Whether the DGC is employed to conserve water or to reduce levels of O<sub>2</sub> surrounding the tissues continues to be intensely investigated. Support for the hygric hypothesis comes from phylogenetic studies that suggest that insects from dry habitats utilize the DGC more often (Marais et al., 2005; White et al., 2007); studies showing that desert insects use the DGC (e.g. Duncan et al., 2002); an acclimation study showing that the respiratory patterns of insects respond to environmental humidity (Schimpf et al., 2009); and studies on insects that are able to vary their respiratory patterns, indicating that respiratory water loss is decreased when the insects use the DGC (Chown and Holter, 2000; Chown and Davis, 2003; Williams et al., 2009).

By contrast, other studies have shown that insects in dry habitats do not use the DGC (Duncan and Dickman, 2009) or utilize the DGC at the wrong time of day for water conservation (Hadley and Quinlan, 1993); insects that vary their respiratory patterns show no reduction in respiratory water loss while using the DGC (Williams et al., 1998; Gibbs and Johnson, 2004; Dingha et al., 2005; Lighton and Turner, 2008); and oxygen availability and metabolic rate influence respiratory pattern, while humidity does not (Terblanche et al., 2008; Contreras and Bradley, 2009). Clearly the results are mixed and the question remains unresolved.

In their examination of respiratory patterns, a number of researchers have employed terrestrial insects from dry habitats, the notion being that under the hygric hypothesis these insects should have an enhanced expression of the DGC. In the present study we sought to explore the reverse side of this hypothesis by examining the respiratory pattern of *Aquarius remigis*, a water strider in the family Gerridae. These insects are referred to as semi-aquatic (Shafer, 2003) because they live on the water surface but not under it. We reasoned that if a major purpose of the DGC is to reduce respiratory water loss, then the DGC pattern should be absent or blunted in these insects, particularly when they are standing on water. We also examined the effects of temperature on respiratory pattern, following the outline of previous studies conducted on strictly terrestrial insects (Contreras and Bradley, 2009; Contreras and Bradley, 2010).

## MATERIALS AND METHODS

### Study animals

Adult water striders, *Aquarius remigis* (Say 1832), were collected from Cajon Creek in San Bernardino County, CA, USA, during late spring/early summer 2009. In the laboratory, *A. remigis* were maintained on a 12 h:12 h light:dark cycle. At least eight insects shared an aquarium (~80 l). Individuals were separated from each

other by being placed in plastic containers within the aquarium. Each container had a small opening low on the sidewalls that allowed water to move freely from that container into the water in the aquarium. The water level within each container was maintained above these openings so that water striders could not escape. An aquarium pump provided a current throughout the aquarium. Insects were fed small and/or medium crickets at least twice a week. Food was removed from the containers 24 h prior to an experiment.

### Experimental design

The rate of CO<sub>2</sub> production ( $\dot{V}_{\text{CO}_2}$ ) was measured using flow-through respirometry on isolated animals using a random block design. Each insect was measured at 10, 20 and 30°C on separate days and in random order. In order to test the effects of humidity on the gas-exchange pattern of this semi-aquatic insect,  $\dot{V}_{\text{CO}_2}$  was measured at each temperature under humid and dry conditions.

For experiments in humid conditions, individuals were weighed and then placed in a 2 ml chamber on the respirometer. The chamber contained ~1 ml of degassed water. The insects were left undisturbed for 5 h prior to experimental measurements. During this time, humid CO<sub>2</sub>-free air was pumped into the chamber at 200 ml min<sup>-1</sup>. After 5 h, CO<sub>2</sub> release from the insect was measured using a Li-Cor model 6262 infrared CO<sub>2</sub> analyzer (Lincoln, NE, USA);  $\dot{V}_{\text{CO}_2}$  data were collected using Expedata data acquisition software (Sable Systems, Las Vegas, NV, USA) controlling an eight-channel multiplexor. A total of four chambers were attached to the multiplexor: a baseline (no insect) and three experimental (containing an insect) chambers. Room air was pumped through a bottle containing a sodium hydroxide solution to be scrubbed of CO<sub>2</sub> before passing through a flask filled with 200 ml of distilled water. Humid air (>95% relative humidity) leaving the flasks passed through a flow controller into the respirometry chamber and then on to the CO<sub>2</sub> analyzer. When an insect was not being measured, its chamber was still perfused with humid CO<sub>2</sub>-free air at a rate equal to the regulated flow entering the measured chamber.

For measurements in dry air, room air was pumped through two silica and one ascarite/drierite column to be scrubbed of water and CO<sub>2</sub>. Airflow through the measured chamber was controlled as above at 200 ml min<sup>-1</sup>. Insects were weighed and placed in a 2 ml vial but were left undisturbed for only 30 min before the trial commenced as they were unable to withstand long periods of time in completely dry conditions. Respirometry measurements were then taken as described above.

Under both humid and dry conditions, airflow through the chamber was controlled at 200 ml min<sup>-1</sup>. As a result, the time constant for volume exchange was 0.6 s for both humid and dry experiments. This time constant ensured that after 3 s, 99% of the air was replaced in the measured chamber.

We were concerned that the presence of water in the experimental chamber during the humid trials might blur or blunt measurements of respiratory pattern because of the dissolution of CO<sub>2</sub> in the water. To test for any such effects, a few experiments were run in which the insects were placed under dry conditions but with a vial containing 1 ml of degassed water added to the system between the experimental chamber and the CO<sub>2</sub> analyzer. Therefore, during a run, air leaving the experimental chamber needed to pass into the vial containing the degassed water before entering the CO<sub>2</sub> analyzer.

Regardless of temperature or humidity, an experimental run lasted 70 min, with two 5 min baselines at the beginning and end of the total run, and one 60 min period measuring air from the experimental chamber. Although drift was very slight, the initial and final

baselines were used to provide baseline values and to correct for any drift. Insects were observed during the experiments for signs of activity.

Measurements made at each temperature were obtained on separate days, and the insects were returned to the colony (room temperature,  $\sim 22^\circ\text{C}$ ) between measurement days.

#### Determining rates of CO<sub>2</sub> release during spiracular closure

We used methods previously described (Contreras and Bradley, 2010) to indicate periods of probable spiracular closure in *A. remigis*. In this method, insects were exposed to hyperoxic conditions at  $10^\circ\text{C}$  while  $\dot{V}_{\text{CO}_2}$  was measured. By placing insects in hyperoxic conditions at low temperatures ( $10\text{--}15^\circ\text{C}$ ) we can obtain extended periods of spiracular closure that can then be used to determine rates of CO<sub>2</sub> release associated with periods of spiracular closure (Lighton et al., 2004; Contreras and Bradley, 2010). To achieve hyperoxic conditions, experiments were carried out as described above but the stream of air was replaced by 100% O<sub>2</sub> (Airgas, Los Angeles, CA, USA). All other aspects of the respirometry procedures were identical to those described above. The lowest  $\dot{V}_{\text{CO}_2}$  values (minimum of 150 s of continuous data) for each individual during these trials were selected using the NADIR function in Expedata. NADIR identifies the lowest values within selected periods of data. These data were averaged and multiplied by a factor of 10 to raise the cut-off threshold above instrumental noise. All data points below this threshold value were considered to be associated with a period of spiracular closure. These criteria were used to determine the proportion of time that the spiracles were closed during a normoxic trial at the three different temperatures being measured. This technique was also used in a previous study (Contreras and Bradley, 2010). In that study, larger insects (hissing cockroaches, *G. portentosa*) were examined and the values identified by NADIR were multiplied by a factor of four. These large insects produced a large signal relative to instrument noise. In the present study we used a multiplication factor of 10 because *A. remigis* are much smaller than adult *G. portentosa* (Table 1). This larger multiplicative factor assured that the data were well above instrument noise and that an appropriate criterion was used to identify periods of burst.

#### Data analysis

Expedata analysis software was used to record and process measurements of  $\dot{V}_{\text{CO}_2}$ . CO<sub>2</sub> levels were recorded in parts per million and, after data were zeroed using baseline values, converted to  $\mu\text{l min}^{-1}$ . Data were then exported into Excel. To determine the effects of temperature (metabolic rate) on the length of spiracular closure, estimates of mass-specific metabolic rates ( $\dot{V}_{\text{CO}_2}$ ,  $\mu\text{l min}^{-1}$ , STP) were determined for all individuals at the three experimental temperatures ( $10$ ,  $20$  and  $30^\circ\text{C}$ ).

Mass-specific  $\dot{V}_{\text{CO}_2}$  values during humid trials were log-transformed before repeated-measures ANOVA was used to test for

significant differences between temperatures. Data are presented as means  $\pm$  s.e.m. Data for dry trials were collected for insects placed at  $20^\circ\text{C}$  and were also log-transformed. Most insects did not survive dry conditions at  $10$  and  $30^\circ\text{C}$ . Therefore, a large amount of missing values prevented the use of robust statistical analysis. These data (dry trials at  $10$  and  $30^\circ\text{C}$ ) were then excluded from the experiment. A paired *t*-test was used to test for significant differences in mass-specific  $\dot{V}_{\text{CO}_2}$  between humid and dry trials at  $20^\circ\text{C}$ .

A Kruskal–Wallis test was used to determine significant differences in the proportion of time that spiracles were closed during humid trials as a function of temperature. Because temperature did have a significant effect, Mann–Whitney *U*-tests were used for *post hoc* analysis.

## RESULTS

Our results indicate that *A. remigis* does show the DGC even when standing on water in humid air. Fig. 1 shows the pattern of CO<sub>2</sub> release for an individual water strider at three temperatures. At  $10^\circ\text{C}$ , when metabolic rate is low, the insect breathes discontinuously. The discontinuous pattern is characterized by extended periods during which the spiracles appear to be closed, as evidenced by extended periods of CO<sub>2</sub> release at rates below the threshold value (dashed line, Fig. 1). In addition, during the open phases,  $\dot{V}_{\text{CO}_2}$  shows an initial rapid rate that declines during the open period. This is the classic pattern observed in lepidopteran pupae during discontinuous gas exchange (e.g. Hetz et al., 1993; Hetz and Bradley, 2005). At  $20^\circ\text{C}$ , the insect uses a cyclic pattern characterized by a failure of  $\dot{V}_{\text{CO}_2}$  to go to zero. At  $30^\circ\text{C}$ , the insect breathes using a continuous pattern.

As expected for a poikilotherm,  $\dot{V}_{\text{CO}_2}$  increased with increasing temperature (Fig. 2). The changes in respiratory pattern are therefore correlated with a change in metabolic rate.

Fig. 1 is an example of the respiratory pattern exhibited by a single insect at the three experimental temperatures. Fig. 3 shows the results obtained when the mean proportion of time the spiracles are closed is compared across these three temperatures for all of the insects examined ( $N=8$ ). A Kruskal–Wallis test of data obtained during humid trials indicated that temperature had a significant effect on the proportion of time that the spiracles remained closed ( $H=19.08$ , d.f.=2,  $P<0.05$ ). Spiracles were maintained closed for a significantly longer period of time at  $10^\circ\text{C}$  compared with  $20^\circ\text{C}$  ( $U=10$ ,  $N_{10^\circ\text{C}}=10$ ,  $N_{20^\circ\text{C}}=12$ ,  $P<0.001$ ). Similarly, spiracles were maintained closed for a significantly longer period of time at  $20^\circ\text{C}$  compared with  $30^\circ\text{C}$  ( $U=33.5$ ,  $N_{20^\circ\text{C}}=N_{30^\circ\text{C}}=12$ ,  $P<0.05$ ).

We also examined the same individuals used in the humid measurements at  $20^\circ\text{C}$  in dry air. The metabolic rate at  $20^\circ\text{C}$  was statistically identical in humid and dry air ( $P>0.05$ ; Fig. 2, Table 1). In addition, no significant differences were observed in the proportion of time the spiracles were closed between humid and dry air at  $20^\circ\text{C}$  ( $U=66$ ,  $N_{\text{humid}}=12$ ,  $N_{\text{dry}}=9$ ,  $P>0.05$ ; Fig. 3).

Table 1. Mass and mean rate of CO<sub>2</sub> release ( $\dot{V}_{\text{CO}_2}$ ) for *Aquarius remigis* during humid and dry trials when placed under three distinct temperatures

Trial	Mass (g)	$\dot{V}_{\text{CO}_2}$ ( $\mu\text{l min}^{-1}$ )	Mass-specific $\dot{V}_{\text{CO}_2}$ ( $\mu\text{l g}^{-1}\text{min}^{-1}$ )	<i>N</i>
Humid				
$10^\circ\text{C}$	0.047 $\pm$ 0.003	0.12 $\pm$ 0.03	2.44 $\pm$ 0.49	11
$20^\circ\text{C}$	0.046 $\pm$ 0.004	0.33 $\pm$ 0.05	6.80 $\pm$ 0.86	12
$30^\circ\text{C}$	0.048 $\pm$ 0.003	0.56 $\pm$ 0.10	10.4 $\pm$ 1.10	12
Dry				
$20^\circ\text{C}$	0.046 $\pm$ 0.004	0.33 $\pm$ 0.06	7.26 $\pm$ 1.34	7

Values are STP-corrected means  $\pm$  s.e.m. *N*, sample size.

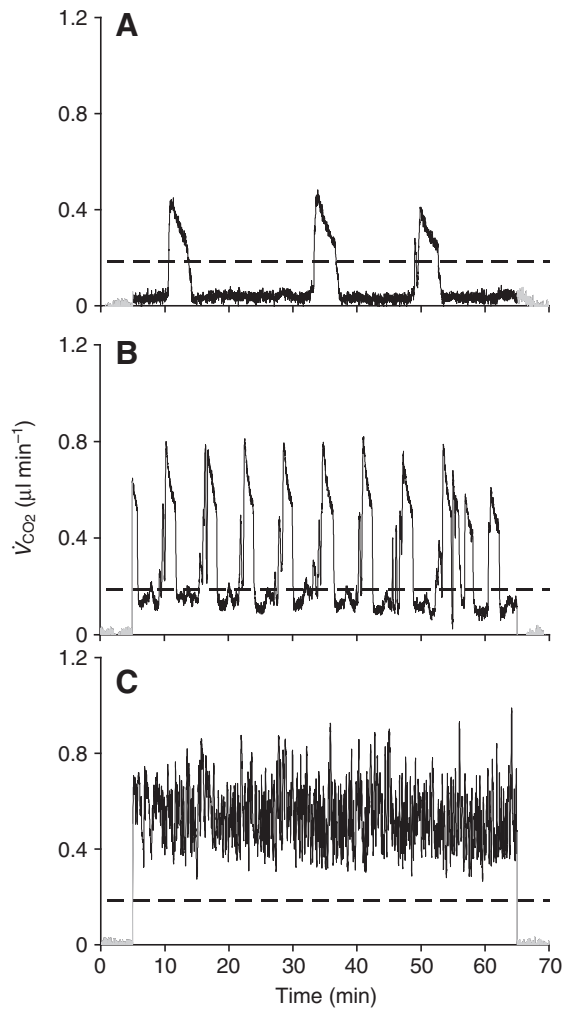


Fig. 1. Sample  $\dot{V}_{CO_2}$  data for an individual *Aquarius remigis* during humid trials at three different temperatures: 10°C (A), 20°C (B) and 30°C (C). During the 70 min experiment, an empty chamber was read twice at 0–5 and 65–70 min (gray) and an experimental chamber (containing an insect) was read continuously for 60 min at 5–65 min (black line). The dashed line (threshold) depicts 10 times the  $\dot{V}_{CO_2}$  levels during spiracular closure as calculated from hyperoxic experiments at 10°C. Any points below this line are considered periods of spiracular closure and points above the line are considered periods when spiracles are not closed.

## DISCUSSION

We found that the semi-aquatic insect *A. remigis* is capable of displaying a variety of  $\dot{V}_{CO_2}$  release patterns, including the DGC. To our knowledge, this is the first time that a semi-aquatic insect was used to study gas-exchange patterns in insects. At 10°C, the pattern shows very clear bursts of  $\dot{V}_{CO_2}$  release with extended periods of greatly reduced  $\dot{V}_{CO_2}$  release in the intervals between bursts (Fig. 1). This pattern was observed despite the fact that the insects were standing on water and in a chamber perfused with humid (>95% relative humidity) air.

As previously noted, differentiation of the C and F phases of the DGC is difficult and many have combined these two phases when describing the different phases of the DGC (Chappel and Rogowitz, 2000). We sought to identify periods of low and steady  $\dot{V}_{CO_2}$  release with the assumption that these are associated with periods of spiracular closure. We characterized the C phase of the DGC using

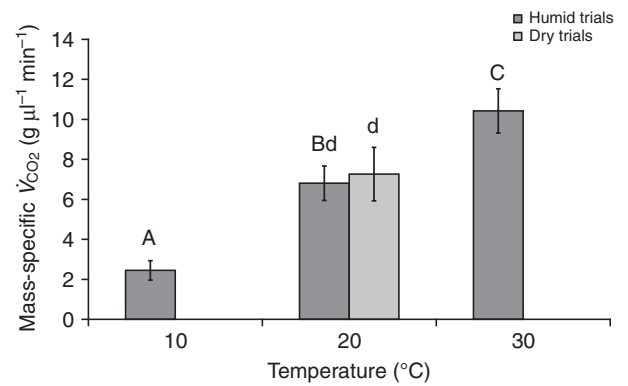


Fig. 2. Mass-specific  $\dot{V}_{CO_2}$  data for *A. remigis* during humid and dry trials for three different temperatures. Data are means + s.e.m. Significant ( $P < 0.05$ ) differences are indicated by different letters above the columns.

data for the release of  $\text{CO}_2$  during hyperoxic conditions (100%  $\text{O}_2$ ) at 15°C. An increase in  $\text{O}_2$  has been shown to not only increase spiracular closure (Lighton et al., 2004) but also to cause the periods of spiracular closure to lengthen, which allows a more accurate determination of  $\dot{V}_{CO_2}$  during the C phase (Contreras and Bradley, 2010). Under these conditions, we identified the lowest  $\dot{V}_{CO_2}$  from each of our individual insects as described in the Materials and methods section. These values, which were never as low as the baseline values from an empty chamber, were multiplied by a factor of 10 to bring the criterion of closure well above instrument noise (dashed line in Fig. 1). Instrument noise can be identified in Fig. 1 in the baseline values at the beginning and end of each graph (gray lines). Note that the threshold line and the bursts produced by the insects are well above the level of instrument noise. If  $\dot{V}_{CO_2}$  was above this value, then that period was defined as being a period when the spiracles were open.

Based on the above criteria, *A. remigis* clearly transitioned from the DGC to a more continuous gas exchange pattern as a response to increasing temperature (Fig. 1). At 10°C, insects employed the DGC (Fig. 1A). Bursts of  $\text{CO}_2$  release were followed by interburst periods where  $\dot{V}_{CO_2}$  values fell below our calculated threshold line and were very close to zero. When temperature increased to 20°C, the proportion of time the spiracles were closed during the experiment decreased. Bursts of  $\text{CO}_2$  release, followed by interburst periods, were still observed at this temperature (Fig. 1B, Fig. 3). However, interburst  $\dot{V}_{CO_2}$  values were not close to zero; instead,  $\dot{V}_{CO_2}$  oscillated around the threshold line ( $0.2 \mu\text{l min}^{-1}$ ). We interpret this to be a cyclic respiratory pattern. Similarly, Chappel and Rogowitz reported that, in the eucalyptus boring beetle,  $\dot{V}_{CO_2}$  never fell to zero when insects were maintained at 20°C (Chappel and Rogowitz, 2000). The absence of a closed phase was also reported for grasshoppers (Hadley and Quinlan, 1993) as a result of increased temperature. In the present study, *A. remigis* employed continuous gas exchange at 30°C. Values of  $\dot{V}_{CO_2}$  stayed well above our calculated threshold line throughout the experiment (Fig. 1C) for most insects and, therefore, the proportion of time that the spiracles were closed during an experiment was very low (Fig. 3).

These results, indicating a transition in respiratory pattern (Figs 1, 3) with increasing metabolic rate (Fig. 2), are similar to those we obtained with the terrestrial insects *R.s. prolixus* and *G. portentosa* (Contreras and Bradley, 2010) and to what others have shown in

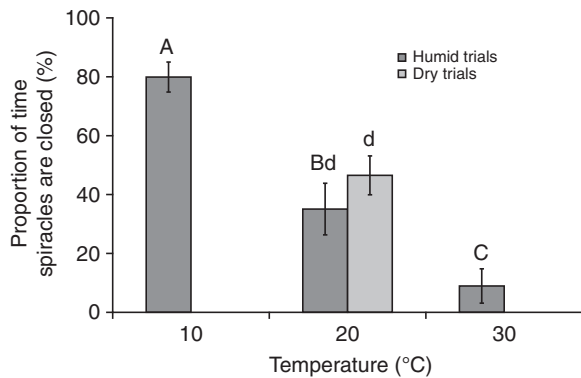


Fig. 3. Proportion of time that spiracles were closed during an experiment at 10, 20 and 30°C for humid and dry trials. Data are means  $\pm$  s.e.m. Significant ( $P < 0.05$ ) differences in spiracular closure are indicated by different letters above the columns.

beetles (Davis et al., 1999; Chappel and Rogowitz, 2000) and ants (Gibbs and Johnson, 2004). Recently, Kaiser et al. showed that increases in metabolic rate, due to endogenous factors related to developmental stages, also cause the C phase of the DGC to shorten (Kaiser et al., 2010).

In this study we found no evidence that humidity affected respiratory pattern. *Aquarius remigis* exhibited DGC even when placed at 10°C in an experimental chamber that contained 1 ml of degassed water while humid (>85% relative humidity) air was pumped through the chamber (i.e. during very humid conditions) (Fig. 1A). When insects were placed at 20°C but ambient humidity was altered, *A. remigis* showed statistically identical gas-exchange patterns and metabolic rates under humid and dry experiments (Figs 2, 3). We found no significant ( $P > 0.05$ ) differences in the proportion of time spiracles were closed as a result of varying ambient humidity (Fig. 3).

Our interpretation of the above result is that the metabolic rate, i.e. the demand for oxygen, determines the respiratory pattern used by the insects. Hetz (Hetz, 2007) and Moerbitz and Hetz (Moerbitz and Hetz, 2010) have suggested that insects display the DGC if spiracular conductance is high relative to their metabolic rate. Support for this hypothesis was provided by changes in respiratory pattern when the spiracles were experimentally occluded. Bradley proposed that the gas-exchange pattern employed by an insect at any particular time is due to the interaction between: (1) the amount of oxygen (both dissolved and intra-tracheal) contained in the insect at the beginning of the closed phase and (2) metabolic rate (Bradley, 2008). The length of the closed phase is determined by the time it takes the insect to lower the oxygen partial pressure to a safe, low level. Oxygen partial pressure is then regulated during the flutter phase by short spiracular openings (Hetz and Bradley, 2005). The length of the closed phase is therefore sensitive to changes in metabolic rate and to changes in the external atmospheric partial pressure of oxygen. The hypotheses proposed by Hetz (Hetz, 2007) and Moerbitz and Hetz (Moerbitz and Hetz, 2010) on the one hand, and Bradley (Bradley, 2008) on the other, are functionally related. They all argue that respiratory pattern is determined by the relationship between oxygen supply and oxygen demand. When the oxygen supply:demand ratio is high, closed phases are required and the DGC will be observed. As this ratio declines, the respiratory pattern shifts from the DGC to cyclic to continuous. Although the metabolic rate of *A. remigis* is in the expected range for insects of

its size at 20°C (Lighton and Fielden, 1995), modulation of the metabolic rate using temperature does affect the respiratory pattern.

The hygric hypothesis and the oxidative damage hypothesis are often presented as opposing and conflicting explanations for the control of insect respiratory patterns (Chown et al., 2006). We are convinced, as has been proposed by Levy and Schneiderman (Levy and Schneiderman, 1966a) and more recently directly demonstrated by Foerster and Hetz (Foerster and Hetz, 2010), that the spiracles of insects are controlled by internal concentrations of oxygen and carbon dioxide. The closed phase is required to lower the internal partial pressure of oxygen, the flutter phase is used to regulate oxygen levels and the open phase is initiated when carbon dioxide accumulates to a critically high partial pressure. This is not to say that the DGC serves no purpose in reducing respiratory water loss. In some insects under some environmental conditions, the DGC has been shown to reduce water loss compared with continuous respiratory patterns (Duncan et al., 2002; Williams, 2010). This effect may have contributed to the retention of the DGC in some insect groups over evolutionary time. What we need to separate, however, are the short-term regulatory mechanisms that induce and control the respiratory patterns of insects from the long-term evolutionary consequences of those patterns. We would argue that the regulatory mechanisms are directed by the internal concentrations of oxygen and carbon dioxide. The long-term adaptive consequences may, in some but clearly not all insects, be a reduction in respiratory water loss.

Marais et al. provided an impressive and comprehensive analysis of the respiratory patterns of insects, which were examined in a phylogenetic context (Marais et al., 2005). The authors concluded that the cyclic respiratory pattern was the basal condition in insects, and that the DGC had evolved at least five times independently in the insect clade. They scored insect orders in which the DGC had not been observed as negative for this trait. Since that time, some new observations have become available. The use of the DGC has been demonstrated for a dipteran (Gray and Bradley, 2006) and for two species of hemipteran (Contreras and Bradley, 2009) (present study). This expands the number of orders in which the DGC has been observed, as well as the proposed number of independent evolutionary events in which it may have arisen, from five to seven. In addition, Gray and Bradley pointed out the importance of the rate of gas perfusion when measuring respiratory patterns using flow-through respiration (Gray and Bradley, 2006). They found that patterns that appear to be cyclic could be resolved to be the DGC at higher rates of gas perfusion. Results presented by Contreras and Bradley (Contreras and Bradley, 2009) as well as in the present study indicate that insects that employ a cyclic respiratory pattern at room temperature will switch to the DGC at lower temperatures. It would therefore be of interest to expand the studies of Marais et al. (Marais et al., 2005) using high flow rates and lower temperatures. It may be the case that many more insect species will display discontinuous patterns under these conditions, perhaps necessitating a re-evaluation of the basal condition. The results would be of great interest, as the evolutionary history of physiological and behavioral traits are best approached in a phylogenetic context (Bradley et al., 2009).

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