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Evidence from mosquitoes suggests that cyclic gas exchange and discontinuous gas exchange are two manifestations of a single respiratory pattern

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Summary

In this paper we demonstrate that the apparent pattern of gas exchange in insects, as observed using flow-through respirometry, is strongly affected by the rate of flow of air through the system. This is true not only because of the time constant of the respiratory chamber in which the insect resides, but also due to the effect of flow rate on the residence time of air as it passes through the detection chamber in the gas analyzer. It is demonstrated that insects respiring with a discontinuous gas exchange pattern can appear to be using a cyclic respiratory pattern. The effects of flow rate on the respiratory pattern discerned are illustrated using the mosquito *Culiseta inornata*. It is demonstrated that these mosquitoes respire discontinuously. They are among the smallest insects to date in which the discontinuous gas exchange cycle has been observed.

Key words: mosquito, *Culiseta inornata*, gas exchange, respiration.

Introduction

Insects show a diversity of gas exchange patterns at rest (Lighton, 1994; Lighton, 1996; Slama, 1999; Hadley, 1994). The most familiar of these is the Discontinuous Gas Exchange Cycle (DGC) (Levy and Schneidermann, 1966; Lighton, 1996; Hetz and Bradley, 2005), which derives from a repeating cycle of spiracular openings and closings that leads to periodic releases of carbon dioxide.

Although the DGC has fascinated insect physiologists and has been the topic of the majority of papers on insect respiratory patterns, it is not the only pattern encountered. Investigators have observed rhythmic patterns of gas exchange in which the release of CO_2 apparently never reaches zero, as well as other patterns of fairly continuous release that show no organized pattern (Lighton, 1996; Gibbs et al., 2003; Gibbs and Johnson, 2004). During periods of very high metabolic activity, such as flight, the spiracles appear to open very widely and remain sufficiently open to supply oxygen at the rate it is needed for aerobic metabolism (Lehmann et al., 2000).

Gibbs and Johnson provided a discrete nomenclature for the types of gas release patterns observed in insects (Gibbs and Johnson, 2004). Their terminology describes the patterns observed in relatively inactive insects. Following the nomenclature of Lighton (Lighton, 1994), they termed the pattern DGC if discrete periods were observed in which the release of CO_2 went to zero. They described a cyclic pattern as one in which there were rhythmic increases and decreases in CO_2 release, but no periods in which the release went to zero. Finally, they reserved the term continuous for patterns in which CO_2 release was never zero and showed no rhythmic pattern of increase and decrease over time.

These descriptive terms (Gibbs and Johnson, 2004) are very useful as a means of classifying the many gas exchange patterns observed in insects. There remains the question, however, of how these distinct patterns are produced and why the transition from one pattern to another may occur.

The most common technique for investigating gas exchange in insects has been flow-through respirometry. In such procedures, air is rendered CO_2- and H_2O-free by passage through absorbing materials. It is then directed, at a precisely controlled rate of flow, through a respiratory chamber containing an individual insect. This air finally flows to a CO_2 analyzer where the partial pressure of CO_2 present is determined through differential infrared absorption. Since the air passing into the respiratory chamber is free of CO_2, the CO_2 measured by the analyzer must have come from the insect. We are providing a description of this now common procedure in order to discuss relevant technical issues associated with the technique.

In recent years, we have been investigating the gas exchange patterns of insects whose body mass is on the order of 1–10 mg, e.g. *Drosophila melanogaster* and various species of mosquitoes (Williams et al., 1997; Williams et al., 1998; Gray and Bradley, 2003; Gray and Bradley, 2005a; Gray and Bradley, 2005b). Insects of this body mass release CO_2 at rates that are near the lower detection limits of the current generation of CO_2 analyzers. In such studies, one faces a trade-off between the need to have the gas flow through the respiratory

equipment at a rate low enough to provide a measurable signal, and the need for that flow to be fast enough to provide insights into the rapid spiracular mechanisms used by the insects.

A major issue related to elucidating the rapid respiratory responses in insects is the time constant of the chamber holding the insects. Variation in the partial pressure of gases in the chamber over time is described by the following equation:

$$(P_{CO_2})_t=(P_{CO_2})_0\,(e^{-F/V(t)})\,.$$

This equation (Bartholomew et al., 1981) can be used to describe the behavior of a flow-through respirometry system under conditions where an insect releases a burst of CO_2 into the chamber. $(P_{CO_2})_0$ represents the instantaneous partial pressure of CO_2 in the chamber at time 0. $(P_{CO_2})_t$ indicates the partial pressure of CO_2 exiting the chamber and heading toward the carbon dioxide detector at time t under conditions where CO_2-free gas is flowing at a rate F (ml min^{-1}) into a chamber with a volume V (ml). The equation also assumes that mixing in the chamber is instantaneous, an assumption we will return to shortly.

A useful value deriving from this equation is the time constant of the chamber under these flow conditions. This is represented as V/F. The units of this value are ml $(ml^{-1}\ min^{-1})^{-1}$=min. Let us assume that release of CO_2 into the chamber occurs as a discrete burst and that mixing is instantaneous. The time required for the partial pressure of CO_2 in the chamber (C_t) to be diluted to 5% of its peak value (C_o) is 3 times the time constant. The time required for the partial pressure of CO_2 to be diluted to 1% of the value at C_o is 5 times the time constant. If, for example, the flow rate of CO_2-free air into the chamber is 100 ml min^{-1} and the chamber has a volume of 1 ml, the time constant of the chamber is $1/100^{th}$ of a minute or 0.6 s. Under these conditions, 99% of the CO_2 released into the chamber in a discrete and instantaneous burst would be cleared from the chamber and sent to the analyzer in 3 s (5× 0.6 s). If this time resolution is insufficient for discerning the respiratory patterns under investigation, the investigator has the option of using a smaller chamber or a higher flow rate to improve the time constant of the chamber and thus the time resolution of his measurements.

The above calculations assume that the mixing of gases in the chamber is close to instantaneous. To this end, investigators have used various methods to ensure that the flow of air in the chamber is turbulent and to avoid any laminar flow that would slow the mixing of gases into the air stream sampled by the gas analyzer, thereby nullifying the assumption of instantaneous mixing. Researchers have been aware of the need for rapid and turbulent flow to reduce the time constant of the respiratory chamber (e.g. Lighton, 1988a; Lighton, 1988b; Davis et al., 2000; Chown and Holter, 2000; Vogt and Appel, 2000). Bartholomew et al. attempted to deal with a long time constant, relative to the respiratory phenomenon being examined, by calculating the instantaneous rate of oxygen uptake assuming a first order response distortion due to washout (Bartholomew et al., 1981). They essentially calculated the asymptote toward which the signal was moving. This calculation (which can also be made using the Z transform on the Sable Systems software, DATACAN), allows one to estimate instantaneous rates even with long time constants.

There is a second issue that profoundly affects the measurements of gas exchange patterns in small insects of which, unlike the time constant, many researchers have not been aware. Carbon dioxide analyzers of the type we, and many others, have used for flow-through respirometry contain an internal detection chamber in which the concentration of CO_2 in the air stream is quantified by differential absorption of infrared light by the CO_2 molecules. The detection chamber is generally a long narrow tube that has a window at each end through which the infrared light is shone. This configuration maximizes the path length over which the infrared light can be absorbed, thereby maximizing the sensitivity of the analyzer. In the analyzer we use (a Li-Cor 6251 IR gas analyzer), this chamber has a cross section of 0.6 cm by 1.3 cm and the chamber is 15.2 cm long. By multiplying these values, one can see that the volume of the detection chamber in the analyzer is 11.86 ml. Detection chambers of similar size are present in all of the commercial CO_2 detectors with which we are familiar (Li-Cor models 6251, 6262, 7000).

It is reasonable to assume, given the long narrow configuration of the detection chamber, that flow through this chamber is laminar. It is inappropriate, therefore, to consider the time constant of this chamber. Instead, we should be thinking of residence time. The value provided by the analyzer at any given time reflects the average partial pressure of CO_2 in the air in the detection chamber at any given instant. For example, if an insect released a burst of CO_2 that exited the chamber in which the insect resided and entered the analyzer, the burst of CO_2 would travel through the detection chamber in the analyzer and would increase the absorbance from the time it entered at the upstream end of the detection chamber until it left the downstream end. Thus this single discrete burst of CO_2 would cause an elevated reading in the analyzer throughout its residence time in the tube. Given a flow rate of 100 ml min^{-1} and a tube volume of 11.86 ml, this residence time would be: 11.86/100 or 0.1186 min (approximately 7 s).

It can be seen that the detection chamber in the analyzer can have a profound effect on the measurements being made, effects that can make irrelevant all the best efforts of the investigators to improve the time constant of the respiratory chamber. The only solution to the problems posed by the volume of the detection chamber is to increase the flow rate such that the residence time of an aliquot of air in the chamber is short compared to the respiratory patterns under investigation.

A classic example of the cyclic pattern of CO_2 release was provided by Gray and Bradley, who reported a rhythmic pattern of CO_2 release in the mosquito *Culex tarsalis* (Gray and Bradley, 2003). The release of CO_2 never went to zero and showed a sinusoidal pattern of release with a periodicity of about 22 s. We wished to investigate further this cyclic, rhythmic pattern, particularly with regard to the physiological basis of the sinusoidal release of CO_2. To this end we

examined mosquitoes at reduced temperatures in order to lower the metabolic rate and lengthen the wavelength of the sinusoidal release pattern. In addition, cognizant of the concerns about residence time in the detection chamber, we examined the mosquitoes at higher rates of air flow. The result was that in many cases, the cyclic pattern of respiratory CO_2 release was found to be an artifact of the respirometry system. We provide evidence that the discontinuous gas exchange cycle may be much more common in small insects than previously thought.

Materials and methods

Larval stages of the mosquito *Culiseta inornata* (Williston) were collected from rain-filled artificial containers in a residential area near the University of California, Irvine, USA, and reared in the laboratory at 20°C and 12 h:12 h L:D (light phase from 06:00 h to 18:00 h). Pupae were placed in pint-size cups and left to emerge, with access to 10% sucrose. Only female mosquitoes aged between 2 and 6 days post-emergence were used for the experiments. *Culiseta inornata* was used because it is a fairly large species. The wet mass of females sampled from the colony was on average 7.68 mg±0.43, mean ± s.e.m., N=11. All experiments were performed between 10:00 h and 17:00 h, during the time when the mosquitoes are resting and relatively inactive.

Measurements of respiratory patterns were performed using flow-through respirometry in a temperature-controlled room. The respirometry equipment consists of an 8-channel multiplexer, a Li-Cor CO_2 infrared gas analyzer (Li-Cor 6251, Lincoln, NE, USA) and a computer running the program Expedata (www.sablesys.com), which simultaneously, *via* a UI-2, controls the multiplexer and acquires data from the gas analyzer. Room air is pumped through columns containing silica gel, Drierite (W. A. Hammond Co., Xenia, OH, USA) and Ascarite (Thomas Scientific, Swedesbora, NJ, USA), in order to remove water and CO_2 from the air stream. A controlled rate of air was passed by the multiplexer alternately through an empty chamber (baseline) or a chamber with a volume of 0.5 ml containing the resting female mosquito (experimental chamber). Air leaving the chamber was directed through the Li-Cor analyzer and the partial pressure of CO_2 was recorded each 1 s using the mean of 74 values obtained over that 1 s interval. The purpose of this averaging is to filter out electrical noise from the measurements. Chambers were flushed with CO_2- and H_2O-free air between measurement intervals.

We performed measurements at a variety of flow rates between 20 and 1000 ml min^{-1}. Since the highest flow rates exceeded the mass controllers available to us, we chose instead to adjust the flow rates using a model R-1 flow control unit (AEI Technologies, Pittsburg, PA, USA), for which the accuracy of the flow rates was verified by measuring volume displacement in calibrated volumetric flasks. All flow rates were further verified using an ADM1000 flowmeter (J&W Scientific, Folsom, CA, USA). Flows are given as ml min^{-1} at the temperature reported and were rechecked at each rate and each temperature used in the studies.

Each series of measurements consisted of recording the respiratory pattern of a mosquito at multiple flow rates. We performed the experiments at different temperatures in order to affect the metabolic rate of the mosquitoes. The temperatures chosen for the measurements were 10, 20 and 30°C. Between each series of experiments we changed the temperature and waited 12 h for the room and equipment to adjust to the new temperature before performing another series of experiments.

On a given day, a mosquito was placed into a small chamber, connected to the multiplexer and left there for approximately 0.5 h to adjust to its new environment. Then the software program was started and repeated for several hours. Each run of the program consisted of two 10 min periods of data collection from the mosquito's chamber with baseline recordings preceding and following each measurement. If the flow rate was changed during an experiment, this occurred between runs or during the baseline interval between experimental recordings. We were thus able to record the pattern of single mosquitoes, undisturbed, at a variety of flow rates.

Data analysis

We used Sable Systems software (www.sablesys.com) for baselining each dataset using linear regression before exporting it to Excel.

Data obtained at high flow rates were in some cases manipulated to mimic slower flow rates in order to observe whether respiratory patterns can be predicted based on residence time in the measurement chamber and flow rate. To do this we first determined the ratio of the volume of the detection chamber in the CO_2 analyzer to the flow rate we wished to mimic. The value obtained, multiplied by 60 (number of measurements min^{-1}), represents the number of points that must be averaged in the original data set. A new dataset was then constructed in which each datum was an average of several consecutive points from the original dataset. These values were then multiplied by the ratio of the old to the new flow rate.

Results

Fig. 1 shows the gas exchange pattern of a female *Culiseta inornata* at 10°C, a relatively low temperature for this species but certainly one that it experiences in its natural environment. In Fig. 1A, the pattern is depicted using flow-through respirometry with a flow rate of 200 ml min^{-1}. The far left and far right portions of all of the figures illustrating respiratory patterns show baseline values, which represent the concentration of CO_2 when the air is passed through an empty chamber. The baseline (zero) value was determined using the Sable Systems DAN software, which allows one to set a zero baseline by randomly picking points over a stretch of baseline values. It can be seen that at this low level of metabolic rate

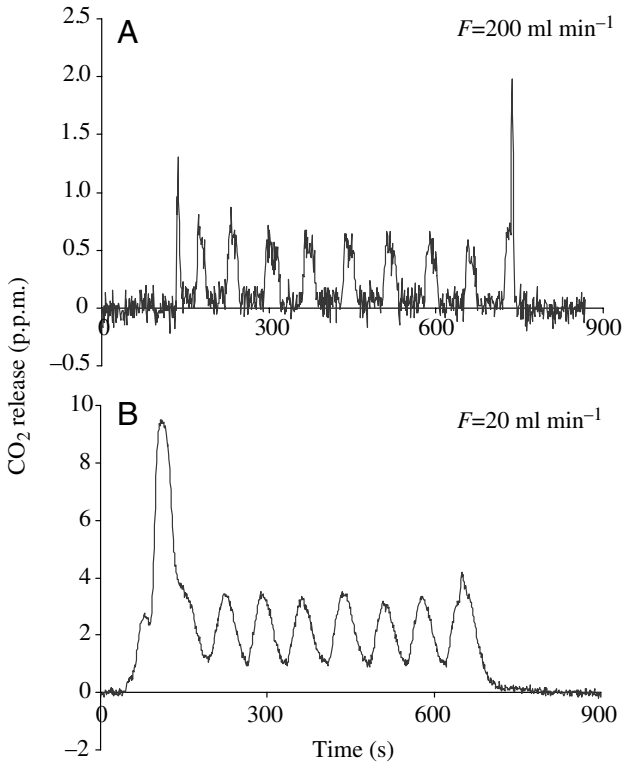

Fig. 1. Example of the respiratory pattern of a female mosquito (*Culiseta inornata*) measured at 10°C at flow rates F=200 ml min^{-1} (A) and 20 ml min^{-1} (B). Values at the far left and the far right of each curve represent baseline measurements obtained with an empty chamber. Note that the concentrations of CO_2 are lower at the higher flow rate due to the effects of dilution. The two flow rates reveal a similar periodicity of peak CO_2 release. In A, the mosquito is engaged in DGC, as shown by the periodic lapses to zero or near-zero rates of CO_2 release. At 20 ml min^{-1} the same respiratory pattern appears as a cyclic pattern of release with no periods of zero release.

and high rate of air flow, the system is operating near its level of resolution. Even with CO_2-free air flowing into the system some noise, primarily electrical (despite signal averaging), is associated with the detector and this is visible as variability in the signal about the zero value. The center of Fig. 1A shows the gas exchange pattern of the mosquito in which one sees a rhythmic release of CO_2. Between the bursts of CO_2 release, the rate of CO_2 release from the insect becomes very low. During some of these periods of low release, the rate is noticeably higher than the baseline values, but at other times it is indistinguishable from baseline. We feel that this pattern reflects the use of the DGC by the insect, characterized by periods of rapid CO_2 release interspersed with prolonged periods, on the order of 30 s, during which release is severely curtailed and/or eliminated. See the Discussion for further comments on whether these low values represent full spiracular closure. We feel, therefore that the combination of low temperatures and high flow rates allows us to demonstrate that this mosquito at this temperature is using the DGC.

Table 1. *Rates of CO_2 release per mosquito and the frequency of CO_2 release peaks at different temperatures, regardless of respiratory pattern*

Temperature (°C)	$\dot{V}_{CO_2}$ (nl min^{-1})	Peaks min^{-1}
10	49.8±4.1	0.6±0.03
20	231.2±17.0	1.1±0.07
30	458.5±11.9	2.5±0.15

Values are means ± s.e.m. (N=4 at 10°C, N=3 at 20°C and 30°C).

Fig. 1B shows the same mosquito in the same experimental run as Fig. 1A, following a tenfold reduction in the flow rate through the respiratory system. Once again, the values on the far left and right sides show baseline values with the zero set by the software. At this flow rate we see two substantial changes in the perceived pattern of CO_2 release. Firstly, the concentrations of CO_2 are higher due to a reduced dilution effect, even though the amount of CO_2 released by the insect is unchanged. Accordingly, the electrical noise is a much smaller percentage of the signal. Secondly, the apparent reaction time of the detector is much delayed. As a result, abrupt changes in the rate of CO_2 release are obscured due to the averaging effect of increased residence time in the detection chamber. In addition, the values do not go to zero because the slow rate of flow does not clear CO_2 out of the detection chamber before the next burst begins to enter. We would emphasize that these changes are not due to the time constant of the respiratory chamber. At this flow rate, 99% of the air in the chamber is cleared in 7 s. By contrast, at this flow rate, the residence time for air in the detection chamber is 35 s; a period of time equal to or longer than the closed period detected at the higher flow rate (Fig. 1A).

At higher temperatures, the metabolic rate of mosquitoes is increased (Table 1). This is reflected in the patterns of CO_2 release from the mosquitoes in two ways; the overall rate of CO_2 release is increased, and the frequency of the bursts of CO_2 release is increased (see Discussion). Fig. 2A shows a female *Culiseta* at 20°C and a flow rate of 1000 ml min^{-1}. Note that the pattern at this higher temperature and higher flow rate resembles that of a mosquito at 10°C and a flow rate of 200 ml min^{-1} (Fig. 1A). Fig. 2B shows the same mosquito in the same experimental run using a flow rate of 20 ml min^{-1}. This pattern is a classic cyclic pattern.

At 30°C and a flow rate of 1000 ml min^{-1} (Fig. 2C), the rate of CO_2 release discerned never appears to go completely to zero, nor to remain at low, flat values for a considerable period of time. This results in part from the increased frequency of the bursts. It might be that a higher flow rate would reveal a pattern more closely resembling DGC. Note, however, the very low concentrations of CO_2, which are produced by such small insects at such a high rate of air flow. The limits of resolution of our CO_2 analyzer are beginning to be reached at this level of analysis. Nonetheless it is clear, even at 30°C, that lower flow rates (Fig. 2D) produce respiratory patterns that are artifactually cyclic and with minimum values of CO_2

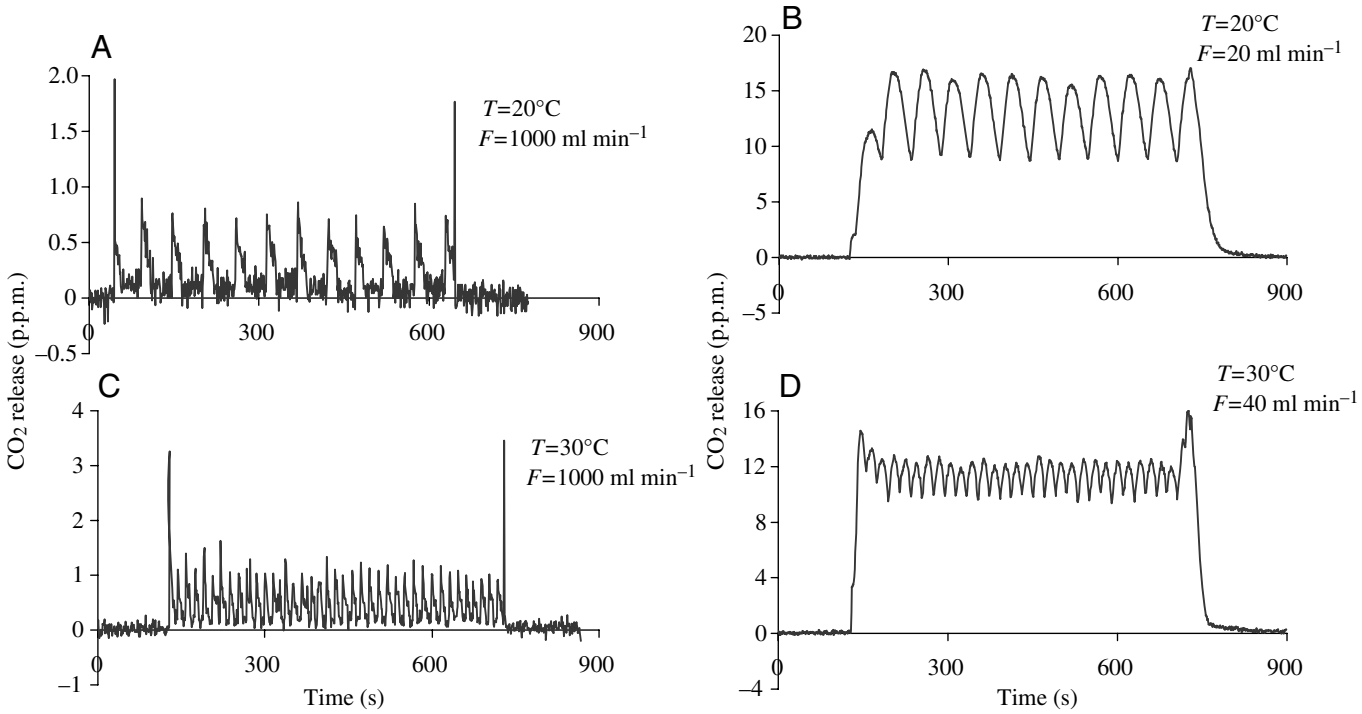

Fig. 2. Example of the respiratory pattern of female mosquitoes at different flow rates (*F*) and different temperatures (*T*). (A,B) Patterns from a single female mosquito at 20°C, (D,E) from a different mosquito at 30°C. Note that as temperature increases, so do metabolic rate and burst frequency. In all cases a lower flow rate results in a less discontinuous (more cyclic) pattern, with an absence of periods of zero release.

release that are substantially above those revealed by higher flow rates.

Fig. 3 shows the pattern of CO_2 release from a female *Culiseta* at 10°C, measured with a flow rate of 20 ml min^{-1} (Fig. 3A) or 200 ml min^{-1} (Fig. 3B). In keeping with our previous results it can be seen that at the lower flow rate, the concentrations of CO_2 are higher, the pattern is more cyclic, as opposed to discontinuous, and the minimum values of CO_2 recorded are substantially higher. We reasoned that the differences in the patterns revealed were due to (1) the effects of dilution, which are proportional to flow rate, and (2) the fact that residence time in the detection chamber affects the true pattern by producing a moving average of all CO_2 values passing through the detector. If these are the only two factors controlling the shape of the pattern recorded we should theoretically be able to mathematically reproduce a slow flow rate pattern based on one obtained at a fast flow rate. We therefore attempted to reproduce these effects by mathematically manipulating the signal produced at the high flow rate to mimic that produced at the low one. Fig. 3C shows the results of such a mathematic manipulation. The residence time of the air in the detection chamber at 20 ml min^{-1} is 35 s. The dilution caused by the lower flow rate is tenfold. We therefore took the experimental 200 ml min^{-1} pattern shown in Fig. 3B and produced a theoretical 20 ml min^{-1} pattern by calculating a moving average of the values. For each time-point, a new value was obtained by averaging 35 points adjacent to it, from the 17th point before it to the 17th point after it. The same procedure was performed on each value. Finally, we multiplied the values by 10 to mimic the concentrating effects of the slower flow rate. The resulting curve is shown in Fig. 3C.

The data in Fig. 4 further explore the differences between patterns gathered at various flow rates and mathematically manipulated data. Fig. 4A,C,E show the data obtained from a single female sampled at 20°C and 100, 50 and 20 ml min^{-1}, respectively. Data from this same female are shown in Fig. 2A, sampled at 1000 ml min^{-1}. We wished to determine whether mathematic manipulation would mimic the curves obtained at the various flow rates. At 1000 ml min^{-1} the residence time for air in the detection chamber is 0.7 s. Since we were sampling at 1 value s^{-1}, the sampling rate of the computer, not the residence time in the detection chamber, determined the time resolution of our measurements. At 100 ml min^{-1} the residence time in the detection chamber is 7 s. We therefore took the data illustrated in Fig. 2A and produced a moving average of those values using an interval of 7 s in order to produce a theoretical 100 ml min^{-1} pattern. The new values were multiplied by 10 to account for the difference in dilution due to flow rate. The resulting curve is shown in Fig. 4B. We used similar reasoning to manipulate the data in Fig. 2A, using a moving average of 15 s and multiplying by 20 to produce Fig. 4D and a moving average of 35 s and multiplying by 50 to produce Fig. 4F. In each case, the figures on the right in Fig. 4 have been manipulated to attempt to match the patterns on the left at that flow rate. If our mathematic manipulations are correct, the

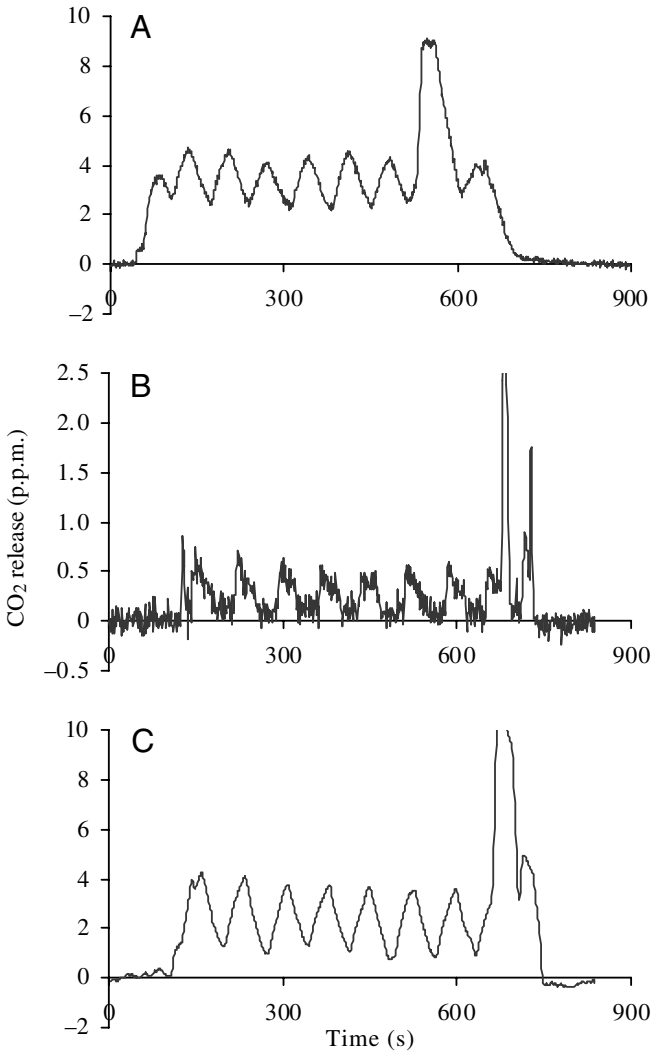

Fig. 3. Examples of the respiratory pattern of a single female mosquito at 10°C. (A) The respiratory pattern measured at flow rate F=20 ml min^{-1}, (B) the same mosquito at F=200 ml min^{-1}. (C) The values in B mathematically manipulated by multiplying by 10 and plotting a sliding average of the values using a window of 35 values. Note the similarity between A and C.

right-hand figures should resemble their experimental counterparts, to their left.

Discussion

The pattern of CO_2 release from a female *Culiseta inornata* at 10°C and 200 ml min^{-1} is illustrated in Fig. 1A. In this figure, the values at the far left and far right of the graph show the release of CO_2 from an empty chamber, and therefore represent zero release of CO_2. The variability in the signal in these regions reflects electrical noise in the signal. If we compare these regions of zero release with the interburst phases in Fig. 1A, we can see that several of the interburst phases have rates of release that are just as low as the 'zero' baseline values. In addition, these periods of low release are often on the order of 30 s in length. Given that the time constant of the respiratory chamber is 0.15 s at this flow rate, and the residence time in the detection chamber is 3.5 s, these lengthy periods of low release clearly represent true periods of very low CO_2 release by the insect. We observed this pattern of release in all of the female mosquitoes investigated at 10°C, and similar respiratory patterns were also seen at 20°C and 30°C (Fig. 2). These mosquitoes are the smallest insects that we are aware of in which DGC has been observed.

The results of our studies are of interest not only in terms of the elucidation of the gas exchange pattern of mosquitoes, but also with regard to the relationship between DGC and cyclic patterns of CO_2 release. Gray and Bradley reported that female mosquitoes of the species *Culex tarsalis* respire with a cyclic pattern of CO_2 release (Gray and Bradley, 2003). We are able to show a similar pattern for a female *Culiseta inornata* if the female shown in Fig. 1A is examined in a slower flow rate (20 ml min^{-1}, Fig. 1B). The pattern which at high flow rates appears to be discontinuous, appears to be cyclic at low flow rates. Similarly, at 20°C and 30°C, use of slower flow rates also produces respiratory patterns that appear less discontinuous and more cyclic. We suggest that these more cyclic patterns are artifacts associated with inadequate rates of air flow through the respirometry system.

Three variables, all of which are under the control of the investigator, affect the temporal resolution that can be achieved in the respirometry system. The first of these is the time interval chosen for recording the data. In modern systems, data collection is computerized and the time interval of collection can be selected. We chose a 1 s interval. Obviously, any events occurring with a time resolution of less than 1 s would not be distinguishable under these conditions. The second variable is the time constant of the respiratory chamber in which the insects reside during the measurements. As discussed in the Introduction of this paper, a period of time equal to 5 times the time constant is sufficient to remove 99% of the gas released into the chamber. With a chamber volume of 0.5 ml and a flow rate of 1000 ml min^{-1}, five times the time constant for this setup is 0.15 s. At this flow rate, the temporal resolution of the data collected is therefore determined by the chosen computerized data interval and not the time constant of the respiratory chamber. At 20 ml min^{-1}, by contrast, five times the time constant is 7.5 s, and the effects of this configuration on the signal obtained must be taken into account. Finally, the temporal resolution of the signal obtained is affected by the residence time of the detection chamber in the CO_2 analyzer. At low flow rates the effects of the slow passage of air through this chamber can have profound effects on the signal obtained.

This is best illustrated by examining Fig. 3. Fig. 3B shows the pattern obtained at 10°C and a flow rate of 200 ml min^{-1}. It can be seen that the insect releases CO_2 in bursts with interburst periods where CO_2 release is very low but measurably above zero. The same insect, exhibiting the same frequency of peak release, is shown in Fig. 3A under conditions where the rate of flow is 20 ml min^{-1}. The signal

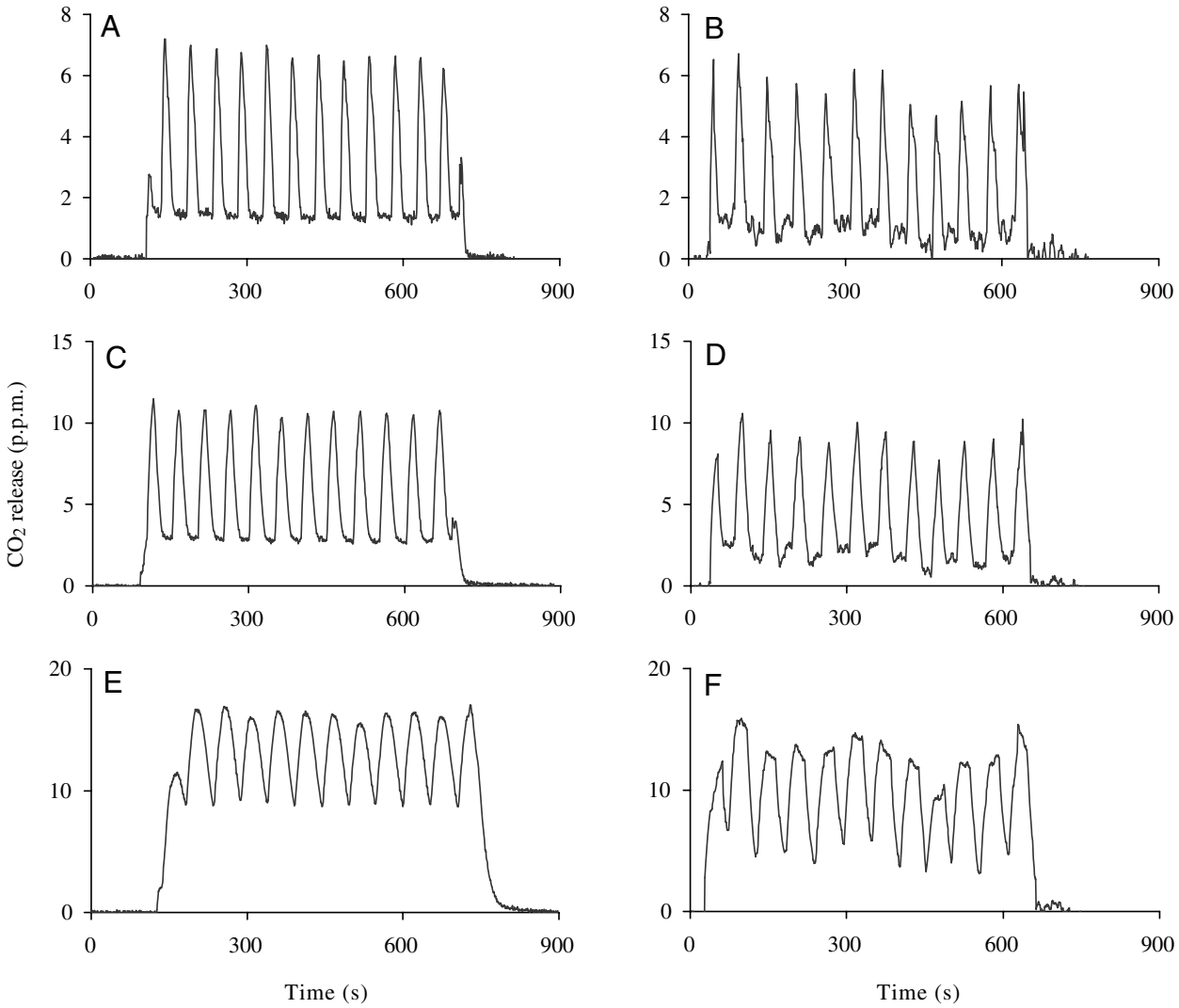

Fig. 4. Examples of the respiratory pattern of a female mosquito at 20°C. The same female was measured at flow rates F=100 (A), 50 (C) and 20 ml min^{-1} (E). The respiratory pattern of this same female at F=1000 ml min^{-1} is shown in Fig. 2A. (B,D,F) Values produced by mathematically manipulating the values from Fig. 2A to mimic the patterns in A,C,E, respectively. Data were manipulated by multiplying by 10 and using a sliding average of 7 (B), multiplying by 20 and using a sliding average of 15 (D) and multiplying by 50 and using a sliding average of 35 (F).

obtained is different with regard to several parameters of interest. Firstly, the mean values and peak values are ten times higher in Fig. 3A than in 3B. The reason for this is the difference in flow rate. The insect is releasing identical amounts of CO_2 into the passing air stream. Since ten times as much air is passing through the system in Fig. 3B as in 3A, the concentration of CO_2 is ten times higher in Fig. 3A. Furthermore, at the slower flow rate used in Fig. 3A, the residence time of the gas in the detection chamber is 35 s, *versus* only 3.5 s in Fig. 3B. This means that the CO_2 analyzer is in essence producing a moving average over 35 points (s) of the CO_2 being released by the insect. This serves to physically average the values obtained, obscuring any rapid changes in CO_2 release, which can be observed in Fig. 3A in that the increases and decreases in CO_2 concentration are faithfully recorded but the full excursion of the values is muted. In addition, increases and decreases occur less abruptly and more smoothly.

In order to test whether our assertions regarding the effects of the detection chamber volume on the signal obtained under lower rates of flow were accurate, we mathematically manipulated the data in Fig. 3B to reproduce the effects of the detection chamber. We did this by producing a moving average of the data obtained in Fig. 3A to mimic the effects of the 20 ml min^{-1} flow rate. We then took the resulting values and multiplied them by 10 to mimic the effects of the slower flow rate on the concentration of CO_2 in the air stream. The results, shown in Fig. 3C, yield a pattern very similar to that obtained in Fig. 3A. We feel that these results support the contention that the detection chamber has marked averaging effects on the

signal output from the analyzer at low rates of air flow, artifactually producing a cyclic pattern of release from an underlying respiratory pattern that can be markedly more discontinuous.

In Fig. 4 we show data obtained from a female mosquito at 20°C, sampled at three different flow rates. This same female is also shown in Fig. 2A at a faster flow rate. In the latter figure it can be seen that this insect has extensive periods of very low rates of CO_2 release. From Fig. 4A,C,E it can be seen that slower rates of flow result in a more cyclic pattern with interburst values that are substantially elevated due both to the multiplying effects of the reduced dilution factor at slow flow rates, and the averaging effects of the residence time in the detection chamber. Fig. 4B,D,F attempt to duplicate these effects by mathematically manipulating data from Fig. 2A through the use of moving averages and multiplication factors appropriate to each flow rate. It can be seen that the figures in Fig. 4B,D,F resemble those in Fig. 4A,C,E, but are not identical to them.

Why are the figures on the right in Fig. 4B,D,F not identical to those in Fig. 4A,C,E? Firstly, at fast flow rates, the CO_2 concentrations are lower, yet the electrical noise occurring in the analyzer remains unchanged. As a result, the signal-to-noise ratio at high flow rates is larger. This can be seen in the comparison of Fig. 4A and B, particularly in the baseline values on the far left and far right. The overall values are very similar. However, since the noise levels were proportionately larger in the data from which Fig. 4B was produced, the noise level in the baseline sections on the far left and far right are also greater. The effects of this noise extend throughout the data, resulting in less uniform values both during peaks and phases of low CO_2 release. As the moving average becomes larger (going from Fig. 4B to D to F) the periods of low CO_2 release between the bursts become shorter and shorter, until finally in Fig. 4F the effects of the large moving average completely obscure them, mimicking the effects of the slow flow rate in Fig. 4E. The low periods between the bursts are replaced by troughs, which transition from falling values to rising values without an intermediate plateau.

We feel that there may be one additional artifact that arises at slow flow rates. The air flowing through the long, narrow detection chamber is very likely to be undergoing laminar flow. As a result, the air near the walls of the chamber would be slowed relative to the air flowing down the center of the chamber. This would tend to 'smear' an aliquot of air flowing through the chamber, producing a temporal average of the signal. This effect would be greatest at slow flow rates since the unstirred layer near the chamber walls would be greatest at slow speeds. We suggest that some of the difference observed between Fig. 4E and F may be due to this additional averaging effect. The result is a more uniform cyclic signal with reduced excursions between the maximum and minimum values at the slower flow rate. This effect is not mimicked by simply taking into account the residence time and dilution effects.

It can be seen in Fig. 4F that the periods of low CO_2 release between the bursts appear to be above a zero level. We are therefore left with the question: if these mosquitoes are, as we assert, engaged in discontinuous ventilation, why does the release of CO_2 not go to zero in the interburst period? Note the very low levels of CO_2 being measured at this high rate of flow. We would suggest that at this very high level of resolution, the low levels of CO_2 exiting the animal may be coming from non-spiracular sources. Insects have a finite rate of CO_2 diffusion across the cuticle. The surface to volume ratio increases exponentially with decreasing size. Mosquitoes also have very extensive cuticle-lined extensions including the legs, wings and proboscis. It is possible, therefore that the release of 0.1 to 0.2 p.p.m. of CO_2 into the air stream is occurring across the cuticle and cannot be shut off during the closed phase. This assertion could be tested in future studies by using high levels of oxygen to force spiracular closure as demonstrated by Lighton et al. (Lighton et al., 2004).

Temperature also has a major effect on our capacity to resolve the gas exchange patterns of insects. Our current understanding of the control of breathing in insects suggests that the burst of CO_2 release associated with each open phase is triggered by the accumulation of a critical concentration of CO_2 in the insect (Levy and Schneiderman, 1966; Lighton, 1996). At higher temperatures, this critical concentration is accumulated more rapidly due to the increased metabolic rate of the insect. Therefore as the metabolic rate increases, the frequency of bursting also increases (Table 1). Several studies have reported that discontinuous gas exchange patterns in insects are replaced by more cyclic or even continuous patterns as temperature increases. Shelton and Appel (Shelton and Appel, 2000) observed that the variability of the gas exchange pattern (expressed as the coefficient of variation) decreased in termites at higher temperatures. Similarly, female alates of the fire ant were found to exhibit more cyclic patterns at higher temperatures (Vogt and Appel, 2000).

Other studies have correlated more cyclic or continuous patterns of respiration with increased metabolic rate, even in the absence of differences in temperature. Marais and Chown (Marais and Chown, 2003) observed that cockroaches in the genus *Perisphaeria*, which had a continuous gas exchange pattern at rest, also had a near twofold higher metabolic rate than those which performed DGC. Gibbs and Johnson (Gibbs and Johnson, 2004) found that queens in the ant species *Pogonomyrmex barbatus* showed a correlation between metabolic rate and respiratory pattern. The discontinuous pattern was associated with the lowest metabolic rate, cyclic with intermediate rates, and continuous with the highest rates. Certainly the pattern of CO_2 release insects does change with metabolic rate. The issues addressed in this paper, however, caution that care must be taken to assure that flow rates are sufficiently high to provide the temporal resolution desired. This issue becomes more critical as the metabolic rate of the insect increases, regardless of the cause.

The issue of flow rate and its effects on the pattern of CO_2 release observed is particularly acute for investigators examining very small insects. This is because the rate of CO_2 release from these insects is very low. Under these conditions

a low flow rate is frequently employed to increase the CO_2 concentration in the air exiting the respiratory chamber, thereby maintaining the concentrations within the detection range of the analyzer and maximizing the signal-to-noise ratio. Increasing the resolution of the CO_2 release pattern in such cases could modify the interpretation of the results and lead to a better understanding of the mechanisms of DGC modulation under different environmental conditions.

Given the confounding effects of flow rate and metabolic rate on the pattern of CO_2 release observed using flow-through respirometry, what flow rate is appropriate to get an accurate depiction of the respiratory pattern? That depends on the degree of temporal resolution required. Let us assume that we would be satisfied with a temporal resolution of 1 s. We would set the time interval for the computer recorder at 1 s. With a detection chamber of volume 11.86 ml, a flow rate of 711 ml min^{-1} would produce a residence time in the detection chamber of 1 s, matching the time resolution of the sampling software. Finally, the time constant of a 1 ml chamber at this flow rate is 0.08 s. Five times this value would therefore be 0.4 s, well below the sampling interval of the computer. Under these conditions, therefore, a flow rate of 711 ml min^{-1} should give an accurate depiction of events occurring with a time course greater than 1 s. If in turn, even more rapid temporal resolution is required, for example for very small insects or higher temperatures, it is important to carry out these calculations and reassess the setup to be certain that no aspect of the analytical system is inappropriately limiting.

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