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CONTROL OF RESTING VENTILATION RATE IN GRASSHOPPERS

SCOTTI L. GULINSON AND JON F. HARRISON*
Department of Zoology, Arizona State University, Tempe, AZ 85287-1501, USA

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Summary

We examined the effect of extracellular acid–base status and tracheal gas levels on the ventilation rate of resting *Romalea guttata* and *Schistocerca americana* grasshoppers. We manipulated haemolymph pH and [HCO_3^-] within normal physiological ranges using injections of HCl, NaOH, $NaHCO_3$ and NaCl into the haemocoel. In contrast to terrestrial vertebrates, there was no evidence that extracellular acidification increases ventilation rate in grasshoppers. Elevation of haemolymph bicarbonate levels (by $NaHCO_3$ injection) increased ventilation rate, while depression of haemolymph bicarbonate levels (HCl injection) had no effect. Injection of $NaHCO_3$ also increased tracheal P_{CO_2}, suggesting that the effect of the $NaHCO_3$ injection might be mediated by a sensitivity of the ventilatory system to tracheal gases. We tested for effects of tracheal gases on ventilation rate by independently manipulating tracheal P_{CO_2} and P_{O_2} using tracheal perfusions. Ventilation rate was positively correlated with tracheal P_{CO_2} and negatively correlated with tracheal P_{O_2}. Increasing tracheal P_{O_2} above normal resting levels or decreasing tracheal P_{CO_2} below normal levels decreased ventilation rate. We conclude that quiescent grasshoppers regulate tracheal P_{CO_2} and P_{O_2} by varying ventilation rate and that both P_{CO_2} and P_{O_2} in the trachea stimulate ventilation in normal, resting grasshoppers.

Key words: grasshopper, pH regulation, regulation of ventilation, *Romalea guttata*, *Schistocerca americana*, ventilation.

Introduction

The abdominal pumping or ventilatory rate of grasshoppers and other insects is known to increase with increasing ambient P_{CO_2} and decreasing ambient P_{O_2} (Miller, 1960*a*; Arieli and Lehrer, 1988). However, it is not known whether sensitivity to internal P_{CO_2} and P_{O_2} is involved in the normal regulation of resting ventilation rate or whether insects regulate their tracheal P_{O_2} or P_{CO_2}. In this study, we tested the hypothesis that grasshoppers regulate their tracheal P_{CO_2} and P_{O_2} levels by varying ventilation rate. It has also been suggested that the ventilatory system participates directly in short-term extracellular acid–base regulation in grasshoppers as it does in terrestrial vertebrates (Harrison, 1989*a*). To test this hypothesis, we examined the effect of extracellular acid–base status on ventilation rate.

In grasshoppers, abdominal pumping is accomplished by contraction of inspiratory and expiratory intersegmental abdominal muscles, which drive convective ventilation (Lewis *et al.* 1973). The coordination of abdominal expansion with opening of the thoracic spiracles, and abdominal compression with opening of the abdominal spiracles, produces a unidirectional thorax-to-abdomen airflow (McCutcheon, 1940; Weis-Fogh, 1967). Abdominal pumping constitutes the most important component of convective ventilation in resting locusts (Miller, 1960*a*; Weis-Fogh, 1967).

The respiratory rhythm of locusts is driven by pacemakers in the metathoracic and abdominal ganglia (Miller, 1960*a*; Lewis *et al.* 1973), with afferent feedback from abdominal stretch receptors (Hughes, 1952; Farley and Case, 1968). The effects of perfusion of local regions of dissected locusts with CO_2 suggest that CO_2 affects the head and thoracic ganglia directly and that locusts lack peripheral afferent nerves sensitive to CO_2 or pH (Miller, 1960*a*). The enhancement of ventilation by hypoxia or hypercapnia may indicate that ventilation rate is controlled by internal P_{O_2} or P_{CO_2} under normal physiological conditions in insects. Alternatively, abdominal pumping rate may be controlled by an endogenous rhythm insensitive to the changes in internal P_{O_2} or P_{CO_2} that occur *in vivo*. Supporting this possibility, internal (tracheal or haemolymph) P_{CO_2} values of resting grasshoppers have been reported to be 2–5 kPa (Krogh, 1913; Harrison, 1988, 1989*a*), and ventilation rate was not significantly stimulated by ambient P_{CO_2} values of 3 kPa (Hustert, 1975; Harrison, 1989*b*). Thus, it is possible that the responsiveness of the ventilatory system to ambient CO_2 and O_2 levels may have evolved in order to help control ventilation in insects living in burrows or other

*Author for correspondence.

microenvironments characterized by hypoxia or hypercapnia. We tested for an effect of tracheal P_{CO_2} or P_{O_2} on resting ventilation rate by independently manipulating tracheal P_{CO_2} and P_{O_2} values above and below normal tracheal levels using perfusions of the tracheal system *via* the metathoracic spiracle.

There is a great deal of correlational data suggesting that the ventilatory system may participate in extracellular pH regulation in insects as it does in vertebrates. In grasshoppers, changes in ventilation rate during hypercapnia or after locomotion are correlated with changes in both haemolymph P_{CO_2} and pH (Harrison, 1989*a*; Harrison *et al.* 1991). Case (1961) demonstrated that the fictive ventilatory rhythm of cockroach ganglia *in vitro* is sensitive to bathing solution pH. In dissected *Nauphoeta cinerea* cockroaches, changes in the pH or P_{CO_2} of fluids irrigating the ventral nerve cord elicited changes in ventilation rate (Snyder *et al.* 1980). However, grasshoppers injected with HCl recover from acidosis without changes in haemolymph P_{CO_2}, suggesting that decreased haemolymph pH does not stimulate ventilation in grasshoppers (Harrison *et al.* 1992). We tested directly for an effect of extracellular acid–base status on ventilation rate using injections of various acid–base solutions into the haemocoel.

Materials and methods

Animals and general protocols

For the experiments examining the effects of haemolymph pH on ventilation rate, we used eastern lubber grasshoppers, *Romalea guttata* Serville, collected from field sites in central Florida, USA. Grasshoppers were maintained in the laboratory as previously described (Harrison and Kennedy, 1994). This large species (mean body mass 4.2 g in males and 8.4 g in females) is particularly useful for such studies, owing to its large haemolymph volume and its passive nature. Since it relies on the secretion of phenol compounds and quinones from the exocrine defensive glands rather than escape tactics to deter predators, it remains calm and relatively immobile under observation (Whitman, 1990). *Romalea guttata* do not have spiracles suitable for cannulating as their spiracles bleed when cannulation is attempted. Therefore, for the experiments involving the manipulation of tracheal gases, we used the desert locust *Schistocerca americana* Drury (body mass range 1.4–2.3 g). All experiments were carried out at room temperature (22–25 °C).

Experiment 1: does haemolymph acid–base status affect resting ventilation rate?

In these experiments, haemolymph acid–base status was manipulated with injections of acid–base solutions into the haemocoel, and we tested for an effect on ventilation rate. Male and female *R. guttata* were weighed, and the haemocoel was cannulated using a 20 cm length of polyethylene tubing (PE 20) filled with 0.5 mol l^{-1} NaCl. Using a Dremel drill (bit diameter 0.4 mm), we drilled a hole into the animal's left side anterior to the metathoracic spiracle. The cannula was inserted into the hole and secured with glue from a hot glue gun. Animals were then placed in clear plastic containers with access to water but not food. Each container was wrapped in Mylar, and a fibre-optic light was shone into it from the top, allowing the Mylar to function as a one-way mirror to avoid disturbance of the grasshoppers. Small wire ladders were glued onto the side of each container for the grasshoppers to climb and be observed in a more natural position. The cannula extended from the animal to the outside of the container, where it was connected to a 50 μl Hamilton gas-tight syringe. The next day (14–20 h post-cannulation), the ventilation rate of each grasshopper was counted visually for 6 min. Each abdominal pumping event, regardless of volume, was counted as a ventilation event. Each animal was then injected through the cannula with one of four 0.5 mol l^{-1} solutions: NaCl, $NaHCO_3$, HCl or NaOH. Females were injected with 50 μl of each solution (to give, on average, 7.2 μl g^{-1} body mass), and males were injected with 30 μl (to give, on average, 5.9 μl g^{-1} body mass). Injection volume was corrected for the dead space of the cannulae to ensure that the correct volume was injected. The effects of acid–base injections into the haemocoel of grasshoppers persist for hours but are most pronounced during the first 10–15 min (Harrison *et al.* 1992). Therefore, we counted ventilation rate for 10 min after each injection, and compared the pre- and post-injection ventilation rates for each animal.

The effect of the injections on haemolymph acid–base status was tested by collecting haemolymph samples from an incision in the ventral cervix (throat) using a glass microcapillary pipette and 10 μl gas-tight Hamilton syringes (Harrison, 1988). Haemolymph samples were collected 6 min after the injections (midway through the period during which ventilation rate was analyzed). Haemolymph sampled from the ventral cervix area should be representative of that bathing the ventral nerve cord. The pH and C_{CO_2} (total [CO_2], mmol l^{-1}) of the haemolymph were analyzed at the body temperature of the animal using a glass capillary pH electrode and gas chromatography as previously described (Harrison and Kennedy, 1994). Haemolymph P_{CO_2} and [HCO_3^-] were calculated using CO_2 solubility coefficients and carbonic acid dissociation constants for locust haemolymph (Harrison, 1988). The NaCl injection was used to control for stress associated with the injection, since a similar injection has been shown to have no effect on haemolymph acid–base status in *Schistocerca gregaria* (Harrison *et al.* 1991).

Experiment 2: do tracheal P_{O_2} *or* P_{CO_2} *affect resting ventilation rate?*

Methods for manipulation of tracheal gas levels

In these experiments, tracheal P_{O_2} and P_{CO_2} were manipulated with gas perfusions *via* a cannula placed into the metathoracic spiracle. *Schistocerca americana* were restrained by rubber bands across the thorax against a metal screen and had cotton packed around their heads to limit their vision. The restraint system did not confine the abdomen or physically impede abdominal pumping. After a few minutes, the animals stopped struggling and appeared to ventilate normally. We allowed the animals 20–40 min to acclimate to the restraint

because this reduced the likelihood that animals would struggle during the cannulation procedure. A heat-stretched piece of polyethylene tubing (approximate tip diameter 0.2 mm) was slid into the spiracle, where it was held in place by the external spiracular valve while it was sealed to the animal using a low-melting-point glue gun. In all cases, we waited 20–40 min after installation of the cannula before performing perfusions to allow animals to recover from the handling stress imposed by the cannulation procedure and because preliminary experiments suggested that ventilatory rates return to resting values during this period. Test gases could be perfused into the trachea *via* the tracheal cannula, and the effect on ventilation rate measured. We perfused the tracheal system for short periods (3–5 s) with a large volume (3–5 ml) relative to normal tracheal volume (less than 1 ml; Harrison, 1989*b*) and minute ventilation rate (about 1 ml min^{-1}; Weis-Fogh, 1967) in an attempt to flush the tracheal system with the test gas. The perfusion did inflate the abdomen, but tracheal pressures returned to normal levels within a few seconds after cessation of the perfusion (Krolikowski and Harrison, 1996). We then measured ventilation rate and tracheal gas levels during the 1 min after cessation of the perfusion (since ventilation rates and tracheal gas levels returned to normal values in approximately 1 min, see Results).

Measuring the effects of tracheal perfusions on tracheal gas levels

Tracheal gas samples were taken *via* the methoracic spiracular cannulae, using a 50 μl Hamilton syringe at a rate of 6–10 $\mu l\,s^{-1}$. In a preliminary experiment, we tested the effect of the size of the tracheal gas samples taken on the CO_2 and O_2 levels measured. Tracheal samples of 10–40 μl had similar levels of CO_2 and O_2, while larger samples had lower CO_2 levels and higher O_2 levels. These data suggest that ambient air may enter the animal through other spiracles when samples larger than 40 μl are taken, hence diluting the sample. Therefore, we used 30 μl samples of tracheal air for all experiments.

The cannulae used had an average dead space of 1.95±0.48 μl (S.E.M., N=8), which probably contained a mixture of ambient and tracheal air. The maximum error in tracheal gas measurement due to dilution with dead space air (if the dead space was completely filled with room air) is 6 %.

Each animal received all of either the O_2-manipulating or CO_2-manipulating perfusions (see below), allowing 5 min between perfusions. We injected one of the gas mixtures and then drew out 30 μl of tracheal air 15, 30, 45 and 60 s after the tracheal perfusion. CO_2, N_2 and O_2 fractions of the air samples were analyzed with a Varian 3400 gas chromatograph and gas-chrom MP-1 column (Varian Analytical Instruments, Walnut Creek, California, USA). Voltage output peak heights from the thermocouple detector were digitized and recorded (Sable Systems, Salt Lake City, Utah, USA). Peak heights were converted to gas composition by calibrating the system with injections of gases of known composition (mixed on a Brooks 5878 mass-flow controller and meters). The coefficient of variation of CO_2 and O_2 values measured on 30 μl samples of calibration gases was approximately 5 %.

Measuring the effect of tracheal perfusions on ventilation rate

Ventilation rate was counted for 1 min before and 1 min after perfusion of the trachea with the test gas, since preliminary experiments indicated that ventilation rates returned to normal values within 1 min after the perfusion (see Results). In the first series of experiments, tracheal P_{CO_2} was varied (18, 10, 4.4, 2.3 or 0.4 kPa) at constant P_{O_2} (15 kPa, balance N_2). In the second series of experiments, we varied tracheal P_{O_2} (0.5, 4.7, 14, 30 or 41 kPa) at constant P_{CO_2} (2.2 kPa, balance N_2). Within a series, each locust received all injections. Gases were mixed using a Brooks 5878 mass-flow controller and Brooks mass-flow meters, with the gas composition of each mix confirmed using the Varian gas chromatograph. Injections were given in random order. All protocols were the same in both series except that, for the CO_2 series, we injected 5 ml of test gas; in the later O_2 series we reduced the volume of test gas perfused to 3 ml, which reduced the number of animals experiencing burst abdomens during perfusions from about 10 % to zero.

Test of the effects of restraint and cannulation on ventilation rate

The restraint and cannulation procedures used in experiment 2 might affect ventilation rate as a result of stress or by changing tracheal gas levels because of spiracle occlusion. Grasshoppers inhale *via* six thoracic spiracles, three on each side. The cannula occluded the left metathoracic spiracle, potentially interfering with gas exchange. Therefore, we tested for effects of restraint and cannulation on resting ventilation rate. Male *S. americana* were placed into separate clear containers inside an opaque box and starved overnight. The front of the box was covered with Mylar, and the grasshoppers were back-lit with a fluorescent bulb to allow the Mylar to function as a one-way mirror as previously described. The next day, the light was turned on 1 h prior to observations. We counted resting ventilation rate visually for 4 min for these completely undisturbed grasshoppers. The animals were then removed from the cups and restrained against the metal screens as described previously. They were left undisturbed for 20–40 min, and a restrained, uncannulated ventilation rate was then counted over 4 min. We then cannulated the animals *via* the metathoracic spiracle as described above, left them undisturbed for 20–40 min, and then counted ventilation rate over a period of 4 min. Finally, the cannula was removed, animals were left undisturbed for 20–40 min, and ventilation rate was counted again. Based on the results from these experiments (see below), a 20–40 min recovery period was allowed between cannulation and measurement of ventilation rates throughout experiment 2.

Experiment 3: does injection of $NaHCO_3$ into the haemocoel raise tracheal P_{CO_2}?

Male *S. americana* were collected and cannulated *via* the metathoracic spiracle as described previously. After 20–40 min, we sampled 30 μl of air *via* the metathoracic cannula and

analyzed its O_2 and CO_2 fraction using gas chromatography. We then injected 30 μl of either 0.5 mol l^{-1} NaCl or $NaHCO_3$ into the haemocoel. We sampled tracheal gases *via* the cannula every 2 min over the 10 min following the injection.

Statistics

Means ± S.E.M. are presented. Data were analyzed using paired *t*-tests (comparing pre-manipulation with post-manipulation values) when individuals received two treatments, or by repeated-measures Huynh–Feldt-corrected analysis of variance (ANOVA) when individuals received more than two treatments. When different individuals received different treatments, statistical analysis was performed using univariate ANOVA or *t*-tests as appropriate. All statistical analyses were performed using SYSTAT (Wilkinson, 1989), with our within-experiment type I error controlled at 5 %.

Results

Experiment 1: does haemolymph acid–base status affect ventilation rate?

The injections had very similar effects on haemolymph acid–base status and ventilation rate in males and females, so data from the two sexes were pooled. The injections significantly affected haemolymph pH (ANOVA, $F_{3,45}$=6.83, P<0.01) and haemolymph [HCO_3^-] (ANOVA, $F_{3,42}$=4.15, P<0.05), but not haemolymph P_{CO_2} (ANOVA, $F_{3,42}$=0.19, P>0.6). We tested whether each injection produced a significant effect on haemolymph pH, [HCO_3^-] or P_{CO_2} relative to the NaCl-injected control group using a two-tailed Dunnett's test. Relative to the NaCl-injected group, injection of $NaHCO_3$ significantly raised haemolymph pH and [HCO_3^-], while injection of HCl significantly reduced haemolymph pH and [HCO_3^-] (Fig. 1). Injection of NaOH had no significant effect on haemolymph acid–base status because of the small sample size (N=2, Fig. 1).

Injections of HCl, NaOH and NaCl had no effect on the ventilation rate averaged over the 10 min after the injection (Fig. 2). Injection of $NaHCO_3$, however, caused a significant 60 % increase in the ventilation rate (Fig. 2). Although the pre-injection ventilation rate for the $NaHCO_3$-injected group appeared low relative to the other treatment groups (Fig. 2), this did not explain the significant effect of the $NaHCO_3$ injection on ventilation rate. The resting ventilation rates of the four treatment groups did not differ significantly (ANOVA, $F_{3,58}$=0.97, P=0.41). The increase in ventilation rate after the $NaHCO_3$ injection occurred in 15 out of 17 animals tested and was significant at each minute during the 10 min after the injection (Fig. 3, *post-hoc* comparisons, repeated-measures ANOVA).

Experiment 2: do tracheal P_{O_2} *or* P_{CO_2} *levels affect resting ventilation rate?*

Effect of tracheal perfusions on tracheal gas composition

Normal resting tracheal P_{O_2} for restrained, cannulated *S. americana* was 18.8±0.62 kPa (N=15). When gases of varying

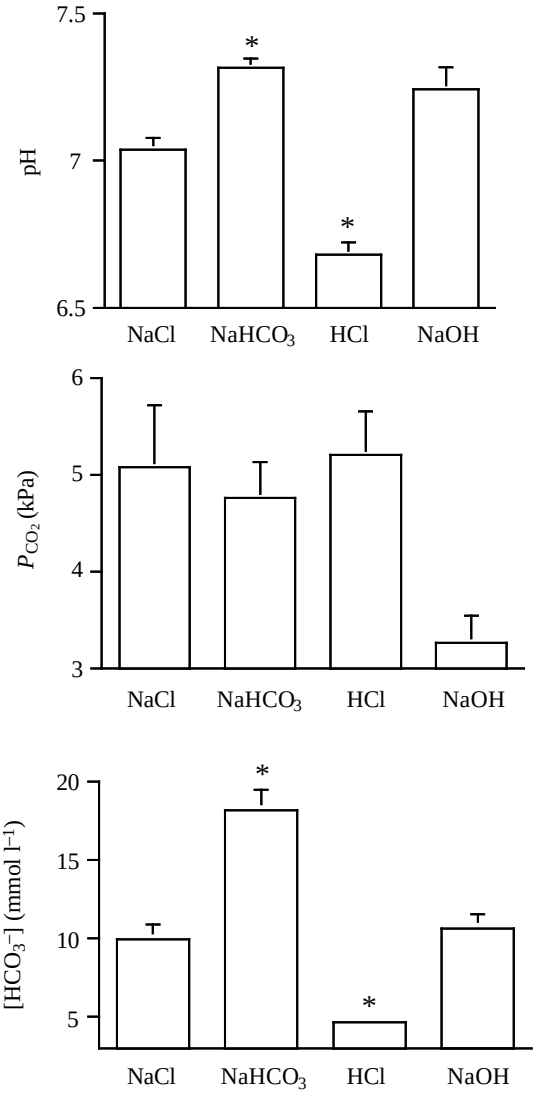

Fig. 1. The effect of injections of 0.5 mol l^{-1} NaCl, $NaHCO_3$, HCl and NaOH on haemolymph acid–base status of *Romalea guttata* measured 6 min after the injection. N=15 for NaCl injections, N=16 for $NaHCO_3$ injections, N=15 for HCl injections, N=2 for NaOH injections. Asterisks indicate a significant difference from the NaCl-injected value (Dunnett's test). Means ± S.E.M. are given in all figures.

P_{O_2} were perfused, in all cases there were transient changes in tracheal P_{O_2}, followed by recovery of tracheal P_{O_2} towards normal levels (repeated-measures ANOVA, P<0.01). Data for two of the perfusion mixtures are shown in Fig. 4. During the first 15 s after perfusion, tracheal P_{O_2} increased with increasing perfusate P_{O_2} (repeated-measures ANOVA, $F_{4,36}$=52.1, P<0.001, Fig. 5), although tracheal P_{O_2} did not match the P_{O_2} of the perfusate. Perfusing the trachea with gases of varying O_2 content (P_{CO_2} constant at 2.2 kPa) generally had little effect on tracheal P_{CO_2} (Table 1). However, in three cases, there were small but significant increases in tracheal P_{CO_2} (Table 1).

Normal tracheal P_{CO_2} for restrained, cannulated *S. americana* was 1.5±0.37 kPa (N=15). When gases of varying P_{CO_2} were perfused through the trachea, we measured transient

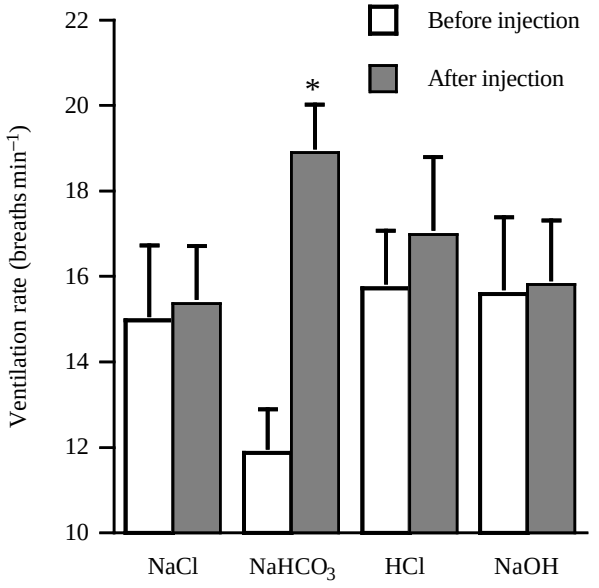

Fig. 2. Average ventilation rate of *R. guttata* during the 10 min after injection of 0.5 mol l^{-1} acid–base solutions into the haemocoel. N=14 for NaCl-injected, N=17 for $NaHCO_3$-injected, N=16 for HCl-injected, N=15 for NaOH-injected animals. An asterisk indicates a significant difference from the pre-injection value (paired t-tests).

Table 1. *The effect of perfusing the tracheal system with gases of varying* P_{O_2} *(perfusate* P_{CO_2} *maintained constant at 2.2 kPa) on tracheal* P_{CO_2} *in* Schistocerca americana

P_{O_2} injected (kPa)	Tracheal P_{CO_2} (kPa)			
		Time after injection (s)		
	Pre-injection	15	30	45
0.5	1.6±0.17	2.4±0.41	2.2±0.36	2.3±0.22*
4.7	1.6±0.14	2.3±0.20*	2.1±0.12	1.9±0.15
14	1.5±0.17	2.1±0.17*	1.7±0.25	1.8±0.13
30	1.4±0.18	1.6±0.15	1.5±0.16	1.6±0.15
41	1.5±0.13	1.2±0.10	1.3±0.09	1.6±0.16

Asterisks indicate significant differences from tracheal P_{CO_2} before injection (*post-hoc* comparisons, repeated-measures ANOVA).

Values are means ± S.E.M., N=6.

changes in tracheal P_{CO_2}, followed by a return towards normal levels (repeated-measures ANOVA, P<0.01). Data for two of the gases are shown in Fig. 6. During the first 15 s after perfusion, tracheal P_{CO_2} increased with perfusate P_{CO_2} (repeated-measures ANOVA, $F_{4,36}$=43.6, P<0.001, Fig. 7), although the change in tracheal P_{CO_2} was much smaller than the change in P_{CO_2} of the perfusing gas. Perfusing the trachea with gases of varying CO_2 content (P_{O_2} constant at 15 kPa) did not affect tracheal P_{O_2} (Table 2).

Effect of tracheal perfusions on ventilation rate

The perfusions caused transient changes in ventilation rates. We show data for the changes in ventilation rate with time after

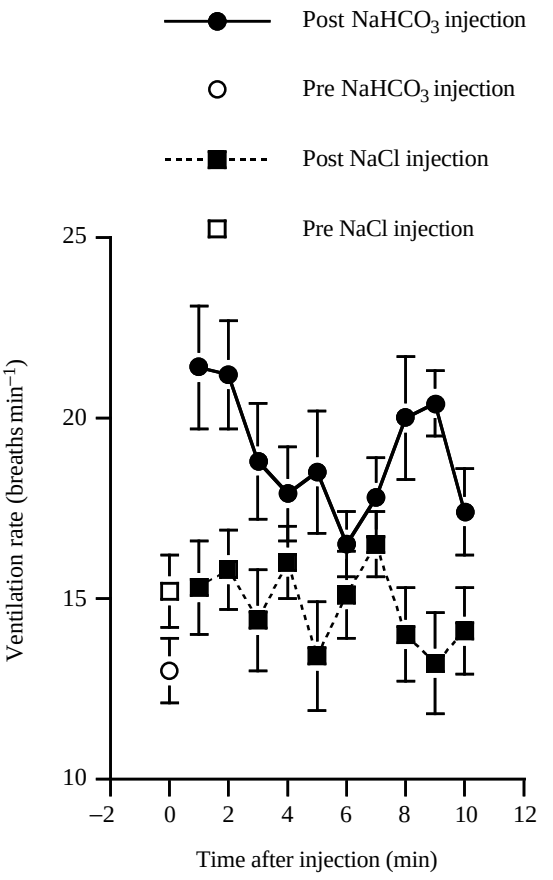

Fig. 3. Ventilation rate of *R. guttata* as a function of time after 0.5 mol l^{-1} $NaHCO_3$ or NaCl injections into the haemocoel. All of the post-injection ventilation rates differed significantly from the pre-injection values for the $NaHCO_3$-injected group (*post-hoc* comparisons for repeated-measures ANOVA, P<0.05). None of the post-injection ventilation rates differed from the pre-injection values for the NaCl-injected group (*post-hoc* comparisons for repeated-measures ANOVA, P>0.05; N=17 for the $NaHCO_3$-injected, N=15 for the NaCl-injected grasshoppers).

Table 2. *The effect of perfusing the tracheal system with gases of varying* P_{CO_2} *(perfusate* P_{O_2} *maintained constant at 15 kPa) on tracheal* P_{O_2} *in* Schistocerca americana

P_{CO_2} injected (kPa)	Tracheal P_{O_2} (kPa)			
		Time after injection (s)		
	Pre-injection	15	30	45
0.4	19.0±0.35	19.7±0.35	19.0±0.58	19.23±0.46
2.3	18.7±0.34	17.7±0.61	19.1±0.36	19.5±0.31
4.4	18.4±0.41	18.4±0.31	19.3±0.32	19.4±0.20
10	18.8±0.53	18.7±0.25	19.5±0.24	19.2±0.18
18	19.2±0.46	18.8±0.30	19.6±0.25	19.2±0.33

Values are means ± S.E.M., N=6.

In no case was tracheal P_{O_2} changed significantly by the P_{CO_2} injection.

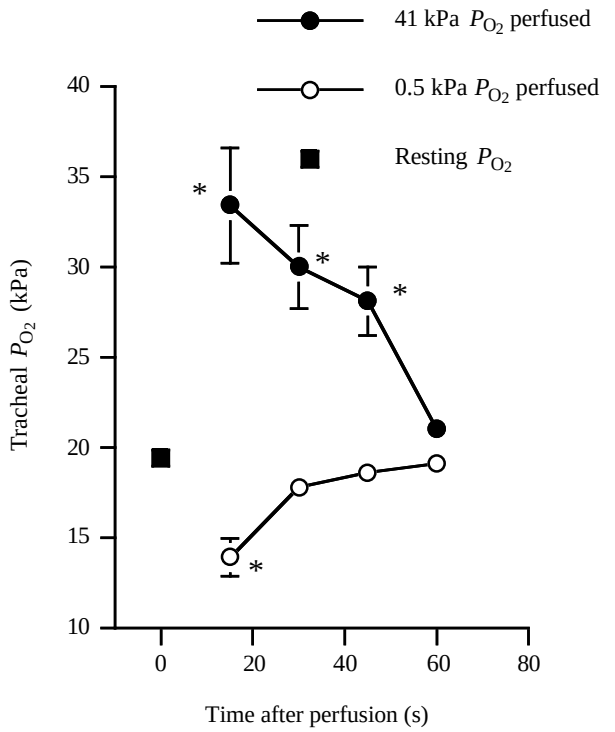

Fig. 4. Effect of time after perfusion on tracheal P_{O_2} for animals perfused with either 0.5 or 41 kPa P_{O_2} (perfusate P_{CO_2}=2.2 kPa) for *Schistocerca americana*. An asterisk indicates that tracheal P_{O_2} differed significantly from the value for unperfused animals (filled square; *post-hoc* comparisons for repeated-measures ANOVA). N=6 for each point.

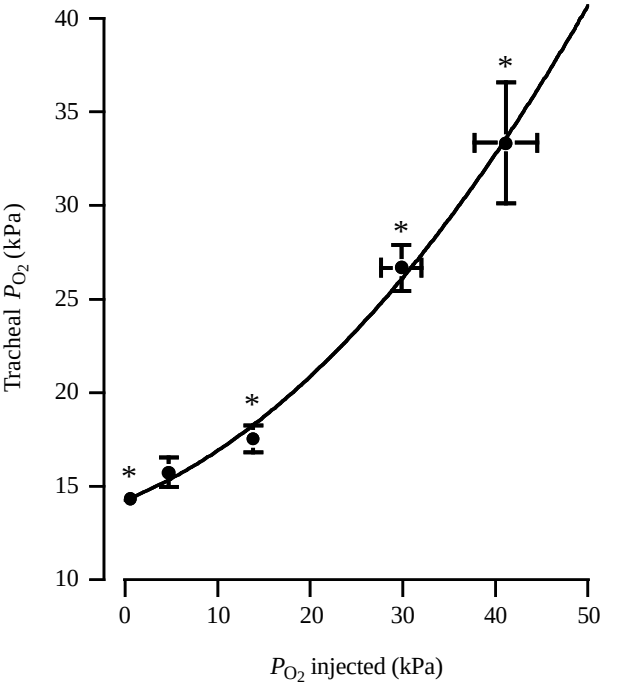

Fig. 5. Tracheal P_{O_2} measured 15 s after cessation of perfusion as a function of the P_{O_2} of the perfused gas for *S. americana*. N=6 for each point. The line shown is $y=0.007x^2+0.198x+14.3$, $r^2=0.99$, $P<0.001$, where y is tracheal P_{O_2} (kPa) and x is perfusate P_{O_2} (kPa). Asterisks indicate that tracheal P_{O_2} differed significantly from the value for unperfused animals (18.8±0.62 kPa, N=15; *post-hoc* comparisons for repeated-measures ANOVA). N=6 for each point.

perfusion for four of the gas mixtures (Figs 8, 9); similar patterns were observed for the other gas mixtures. Ventilation rate during the 15 s after perfusion was significantly affected by the P_{O_2} of the perfusate (repeated-measures ANOVA, $F_{4,28}$=10.3, $P<0.001$), and mean ventilation rate was negatively correlated with the mean tracheal P_{O_2} (Spearman's rank correlation coefficient = 0.9, $P<0.05$, Fig. 10). Ventilation rate during the 15 s after perfusion was also significantly affected by the P_{CO_2} of the perfusate (repeated-measures ANOVA, $F_{4,24}$=22.8, $P<0.001$), and mean ventilation rate was positively correlated with the mean tracheal P_{CO_2} (Spearman's rank correlation coefficient=1.0, $P<0.05$, Fig. 11).

Does restraint or tracheal cannulation affect resting ventilation rate?

Ventilation rate did vary with treatment during the analysis of the effects of restraint and cannulation on ventilation rate (repeated-measures ANOVA, $F_{3,24}$=5.69, $P<0.01$), as ventilation rate decreased with each successive treatment (Table 3). However, ventilation rate did not differ significantly between unrestrained and restrained locusts (Table 3, group 1 *versus* group 2, *post-hoc* comparisons, repeated-measures ANOVA). The ventilation rate of the cannulated animals did not differ from the mean ventilation rate of the pooled pre- and post-cannulation animals (group 3 *versus* groups 2 and 4 pooled, *post-hoc* comparisons, repeated-measures ANOVA). We conclude that neither restraint nor tracheal cannulation had large effects on the ventilation rate of our grasshoppers.

Experiment 3: does injection of $NaHCO_3$ increase tracheal P_{CO_2}?

Time after injection (up to 10 min) did not significantly affect the tracheal P_{CO_2} measured for either the $NaHCO_3$- or NaCl-injected animals (repeated-measures ANOVA). Animals injected with $NaHCO_3$ showed a significant 23 % increase in tracheal P_{CO_2} levels averaged over the 10 min following injection (N=11, pre-injection P_{CO_2} 1.6±0.17 kPa, post-injection P_{CO_2} 2.0±0.14 kPa; paired t-test, t=3.06, P=0.014).

Table 3. *The effects of restraint and tracheal cannulation on ventilation rate in* Schistocerca americana

	Treatment	Ventilation rate (breaths min^{-1})
1	Unrestrained	49.9±5.3
2	Restrained	52.6±4.5
3	Restrained and cannulated	41.4±4.6
4	Restrained	39.9±4.2

Numbers on the left refer to the sequence of the measurements, see text for details.

Values are means ± S.E.M., N=9.

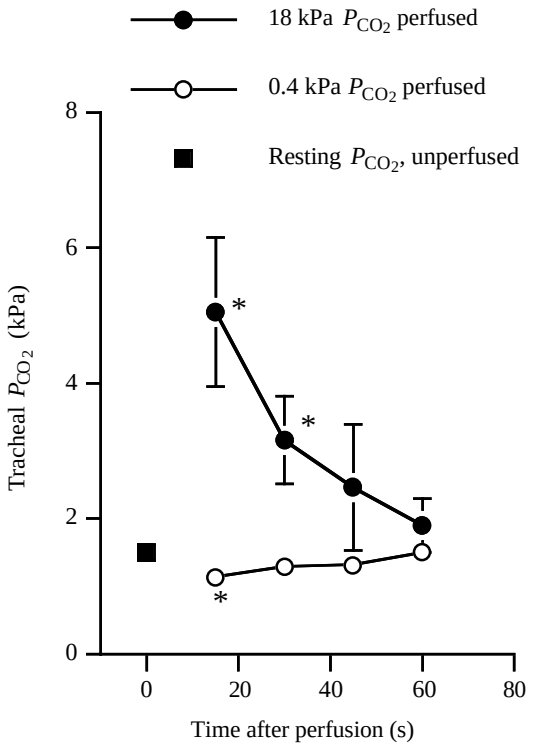

Fig. 6. Effect of time after perfusion on tracheal P_{CO_2} for animals perfused with either 0.4 or 18 kPa P_{CO_2} (perfusate P_{O_2}=15 kPa). Asterisks indicate that tracheal P_{CO_2} differed significantly from the value for unperfused animals (*post-hoc* comparisons for repeated-measures ANOVA). N=6 for each set of perfused animals, N=15 for unperfused animals.

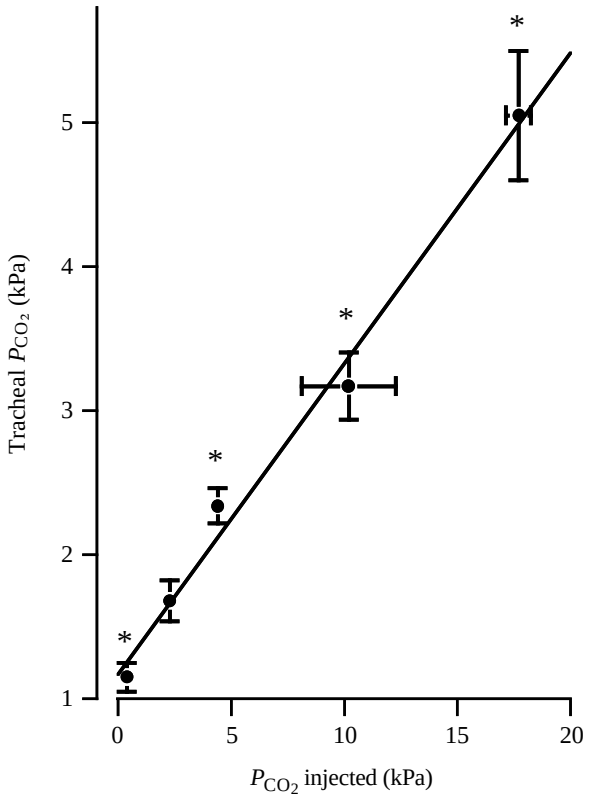

Fig. 7. Tracheal P_{CO_2} measured 15 s after cessation of perfusion as a function of the P_{CO_2} of the perfused gas for *S. americana*. Asterisks indicate that tracheal P_{CO_2} differed significantly from the value for unperfused animals (1.5±0.37 kPa, N=15; *post-hoc* comparisons for repeated-measures ANOVA). N=6 for each point. The line shown is y=0.216 x+1.17, r^2=0.99, P<0.001, where y is tracheal P_{CO_2} (kPa) and x is perfusate P_{CO_2} (kPa).

Injection of NaCl did not significantly affect tracheal P_{CO_2} (N=10, pre-injection P_{CO_2} 1.9±0.32 kPa, post-injection P_{CO_2} 2.1±0.29 kPa; t=0.94, P>0.05).

Discussion

In grasshoppers, haemolymph acidification does not increase resting ventilation rate. Resting ventilation rate is, however, strongly affected by tracheal P_{O_2} and P_{CO_2}. Our data suggest that grasshoppers homeostatically regulate the levels of both tracheal P_{O_2} and P_{CO_2} by varying abdominal pumping rate. Ventilation rate is markedly reduced when the tracheal P_{O_2} rises above or P_{CO_2} falls below normal values (Figs 10, 11). These results strongly suggest that the levels of P_{O_2} and P_{CO_2} in the trachea stimulate ventilation rate in normal, resting grasshoppers.

Relationship between haemolymph acid–base status and resting ventilation rate

Ventilation rate is unaffected by a reduction in haemolymph pH in *R. guttata*. We manipulated haemolymph pH within normal physiological ranges without affecting ventilation rate. In jumping grasshoppers, pH changes by 0.1–0.2 units; while with temperature changes, haemolymph pH varies by 0.17 units 10 °C^{-1} (Harrison, 1988, 1989*b*). In the present study, we reduced haemolymph pH by up to 0.37 units without any effect on ventilation rate. These results strongly suggest that locusts lack chemoreceptors for pH in the haemolymph. It is possible that ventilation volume, but not rate, is affected by haemolymph pH. However, if ventilation volume is increased relative to metabolic rate in response to a decrease in extracellular pH, then haemolymph acidosis should be accompanied by a temporary decrease in haemolymph P_{CO_2}. This is not the case in grasshoppers (Fig. 1, Harrison *et al.* 1992), suggesting that neither ventilation volume nor ventilation rate is affected by haemolymph acidosis.

The relationship between haemolymph [HCO_3^-] and ventilation rate is less clear. $NaHCO_3$ injection increased ventilation rate and haemolymph [HCO_3^-]; however, decreases in haemolymph [HCO_3^-] (HCl injection) had no effect on ventilation rate. $NaHCO_3$ injection also increased tracheal P_{CO_2} by 0.4 kPa, presumably as a result of the actions of intracellular carbonic anhydrase, perhaps explaining the stimulatory effect of this injection on ventilation rate. An increase in tracheal P_{CO_2} of 0.4 kPa elevated ventilation rate by approximately 15 % in the tracheal perfusion experiments

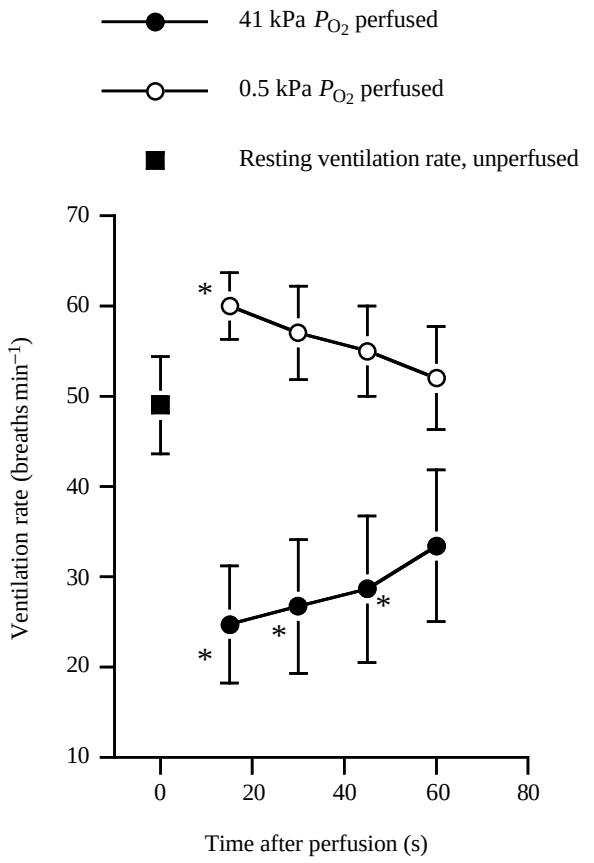

Fig. 8. Time course of changes in ventilation rate after perfusion of 0.5 kPa P_{O_2} (N=9) or 41 kPa P_{O_2} (N=10) with a perfusion P_{CO_2} of 2.2 kPa for *S. americana*. Time after perfusion significantly affected ventilation rate for both the 41 kPa P_{O_2} perfusion (repeated-measures ANOVA, $F_{4,20}$=12.9, P<0.001) and the 0.5 kPa P_{O_2} perfusion (repeated-measures ANOVA, $F_{4,20}$=4.8, P<0.01). Asterisks indicate that the ventilation rate at a given time differed significantly from the value for unperfused animals (N=15, *post-hoc* comparisons for repeated-measures ANOVA).

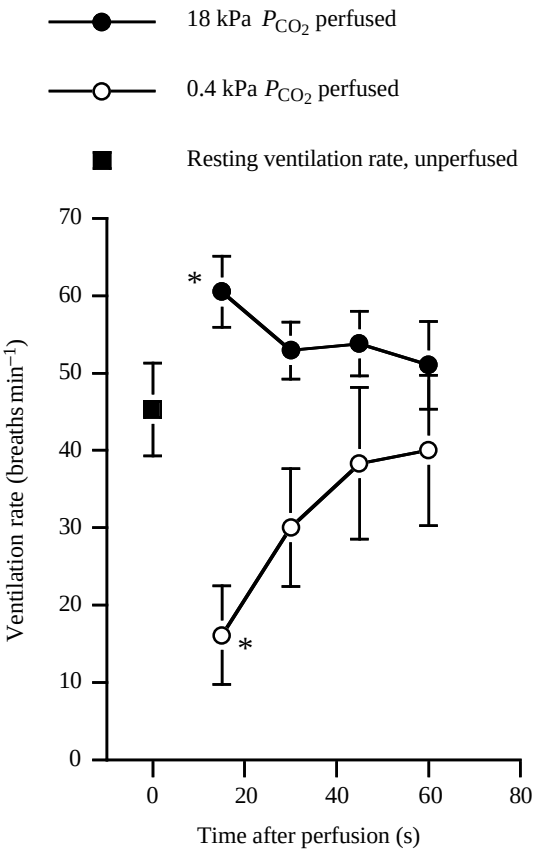

Fig. 9. Time course of changes in ventilation rate after injection of 0.5 or 18 kPa P_{CO_2} with perfusion P_{O_2} kept constant at 15 kPa for *S. americana*. Time after perfusion significantly affected ventilation rate for both the 18 kPa P_{CO_2} perfusion (repeated-measures ANOVA, $F_{4,20}$=12.9, P<0.001) and the 0.4 kPa P_{CO_2} perfusion (repeated-measures ANOVA, $F_{4,20}$=4.8, P<0.01). Asterisks indicate that the ventilation rate at a particular time differed significantly from the value for unperfused animals (*post-hoc* comparisons for repeated-measures ANOVA). N=7 for each perfused group, N=15 for unperfused animals.

(Fig. 11), while $NaHCO_3$ injection increased ventilation rate by approximately 50 % (Fig. 2). The disparity in these figures may be due to (1) the more long-lasting effects of the $NaHCO_3$ injections than the perfusions on tracheal P_{CO_2} and ventilation rate, (2) a more effective increase in the tracheal P_{CO_2} near the ganglia in response to the $NaHCO_3$ injection than in response to the tracheal perfusion, or (3) a direct effect of high haemolymph [HCO_3^-] on neuronal cells which generate or modulate the ventilatory rhythm. We were unable to manipulate haemolymph P_{CO_2} successfully with our injections (Fig. 1), presumably because CO_2 can move rapidly between the tracheal, intra- and extracellular compartments as a result of the actions of intracellular carbonic anhydrase. Therefore, we cannot rule out the presence of chemoreceptors which sense haemolymph P_{CO_2}.

Our results differ from those presented by Snyder *et al.* (1980) for the cockroach *Periplaneta americana* in which the nerve cord of a dissected cockroach was irrigated with fluids varying in pH, P_{CO_2} and P_{O_2}. In their study, changes in saline pH had strong effects on ventilation rate. It is not clear whether the differences between these results and those reported here are due to the use of different animals or different methods. It is possible that, in *P. americana*, haemolymph pH does affect ventilation rate. Alternatively, the low pH of the saline could have stimulated CO_2 formation from tissue HCO_3^-, causing an elevation of tracheal P_{CO_2} which stimulated ventilation rate. Also, because the cockroaches were dissected open and pinned, with potential disruption of the tracheal system, results from this preparation may differ from those using intact organisms.

In summary, our data suggest that the widely held assumption that ventilatory rate is sensitive to extracellular pH in insects, as in terrestrial vertebrates, should be re-examined. However, this question clearly needs to be addressed using a wider variety of insect species and with further study on the direct responses of the chemosensory cells to their microenvironment.

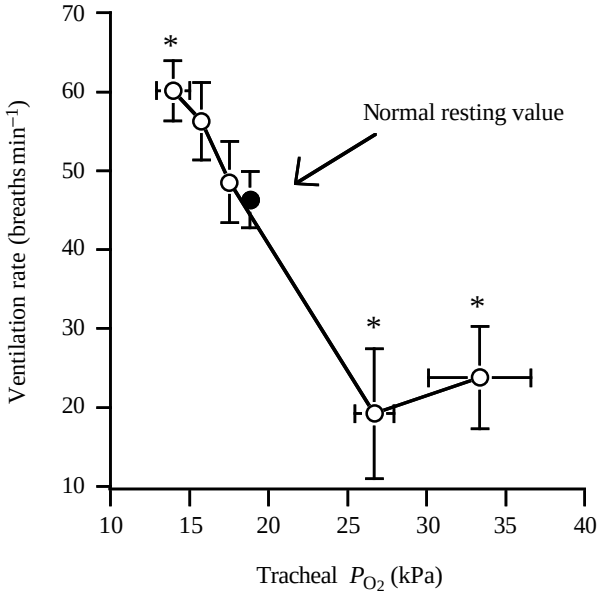

Fig. 10. Relationship between tracheal P_{O_2} and ventilation rate for *S. americana*, with both measured during the 15 s after perfusion. Asterisks indicate that ventilation rate differed significantly from the value for unperfused animals (*post-hoc* comparisons for repeated-measures ANOVA). N=9 for 0.5 kPa P_{O_2}, N=5 for 30 kPa P_{O_2}, and N=10 for the remaining perfused groups, N=15 for unperfused animals.

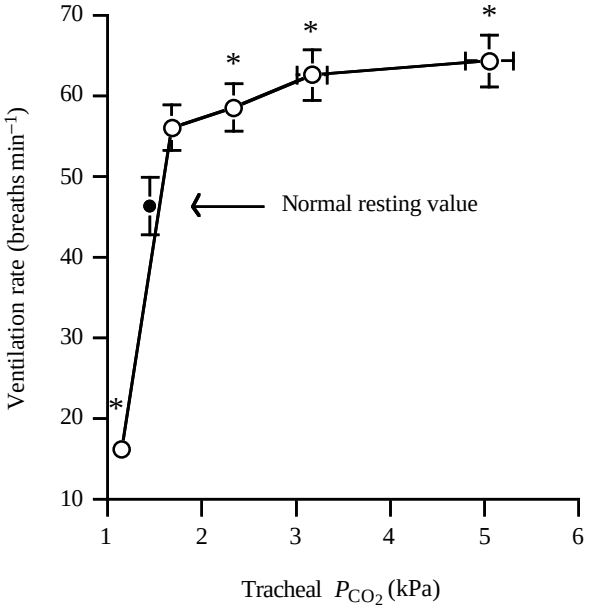

Fig. 11. Effect of tracheal P_{CO_2} on ventilation rate for *S. americana*, with both variables measured during the 15 s after perfusion. Asterisks indicate that ventilation rate differed significantly from the value for unperfused animals (*post-hoc* contrasts for repeated-measures ANOVA). N=7 for each perfused group, N=15 for unperfused animals.

The effect of the tracheal perfusions on tracheal gas levels

While our tracheal perfusions were successful in manipulating tracheal P_{O_2} and P_{CO_2}, we were unable to manipulate tracheal gas levels to values identical to those of the perfused gases, despite our rapid perfusion rate (Figs 5, 7). Tracheal P_{O_2} values were 15–20 % lower than perfusate P_{O_2} when hyperoxic gases were perfused, and tracheal P_{O_2} was 14 kPa 15 s following the perfusion of 0 kPa P_{O_2} (Fig. 5). These data suggest that our tracheal perfusions were accompanied by (1) incomplete flushing of the tracheal system (particularly the contralateral side to the cannulae and the tracheoles), followed by mixing of these unflushed gases with the perfusion gases, and (2) rapid removal of the perfusate gas *via* the spiracles. The rapid recovery of tracheal gases to normal levels (Figs 4, 6) suggests that grasshoppers completely renew their tracheal gases within 1 min under these conditions.

Tracheal P_{CO_2} values were only 25 % of the perfusate P_{CO_2} when hypercapnic gases were perfused and were 1.1 kPa 15 s after perfusion with 0.5 kPa P_{CO_2} (Fig. 7). The lower tracheal-to-perfusate ratio for CO_2 than for O_2 can be explained by the much higher solubility of CO_2 than O_2 in body fluids. Changes in P_{CO_2} in the trachea cause rapid changes in P_{CO_2} of the body fluids (Krolikowski and Harrison, 1996), damping any variation in tracheal P_{CO_2}.

Regulation of tracheal P_{O_2} *and* P_{CO_2} *by ventilation rate*

Ventilation rate was negatively correlated with tracheal P_{O_2} and positively correlated with tracheal P_{CO_2} (Figs 10, 11). Although there were small changes in tracheal P_{CO_2} with some of the tracheal P_{O_2} manipulations (Table 1), these were inconsistently related to the changes observed in ventilation rate. Elevation of tracheal P_{O_2} above normal levels or depression of P_{CO_2} below normal levels decreases ventilation rate (Figs 10, 11), suggesting that the levels of O_2 and CO_2 in the trachea stimulate ventilation rate in normal, resting grasshoppers. Approximately 30 % of the animals perfused with 0.5 kPa CO_2 completely ceased abdominal pumping during the first 15 s after the injection. These results strongly suggest that resting locusts regulate tracheal gas levels through variation in abdominal pumping rate. An effect of tracheal P_{O_2} and P_{CO_2} on ventilation rate is also supported by the similar time courses of the changes in tracheal P_{CO_2} and P_{O_2} and ventilation rate after perfusions.

Role of the ventilatory system in haemolymph pH regulation

In terrestrial vertebrates, extracellular acid–base status has strong effects on ventilation which are mediated by extracellular chemoreceptors, and this chemosensitivity allows the respiratory system to be an important component of short-term pH regulation (Truchot, 1987). It might be expected that terrestrial insects would have similar chemoreceptors and ventilatory responses, since the high oxygen capacity of the respiratory medium (air) and the efficiency of tracheal gas exchange should allow flexibility in ventilation requirements. Our study suggests that the ventilatory systems of terrestrial insects and vertebrates differ in their sensitivity to extracellular pH. Why do terrestrial insects and vertebrates differ in this way?

One possibility is that extracellular acid–base chemoreceptors evolved in vertebrates to ensure a proper acid–base environment for haemoglobin. In vertebrates, blood pH affects haemoglobin oxygen-affinity and therefore gas exchange. In insects, haemolymph pH should have relatively small effects on gas exchange, since O_2 and CO_2 move primarily *via* the tracheae. Since gases exchange through the tracheae rather than *via* the blood as in vertebrates, there may not be a need for the ventilatory system to receive direct sensory information on haemolymph pH in insects.

In terrestrial vertebrates, both the ventilatory and renal systems respond to extracellular pH and are involved in blood pH regulation (Truchot, 1987). Our data suggest a more definite division between the systems involved in acid–base homeostasis in grasshoppers, with the ventilatory system responsible for regulating tracheal P_{CO_2} and the renal system regulating non-volatile acid–base equivalents and responding to haemolymph pH (Harrison, 1994). The grasshopper alimentary canal and renal system have a high capacity for acid–base transport, and excretion rates of both titratable acid and ammonium vary with haemolymph acid–base status (Harrison and Phillips, 1992; Harrison and Kennedy, 1994; Phillips *et al.* 1994). In grasshoppers, changes in blood pH with temperature are also primarily driven by non-respiratory mechanisms, consistent with the hypothesis that ionic rather than respiratory mechanisms predominate in the regulation of extracellular acid–base status in locusts (Harrison, 1988, 1989*b*).

The ventilatory system participates indirectly in extracellular acid–base balance by maintaining a relatively constant tracheal P_{CO_2}. Owing to the extensive diffusing capacity of the tracheoles and the action of intracellular carbonic anhydrase (Buck and Friedman, 1958), tracheal P_{CO_2} strongly influences, but is not identical to, haemolymph P_{CO_2} (Krolikowski and Harrison, 1996). In the case of an acute extracellular acid load, the respiratory and bicarbonate buffer systems participate in ameliorating extracellular pH changes (Harrison *et al.* 1990, 1992). Extracellular protons combine with bicarbonate, producing CO_2 which diffuses from the haemolymph into the tracheae. This elevates tracheal P_{CO_2}, stimulating ventilation and hence the convective removal of CO_2.

Mechanism of tracheal gas effects on ventilation rate

Since our data and that of Krolikowski and Harrison (1996) suggest that CO_2 moves rapidly between the tracheae and the haemolymph, we cannot determine whether P_{CO_2} or P_{O_2} levels are detected in the trachea, haemolymph or body fluids. Resolution of this question will require a study of the microenvironment and responses of identified sensory cells.

Similarly, the cellular mechanism by which tracheal P_{CO_2} controls ventilation rate remains to be determined. Case (1961) demonstrated that the discharge rhythm of ganglia of the cockroach is sensitive to CO_2 levels and pH changes *in vitro*. He suggested that rises in external P_{CO_2} affected this rhythm by reducing the pH within the ganglia. Case (1961) also showed that a variety of weak acids could increase the ventilatory discharge rate of cockroach ganglia *in vitro*, indicating that undissociated weak acids penetrate the nerve cell membrane and change intracellular pH. While we have shown that decreases in haemolymph pH have no effect on ventilation rate in grasshoppers, it is possible that the actual transduction mechanisms involve changes in the pH of nerve cytoplasm or perineural fluid.

Functional significance of the regulation of resting ventilation rate by tracheal gases

A rise in ventilation rate of grasshoppers after locomotion is not affected by tracheal P_{O_2} or P_{CO_2}, suggesting that the sensitivity of ventilation rate to tracheal O_2 and CO_2 levels does not function to allow the respiratory system to respond to the increased demands for gas exchange associated with exercise (Krolikowski and Harrison, 1996). Regulating tracheal P_{O_2} at a higher level or P_{CO_2} at a lower level would increase both evaporative water loss and the energy costs of ventilation. However, it is less clear why the trans-spiracular gradients for O_2 and CO_2 are so low. There is no evidence that maintenance of tracheal P_{O_2} at 18 kPa (the value we report for unmanipulated grasshoppers) is necessary for adequate O_2 delivery. Metabolic rate in resting grasshoppers does not decrease until ambient P_{O_2} falls below 3.4 kPa (Arieli and Lehrer, 1988). However, the large store of O_2 in the tracheal air sacs (created by regulating a high tracheal P_{O_2}) may provide an important oxygen source during locomotion. In *Melanoplus bivittatus*, 40 % of the O_2 used during a few minutes of jumping comes directly from within the tracheae rather than from freshly inspired air (Harrison *et al.* 1991). It is also possible that a large O_2 gradient enhances O_2 supply to the leg muscles during burst locomotion.

Comparison of the control of abdominal pumping with the control of spiracular opening

Ventilation in large insects is regulated jointly by control of spiracular opening and abdominal pumping, with the two systems tightly coordinated (McCutcheon, 1940; Miller, 1960*b*). Control of spiracular opening appears to be primarily regulated by tracheal gas levels, rather than by extracellular pH (Case, 1957; Hoyle, 1960). During discontinuous ventilation in pupae of the *Cecropia* silkworm, ‘within the insect the normal stimulus for cyclical valve activity is a combination of decreasing internal tension of O_2 and increasing tension of CO_2 during the interburst period’ (Schneiderman, 1956). A similar mechanism appears to regulate abdominal pumping in quiescent locusts. An accumulation of CO_2 or a lack of O_2 in the grasshopper tracheal system causes the animal to pump its abdomen and open its spiracles, increasing the delivery of fresh air and restoring tracheal O_2 and CO_2 to their regulated levels.

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