The Journal of Experimental Biology 201, 2843–2855 (1998)
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JEB1469

ACID–BASE AND RESPIRATORY RESPONSES TO HYPOXIA IN THE GRASSHOPPER *SCHISTOCERCA AMERICANA*

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Accepted 16 July; published on WWW 22 September 1998

Summary

How do quiescent insects maintain constant rates of oxygen consumption at ambient P_{O_2} values as low as 2–5 kPa? To address this question, we examined the response of the American locust *Schistocerca americana* to hypoxia by measuring the effect of decreasing ambient P_{O_2} on haemolymph acid–base status, tracheal P_{CO_2} and CO_2 emission. We also tested the effect of hypoxia on convective ventilation using a new optical technique which measured the changes in abdominal volume during ventilation. Hypoxia caused a progressive increase in haemolymph pH and a decrease in haemolymph P_{CO_2}. A Davenport analysis suggests that hypoxia is accompanied by a net transfer of base to the haemolymph, perhaps as a result of intracellular pH regulation. Hypoxia caused a progressive increase in convective ventilation which was mostly attributable to a rise in ventilatory frequency. Carbon dioxide conductance ($\mu mol\,h^{-1}\,kPa^{-1}$) across the spiracles increased more than threefold, while conductance between the haemolymph and primary trachea nearly doubled in 2 kPa O_2 relative to room air. The rise in trans-spiracular conductance is completely attributable to the elevations in convective ventilation. The rise in tracheal conductance in response to hypoxia may reflect the removal of fluid from the tracheoles described by Wigglesworth. The low critical P_{O_2} of quiescent insects can be attributed (1) to their relatively low resting metabolic rates, (2) to the possession of tracheal systems adapted for the exchange of gases at much higher rates during activity and (3) to the ability of insects to rapidly modulate tracheal conductance.

Key words: acid–base, ventilation, tracheal system, hypoxia, gas exchange, grasshopper, *Schistocerca americana*.

Introduction

It is well known that quiescent insects can maintain constant rates of oxygen consumption in extremely low oxygen concentrations. For example, the critical P_{O_2} values (the P_{O_2} below which oxygen consumption begins to fall) for some resting insects are as follows: *Tenebrio molitor* pupae, <5 kPa (Gaarder, 1918); adult *Aedes aegypti* mosquitoes, 3–4 kPa (Galun, 1960); adult *Phormia regina* flies, 2–5 kPa (Keister and Buck, 1961); adult *Termopsis navidensis* termites, 2–5 kPa (Cook, 1932) and adult *Locusta migratoria*, 3–4 kPa (Arieli and Lehrer, 1988). While these data demonstrate that the safety margin for oxygen delivery in resting insects is large, the physiological mechanisms responsible for this remain unclear. One hypothesis is that the conductance (the quantity of gas transferred divided by the partial pressure gradient) of the tracheal system is so high at rest that no response is needed to hypoxia. Alternatively, insects may need to increase the conductance of the tracheal system in proportion with the fall in atmospheric oxygen. If tracheal conductance does increase, by what mechanisms is this accomplished? We investigated these questions by testing the effects of hypoxia on the tracheal physiology of the grasshopper *Schistocerca americana*.

Together, the tracheal morphology, mechanisms of gas exchange and the neural control of the ventilatory system have been better studied in grasshoppers than in any other insect. Large longitudinal trunks run along each side of the animal connecting all ipsilateral spiracles and branching into a system of air sacs and secondary and tertiary tracheae, further branching into tracheoles which are the sites of gas exchange in the tissues (Weis-Fogh, 1964, 1967). Convective gas exchange in non-flying grasshoppers is accomplished mostly by abdominal pumping, which includes both dorso-ventral contractions and longitudinal telescoping movements (Miller, 1960*a*; Weis-Fogh, 1967). Abdominal pumping is initiated by a pacemaker in the metathoracic ganglion (Miller, 1960*a*; Hoyle, 1959) and is synchronized with spiracular opening so that inspiration occurs through the first four pairs of spiracles and expiration through the last six pairs of abdominal spiracles, producing a largely unidirectional flow of air through the grasshopper (McCutcheon, 1940; Weis-Fogh, 1967). Abdominal pumping is stimulated by hypoxia (Miller, 1960*a*), but ventilatory frequency is reported not to be stimulated by hypoxia until the P_{O_2} falls below the point at which the rate of oxygen consumption drops (Arieli and

Lehrer, 1988). However, when tracheal P_{O_2} is varied at constant tracheal P_{CO_2}, ventilatory frequency is inversely related to tracheal P_{O_2}, and evidence suggests that resting grasshoppers in room air actively maintain tracheal P_{O_2} at approximately 18 kPa (Gulinson and Harrison, 1996).

The complex respiratory system of an insect can be viewed as two steps in series: (1) a trans-spiracular step, between the large trachea and air across the spiracles; and (2) a tracheolar step, between the secondary and tertiary tracheae and the cells (Harrison, 1997). The conductance at each step can be estimated from the gas flow divided by the gradient for gas diffusion at that step. Technical problems make measurements of the O_2 gradients difficult, but techniques for the measurement of haemolymph and tracheal P_{CO_2} and CO_2 emission are now established (Harrison, 1988; Gulinson and Harrison, 1996). In the present paper, we investigate the effect of hypoxia on the conductance of the trans-spiracular and tracheolar steps for CO_2 as a first step in understanding the tracheal responses to hypoxia. To address the mechanisms responsible for variations in spiracular conductance, it is necessary to be able to quantify the bulk flow of air accomplished by abdominal pumping. This has been done by Weis-Fogh (1967), who measured the flow of air from the most terminal abdominal spiracles after sealing all the other abdominal spiracles with wax. This procedure is quite invasive and provides poor temporal resolution. We have, therefore, developed a new optical method for quantifying insect convective ventilation from measurements of abdominal volume changes.

Materials and methods

Animals

Schistocerca americana Drury were reared from eggs in culture at Arizona State University as previously described (Harrison and Kennedy, 1994). We used only adult females at least 1 week past the last moult in all experiments, as their larger abdomens and greater haemolymph volumes facilitated our measurements. All animals were unfed but provided with water for at least 12 h prior to measurements. Air temperature was 25.4 °C (range 24.4–26.3 °C). Immediately prior to all experiments, the wings were clipped 2 mm from the base to provide a clear view of the abdomen, and the animals were weighed.

Effects of ambient P_{O_2} *on tracheal* P_{CO_2} *and haemolymph acid–base status*

Metathoracic spiracles were cannulated using a 30 mm section of heat-stretched Intramedic PE-60 tubing (Clay Adams, Parsippany, NJ, USA) sealed to the cuticle with hot glue (Gulinson and Harrison, 1996). Animals were placed in a 10 cm length of Plexiglas tubing which served as the respirometry chamber (18.25 mm i.d., 26 ml volume); the spiracular cannula exited the chamber *via* a small hole drilled in the chamber wall. We restrained grasshoppers in the chamber by tightly enclosing the head of the animal with cotton and a metal mesh helmet anchored in the rubber stopper which sealed one end of the chamber. This prevented the animals from moving, but enabled the abdomen to pump freely. The cotton covering the eyes prevented visual input, which helped keep the grasshopper calm and kept ventilatory frequencies statistically identical to those of unrestrained grasshoppers (Gulinson and Harrison, 1996).

Gas mixtures were generated with a Brooks 5878 mass flow controller and Brooks mass flow meters (Brooks Instruments, Hatfield, PA, USA). Gas mixtures (21,10, 5 or 2 kPa O_2, balance N_2) were perfused through the chamber at a rate of 200 ml min^{-1}. Partial pressures of air were calculated from the percentage of oxygen in the mixture and the total barometric pressure, which was measured daily to the nearest 13 Pa. After 30 min of exposure to a gas mixture, two 30 µl tracheal gas samples were withdrawn sequentially from the spiracular cannula using a 100 µl Hamilton gas-tight syringe (Gulinson and Harrison, 1996). We injected these samples into a Varian 3400 gas chromatograph and gas chrom MP1 column (Varian Analytical Instruments, Walnut Creek, CA, USA) for analysis of CO_2, as previously described (Gulinson and Harrison, 1996). We then quickly removed the rubber stopper and attached grasshopper from the chamber and, within 5 s, a small incision was made in the ventral neck cuticle and a 50 µl haemolymph sample was taken using a 100 µl Hamilton gas-tight syringe, as described previously, keeping CO_2 loss statistically insignificant (Harrison, 1988; Gulinson and Harrison, 1996). We analyzed 45 µl of this sample for total carbon dioxide content (C_{CO_2}, mmol l^{-1}) using a Varian 3400 gas chromatograph as described by Boutilier *et al.* (1985).

The pH of the remaining 5 µl of haemolymph was measured using a new technique for the measurement of pH in small samples without CO_2 loss. Haemolymph samples were expelled from the gas-tight syringe into a 4 mm length of PE-50 tubing placed snugly on the end of a KCl–agar bridge made of PE-10 tubing held in place by a clamp attached to a micromanipulator. We constructed Hinke-type pH-sensitive electrodes using methods described by Harrison *et al.* (1990). We viewed the pH microelectrode through a microscope and lowered it into the haemolymph sample using another micromanipulator. The voltage difference between the haemolymph and the calomel reference electrode was measured using a Biologic IS-100 dual electrometer and amplified using a Biologic VF 102 dual microelectrode amplifier (Biologic, France).

We calculated haemolymph P_{CO_2} (kPa) using the equation:

$$P_{CO_2} = C_{CO_2}/\alpha[10^{(pH-pK)} + 1], \quad (1)$$

where α is the solubility of CO_2 in haemolymph, with pK and α calculated from Harrison (1988).

Effect of ambient P_{O_2} *on convective ventilation and* CO_2 *emission*

Protocols

We exposed grasshoppers to test gases (21, 10, 5 and 2 kPa O_2, balance N_2) in ascending or descending order for 40 min. During the last 10 min, we concurrently measured the carbon

dioxide emission rate ($\dot{M}_{CO_2}$, $\mu mol\,g^{-1}\,h^{-1}$) and convective ventilation ($ml\,min^{-1}$). Grasshoppers were secured in the respirometry chamber exactly as described above, except that animals were not cannulated, and no hole was drilled in the chamber.

Respirometry

Gas mixtures were drawn through the chamber and through a magnesium perchlorate column (to remove water), a LI-6252 CO_2 analyzer (Li-Cor, Lincoln, NE, USA), an Ascarite column (to remove CO_2) and an AMETEK S3A/I oxygen analyzer (AMETEK, Pittsburgh, PA, USA) at $147\,ml\,min^{-1}$ ATP by a downstream AMETEK R-1 pump. The output of the gas analyzers was digitized and recorded (Sable Systems, Las Vegas, NV, USA). We calculated the CO_2 emission rate by multiplying the flow rate and the expired CO_2 fraction since incurrent CO_2 was zero and the volumes of CO_2 and water vapour produced by the grasshopper were negligible relative to the flow rate of air through the chamber. Water production for grasshoppers at this temperature is reported to be $0.0147\,ml\,min^{-1}$ (Loveridge and Bursell, 1975; Prange, 1990); CO_2 production was measured in this study and was less than 0.4 % of the flow rate (see Results).

Optical measurement of abdominal volume changes

The Plexiglas respirometry chamber was placed within an optical bench for measurement of the volume of air moved by abdominal pumping (Fig. 1). The respirometry chamber was surrounded by an array of three silicon solar cells (Radio Shack, USA). Three 12 V 55 W quartz–halogen H3 light bulbs (Blazer International Corp., Franklin Park, IL, USA) were mounted at different heights and each was powered by its own low-noise, single-output linear open-frame direct-current power source (Sola 12 V, 10 A regulated supply, Newark Electronics, Chicago, IL, USA) with line regulation (0.1 % for 10 % change), load regulation (0.1 % for 90 % change) and ripple control (0.1 % P–P_{max} PARD). The light beams were diffused using convex lenses (Edmund Scientific, Barrington, NJ, USA) and polarized and directed by mirrors towards the solar cells. One light beam travelled from directly above the animal, casting a 'top-view shadow' of the abdomen onto a solar cell placed directly beneath the chamber. A second light beam travelled horizontally towards the animal, casting a 'side-view shadow' of the abdomen onto a solar cell placed vertically beside the chamber. The third solar cell was placed at 45 ° to the other two solar cells and was used to record the length of the abdomen. The light beam for length recording travelled through a metal plate into which was machined a 2 mm×10 mm rectangular slit, then onto a mirror, and finally past the grasshopper to the solar cell, which was covered with a piece of cardboard with a slit dimensionally identical to the one in the metal plate. We mounted the lights, mirrors and solar cell array on a 61 cm×30.5 cm optical bench (Edmund Scientific, Barrington, NJ, USA; Fig. 1) and covered the entire structure with an opaque box to prevent interference from outside light.

The current output of these solar cells was linearly related to the energy of the light received by the cell. Because our data acquisition system is responsive to voltage variations, we

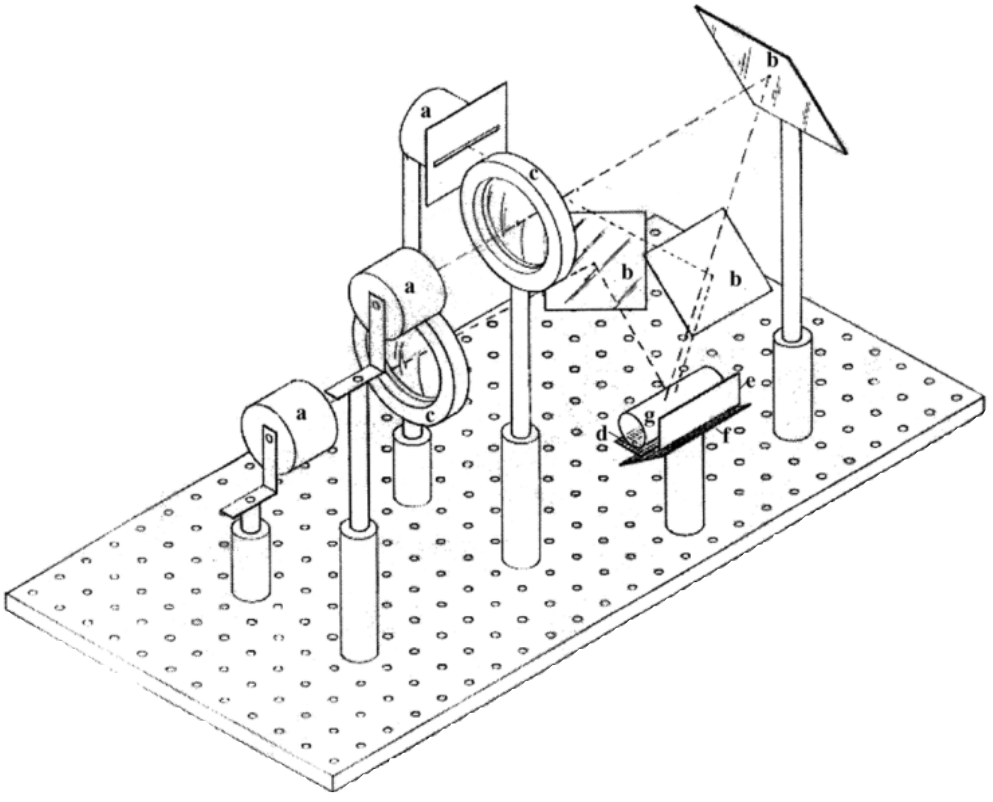

Fig. 1. Diagram of the optical apparatus for recording ventilation volume. The drawing is not to scale as the positioning of the mirrors has been altered to allow complete viewing of the system components. a, light sources; b, mirrors; c, diffusing lenses; d, top view solar cell; e, side view solar cell; f, length solar cell; g, chamber. See text for details.

recorded the voltage generated by the solar cell output across 10 Ω resistors. The voltage output from each of the three solar cells was amplified using a custom-designed signal conditioner and digitized and recorded on separate channels using Sable Systems DATACAN hardware and software. Output from each solar cell was linearly related to shadow area. Volumes of solid aluminium square prisms (V_{prism}) placed within the respirometry system could be directly plotted on a fourth channel where:

$$V_{prism} = (tva/l)(sva/l)l\,, \quad (2)$$

where *tva* is top view area, *sva* is side view area and *l* is length. Calculated volumes of prisms were linearly related to actual volumes, with the r^2 for standard curves routinely greater than 0.99. The shape of the abdomen of a grasshopper is complex, however, and more closely approximates an elliptical cone (with a volume V_{cone}) than a square prism. Therefore, we calculated V_{cone} for painted, plaster casts of grasshopper abdomens using the equation:

$$V_{cone} = 0.333\pi l\{[(tva/l) + (sva/l)]^2\}/2\,, \quad (3)$$

To make the casts, grasshoppers were frozen in liquid N_2. We made negative moulds of the frozen abdomens by inserting them into a mixture of Alginate (JB Dental Supply, Tempe, AZ, USA) and water. Once the mixture had set, the abdomens were removed, and the resulting holes filled with plaster of Paris and a handling wire. We removed the dry plaster casts from the Alginate molds and coated them with clear nail polish, for sealing, and charcoal grey acrylic paint, for glare reduction. The volume of each cast was first measured by water displacement. The handling wire was then anchored in a rubber stopper, the cast placed in the respirometry chamber, and V_{cone} of the abdomen cast recorded.

The calculated values for V_{cone} overestimated actual

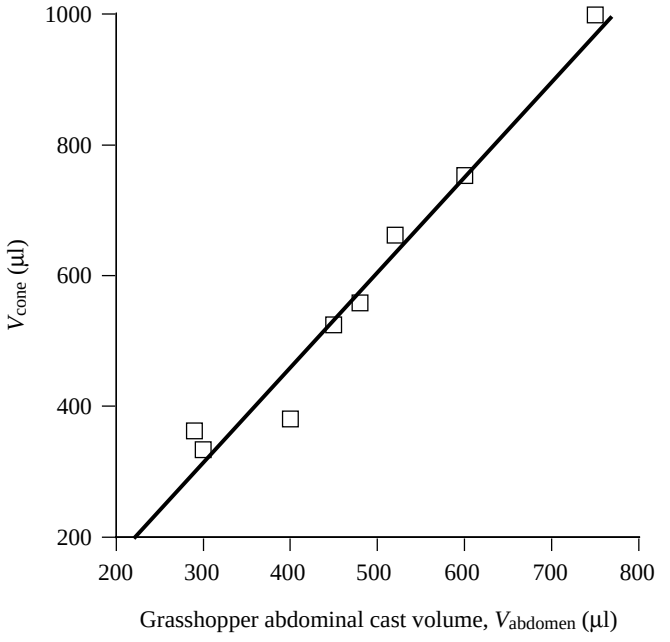

Fig. 2. The relationship between V_{cone} (optically measured and calculated volume of a cast) and the actual volume of grasshopper abdominal casts ($V_{abdomen}$) (y=1.459x–119.585, r^2=0.966, P<0.001, N=8).

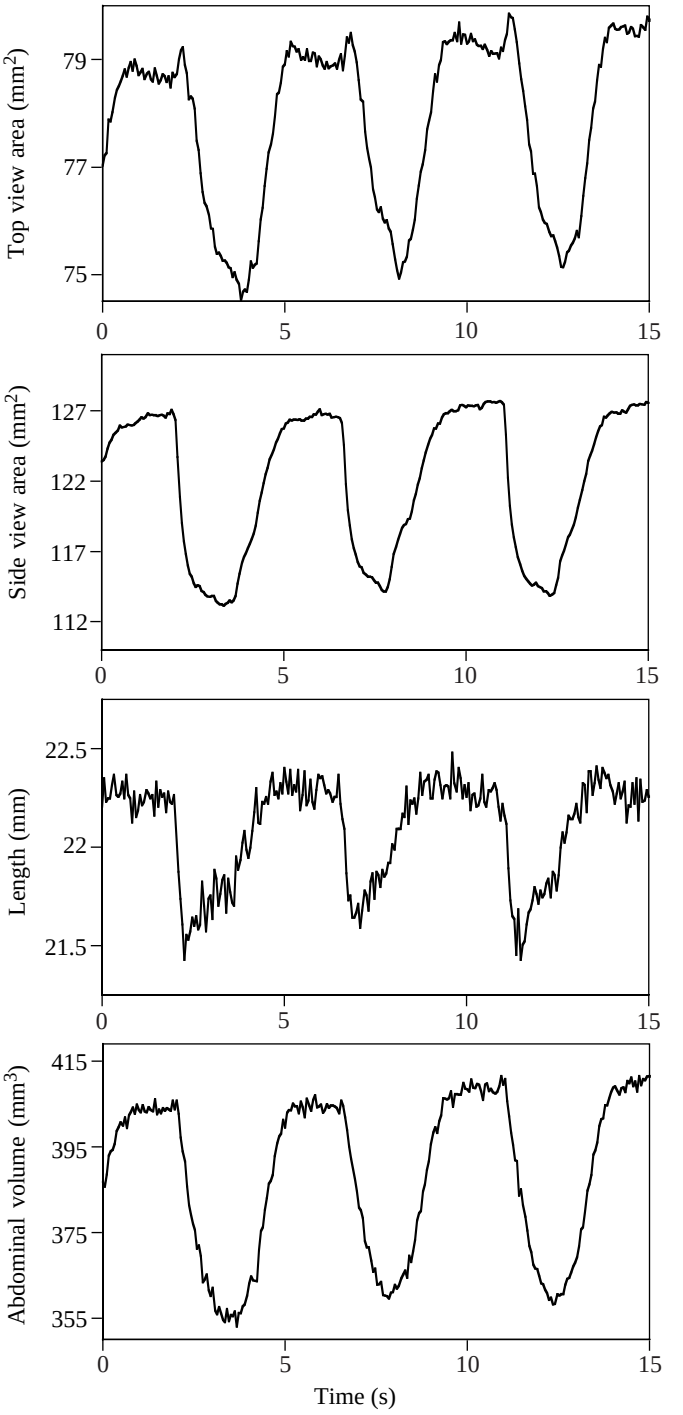

Fig. 3. Abdominal area (top and side views), length and volume changes of a typical *Schistocerca americana* in 21 kPa O_2. Mean tidal volume for this animal over five 30 s intervals was 162 µl, mean ventilatory frequency was 14 breaths min^{-1} and mean convective ventilation was 2.27 ml min^{-1}.

grasshopper abdomen volumes but, abdominal volumes ($V_{abdomen}$) were linearly related to V_{cone}, as:

$$V_{abdomen} = 0.6854(V_{cone} + 119.59), \quad (4)$$

(r^2=0.966, Fig. 2). This equation was therefore used to calculate the abdominal volumes of living grasshoppers from solar cell output and V_{cone}. The outputs of the three solar cells and the calculated abdominal volumes for a grasshopper in 21 kPa O_2 and 2 kPa O_2 are shown in Figs 3, 4. We calculated ventilatory frequency from peak frequency and tidal volume (abdominal volume changes per breath, μl) from the mean difference between the maxima and minima of the abdominal volume peaks averaged over 10–15 breaths. Convective ventilation (ml min^{-1}) was calculated as the product of ventilatory frequency and tidal volume.

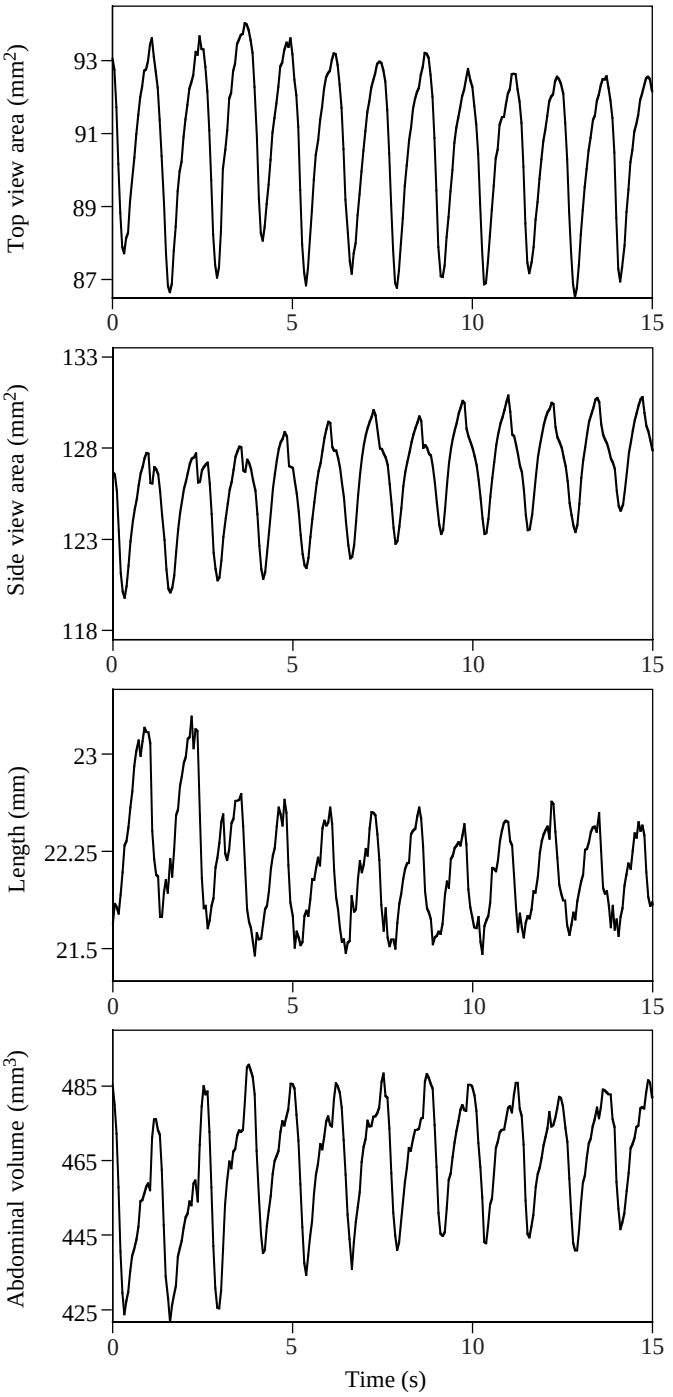

Fig. 4. Abdominal area (top and side views), length and volume changes of the same specimen of *Schistocerca americana* as that shown in Fig. 3 in 2 kPa O_2. Mean tidal volume over five 30 s intervals was 86 μl, mean ventilatory frequency was 51 breaths min^{-1} and mean convective ventilation was 4.39 ml min^{-1}.

Statistics

Mean values ± S.E.M. are shown throughout. Unless otherwise stated, statistical analysis was performed using SYSTAT (Wilkinson, 1989), with our within-experiment, type I error less than or equal to 5 %. Haemolymph acid–base status and tracheal gas data were analyzed by linear regression (GLM, general linear model), since different individuals were used and atmospheric P_{O_2} was manipulated in a graded fashion. Plots of residuals *versus* ambient P_{O_2} yielded lines with slopes and intercepts equal to 0, indicating that no assumptions of linear regression were violated (Sokal and Rohlf, 1995). Regression analyses performed including $(P_{O_2})^2$ terms were non-significant, lending further support for the use of linear regression. Ventilation data were analyzed using repeated-measures analysis of variance (ANOVA), since individuals were measured at multiple P_{O_2} values.

Results

Effect of hypoxia on haemolymph acid–base status and tracheal P_{CO_2}

Haemolymph pH became increasingly alkaline as ambient P_{O_2} decreased from 21–2 kPa (Fig. 5). The total CO_2 in the haemolymph did not change significantly with reductions in inspired P_{O_2} (Fig. 5). The calculated value for haemolymph P_{CO_2} decreased nearly threefold when ambient P_{O_2} decreased 10-fold (Fig. 5). The variation in tracheal P_{CO_2} appeared to decrease as inspired P_{O_2} decreased; however, further statistical analysis showed no significant heterogeneity in variances (Scheffe box test; Sokal and Rohlf, 1995; $F_{3,8}$=1.88, 0.05<P<0.1). Tracheal P_{CO_2} also decreased strongly in response to hypoxia (Fig. 6), and the P_{CO_2} gradient from haemolymph to trachea, calculated within individuals, was more than halved by hypoxia (Fig. 6).

Effect of hypoxia on ventilation and $\dot{M}_{CO_2}$

Ventilatory frequency, tidal volume and convective ventilation were not affected by the order of exposure to test gases as analyzed by a multi-factor ANOVA (Table 1). Since the order of test gas exposure did not affect ventilatory frequency, tidal volume or convective ventilation, data from the ascending and descending P_{O_2} protocols were pooled for the subsequent statistical analysis of these variables. In response to a 10-fold reduction in ambient P_{O_2}, ventilatory

Table 1. *Results of repeated-measures multi-factor ANOVA for experiments examining the effects of ambient* P_{O_2} *on ventilatory and gas exchange variables*

Dependent variable	Factors			
	Order; d.f.=1,10		P_{O_2}; d.f.=1,10	
	F	*P*	*F*	*P*
Convective ventilation	0.67	0.43	10.57	<0.001
Tidal volume	0.67	0.43	1.37	0.27
Ventilatory frequency	0.60	0.46	51.31	<0.001
$\dot{M}_{CO_2}$	9.84	0.01	3.48	0.028

Grasshoppers were exposed to various P_{O_2} values in either ascending or descending order.

frequency more than doubled, tidal volume did not change significantly and convective ventilation increased significantly by aproximately fourfold (Table 1; Fig. 7).

$\dot{M}_{CO_2}$ (μmol g^{-1} h^{-1}) was affected by the order of exposure to gases and by P_{O_2} (Table 1; Fig. 8). When analyzed separately, neither ascending nor descending P_{O_2} protocol animals experienced a significant change in $\dot{M}_{CO_2}$ in response to decreased ambient P_{O_2}. While mean $\dot{M}_{CO_2}$ at 21 kPa was significantly higher in animals with prior exposure to hypoxia (Fig. 8, Tukey HSD multiple comparisons, P=0.001), mean $\dot{M}_{CO_2}$ at all other P_{O_2} values did not differ between animals exposed to ascending or descending levels of P_{O_2} (Fig. 8, Tukey HSD multiple comparisons; 10 kPa, P=1.0; 5 kPa, P=0.61; 2 kPa, P=0.97).

Effects of hypoxia on expired P_{CO_2} *and ventilatory conductance*

We calculated expired P_{CO_2} (kPa) for each individual from measurements of $\dot{M}_{CO_2}$ and convective ventilation for that individual, using the equation:

$$\text{Expired } P_{CO_2} = \frac{\dot{M}_{CO_2} \times M_b \times 0.0003733 \times F \times 101.3}{\text{convective ventilation}}, \quad (5)$$

where M_b is animal mass (g), 0.0003733 is the value that converts $\dot{M}_{CO_2}$ from μmol h^{-1} to ml min^{-1} (STP), F is the ATP to STP conversion factor and convective ventilation is in ml min^{-1}. We calculated spiracular conductance (μmol h^{-1} kPa^{-1}) as $\dot{M}_{CO_2}$/(expired P_{CO_2}) and tracheolar conductance (μmol h^{-1} kPa^{-1}) as $\dot{M}_{CO_2}$/(expired P_{CO_2} minus mean haemolymph P_{CO_2}). Ambient P_{O_2} significantly affected spiracular conductance ($F_{3,33}$=12.9, P<0.001), but not expired P_{CO_2} (P=0.054) or tracheolar conductance (P=0.76).

However, two of the animals had very high calculated expired P_{CO_2} values relative to the other animals (6–7 kPa in room air) and low conductances and were, therefore, excluded from further statistical analyses of expired P_{CO_2} and both conductance variables on the basis of Dixon's test for outliers (Sokal and Rohlf, 1995). According to this test, these two animals were not outliers for any of the other variables measured (ventilatory frequency, tidal volume, convective

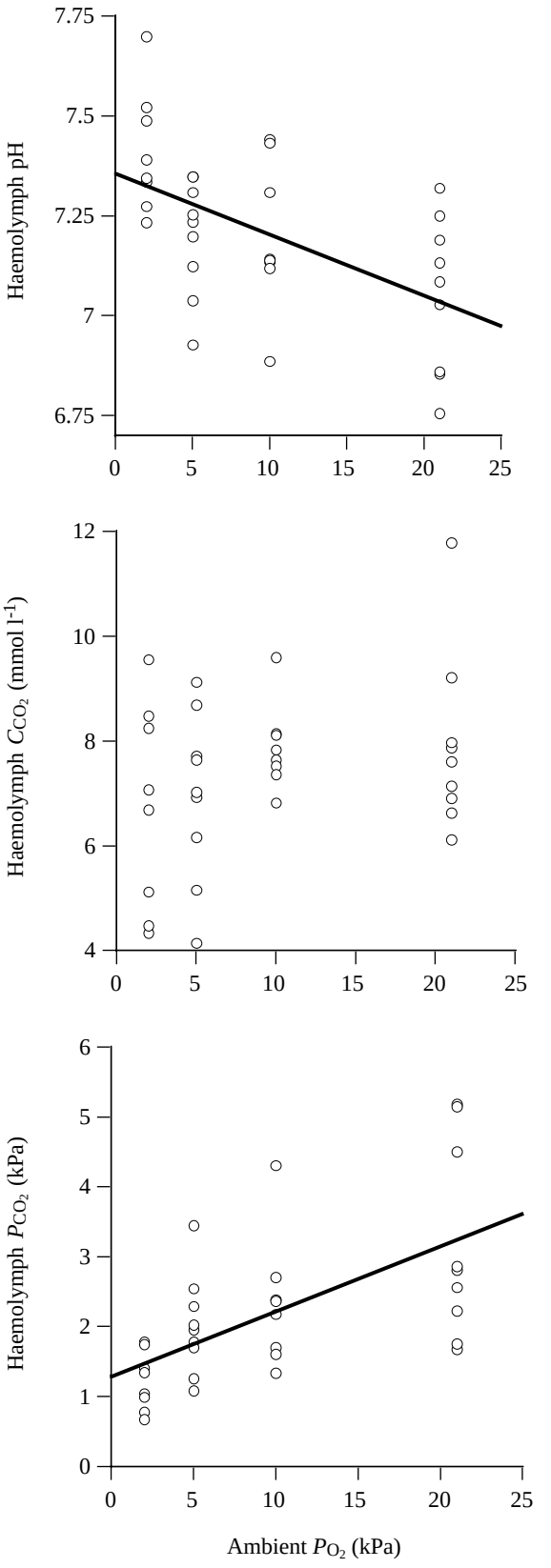

Fig. 5. Haemolymph pH (y=–0.015x+7.359, r^2=0.30, P<0.001), C_{CO_2} (0.05<P<0.1) and P_{CO_2} (y=0.093x+1.309, r^2=0.361, P<0.001) as a function of ambient P_{O_2}. N=8 for 2 and 10 kPa O_2; N=9 for 5 and 21 kPa O_2.

ventilation and $\dot{M}_{CO_2}$), so these animals were not excluded from any other statistical analyses. With these two outliers removed, expired P_{CO_2} decreased strongly as ambient P_{O_2} decreased, with expired P_{CO_2} at 2 kPa being 27 % of the value for animals in room air (Fig. 9B). Spiracular conductance increased nearly fourfold over the 10-fold decrease in ambient P_{O_2} (Fig. 10). Mean tracheolar conductance nearly doubled as inspired P_{O_2} decreased, although the effect was not significant by ANOVA (Fig. 10).

The effects of ambient P_{O_2} on calculated expired P_{CO_2} and measured tracheal P_{CO_2} were compared using analysis of covariance (ANCOVA) since there was no significant difference in the slopes of these lines (Fig. 9A, GLM, $F_{1,70}$=2.62, P=0.110). The intercepts of these lines also did not differ (ANCOVA, $F_{1,69}$=0.21, P=0.65), indicating that these independent measurements of tracheal P_{CO_2} yield similar values. The slope of the haemolymph P_{CO_2} *versus* ambient P_{O_2} regression line differed from slopes of regression lines calculated for expired P_{CO_2} and tracheal P_{CO_2} *versus* ambient

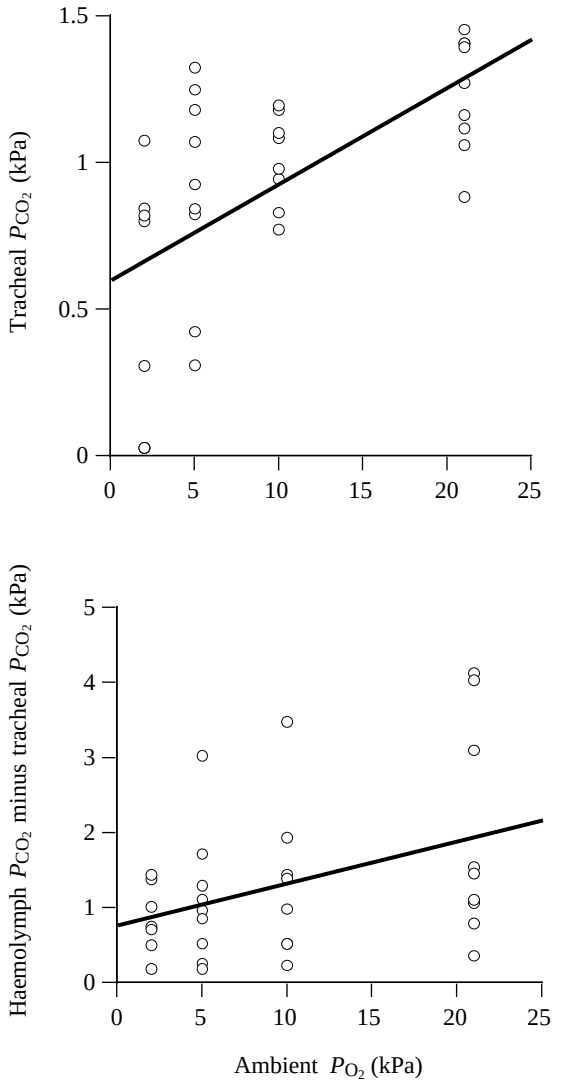

Fig. 6. Tracheal P_{CO_2} (y=0.033x+0.604, r^2=0.371, P<0.001) and the P_{CO_2} gradient from haemolymph to trachea (y=0.06x+0.705, r^2=0.171, P=0.015) as a function of ambient P_{O_2}. N=8 for 2 and 10 kPa O_2; N=9 for 5 and 21 kPa O_2.

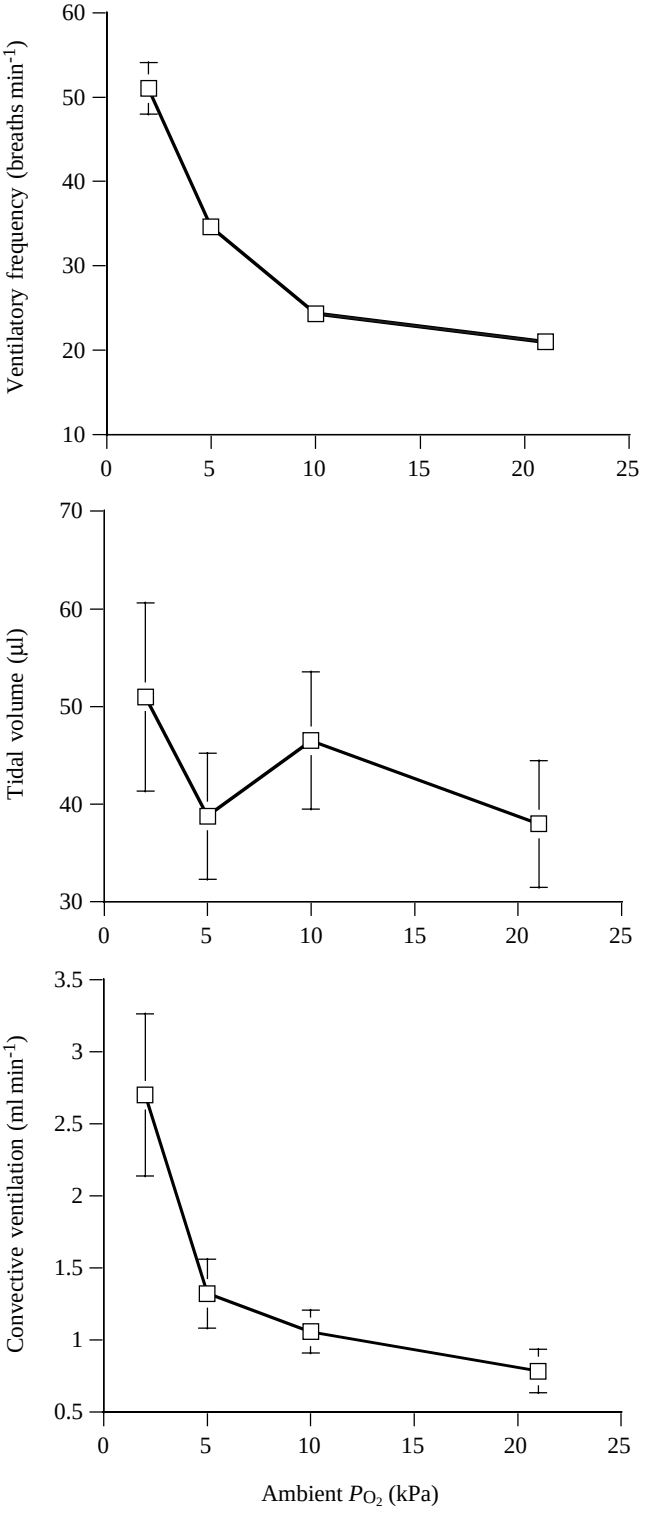

Fig. 7. The effect of ambient P_{O_2} on ventilatory frequency ($F_{1,10}$=94.64, P<0.001), tidal volume ($F_{1,10}$=0.81, P=0.39) and convective ventilation ($F_{1,10}$=11.48, P=0.007) for *Schistocerca americana*. Values are means ± 1 S.E.M., N=12 for all gas mixtures.

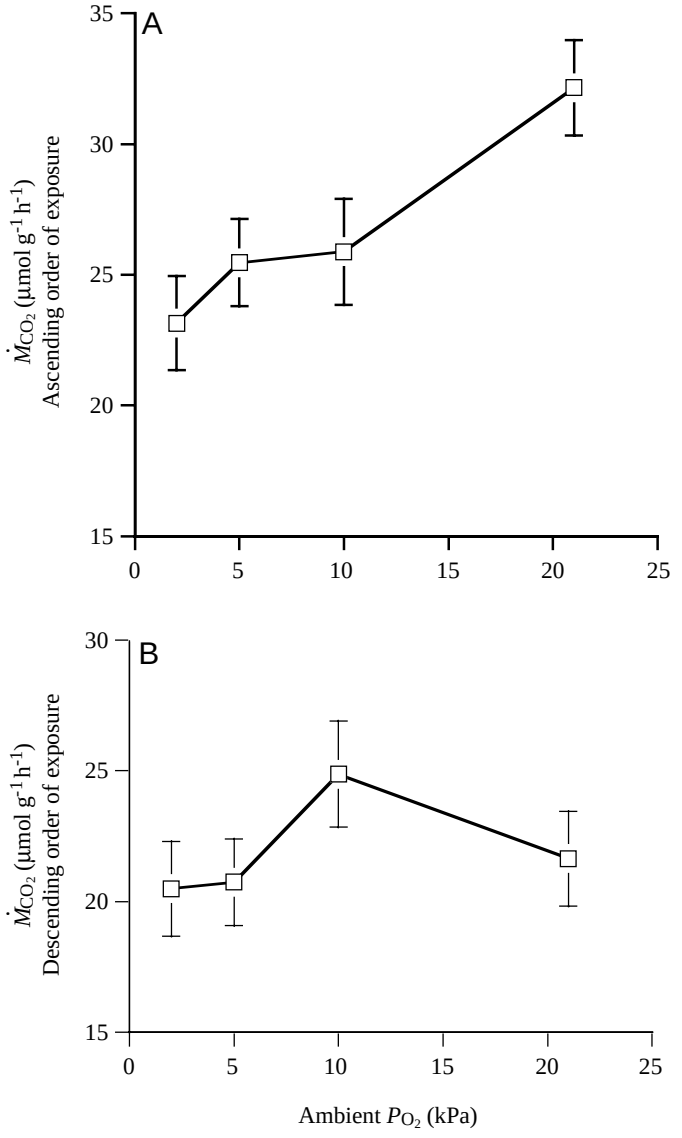

Fig. 8. The effect of ambient P_{O_2} on $\dot{M}_{CO_2}$ for *Schistocerca americana* exposed to ascending (A) (P_{O_2} effect, $F_{3,15}$=3.19, P=0.054; linear test of order, $F_{1,5}$=4.18, P=0.08) or descending (B) (P_{O_2} effect, $F_{3,15}$=3.20, P=0.054; linear test of order, $F_{1,5}$=1.82, P=0.24). Values are means ± 1 S.E.M., N=12 for all gas mixtures.

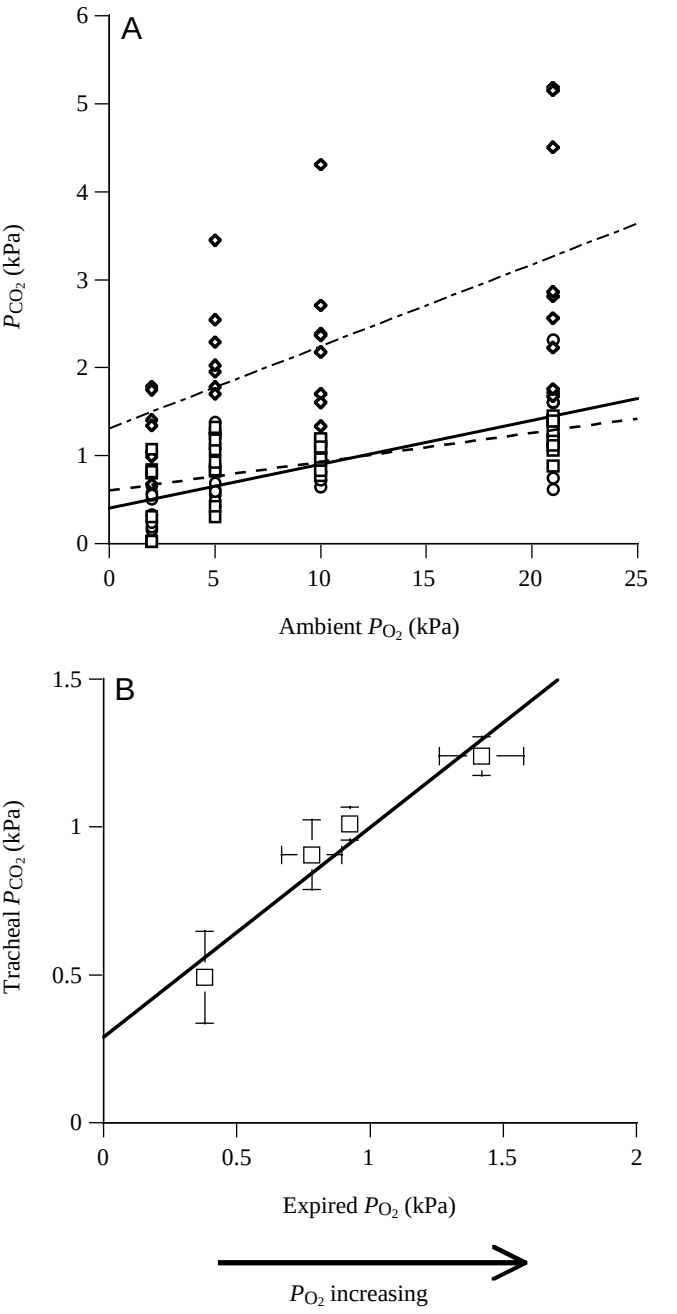

Fig. 9. (A) The effect of inspired P_{O_2} on calculated expired P_{CO_2} ($F_{3,27}$=19.58, P<0.001, squares, solid line, y=0.050x+0.403, r^2=0.939), tracheal P_{CO_2} (circles, dashed line) and haemolymph P_{CO_2} (diamonds, dashed and dotted line). Values are means ± 1 S.E.M., N=10 for all gas mixtures for calculated expired P_{CO_2}; N=8 for 2 and 10 kPa O_2; N=9 for 5 and 21 kPa O_2 for tracheal and haemolymph P_{CO_2}. Note that not all symbols are visible due to overlapping. (B) The relationship between mean calculated expired P_{CO_2} and mean tracheal P_{CO_2} as P_{O_2} was varied from 2 to 21 kPa (y=0.710x+0.290, r^2=0.946, P<0.001).

P_{O_2} (Fig. 9A; GLM, haemolymph P_{CO_2} *versus* expired P_{CO_2}, slope effect $F_{1,70}$=3.97, P=0.05; GLM, haemolymph P_{CO_2} *versus* tracheal P_{CO_2}, slope effect $F_{1,64}$=6.79, P=0.011).

Discussion

Validation of a new technique for optical measurement of abdominal volume changes

Convective ventilation is one of the most important parameters used in investigating any respiratory system. Our optical method of recording abdominal volume changes allows the first continuous estimates of tidal volume and convective ventilation in an unimpeded tracheal system. Because of the chamber design, we were able simultaneously to perform respirometry and manipulate ambient gases. This method should be widely applicable to insects of various sizes and shapes and may, perhaps, be used during tethered

flight if the animal can be restrained in the chamber and the size of the insect relative to the photo-sensing system is maintained. We have successfully measured convective ventilation in a juvenile *S. americana* grasshopper (0.16 g, abdomen length 9.51 mm) within our apparatus, demonstrating that this apparatus could be used without alteration over an order of magnitude size range. However, to record volume changes of an insect with differing abdominal morphology, the shape of the abdomen must be assessed, and the relationship between shadow area and abdominal volume determined empirically.

We were not certain that the dimensional changes in abdominal volume during breathing would match the dimensional variation among individuals under dynamic conditions, an assumption implicit in our calibration method. In addition, our method ignores ventilation due to movements of the neck and prothoracic regions, which, according to Miller (1960*a*), can account for up to 14 % of convective ventilation. It is also important to note that our technique actually measures abdominal volume changes, not convective ventilation. Conceivably, there might not be either sufficient time between breaths or a low enough spiracular resistance (especially at high ventilatory frequencies) for convective ventilation to match abdominal volume changes. However, the close match between calculated expired P_{CO_2} and measured tracheal P_{CO_2} across a variety of ventilatory frequencies (Fig. 9B) strongly supports the hypothesis that our optical method accurately measured convective ventilation. At 21 kPa O_2, our measurement of convective ventilation of 0.78±0.15 ml min^{-1} (mean ± S.E.M., Fig. 7, mean mass for the *S. americana* used in this study was 1.04 g) was very similar to that measured by Weis-Fogh (1967, 0.7 ml g^{-1} min^{-1}) for *S. gregaria* using a completely different method, further supporting the accuracy of this method. The response of ventilatory frequency to hypoxia was also similar in this study to that found by Arieli and Lehrer (1988).

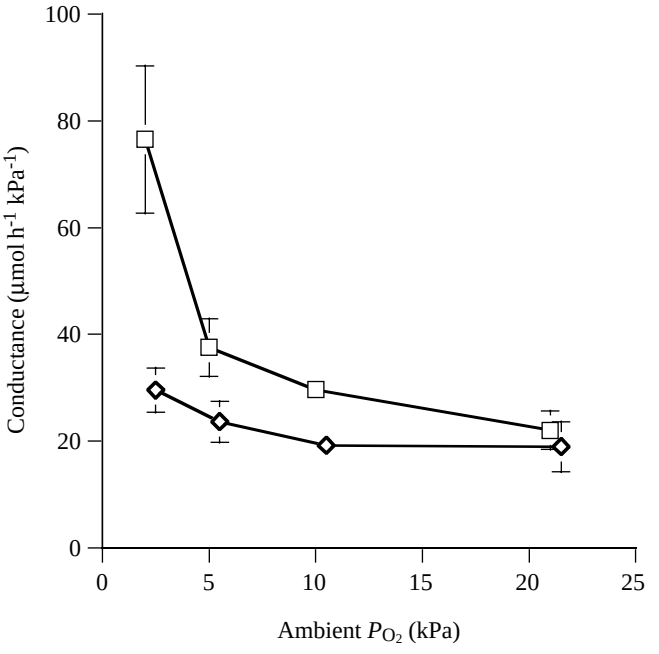

Fig. 10. The effect of inspired P_{O_2} on spiracular ($F_{3,27}$=12.90, P<0.001, squares) and tracheolar ($F_{3,27}$=2.92, P=0.052, diamonds) conductances. Values are means ± 1 S.E.M., N=10 for all gas mixtures.

Critique of methods

Our calculations of spiracular and tracheolar conductances have a number of assumptions and limitations which should be admitted. For our measurements of tracheolar conductance of P_{CO_2}, we assume that haemolymph P_{CO_2} is representative of cellular P_{CO_2}. One would expect cellular P_{CO_2} to be higher than haemolymph P_{CO_2}, since cells are the site of CO_2 production. In addition, CO_2 produced in the cells may move *via* the haemolymph to enter the tracheal system at other points. To the extent that haemolymph P_{CO_2} underestimates cellular P_{CO_2}, we are overestimating tracheolar CO_2 conductance. However, since carbonic anhydrase occurs in cells but not in haemolymph for most insects (Darlington *et al.* 1985), CO_2 exchange is likely to occur between the cells and tracheae but not between the haemolymph and tracheae, especially during dynamic conditions. Supporting this assumption, calculated whole-body P_{CO_2} is similar to haemolymph P_{CO_2} for resting grasshoppers (Harrison, 1988).

For our calculation of spiracular conductance, we used expired P_{CO_2} values calculated according to equation 5, which were very similar to tracheal P_{CO_2} values measured directly *via* the metathoracic spiracle (Fig. 9). We suspected that expired P_{CO_2} would be higher than the P_{CO_2} measured from thoracic

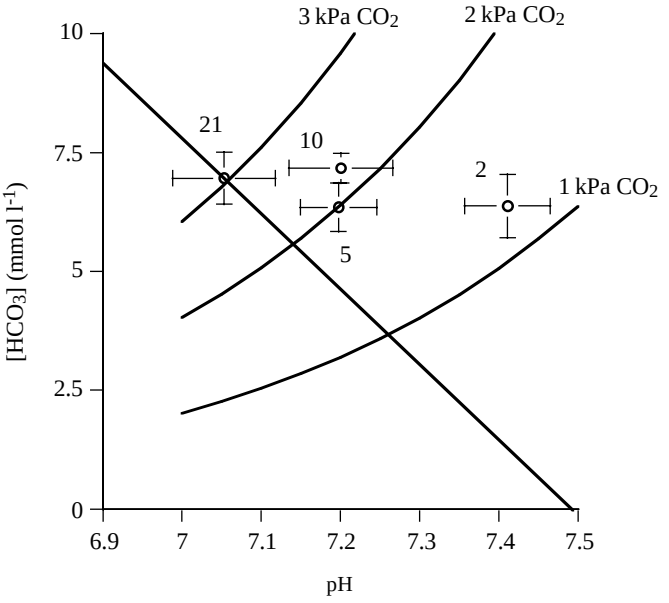

Fig. 11. Davenport diagram comparing the changes in pH and bicarbonate concentration during hypoxia with the non-bicarbonate buffer line for *Schistocerca gregaria* haemolymph (Harrison *et al.* 1990). Numbers next to symbols indicate the partial pressure (in kPa) of ambient O_2. Values are means ± 1 S.E.M., N=8 for 2 and 10 kPa O_2; N=9 for 5 and 21 kPa O_2.

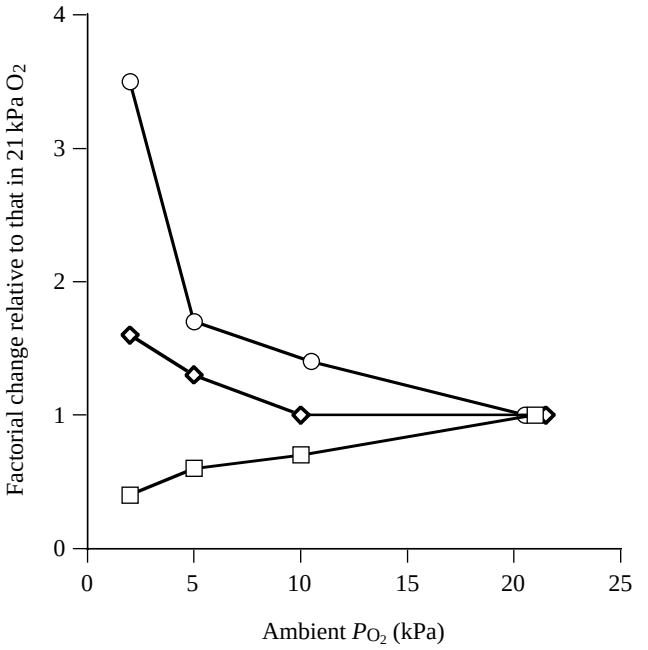

Fig. 12. The effect of P_{O_2} on the haemolymph-to-air CO_2 gradient (squares), spiracular conductance (circles) and tracheolar conductance (diamonds) expressed relative to the values occurring in room air.

tracheal samples, since inspiration occurs *via* the thoracic spiracles. It is possible that cannulation impeded inspiration, elevating tracheal P_{CO_2} to levels equivalent to expired P_{CO_2} of uncannulated animals. However, cannulation does not affect resting ventilatory frequency (Gulinson and Harrison, 1996), suggesting that this is not the case. Our data suggest that the geometry of the insect tracheal system is such that thoracic and expired P_{CO_2} do not differ appreciably in resting grasshoppers except, perhaps, under conditions of very high convective ventilation (see below).

Is it possible that our 30 min of exposure to test P_{O_2} values did not allow us to examine steady-state conditions? Total tracheal volume is 500–950 µl in *Schistocerca gregaria* (Weis-Fogh, 1964; Harrison, 1989). Assuming tidal flow and complete mixing, at the slowest convective ventilation rate (21 kPa: 780 µl min^{-1}) and the largest possible tracheal volume (950 µl), the system would be 99 % equilibrated in 5.8 min. These assumptions are conservative, since the system is not tidal (Miller, 1960*b*), but at least partially unidirectional, which would cause complete flushing to occur considerably faster. Thus, the 30 min equilibration time should have been more than ample to allow tracheal P_{CO_2} levels to reach a steady state. Since the haemolymph pool is large (approximately 500 µl in adult female *S. americana*; Harrison, 1988) and haemolymph lacks carbonic anhydrase, the slowest-reacting CO_2 pool would be the haemolymph, and it is possible that haemolymph P_{CO_2} would not reach a steady state after 30 min even if tracheal P_{CO_2} were equilibrated. Preliminary experiments indicated a transient elevation in the rate of carbon dioxide emission upon exposure to hypoxia, consistent with a small decrease in animal CO_2 content in response to hypoxia. However, visual inspection of the $\dot{M}_{CO_2}$ traces indicated that such elevations in $\dot{M}_{CO_2}$ disappeared within minutes, suggesting that the washout of CO_2 from the grasshopper is rapid and complete in well under 30 min.

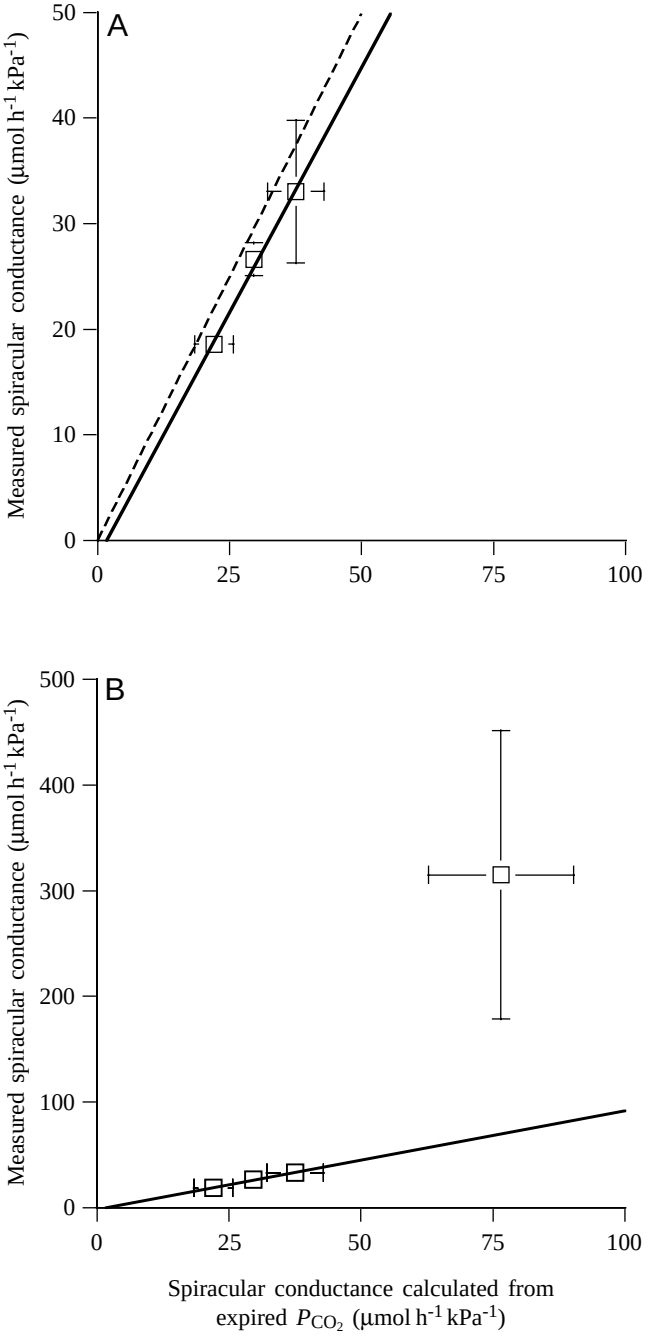

Fig. 13. Spiracular conductance measured from tracheal P_{CO_2} plotted *versus* spiracular conductance calculated from expired P_{CO_2}. (A) Values plotted for grasshoppers in 21, 10 and 5 kPa O_2 (solid line, regression equation for these points: y=0.931x–1.608, r^2=0.994, P=0.025). The broken line is the line of equivalence. (B) Values in 2 kPa O_2 included in the regression equation for all points: y=5.813x–142.581, r^2=0.951, P=0.051). The regression line from A is shown for comparison. Values are means ± 1 S.E.M., N=3 (A) and N=4 (B).

Effects of hypoxia on acid–base status

Hypoxia causes a pronounced alkalization of the haemolymph (Fig. 5), which is partly due to the reduction in haemolymph P_{CO_2} associated with the hyperventilation induced by hypoxia (Figs 5, 6, 7). However, a Davenport analysis (Fig. 11) demonstrates that haemolymph pH and bicarbonate values observed under hypoxic conditions are much higher than predicted given the measured decrease in haemolymph P_{CO_2}. The process that leads to greater extracellular alkalization than predicted by ventilatory changes alone was not examined in this study. One hypothesis is that grasshoppers attempt to conserve intracellular pH by transferring net acid from the haemolymph compartment to the intracellular compartment when intracellular pH is increased by declining P_{CO_2} during hypoxia exposure. Although we have no direct evidence to support this suggestion, intracellular pH has been shown to be regulated independently of haemolymph pH when body temperature varies (Harrison, 1988). An alternative hypothesis is that hypoxia could inhibit acid–base transport processes either in general and/or in the gut epithelia. For example, apparent base secretion by the midgut lumen (Harrison *et al.* 1992) could be highly sensitive to hypoxia.

Effect of order of gas exposure on $\dot{M}_{CO_2}$

Many studies have reported that insects tolerate exposure to hypoxia or anoxia with little or no detectable oxygen debt (Park and Buck, 1960; reviewed by Keister and Buck, 1974). Our data suggest that exposure to hypoxia does have some effect on metabolism, since the $\dot{M}_{CO_2}$ values of animals in room air previously exposed to hypoxia were elevated by approximately 45 % relative to animals without prior hypoxic exposure (Fig. 8). One possible explanation for this effect is that the increase in $\dot{M}_{CO_2}$ represents a classic oxygen debt, associated with clearance of lactate produced by grasshopper leg muscles (Gade, 1975; Zebe and McShaw, 1957; Harrison *et al.* 1991) in response to hypoxia. If so, the $\dot{M}_{CO_2}$ we measured under hypoxic conditions may not accurately reflect the oxygen consumption rate since CO_2 buffering of metabolic acid is expected to occur under these conditions. Arieli and Lehrer (1988) measured oxygen consumption of *Locusta migratoria* grasshoppers and found that the rate of oxygen consumption decreased at P_{O_2} values of less than 4 kPa. Thus, if CO_2 buffering of metabolic acid occurred, it is most likely to have occurred at 2 kPa O_2 and not at 5 kPa O_2.

Mechanisms for tolerance to hypoxia

Since resting grasshoppers maintain a high tracheal P_{O_2} (Gulinson and Harrison, 1996), and prior studies have suggested that ventilatory frequency does not increase until P_{O_2} approaches 5 kPa in grasshoppers (Arieli and Lehrer, 1988), it was conceivable that *S. americana* would simply tolerate the fourfold lower oxygen gradient from air to mitochondria in response to a switch from 21 to 5 kPa O_2 without any active change in tracheal function. In contrast to this view, our study demonstrates that *S. americana* grasshoppers respond to hypoxia with increases in the conductance of the tracheal system at each progressive decrease in ambient P_{O_2} (Figs 10, 12). Our study also demonstrates that the conductance of the tracheal system of *S. americana* increases because of increases in both spiracular and tracheolar conductances (Figs 10, 12). Between 21 and 5 kPa O_2, the increases in spiracular and tracheolar conductances are similar (Figs 10, 12); whereas at 2 kPa O_2, it is primarily spiracular conductance which increases further. Since the rate of oxygen consumption falls in this range (Arieli and Lehrer, 1988), our data are consistent with the hypothesis that tracheolar conductance in this range (<5 kPa O_2) cannot be appreciably increased and becomes limiting for oxygen delivery. As ambient P_{O_2} drops from 21 to 2 kPa, the haemolymph-to-air CO_2 conductance increases approximately half as much as required for perfect compensation for the hypoxia; thus, the maintenance of constant metabolic rates in the face of severe hypoxia is probably also facilitated by tolerance of lower cellular P_{O_2} values.

Spiracular conductance

Our results strongly suggest that convection is the major mechanism by which spiracular conductance is increased during hypoxic exposure. If diffusion played a large role in spiracular gas exchange, the expired P_{CO_2} calculated according to equation 5 would have been higher than that measured directly from the trachea. In fact, calculated expired P_{CO_2} and directly measured thoracic tracheal P_{CO_2} were statistically identical (Fig. 9). In addition, at least between 21 and 5 kPa O_2, the measured rise in spiracular conductance was linearly related with a slope of approximately 1 to the rise in spiracular conductance calculated using expired P_{CO_2} values derived from equation 5 (Fig. 13), further supporting the argument that the rise in spiracular conductance in response to hypoxia is almost completely due to an increase in bulk flow. At 2 kPa O_2, the spiracular conductance calculated from measurements of thoracic tracheal gases was very high relative to that calculated from $\dot{M}_{CO_2}$ and convective ventilation (five times greater, Fig. 13B), owing to very high calculated spiracular conductances for a few animals with near-zero thoracic tracheal P_{CO_2} values. Since inspiration occurs *via* the thoracic spiracles, it is possible that for some animals the convective ventilation is so high during severe hypoxia that the P_{CO_2} of thoracic tracheal gases closely approximates ambient P_{CO_2}.

Previous studies have suggested that in room air a considerable proportion (50 %) of the CO_2 emission of quiescent grasshoppers can be independent of abdominal pumping (Harrison, 1997). For the two animals excluded from the statistical analysis, calculated expired P_{CO_2} values were very high and convective ventilations were low, yet $\dot{M}_{CO_2}$ values were normal. These data, together with previous studies (Harrison, 1997; Harrison *et al.* 1995), suggest that adequate gas exchange can occur when visible abdominal pumping is absent in resting grasshoppers. In these animals, other mechanisms, such as convection associated with miniature ventilations (Hustert, 1975) or diffusion, may be important.

Tracheolar conductance

There is some evidence that tracheolar conductance for CO_2 increased in response to hypoxia, on the basis of the significant heterogeneity of slopes observed when comparing the ambient P_{O_2} *versus* haemolymph P_{CO_2} and the ambient P_{O_2} *versus* expired P_{CO_2} regressions (Fig. 9) with the nonsignificant (P=0.052) doubling of calculated tracheolar conductance (Fig. 10). The most likely explanation for these data is that the fluid levels in the grasshopper tracheoles decrease in response to hypoxia as reported by Wigglesworth (1931) for *Ceratophyllus fasciatus* fleas, adult *Aedes argenteus* mosquitoes, *Tenebrio molitor* mealworm larvae and *Blattella germanica* cockroaches. However, the increase in tracheolar conductance could also be due to increased convection between the large longitudinal trachea and the tracheoles, driven by the abdominal pressure pulsations. Tests of these alternative mechanisms will require observations or measurement of fluid levels in grasshopper tracheoles, the repetition of experiments such as these in animals for which tracheolar fluid levels can be observed or the use of methods to differentiate between the importance of diffusion and convection in tracheolar conductance (such as the use of varying inert gases or barometric pressure).

Conductances for oxygen

The spiracular conductance for oxygen is likely to be very similar to that measured for CO_2 since, at least in these experiments, spiracular conductance was primarily convective. Tracheolar conductance is likely to occur at least partially by diffusion, first in the gas phase down the blind-ended tracheoles (within which oxygen will move 1.2 times faster than CO_2) and then through tracheolar fluids and tissue (within which oxygen will move 36 times slower than CO_2; Krogh, 1919). Most authors have assumed that the conductance of oxygen from the tracheoles to the tissues will be much lower than the conductance of CO_2 (Buck, 1962; Kestler, 1985; Wigglesworth, 1983), given the much lower solubility of oxygen than of CO_2 in tissues. However, at 2 kPa O_2, haemolymph P_{CO_2} was 1.2 kPa (and cellular P_{CO_2} may be higher), while the maximum tracheal P_{O_2} under these conditions is 2 kPa O_2 and, thus, the tracheolar conductance for oxygen cannot be less than 60 % of that for CO_2 under these conditions. At least under severe hypoxic conditions, therefore, transport of oxygen to the mitochondria must occur mostly in the gas phase. However, if the fluid levels in the tracheae of grasshoppers vary with P_{O_2} as they do in other terrestrial insects (Wigglesworth, 1983), then the tracheolar conductance for O_2 may be much more affected by P_{O_2} variation than the tracheolar conductance for CO_2.

Why is the safety margin for hypoxia in insects so high?

Two possible reasons for the high tolerance of insects to extremely low ambient oxygen concentrations can be suggested. The first possibility is that hypoxia tolerance has evolved to allow insects to survive rare, but potentially deadly, exposure to low oxygen levels such as might occur when an insect is temporarily trapped in a small space. Alternatively, the tolerance to hypoxia might simply reflect an adaptation of the insect tracheal system to high metabolic rates during activity. For example, at 35 °C, both jumping and feeding grasshoppers consume oxygen at rates over 10 times that of an unfed, quiescent grasshopper at 22 °C (Harrison *et al.* 1991; Harrison and Fewell, 1995), and in-flight oxygen consumption rates can be 40–50 times greater than that of a quiescent grasshopper (Krogh and Weis-Fogh, 1951). To meet the gas exchange requirements of activity, insects in general, and grasshoppers in particular, have evolved the capacity to greatly increase the conductance of the tracheal system. Thus, the ability of these animals to maintain constant oxygen consumption rates in the face of very low oxygen availability probably reflects (1) the low metabolic rates of resting insects relative to those occurring in active insects, (2) the evolution of tracheal morphology and ventilatory mechanisms to exchange gases at some of the highest rates known in the animal kingdom during activity and (3) the ability of insects to rapidly modulate tracheal conductance.

Support for this research was provided by NSF IBN-9317784 to J.F.H. We would like to thank Eric Wilkinson and Katie Krolikowski for their efforts in the design and construction of the optical ventometer, B. J. Behm for his rendering of the apparatus and Stephen Roberts and two anonymous reviewers for helpful comments on the manuscript.

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