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HAEMOLYMPH ACID–BASE STATUS, TRACHEAL GAS LEVELS AND THE CONTROL OF POST-EXERCISE VENTILATION RATE IN GRASSHOPPERS

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Summary

In grasshoppers, ventilation rate increases after jumping, in association with decreases in haemolymph pH and tracheal P_{O_2} and increases in haemolymph and tracheal P_{CO_2}. Are these changes in haemolymph acid–base status or tracheal gas composition causally responsible for the increases in post-locomotion ventilation rate? To answer this question, we manipulated haemolymph acid–base status with injections into the haemocoel and independently manipulated tracheal P_{O_2} and P_{CO_2} with tracheal perfusions. Using a new technique, we continuously monitored ventilation rate and ventilatory pressures on virtually unrestrained insects. Changes in haemolymph acid–base status or tracheal P_{CO_2} did not affect post-exercise ventilation rate, clearly demonstrating that the ventilatory stimulus associated with locomotion is not dependent on negative feedback from these variables. Post-exercise ventilation rate varied with tracheal P_{O_2}, with the lowest ventilation rates observed at the lowest tracheal P_{O_2} values, a result opposite to that expected if negative feedback from internal P_{O_2} levels were to drive the increase in ventilation rate. Particularly after activity, there was considerable heterogeneity in unperfused animals between tracheal and haemolymph P_{CO_2}, and between tracheal P_{CO_2} in the thorax and leg, consistent with unidirectional airflow and a considerable role for diffusion gradients in the gas exchange of grasshoppers.

Key words: chemosensory feedback, grasshopper, locomotion, *Melanoplus differentialis*, pH, regulation of ventilation, *Schistocerca americana*, ventilation.

Introduction

Ventilation plays an important role in meeting tissue requirements for O_2 delivery and CO_2 excretion, especially in response to locomotor activity. Insects commonly increase their convective ventilation by increasing their rate of abdominal pumping during and after locomotion (Miller, 1960*b*, 1966; Weis-Fogh, 1967; Ramirez and Pearson, 1989; Harrison *et al.* 1991). In grasshoppers, increased ventilation rate (the frequency of abdominal pumping) after exercise is correlated with decreased haemolymph pH and tracheal P_{O_2} and with increased haemolymph and tracheal P_{CO_2} (Krogh, 1913; Harrison *et al.* 1991). Chemosensory feedback from the levels of blood pH, K^+, CO_2 and O_2 appears to be important in the control of locomotion-associated increases in ventilation rate in vertebrates (Griffiths *et al.* 1986; Pandit and Robbins, 1992; Paterson, 1992). In this study, we tested whether the post-exercise rise in ventilation rate in grasshoppers is caused by chemosensory feedback from haemolymph acid–base status or tracheal gas levels, using the differential grasshopper *Melanoplus differentialis* and the American locust *Schistocerca americana*.

Stimulation of ventilation rate in resting insects by ambient hypercapnia or hypoxia is well known (Miller, 1966; Arieli and Lehrer, 1988). In a companion paper, we have demonstrated that ventilation rate in quiescent grasshoppers is strongly affected by tracheal P_{O_2} and P_{CO_2}, but not by haemolymph acid–base status (Gulinson and Harrison, 1996). Does the chemosensory response of the insect ventilatory system function during activity, providing a mechanism by which insects modulate ventilation rate to match the changing requirements for gas exchange associated with locomotion? Because ventilation rate can also be stimulated by enhanced activity (Ramirez and Pearson, 1989), we also investigated the effect of haemolymph acid–base status and tracheal P_{O_2} on locomotory performance.

To investigate these phenomena, we modified a technique from Lighton (1988) for monitoring ventilation rate and ventilatory pressures in virtually unrestrained insects. Previous measures of tracheal pressures required rigid restraint of the insect (McCutcheon, 1940; Weis-Fogh, 1967). Our technique of measuring tracheal pressures using a polyethylene cannula placed into the metathoracic spiracle allowed us to measure ventilation rate and tracheal pressures continuously from

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virtually unrestrained grasshoppers at rest, during jumping and during recovery from activity.

An important issue related to the control of ventilation rate during activity is the degree of homogeneity of humoral factors that might serve as ventilatory stimulants. Specifically, are levels of CO_2 and other factors produced by the exercising muscle similar in the leg and around the metathoracic ganglia which control ventilation rate? Are haemolymph and tracheal gas levels identical? This is a particularly important issue in locusts, because the available data suggest that these insects lack peripheral chemoreceptors for CO_2 (Miller, 1960*a*). In the present study, we have compared the P_{CO_2} of haemolymph sampled from the neck with the P_{CO_2} of tracheal samples from the leg and thorax, at rest and after exercise.

Materials and methods

Animals

All animals were reared from eggs in culture at Arizona State University as previously described (Harrison and Kennedy, 1994). Only males at least 2 weeks past the moult to adulthood were used. Animals were isolated, starved and provided with water for 15 h overnight at 35 °C prior to the experiments. Most grasshoppers prefer temperatures in the range 30–40 °C (Chappell and Whitman, 1990), so all the experiments were conducted at 35 °C.

Measurement of ventilation rate

Ventilation rate was recorded by monitoring pressure changes within the tracheal system using an air-filled cannula of heat-stretched Intramedic PE-20 tubing (Clay Adams, Parsippany, NJ, USA) inserted into the metathoracic spiracle (Gulinson and Harrison, 1996). The cannula was sealed to the cuticle with hot glue and connected to an Omega (PX164005 BD 5V, Omega Inc., Stamford, CT, USA) pressure transducer. Output from the pressure transducer was amplified with the circuitry described by Lighton (1988). The voltage outputs corresponding to pressure changes were digitized and recorded (DATACAN V, Sable Systems, Salt Lake City, UT, USA). Visually observed abdominal pumps (breaths) were always correlated with easily identifiable pressure fluctuations. *Melanoplus differentialis* Thomas were placed in 360 ml opaque containers for 15 min prior to measurements to allow recovery from handling stress. With this method, we were able to count tracheal pressure fluctuations while the grasshopper was in complete isolation. Animals were free to move with minimal restraints as the container dimensions were smaller than the cannula length (approximately 15 cm).

Cannula volumes (<50 μl) were small relative to locust tracheal volumes (approximately 750 μl, Weis-Fogh, 1967; Harrison, 1989), minimizing the diminution of tracheal pressure changes due to enlargement of effective tracheal volume by the cannula dead space. To control further for this type of error, cannulae were calibrated by measuring the pressure fluctuations created by using a 10 μl Hamilton syringe to inject volumes of air into a cylinder with a volume similar to locust tracheal volume (750 μl). Voltage output varied linearly with changes in chamber pressure, as calculated from the ideal gas law, and was similar for all cannulae. The effect of frequency-damping on the response of each cannula was measured by recording the voltage response of the cannulae to similar pressure fluctuations at frequencies ranging from 0.1 to 1 Hz. Voltage output varied non-linearly with changes in frequency and varied among the cannulae. For all the cannulae used for tracheal pressure measurements, exponential equations fitted to these data explained at least 97 % of the variation in voltage with frequency. The best-fitting exponential equation for each individual cannula was used to calculate the tracheal pressure changes associated with abdominal pumping over a range of breaths during which visual inspection of the data indicated that ventilatory frequency was constant.

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

Manipulation of haemolymph acid–base status

Melanoplus differentialis were cannulated through the metathoracic spiracle, connected to the pressure transducer, and replaced in isolation for 15 min. Resting ventilation rate was then recorded over 5 min. Each grasshopper was removed from isolation and given a 20 μl injection of 0.5 mol l^{-1} NaOH, $NaHCO_3$ or NaCl, or 0.25 mol l^{-1} HCl, through the second abdominal inter-segmental membrane into the haemocoel. One group received no injection. Each animal was used only once and received only one injection. We then forced each animal to hop for 2 min by constant prodding. The animal was replaced in isolation and ventilation rate was recorded during a 15 min recovery period. Most animals lived for several weeks after receiving the injections, suggesting that they could completely recover from the acid–base disturbance caused by the injections and the injection and cannulation procedures. Both *Melanoplus* and *Schistocerca* species recover from mild extracellular acid–base disturbances (changes in haemolymph pH of less than 0.5 units) caused by injecting HCl or NaOH into the haemocoel within 4–8 h (Harrison *et al.* 1992; Harrison, 1995).

Measurement of haemolymph acid–base status

The effect of the acid–base injections on haemolymph acid–base status was measured by sampling haemolymph from a separate group of *M. differentialis* which were treated identically except that these animals did not receive tracheal cannulae. Each animal received at most one injection and was used only once. Haemolymph was sampled through a ventral neck incision immediately after the cessation of exercise, and haemolymph pH and total CO_2 (C_{CO_2}, mmol l^{-1}) were measured as previously described (Harrison and Kennedy, 1994). Haemolymph P_{CO_2} and [HCO_3^-] were calculated using the CO_2 solubility coefficient and carbonic acid dissociation constants for locust haemolymph (Harrison, 1988).

Jumping performance

The effect of the acid–base injections on jumping performance was tested by measuring the jump frequency during a 2 min forced exercise bout for animals injected with 20 μl of 0.5 mol l^{-1} NaOH, $NaHCO_3$ or NaCl or 0.25 mol l^{-1} HCl. These experiments were performed with a separate group of *M. differentialis*, treated identically but without tracheal cannulae. Each animal's performance was measured in jumps min^{-1} over a 2 min exercise period for each treatment. Over a period of 4 days, each animal received one treatment each day, with injections given in random order.

Experiment 2: do tracheal gas levels affect post-jumping ventilation rate?

Manipulation of tracheal gas levels

Melanoplus differentialis were cannulated through the metathoracic spiracle, allowed to recover in isolation for 15 min, and then a resting ventilation rate was recorded for 2 min. The grasshopper was removed from isolation and induced to jump for 2 min. Throughout the activity period, gas was perfused through the tracheal system *via* the metathoracic cannula at a rate of 3.5–5 ml min^{-1}. This high perfusion rate was chosen in an attempt to flush the tracheal system. The volume perfused was large relative to tracheal volume (<1 ml; Harrison, 1989) and minute ventilation rate (approximately 1 ml min^{-1}; Weis-Fogh, 1967). Tracheal pressures returned to normal levels within seconds after cessation of the perfusion. Gas mixtures varied in O_2 level (series 1: 1, 14.5 or 35.6 kPa P_{O_2}) at a constant CO_2 level (1.6 kPa) or varied in CO_2 level (series 2: 0.7, 1.6 or 4.4 kPa P_{CO_2}) at a constant O_2 level (14.5 kPa). The mixture containing 1.6 kPa P_{CO_2} and 14.5 kPa P_{O_2} closely matches the normal gas levels in the trachea and thus serves as a control for the effects of the perfusion. In all mixtures, the remaining gas volume consisted of N_2. Each animal was only used once and received only one perfusion. The gases were mixed using a Brooks model 5878 mass-flow controller and Brooks flow meters (Brooks Instruments, Hatfield, PA, USA).

Metathoracic airsac gas sampling

We measured the P_{O_2} and P_{CO_2} of tracheal gas samples obtained from the thoracic airsacs of resting, post-active and gas perfused/post-active *M. differentialis*. For tracheal gas sampling, the cannulae were shortened to reduce dead space volume to less than 5 % of the sample volume. A 30 μl gas sample was drawn out through the cannula using a 50 μl Hamilton gas-tight syringe as previously described (Gulinson and Harrison, 1996). For post-active animals, the sample was taken between 10 and 20 s post-exercise. The samples were analyzed with a gas-chrom MP-1 column (Alltech, Deerfield, IL, USA) using a Varian 3400 gas chromatograph as previously described (Gulinson and Harrison, 1996). Each animal was only used once and received only one perfusion.

Simultaneous sampling of leg and thoracic tracheal gases

We measured leg and thoracic tracheal P_{CO_2} for resting locusts and for locusts immediately after 2 min of forced exercise. Male *Schistocerca americana* Drury were used in this experiment because the larger size of this species facilitated measurement of leg gas levels. Animals were cannulated through the metathoracic spiracle as previously described. For resting gas measurements, the animals were kept in isolation for 15 min prior to sampling. The animals were then removed from isolation, and a metathoracic gas sample was taken within 5 s as described above. One hind leg was simultaneously pinched off at the coxal joint and removed. The leg was placed in acidified water to slow diffusion of CO_2 from the sample, and a gas bubble (usually 5–10 μl) was squeezed from the medial portion of the leg. The bubble was captured and transferred with the surrounding acidified water into a 50 μl Hamilton gas-tight syringe and the volume of the bubble was measured. The bubble was then transferred to a helium-flushed 5 ml Hamilton gas-tight syringe. The contents of this syringe were then injected through a drying column (magnesium perchlorate) into the gas chromatograph, and P_{CO_2} was measured as described above.

Effect of tracheal P_{CO_2} *manipulations on haemolymph* P_{CO_2}

We performed only one experiment to test whether the tracheal perfusions also changed the gas tensions in the haemolymph. *Melanoplus differentialis* with metathoracic cannulae were perfused with 0.7 kPa P_{CO_2}, 14.5 kPa P_{O_2} during exercise. Immediately after cessation of activity, the acid–base status of haemolymph samples was analyzed as described above. We compared the haemolymph acid–base status of the group of animals perfused with 0.7 kPa P_{CO_2} with haemolymph values from a separate group of unperfused animals.

The effect of perfusate oxygen level on locomotory performance

The effect of tracheal P_{O_2} levels on jumping performance was tested by counting the jumping rate of *S. americana* for which the tracheal system was perfused during exercise as described above. *Schistocerca americana* were used, owing to a shortage of *M. differentialis*. Animals were forced to hop for 3 min with the tracheal system perfused at approximately 5 ml min^{-1}. The gas mixtures used contained 1.6 kPa CO_2 and 1, 14.5 or 35.6 kPa O_2 (balance N_2). Eight animals received each of the perfusions in random order, with 2 h between tests of jumping frequency.

Statistics

Means ± S.E.M. are shown throughout. When individuals received multiple treatments, data were analyzed using paired *t*-tests when only two groups were compared, or by repeated-measures Huynh–Feldt-corrected analysis of variance (ANOVA) when individuals received more than two treatments. When different individuals received the different treatments, statistical analyses were performed using univariate ANOVA or *t*-tests, as appropriate. We compared means among multiple treatment groups using a Tukey *a*

posteriori test (for independent data) or using *post-hoc* contrasts for repeated-measures ANOVA. All statistical analysis was performed using SYSTAT (Wilkinson, 1989), with our within-experiment type I error controlled at 5 %.

Results

The effect of activity on haemolymph acid–base status and tracheal gases

In *M. differentialis*, 2 min of hopping caused a reduction in haemolymph pH and a rise in haemolymph P_{CO_2} (Table 1). Tracheal P_{O_2} was significantly elevated in post-exercise *M. differentialis*, while tracheal P_{CO_2} was identical in resting and post-exercise animals.

Haemolymph pH and P_{CO_2} of quiescent *S. americana* were similar to those of *M. differentialis* (Table 1). P_{CO_2} of the haemolymph and leg trachea significantly exceeded those measured *via* the metathoracic spiracle (independent *t*-tests), and thorax-to-leg tracheal P_{CO_2} heterogeneity increased post-activity (paired *t*-tests, Table 1). Thoracic tracheal P_{CO_2} did increase significantly after exercise in *S. americana* (paired *t*-tests, Table 1).

The effect of activity on ventilation rates and tracheal pressures

For resting and post-exercise *M. differentialis*, the pressure fluctuations recorded from the metathoracic spiracle corresponded to observable abdominal pulsations. Tracheal pressures fluctuated sinusoidally in resting and post-exercise grasshoppers, with each abdominal compression accompanied by an increase in tracheal pressure (Fig. 1). The pressure fluctuations recorded during jumping were erratic and did not correspond to observable abdominal movements. Therefore, ventilation rate was not measured during exercise. Ventilation rate was 4–6 times resting values immediately post-exercise, declining to resting values in 5.5 min in unmanipulated *M. differentialis* (Fig. 2, *post-hoc* contrasts for repeated-measures ANOVA).

Tracheal pressures varied considerably among animals, and we can draw only qualitative conclusions about the effects of activity on tracheal pressures because we were only able to calibrate three of our cannulae satisfactorily after

Table 1. *The effect of forced jumping activity on haemolymph acid–base status and tracheal gas levels in* Melanoplus differentialis *and* Schistocerca americana

	Resting value	*N*	Post-exercise value	*N*
Melanoplus differentialis				
Haemolymph pH	6.99±0.020	13	6.88±0.016*	15
Haemolymph P_{CO_2} (kPa)	3.3±0.23	8	4.9±0.31*	9
Haemolymph [HCO_3^-] (mmol l^{-1})	6.8±0.87	8	7.0±0.02	9
Metathoracic tracheal P_{CO_2} (kPa)	1.9±0.20	19	1.9±0.19	18
Metathoracic tracheal P_{O_2} (kPa)	17.1±0.38	19	18.4±0.22*	18
Schistocerca americana				
Haemolymph pH	6.93±0.023	8		
Haemolymph P_{CO_2} (kPa)	3.5±0.34	8		
Haemolymph [HCO_3^-] (mmol l^{-1})	5.6±0.84	8		
Metathoracic tracheal P_{CO_2} (kPa)	1.5±0.15	8	2.1±0.22*	8
Leg tracheal P_{CO_2} (kPa)	3.7±0.45	8	9.5±1.27*	8

Asterisks indicate that a value differs significantly between quiescent and post-active animals (*t*-tests).

Values are mean ± S.E.M.

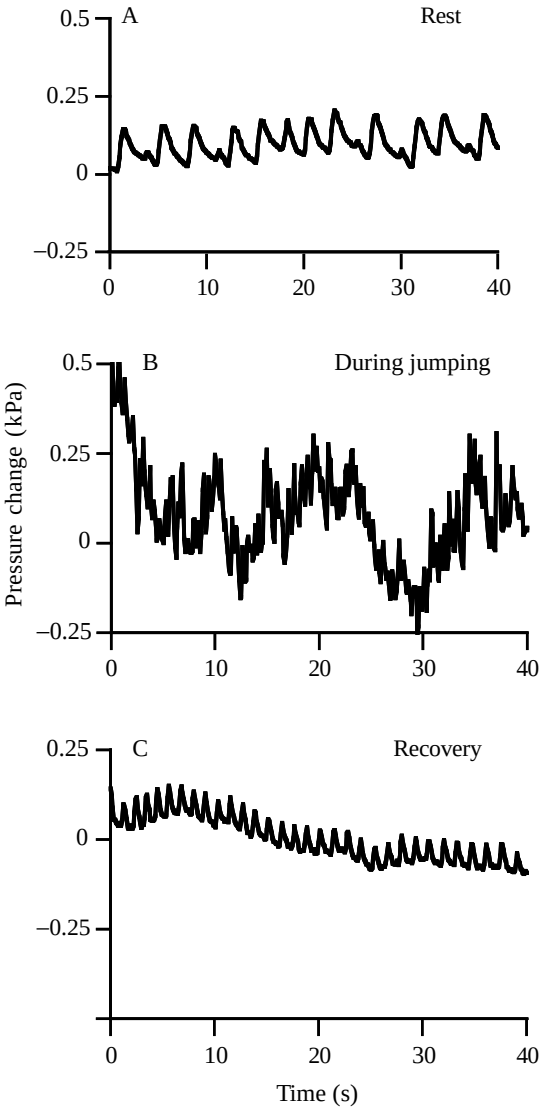

Fig. 1. Pressure fluctuations recorded from the metathoracic spiracle in a single *Melanoplus differentialis* at rest (A), during jumping (B) and post-exercise (C).

Table 2. *Tracheal pressure fluctuations at rest, during exercise and during recovery from jumping in* Melanoplus differentialis

	Maximum pressure per breath (kPa)	Range	Force duration per breath (kPa min)	Range	Rate of pressure generation (kPa min^{-1})	Range	Ventilation rate (breaths min^{-1})	Range
Rest	1.6	0.3–3.5	0.4	0.06–0.9	4.8	0.8–10.6	15	12–20
During exercise	1.4	0.8–2.1	0.03	0.01–0.06	3.7	1.3–42	122	104–142
First minute of recovery	2.7	0.1–6.5	0.02	0.004–0.003	1.9	0.4–3.9	93	56–116
Eighth minute of recovery	1.2	0.2–2.5	0.3	0.03–0.7	3.3	0.4–7.2	13	10–16

N = 3 for each, values are means.
During hopping, 'breaths min^{-1}' refers to the frequency of pressure fluctuations (these did not correspond to observable abdominal pumping).

removing them from the animals. While ventilation rate and maximum pressure per breath increased in all 1 min post-exercise animals relative to quiescent animals (Table 2), the value of the integrated pressure–time curve per breath (kPa min $breath^{-1}$) and the rate of convective pressure generation (kPa min^{-1}) were lower during the first minute after activity than during rest for all three animals (Table 2). During jumping, the pressure fluctuations were relatively small but of high frequency. These erratic, high-frequency pressure fluctuations created tracheal pressure generation rates similar to those measured in animals exhibiting abdominal pumping (Table 2).

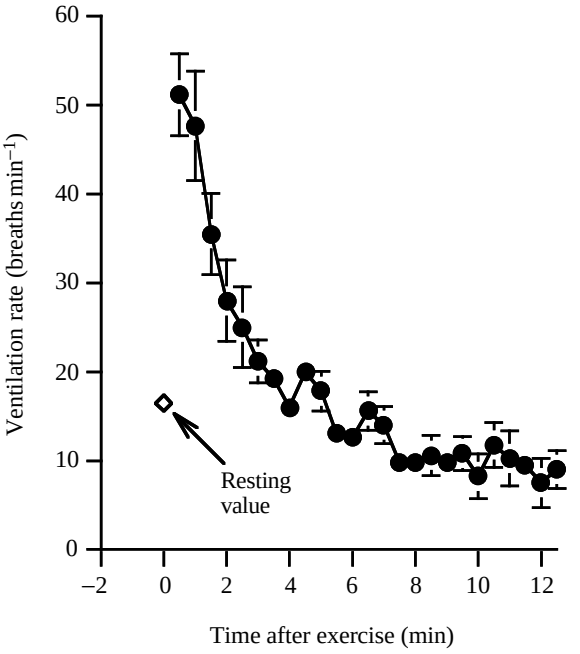

Fig. 2. Ventilation rate during recovery from a 2 min bout of forced jumping for *M. differentialis*. Ventilation rates were significantly elevated above rest until 5.5 min post-exercise (*post-hoc* comparisons for repeated-measures ANOVA, $P \leqslant 0.05$). Values are means ± S.E.M. N=15–18 at each point.

Experiment 1: are changes in haemolymph acid–base status responsible for the increase in post-exercise ventilation rate?

The effect of the acid–base injections on haemolymph acid–base status

The haemolymph acid–base status of post-exercise, NaCl-injected *M. differentialis* was not significantly different from that of post-exercise, uninjected animals, indicating that stress associated with the injections did not significantly affect haemolymph acid–base status (Table 3). The HCl, NaOH and $NaHCO_3$ injections caused significant variation in haemolymph acid–base status among post-exercise animals (Table 3). The NaOH injection increased the haemolymph pH of post-active animals to a level not statistically different from that of quiescent animals (Table 3).

Relationships between haemolymph acid–base status and post-exercise ventilation rate

All treatment groups showed similar increases in post-exercise ventilation rate (Table 3). The mean resting ventilation rates of the various treatment groups did not differ significantly (ANOVA, $F_{4,33}$=2.01, P>0.1). The treatment groups also did not differ significantly in their ventilation rate during the first 30 s post-exercise (Table 3; ANOVA, $F_{4,25}$=1.45, P>0.1). For all groups, post-exercise ventilation rate was significantly higher than resting values for the first 5 min of recovery (*post-hoc* comparisons, repeated-measures ANOVA). The ventilation rates of the NaCl-injected, uninjected and $NaHCO_3$-injected animals all returned to resting ventilation rates by 6 min post-exercise (*post-hoc* comparisons, repeated-measures ANOVA). The ventilation rates of both the NaOH- and HCl-injected animals returned to resting levels after 16 min of recovery (*post-hoc* comparisons, repeated-measures ANOVA). There were no significant correlations (Spearman's correlation coefficients, P>0.5) between post-exercise ventilation rate measured 30 s or 1 min post-exercise and haemolymph pH, haemolymph P_{CO_2} or haemolymph [HCO_3^-]. The acid–base injections also did not affect jump frequency (Table 3, repeated-measures ANOVA, P>0.5).

Table 3. *The effect of injections of acid–base solutions made immediately before 2 min of forced jumping on post-exercise haemolymph acid–base status, ventilation rate and locomotory performance in* Melanoplus differentialis

Injection	Haemolymph acid–base status			Ventilation rate (breaths min^{-1})		Locomotion (jumps min^{-1})
	pH	P_{CO_2} (kPa)	[HCO_3^-] (mmol l^{-1})	Resting	Post-exercise	
NaOH-injected	7.02±0.028^{c}	5.3±0.41*^{,a,b}	10.4±0.72*^{,c}	17.5±6.9	39.0±9.3†	28.4±3.06
HCl-injected	6.57±0.030*^{,a}	6.2±0.56*^{,a,b}	4.3±0.35*^{,a}	23.1±9.3	36.3±12.4†	33.5±2.63
$NaHCO_3$-injected	7.42±0.030*^{,d}	6.8±0.75*^{,b}	35.6±2.41*^{,d}	24.8±6.7	54.2±17.0†	38.5±2.22
NaCl-injected	6.82±0.008*^{,b}	4.8±0.32*^{,a}	5.9±0.62^{b}	19.2±4.7	49.3±6.7†	33.5±2.63
Uninjected	6.88±0.016*^{,b}	4.9±0.31*^{,a}	7.0±0.02^{b}	16.5±3.2	51.2±11.3†	34.2±2.88

Haemolymph acid–base values were measured within 10 s after cessation of the 2 min exercise bout.

Asterisks indicate an acid–base level different from those of uninjected, resting *M. differentialis* from the same experiment (Tukey HSD test, $P<0.05$, data given in Table 1) and a common superscripted letter indicates that the haemolymph acid–base value did not differ among the post-exercise treatment groups from the same experiment (Tukey HSD test).

Ventilation rates were measured during rest and during the 30 s post-exercise for animals in each treatment group.

Daggers indicate a significant difference between post-exercise and resting values (*post-hoc* comparisons, repeated-measures ANOVA). The treatment groups did not differ significantly in either resting or post-exercise ventilation rates (ANOVA, $P>0.1$).

Locomotory performance was measured during 2 min of forced exercise. Treatment did not significantly affect jump rate (repeated-measures ANOVA, $P>0.5$).

All values are means ± S.E.M., N = 6–8 for each.

Table 4. *The effect of perfusate composition on post-exercise tracheal gas levels, ventilation rates and jump performance*

Series	Perfusion gas (kPa)		Tracheal gas (kPa)		Ventilation rate (breaths min^{-1})		Locomotion (jumps min^{-1})
	P_{O_2}	P_{CO_2}	P_{O_2}	P_{CO_2}	Resting	Post-exercise	
1	1	1.6	11.7±1.5^{a}	1.7±0.05^{a}	29.1±5.9	43.2±11.83^{a,*}	7.6±1.89^{a}
1, 2	14.5	1.6	17.8±0.20^{b,e}	1.7±0.09^{a,c}	43.6±13.7	91.6±8.0^{b,c,*}	16.9±3.02^{b}
1	35.6	1.6	22.3±2.40^{c}	1.9±0.34^{a}	21.75±1.4	83.8±15.6^{b,*}	18±3.45^{b}
2	14.5	0.7	19.9±0.29^{d}	1.5±0.13^{b}	36.3±3.5	101.1±14.1^{c,*}	NM
2	14.5	4.4	17.6±0.24^{e}	2.1±0.10^{d}	24.4±5.9	87±16.6^{c,*}	NM
	Unperfused		18.4±0.22	1.9±0.19	35.2±5.3	108.0±5.2*	NM

Perfusions were made *via* the metathoracic spiracle during 2 min of forced exercise. Tracheal air samples were collected immediately after cessation of jumping for *Melanoplus differentialis*. Within each series (P_{O_2} or P_{CO_2} varied with the level of the other gas held constant), common letters indicate that treatment groups do not differ significantly (Tukey HSD test).

Ventilation rates were measured at rest and during 30 s post-exercise for *M. differentialis*. Asterisks indicate that the ventilation rate post-exercise was significantly higher than that for the resting animals (*post-hoc* comparisons, repeated-measures ANOVA). Within a series, common letters indicate that treatment groups did not differ significantly in post-exercise ventilation rate (Tukey HSD test).

For locomotory performance, jumps were counted during the entire exercise bout for *Schistocerca americana*. Common letters indicate that treatment groups did not differ significantly in jump rate (Tukey HSD test).

Means ± S.E.M., N = 6-8. NM, not measured.

Experiment 2: are changes in tracheal gas levels responsible for the elevation of post-hopping ventilation rate?

Effect of tracheal injections on tracheal gases

Post-exercise tracheal P_{O_2} and P_{CO_2} were successfully manipulated by the gas perfusions (Table 4), but thoracic tracheal P_{CO_2} and P_{O_2} were not equivalent to the levels in the injected gases. For gas samples obtained from the metathoracic spiracle 10–20 s after cessation of hopping within series 1 (P_{O_2} manipulation), tracheal P_{O_2} (P_{O_2t}) was related to perfusate P_{O_2} (P_{O_2p}) by the regression line:

$$P_{O_2t} = 12.156 + 0.296P_{O_2p}$$

(r^2=0.95, $P<0.001$, N=19). For series 2 (P_{CO_2} manipulation), tracheal P_{CO_2} (P_{CO_2t}) was related to perfusate P_{CO_2} (P_{CO_2p}) by the regression line:

$$P_{CO_2t} = 1.403 + 0.163P_{CO_2p}$$

(r^2=0.99, $P<0.001$, N=20).

When perfusate P_{O_2} was varied (series 1), tracheal P_{CO_2} was maintained statistically unchanged (Table 4). When perfusate P_{CO_2} was varied, there was a significant effect on tracheal P_{O_2}, despite the fact that perfusate P_{O_2} was maintained constant at 14.5 kPa. The group perfused with the lowest P_{CO_2} had a significantly higher tracheal P_{O_2} than the other series 2 groups (Tukey HSD tests, Table 4).

Effect of tracheal P_{CO_2} manipulation on haemolymph P_{CO_2}

Perfusion of the tracheal system of *M. differentialis* during forced exercise with 0.7 kPa P_{CO_2}, 14.5 kPa P_{O_2} significantly lowered post-exercise haemolymph P_{CO_2} and raised haemolymph pH relative to unperfused, post-exercised animals (for perfused animals, haemolymph pH=7.009±0.033, haemolymph P_{CO_2}=3.4±0.30 kPa, N=6, data for unperfused animals in Table 1; independent t-tests; P_{CO_2} comparison, t=3.1, P<0.05; pH comparison, t=5.1, P<0.05, d.f.=1,13 for each). Neither haemolymph pH nor P_{CO_2} of these post-exercise perfused animals differed significantly from those of unperfused, resting animals (independent t-tests, P>0.1).

The effect of tracheal gas levels on post-exercise ventilation rate

Decreasing tracheal P_{O_2} levels did not increase post-exercise ventilation rate as would be predicted from stimulation of ventilation rate by negative feedback from a reduction in internal P_{O_2} (Table 4). Post-exercise ventilation rate was significantly affected by tracheal P_{O_2} (ANOVA, $F_{2,13}$=3.91, P<0.05). Post-exercise ventilation rates were similar at control and elevated tracheal P_{O_2} values but were decreased at low tracheal P_{O_2} (Table 4, Tukey HSD tests). Manipulation of tracheal P_{CO_2} levels did not affect post-exercise ventilation rate (Table 4, ANOVA, F=0.27, P=0.77). The post-exercise ventilation rate increased significantly relative to the resting value for all treatment groups except the group perfused with 1 kPa P_{O_2},1.6 kPa P_{CO_2} (Table 4). Resting ventilation rates of the various groups did not differ significantly (Table 4, ANOVA, $F_{5,28}$=1.55, P=0.21).

Effect of tracheal P_{O_2} on hopping performance

The P_{O_2} of the perfusate significantly affected the frequency of jumping for *S. americana* (Table 4, ANOVA, $F_{1,22}$=6.0, P=0.022), with the group injected with the lowest P_{O_2} having a significantly lower jump frequency than the other groups (Tukey HSD test).

Discussion

Tracheal pressures, gas heterogeneity and the ventilatory mechanism

With each abdominal pump of *M. differentialis*, defined as a visible longitudinal telescoping and/or dorso-ventral contraction of the abdomen, we measured an increase in tracheal pressure at the metathoracic spiracle of 0.1–3 kPa (Fig. 1; Table 2), similar to the increase in expiratory pressure measured in the haemocoel of *S. gregaria* during abdominal pumping (Weis-Fogh, 1967). The increase in pressure occurs during the compression phase, when the abdomen contracts during a period of complete spiracular closure (McCutcheon, 1940). Tracheal pressures decrease as first the abdominal spiracles open for expiration and then the thoracic spiracles open for inspiration (McCutcheon, 1940). We never measured subatmospheric pressures in the thoracic trachea. Since air enters the animal through the thoracic spiracles, there must be a negative pressure in the metathoracic airsacs during inspiration which was too small or transient for detection using our methods.

The rate of pressure generation (kPa min^{-1}) available to drive convection does not appear to be greater in post-exercise grasshoppers than in resting animals (Table 2), despite the fact that post-active *M. differentialis* take more frequent, larger breaths. It seems likely that spiracular aperture size also increases after exercise, reducing the pressure gradient produced by abdominal compression and allowing a larger volume of air to be moved by a smaller pressure gradient.

Coordination of abdominal pumping with spiracular opening allows unidirectional thorax-to-abdomen airflow in grasshoppers (McCutcheon, 1940; Weis-Fogh, 1967). Consistent with this model of tracheal function, we found that P_{CO_2} was lower in the thoracic trachea than in the leg trachea or haemolymph (Table 1). P_{CO_2} heterogeneity increased post-exercise, supporting the argument that increases in the partial pressure gradients driving diffusion serve as major components of the increase in gas exchange in grasshoppers during exercise (Harrison *et al.* 1991).

Our recordings of tracheal pressures during jumping show no evidence of the cyclic, smooth pressure pulsations associated with abdominal pumping. Either the pressure fluctuations associated with abdominal pumping are masked by the effects of jumping, or perhaps abdominal pumping does not occur during forced exercise in *M. differentialis*, as previously hypothesized (Harrison *et al.* 1991). Considerable erratic pressure gradients did occur during exercise which could drive convection (Fig. 1; Table 2). These pressure gradients may be due to cuticular deformations associated with jumping and landing, or possibly to the thoracic or neck ventilatory mechanisms which have been observed in grasshoppers subject to high ambient P_{CO_2} (Miller, 1960*a*). Our data suggest that the pressures generated during activity are likely to be relatively ineffective in driving convective gas exchange since (1) internal P_{CO_2} increases during activity in both *M. differentialis* and *S. americana* (Table 1), (2) internal P_{CO_2} heterogeneity increases in *S. americana* (Table 1) after activity and (3), in *M. bivittatus*, the haemolymph-to-air CO_2 conductances are similar in resting and active grasshoppers, but much higher in post-exercise animals (Harrison *et al.* 1991).

Post-exercise ventilation rate is not regulated by changes in haemolymph acid–base status

Post-exercise ventilation rate was not affected by changes in haemolymph pH. Haemolymph pH drops by 0.1 units over a few minutes of activity and returns to resting levels in conjunction with ventilation rate in untreated animals (Table 1; Harrison *et al.* 1991). We would expect that if the reduction in pH were to drive the increase in ventilation rate after exercise, then the injections of NaOH, which eliminated the post-activity reduction in haemolymph pH, would also prevent the rise in ventilation rate. However, ventilation rate did not differ significantly between post-active, uninjected and post-active,

NaOH-injected animals (Table 4). In general, there was no systematic relationship between haemolymph pH and ventilation rate when haemolymph acid–base status was manipulated by injections of acids or bases into the haemocoel (Table 4).

Similarly, when haemolymph acid–base status was manipulated, post-exercise ventilation rate was not correlated with post-exercise haemolymph P_{CO_2}. In unmanipulated animals, haemolymph P_{CO_2} rises by 1.6 kPa during forced exercise (Table 1), and haemolymph P_{CO_2} and ventilation rate return to resting levels after a similar period of recovery (Table 1; Harrison *et al.* 1991). If the rise in haemolymph P_{CO_2} causes the rise in ventilation rate post-exercise, ventilation rate after activity should be positively correlated with haemolymph P_{CO_2}, and the rise in ventilation rate after exercise should be eliminated by manipulations which block the rise in haemolymph P_{CO_2}. However, after exercise and haemolymph acid–base manipulation, ventilation rate was not correlated with haemolymph P_{CO_2} (Table 3). Perfusing the trachea with gas containing 0.7 kPa CO_2 blocked the rise in haemolymph P_{CO_2} with activity, but had no affect on post-exercise ventilation rate. Clearly, the rise in ventilation rate post-activity in grasshoppers is independent of haemolymph P_{CO_2}.

The results from the haemolymph manipulation experiments show that changes in haemolymph acid–base status are not likely to be responsible for the rise in ventilation rate after exercise in grasshoppers. Similarly, for resting grasshoppers, manipulation of haemolymph acid–base variables does not affect ventilation rate in *Romalea guttata* (Gulinson and Harrison, 1996). *Schistocerca gregaria* recover from haemolymph acidosis at constant haemolymph P_{CO_2} (Harrison *et al.* 1992). Together, the results from these studies suggest that, in general, grasshoppers do not utilize chemosensory feedback from haemolymph pH to regulate ventilation rate.

Post-exercise ventilation rate is not regulated by changes in tracheal gas levels

We successfully independently manipulated tracheal P_{O_2} and P_{CO_2} above and below normal values for post-exercise *M. differentialis*. The tracheal gas levels measured generally did not match the values for the perfusate (Table 4), probably because of (1) rapid removal of the perfused gases, (2) transfer of gases between the perfused side and the unperfused side contralateral to the cannula, (3) transfer of gases between the tracheal, cellular and haemolymph compartments. In resting *S. americana*, perfusions similar to those used in this study caused transient changes in tracheal gas composition, with values returning to normal levels within 1 min (Gulinson and Harrison, 1996). In this study, perfusion of the trachea of *M. differentialis* during exercise with gas containing 0.7 kPa P_{CO_2} decreased the P_{CO_2} of haemolymph from the normal post-exercise value of 4.9 kPa to 3.4 kPa, indicating that gases move quickly between the tracheal and extracellular compartment. Because the gas tensions within the animal varied with site and compartment (Table 1), it is unlikely that the ipsilateral tracheal gas tensions we report were exactly those experienced by the central chemoreceptors. Our perfusions had large effects on the gas tensions measured on the ipsilateral side and also strongly affected the gas composition of haemolymph sampled from the ventral neck region. Therefore, we feel confident that we successfully manipulated internal gas levels and were able to test whether post-exercise ventilation rate is affected by tracheal gas levels.

Since CO_2 production rises during activity, and tracheal CO_2 levels may generally rise during activity in insects (Krogh, 1913; Downer and Matthews, 1977; Bartholomew and Barnhart, 1984), tracheal P_{CO_2} might be expected to provide a good signal for increasing ventilation rate during locomotion in insects. In addition, we have recently shown that tracheal P_{CO_2} and P_{O_2} both affect resting ventilation rate in locusts (Gulinson and Harrison, 1996). However, in the present study, when we manipulated post-active tracheal and haemolymph P_{CO_2} levels, there was no effect on post-exercise ventilation rate (Table 4). The post-exercise rise in ventilation rate does not appear to be a consequence of a rise in internal P_{CO_2}.

Results from the experiments in which tracheal P_{O_2} was manipulated were not consistent with the hypothesis that a drop in tracheal P_{O_2} after exercise is responsible for the increase in ventilation rate after exercise. Post-exercise ventilation rates were unaffected by perfusions with gases with a high P_{O_2} and were lower when the perfusate had a low P_{O_2} (Table 4). In contrast, a variety of studies with grasshoppers and other insects have shown that resting ventilation rate is negatively correlated with ambient or tracheal P_{O_2} (Miller, 1966; Arieli and Lehrer, 1988; Gulinson and Harrison, 1996). The reduction in post-exercise ventilation rate in *M. differentialis* perfused with 1 kPa P_{O_2} gas could be due to a direct suppressant effect of low tracheal or cellular P_{O_2} on the neuronal or muscular systems. Supporting this suggestion, *S. americana* perfused with 1 kPa P_{O_2} gas had a lower jumping frequency than animals perfused with gases containing more oxygen (Table 4).

Why does the ventilatory system respond to levels of tracheal gases at rest but not after locomotion in grasshoppers? Evidence to date suggests that the ventilatory CO_2 receptors are all centrally located within the thoracic and head ganglia, near to the inspiratory spiracles (Miller, 1960*a*; Weis-Fogh, 1967). The head and thoracic ganglia are poorly located to function as good sensors of rapid changes in tracheal gas levels, especially those occurring in the long metathoracic legs of grasshoppers. The CO_2-sensitive regions of the nervous system experience diminished and possibly time-delayed changes in tracheal gas levels relative to those experienced by the working muscle (Table 1). Neuronal or hormonal feedforward mechanisms may allow more rapid and precise matching of locomotion and ventilation in grasshoppers. It seems possible that chemosensory feedback might be more effective in matching ventilation with locomotion when the locomotory muscles are nearer to the central CO_2 receptors, as during flight for all insects or in smaller insects during terrestrial locomotion.

At present, the mechanism that controls the rise in

ventilation rate after locomotion is unclear. Both feedforward and humoral feedback mechanisms seem possible. In locusts, Ramirez and Pearson (1989) found interneurones which affected both flight behaviour and abdominal pumping rate. It is reasonable to predict that there could be similar interneurone-mediated, feedforward regulation of ventilation rate during jumping, with one region of the nervous system stimulating both locomotion and respiration. Neuromodulators such as octopamine, whose levels increase during activity (Goosey and Candy, 1980), might also affect ventilation rate. It is also possible that some other factor released from the working muscle (e.g. K^+) stimulates ventilation rate during and after activity.

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