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Development of respiratory function in the American locust *Schistocerca americana*

I. Across-instar effects

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Summary

We tested the hypothesis that oxygen delivery from the atmosphere to the tissues becomes more difficult as grasshoppers increase in body size throughout development due to increases in tracheal length. If this is true, then older, larger grasshoppers should have smaller safety margins [higher critical oxygen partial pressures (P_{O_2}s)] for oxygen delivery than younger, smaller grasshoppers. We exposed grasshoppers of first, third and fifth instars and adults to decreasing levels of atmospheric O_2 and measured their ventilatory responses. Contrary to our prediction, we found that larger grasshoppers had critical P_{O_2}s eight times lower than juveniles due in part to their threefold lower mass-specific metabolic rates and their ability to quadruple convective gas exchange. Adults more than doubled abdominal pumping frequency and increased tidal volume by 25% as P_{O_2} decreased fourfold, whereas the youngest juveniles showed no such responses. This study indicates that juveniles may be more susceptible to hypoxia in natural situations, such as exposure to high altitude or restricted burrows. Also, larger size is not necessarily correlated with a smaller safety margin for oxygen delivery in insects.

Key words: ontogeny, insect, grasshopper, *Schistocerca americana*, ventilation, gas exchange, hypoxia.

Introduction

The development of physiological traits is a critical aspect of evolutionary and ecological physiology (Burggren, 1991). Most natural selection probably occurs during the juvenile stages, because young animals generally have much higher rates of mortality than fully developed, adult organisms (insects – Estevez and Gonzalez, 1991; reptiles – Iverson, 1991; arachnids – Tanaka, 1992; birds – Currie and Matthysen, 1998; fish – Molles, 1999; mammals – Durant, 2000). Therefore, knowledge of the physiology of juveniles and developmental patterns of physiological traits is important in understanding ecological success and evolution.

Adult and pupal insects tolerate low levels of oxygen (<5 kPa O_2), at which most mammals would die (Wegener, 1993), and generally have resting critical oxygen partial pressures [P_c; the oxygen partial pressure (P_{O_2}) below which metabolism can no longer be maintained] well below those of vertebrates and perhaps below those of non-tracheate invertebrates (Table 1; Hoback and Stanley, 2001). P_c is the point at which an animal switches from being an oxy-regulator to an oxy-conformer (Yeager and Ultsch, 1989; Portner and Grieshaber, 1993), and thus it defines the safety margin for O_2 delivery (the range of P_{O_2}s across which metabolism is constant; Kam and Lillywhite, 1994). For this reason, organisms with low P_cs tend to tolerate hypoxia better than animals with higher P_cs (Ultsch, 1973, 1974). To our knowledge, P_c has not yet been compared across the developmental stages of an insect.

One prominent hypothesis suggests that as insects become larger, gas exchange becomes more difficult due to increases in tracheal system lengths (Graham et al., 1995; Dudley, 1998). This logic is the basis for the hypothesis that increases in atmospheric O_2 during the Paleozoic era facilitated insect gigantism (Graham et al., 1995; Dudley, 1998). Alternatively, larger insects could have greater tracheal diameters or could increase the use of ventilation to compensate for increased tracheal lengths. We tested this hypothesis using developing grasshoppers. If older, larger grasshoppers have smaller safety margins (higher P_cs) for oxygen delivery than younger, smaller grasshoppers, this would support the hypothesis of Graham et al. (1995).

Development of the respiratory system is particularly interesting, because all organisms grow in size and increase total oxygen demand with age. P_c changes during ontogeny will depend on whether the ratio of maximal tracheal

Table 1. *Summary of critical oxygen partial pressures (P_c) for metabolic rates of vertebrates and invertebrates*

Species	Common name/ developmental stage	P_c	Reference
Invertebrates: non-insect			
Metridium senile	Sea anemone	<6 kPa	Mangum and Van Winkle (1973)
Caenorhabditis elegans	Nematode	3.6 kPa	Van Voorhies and Ward (2000)
Nassarius obsoletus	Mud snail	<12 kPa	Mangum and Van Winkle (1973)
Nephrops norvegicus	Adult lobster	5 kPa	Spicer (1995)
Invertebrates: insect			
Tenebrio molitor	Beetle pupae	<5 kPa	Gaarder (1918)
Aedes aegypti	Adult mosquito	3–4 kPa	Galun (1960)
Phormia regina	Adult fly	2–5 kPa	Keister and Buck (1961)
Termopsis navidensis	Adult termite	2–5 kPa	Cook (1932)
Locusta migratoria	Adult grasshopper	3–4 kPa	Arieli and Lehrer (1988)
Schistocerca americana	Adult grasshopper	2–5 kPa	Greenlee and Harrison (1998)
Vertebrates			
Rana temporaria	Adult frog	5–10 kPa	Tattersall and Boutilier (1999)
Pseudemys nelsoni	Snake embryo	8–15 kPa	Kam and Lillywhite (1994)
Coturnix coturnix japonica	Adult quail	11 kPa	Weathers and Snyder (1974)
	22 small rodent species	16–18 kPa	Rosenmann and Morrison (1975)

conductance to metabolic rate changes with age. Maximal tracheal system conductance (G_{max}; μmol g^{-1} h^{-1} kPa^{-1}) will depend on tracheal system structure (e.g. spiracle size and dimensions and density of tracheae and tracheoles) and function (e.g. the magnitude of convective gas exchange). The scaling exponents for metabolic rates during insect development vary widely and may exceed 1.0 (Casey and Knapp, 1987; Vogt and Appel, 1999).

In the present study, we quantify developmental changes in metabolic rate, maximal tracheal system conductance and several aspects of tracheal system function (ventilation frequency and abdominal pumping height, an index of tidal volume) for the American locust *Schistocerca americana*. We also compare the P_c for oxygen consumption and carbon dioxide emission to verify that our P_c for CO_2 emission truly reflected the P_c for oxygen delivery. In addition, we examine the effect of hypoxia exposure time on the P_c for CO_2 emission, since we wanted to be sure that our calculated P_c values were not strongly affected by the duration of hypoxia exposure in our major experiment.

Materials and methods

Animals

Schistocerca americana Drury were reared from eggs in culture at Arizona State University as previously described (Harrison and Kennedy, 1994). For these experiments, it was important to have grasshoppers of known age and body size. We took 200 grasshoppers that hatched on the same day and marked them with paint (Testors, Rockford, IL, USA). We monitored the colony each day, and, when an animal molted, we applied a new color of paint. All animals that molted on a given day had the same paint color, so we knew the age in days of each individual. Experimental animals were randomly selected from the animal care facility in the morning and kept in a lit, 35°C incubator with access to green leaf lettuce for 0.5–6 h before measurements were made. We recorded body mass (M_b) of all animals, weighing them to the nearest 0.001 g using an analytical balance (Mettler Instruments, Hightstown, NJ, USA). For measurements of adult insects, we used only males, but for measures of juveniles, sex was not determined.

Comparison of P_c values for O_2 consumption and CO_2 emission

Adult animals were placed in a 26 ml Plexiglas cylinder sealed with rubber stoppers. Inside the chamber, we placed cotton anterior and posterior to the grasshopper to prevent movement and around the animal's head to prevent visual stimulation. Test animals sat in the chamber for 20 min before measurements were made, a procedure that kept ventilatory frequencies similar to those of unrestrained grasshoppers behind one-way mirrors (Gulinson and Harrison, 1996). Each grasshopper was exposed to 11 different levels of P_{O_2} (21, 16, 13, 9, 7, 5, 3, 2, 1, 0.5 and 0 kPa O_2) for at least 15 min in descending order to prevent increases in metabolic rate associated with prior hypoxic exposure (Greenlee and Harrison, 1998).

Gas mixtures were made by diluting dry, CO_2-free air with N_2 (Balston purge-gas generator; Havervill, MA, USA). The ratio of air to N_2 was controlled by a Brooks 5878 mass flow controller and mass flow meters (Brooks Instruments, Hatfield, PA, USA). Gas mixtures were pushed through three identical chambers at 223–326 ml min^{-1}. Flow from one of these chambers was directed to the reference cells of the gas analyzers. The sample cells of the gas analyzers received output either from the animal chamber or an empty baseline chamber. All three chambers emptied into 60 ml syringes

from which excurrent gases were subsampled. The reference stream and the sample stream were pulled through the CO_2 analyzer (LI-6252; Li-Cor, Lincoln, NE, USA) and then the O_2 analyzer (Sable Systems Oxzilla, Las Vegas, NV, USA) by an AMETEK R-1 flow controller (Pittsburgh, PA, USA). Water and CO_2 were removed between the two analyzers using columns of drierite and ascarite. The output of both gas analyzers was digitized and recorded (Sable Systems).

We calculated the rates of CO_2 emission and O_2 consumption ($\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$, respectively; μmol g^{-1} h^{-1}) using the following equations:

$$\dot{M}_{O_2} = \frac{\dot{V}_{in} \times (F_{IO_2} - F_{EO_2}) \times 2678.57}{(1 - F_{EO_2}) \times M_b}, \quad (1)$$

$$\dot{M}_{CO_2} = \frac{\dot{V}_{in}\,(F_{ECO_2} - F_{ICO_2}) - (\dot{V}_{O_2} \times F_{ECO_2}) \times 2678.57}{(1 - F_{ECO_2}) \times M_b}, \quad (2)$$

where $\dot{V}_{in}$ is the flow rate measured upstream of the animal chamber (ml min^{-1} STP; Omega mass flow meter, Stamford, CT, USA), $\dot{V}_{O_2}$ is the rate of O_2 consumption, F_{ICO_2} and F_{IO_2} are the incurrent CO_2 and O_2 fractions, respectively, and F_{ECO_2} and F_{EO_2} are the excurrent CO_2 and O_2 fractions, respectively. Additionally, 2678.57 is the conversion factor used to convert ml g^{-1} min^{-1} to μmol g^{-1} h^{-1} (1000 μl ml^{-1}, 60 min h^{-1} and 22.4 μl μmol^{-1}). Although flow rate was measured upstream from the chamber, the added volume flow due to water production by the animal was negligible, introducing an error of less than 0.08% (Greenlee and Harrison, 1998).

Effect of duration of hypoxic exposure on $\dot{M}_{CO_2}$

We wanted to be sure that our calculated P_c values were not strongly affected by the short-duration hypoxia exposure in our major experiment. Therefore, we exposed first-instar juvenile and adult grasshoppers for 1 h to each of seven levels of P_{O_2} (adults – 21, 5, 3, 2, 1, 0.5 and 0 kPa O_2; juveniles – 21, 16, 13, 9, 7, 3, 1 and 0 kPa O_2) and measured CO_2 emission rates. Animals were treated as described above, except that small animals were placed in glass cylinders and larger animals were in Plexiglas cylinders. In all cases, the inner diameter of the chamber was just large enough to insert the animal but small enough to inhibit movement. Gas mixtures were made as described above and were sub-sampled from a 60 ml syringe using an AMETEK R-1 flow controller, which pushed the mixture through the respirometry chamber and then through the CO_2 analyzer only. Flow rates ranged from 30 ml min^{-1} to 300 ml min^{-1}, increasing with the size of the animal. The weakest signal-to-noise ratio (average p.p.m. CO_2 in excurrent air relative to peak-to-peak system noise) was 13:1 during anoxia for first-instar grasshoppers. The signal-to-noise ratio improved as oxygen level and animal body sizes increased (e.g. signal-to-noise ratio was 84:1 in normoxia for first-instar grasshoppers and 127:1 for adults in anoxia). $\dot{M}_{CO_2}$ was calculated at various time periods ranging from 3 min to 60 min after exposure to a new gas mix using the following equation:

$$\dot{M}_{CO_2} = \frac{\dot{V}_{in}\,(F_{ECO_2} - F_{ICO_2}) \times 2678.57}{M_b}. \quad (3)$$

For these calculations, we assumed that the respiratory exchange ratio (RER) was 1; the misestimation of flow due to possible variations in RER was negligible, as variation between 1.0 and 0.7 would have produced a variation in calculated $\dot{M}_{CO_2}$ of less than 0.026%.

Ontogenetic change in the responses of $\dot{M}_{CO_2}$ *and ventilation frequency to progressive hypoxia*

Respirometry chambers, as described above, were placed in a 35°C water bath, the preferred field body temperature for most grasshoppers (Uvarov, 1966). First-, third- and fifth-instar and adult grasshoppers were each exposed to 12 different levels of P_{O_2} (21, 16.2, 13.2, 9.1, 7.5, 6.2, 5.1, 3.8, 2.4, 1.3, 0.7 and 0 kPa O_2) for 3–4 min at each P_{O_2}. We counted ventilatory frequency at each P_{O_2} while viewing the animals through a dissecting microscope. We calculated CO_2 emission rate using equation 3 above.

Determination of P_c *for gas exchange and abdominal pumping*

We determined individual P_c values for gas exchange by statistically identifying the P_{O_2} at which CO_2 emission or O_2 consumption dropped (Fig. 1, box A). We compared 95% confidence intervals (CI) created using the mean ± (t×S.E.M.) where t=1.96 (Zar, 1999). There were three criteria for designating a P_{O_2} as critical: the 95% CI at the P_c could not overlap and had to be (1) less than the CI of the previous P_{O_2} (Fig. 1, box B), (2) less than the CI of all the previous P_{O_2}s combined (Fig. 1, box C) and (3) greater than or equal to the CI of all subsequent P_{O_2}s. The mean P_{O_2} between the P_{O_2} at which CO_2 emission dropped significantly and the next higher P_{O_2} was designated as the P_c (Fig. 1, box B; 1.3% O_2). The P_c for abdominal pumping frequency was determined for each individual as the first P_{O_2} at which abdominal pumping frequency dropped below that in normoxia and continued to decrease.

Magnitude of abdominal pumping

We obtained indices of tidal volume for first-instar juvenile and adult grasshoppers by measuring their abdominal height changes in response to hypoxia using videography. Each grasshopper was exposed to six levels of P_{O_2} (adults – 21, 13, 5, 2, 1 and 0.5 kPa O_2; juveniles – 21, 16, 13, 9, 5 and 1 kPa O_2) at flow rates of 500 ml min^{-1}. The grasshopper's abdomen was magnified with a dissecting microscope and the image was digitized with a Hitachi 3CCD camera (Tokyo, Japan) and displayed on a television monitor. We adjusted the magnification so that the abdomen nearly filled the monitor. Abdominal pumping was then video-recorded with a Panasonic SVHS (Desktop Editor Pro-Line, Secaucus, NJ, USA). Afterwards, the recording was analyzed frame by frame,

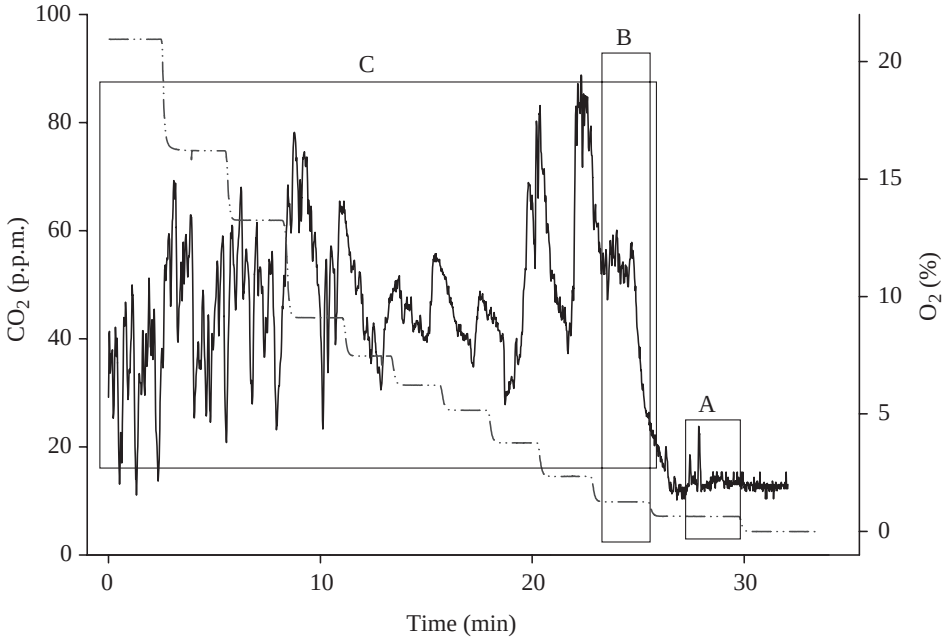

Fig. 1. Determination of critical oxygen partial pressure (P_c). This is the raw data file from an individual grasshopper, showing CO_2 emission (solid line) and atmospheric O_2 (broken line). As oxygen partial pressure (P_{O_2}) drops incrementally, CO_2 transiently increases. Briefly, we compared 95% confidence intervals constructed around the mean CO_2 p.p.m. in each box. Box A is the P_{O_2} where CO_2 was significantly less than at the next higher P_{O_2} (box B) and less than the average CO_2 at all higher P_{O_2}s (box C).

and the height of the abdomen was measured at the fourth abdominal segment using a digital micrometer (Mitutoyo Corporation, Kawasaki, Japan). We recorded maximal height (inspiration) and minimal height (expiration) and calculated the percent difference using the following equation:

$$\%\ \text{Change in abdominal height} = 100 \times \frac{(\text{Height}_{max} - \text{Height}_{min})}{\text{Height}_{min}} . \quad (4)$$

This measure provided an index of tidal volume, where larger percentage differences indicated higher tidal volumes. We calculated a mean value at each P_{O_2} for each animal from three breaths and used this mean as a data point. Since we measured this index of tidal volume at relatively few P_{O_2}s, we did not calculate P_c values for this parameter.

Calculation of maximal tracheal system conductance

We calculated mass-specific tracheal system conductance for oxygen delivery (G_{max}; μmol g^{-1} h^{-1} kPa^{-1}; from air to mitochondria, incorporating both diffusive and convective conductances) as:

$$G_{max} = \frac{\dot{M}_{CO_2}}{P_{O_2}} . \quad (5)$$

We assumed that at the P_c, tracheal system conductance would be maximized, since animals should have their spiracles opened wide (Case, 1956; Miller, 1960), ventilation maximized (Greenlee and Harrison, 1998) and tracheolar fluid removed (Wigglesworth, 1983). This calculation also assumes that mitochondrial P_{O_2} is near zero at the P_c (Richmond et al., 1999) and that RER is 1 (see Results). Since P_c was calculated as an average of two P_{O_2}s, we used the value of $\dot{M}_{CO_2}$ at the P_{O_2} just above the P_c.

Statistics

Mean values ± S.E.M. are presented for parametric data, and median values are shown for nonparametric data. Statistical analyses were performed using SYSTAT 10.2, with our within-experiment type I error less than or equal to 5%. For our analyses, the adult instar was designated as instar 7. We used repeated-measures analysis of variance (ANOVA) to compare the hypoxia responses of each instar, since individuals were measured at multiple levels of P_{O_2}; N=8 for all treatment groups. All P_c values were statistically analyzed as nonparametric data, since these are discrete variables. We used the Kruskal–Wallis test, a single-factor analysis of variance in SYSTAT 10.2 and also calculated nonparametric multiple comparisons as described in Zar (1999). To determine whether there was a linear effect of instar on P_c, we also compared those values using nonparametric regression, Kendall's robust line-fit method and rank correlation coefficient (Sokal and Rohlf, 1995).

Results

Developmental effects on mass and normoxic CO_2 emission

Body mass (M_b) increased over 50-fold from hatching to early adulthood (Fig. 2). Metabolic rates increased with body mass (metabolic rate=$0.005\times M_b^{0.77}$; r^2=0.95). Third instar CO_2 emission rate was higher than predicted by the general scaling relationship for $\dot{M}_{CO_2}$ and M_b (Fig. 3).

Comparison of the P_c for $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$

In adult grasshoppers, the P_c for $\dot{M}_{O_2}$ and the P_c for $\dot{M}_{CO_2}$ did not differ significantly (Table 2; Fig. 4; Mann–Whitney U test, U=32, P=1.0). Respiratory exchange ratios remained near one until the P_c was reached, below which they increased dramatically (Fig. 4 inset; repeated-measures ANOVA, $F_{7,49}$=6.1, P<0.001).

Effect of the duration of hypoxia exposure on the P_c for $\dot{M}_{CO_2}$

Duration of exposure (3 min or 1 h) had no significant effect

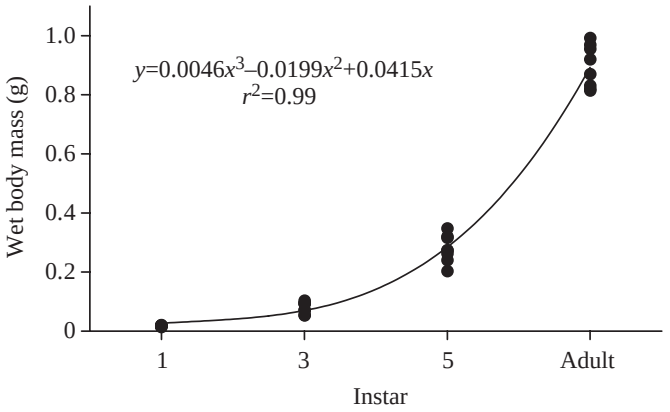

Fig. 2. Wet body mass increased with instar for *Schistocerca americana*.

Table 2. *Summary of the median* P_c *values found in our experiments*

Variable	$\dot{M}_{CO_2}$			$\dot{M}_{O_2}$	Abdominal pumping
Exposure duration	3 min	15 min	60 min	15 min	3 min
Instar					
1	14.1		18.5		2.3
3	9.7				3.0
5	4.5				1.3
Adult	1.8	3.25	4.0	3.25	0.95

$\dot{M}_{O_2}$, mass-specific rate of oxygen consumption; $\dot{M}_{CO_2}$, mass-specific rate of CO_2 emission.

on the P_c of first-instar grasshoppers (Table 2; Fig. 5). Adults exposed for 1 h at each P_{O_2} had lower CO_2 emission rates than those animals exposed for 3 min, and longer exposures slightly (but significantly) increased the P_c for $\dot{M}_{CO_2}$ (Table 2; Fig. 5; Mann–Whitney *U* test, U=4.5, P=0.002).

Across-instar developmental effects on the P_c *for* $\dot{M}_{CO_2}$

There was a significant interaction between P_{O_2} and instar in the repeated-measures ANOVA, indicating that the decrease in $\dot{M}_{CO_2}$ in response to hypoxia differed in different instars ($F_{27,252}$=10.5, P<0.001). As has been previously shown (Greenlee and Harrison, 1998), adult grasshoppers exposed to hypoxia were able to maintain constant CO_2 emission down to a median P_{O_2} of 1.8 kPa (Fig. 6). However, first-instar grasshoppers appeared to be oxygen limited across P_{O_2} levels lower than the median P_c of 14 kPa, as metabolic rate dropped virtually continuously with P_{O_2} below 14 kPa (18 kPa for 1 h measures). Third and fifth instars showed intermediate responses, with median P_c values of 9.7 kPa and 4.5 kPa, respectively (Figs 6, 7). Overall, P_c decreased linearly over sevenfold from hatchling to adult grasshopper (Fig. 7; Kendall's rank correlation coefficient, τ=–0.58, P<0.01).

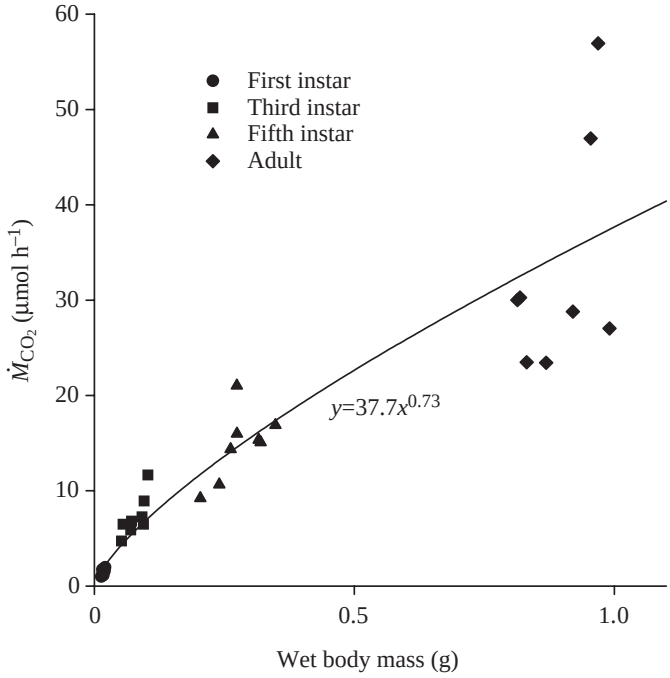

Fig. 3. Absolute CO_2 emission ($\dot{M}_{CO_2}$) in normoxia as a function of wet body mass. $\dot{M}_{CO_2}$ scaled with body mass to the 0.73 power; r^2=0.77, P<0.001.

Across-instar developmental effects on the response of ventilatory frequency to hypoxia

For all instars, breathing frequency plummeted at some low level of ambient oxygen (Fig. 8). In contrast to the P_c for $\dot{M}_{CO_2}$, the P_c for abdominal pumping was very low across all instars (Table 2; Fig. 7). However, there was still a significant effect of instar on the P_c for abdominal pumping (Kruskal–Wallis test statistic=14.3, P<0.01). Nonparametric multiple comparisons revealed that only third instars and adults differed in their P_c for abdominal pumping, with the third-instar P_c threefold higher than that of adults (q=4.7, P<0.05).

In response to hypoxia, adults more than doubled their ventilatory frequencies, while the first instars showed no change (Fig. 8). The response of abdominal pumping frequency to hypoxia varied significantly with instar, as indicated by a significant interaction between P_{O_2} and instar (repeated-measures ANOVA, $F_{27,252}$=10.5, P<0.001). We used analysis of covariance (ANCOVA) to determine which instars had different responses to hypoxia based on the slope of the line created when abdominal pumping was regressed on P_{O_2} at all P_{O_2}s above the P_c for abdominal pumping (21 kPa to 3 kPa). We limited our test to these P_{O_2}s because, below the P_c, abdominal pumping decreased dramatically. This analysis revealed that the first-instar grasshoppers had no response to hypoxia above the P_c (slope=–0.114, P=0.9; Fig. 8). Pair-wise comparisons of the slopes of the other instars indicated that all were different from the first-instar slope but not different from each other (first instar *versus* other instars: all P≤0.011; comparisons between third, fifth and adults: all P>0.6). Thus,

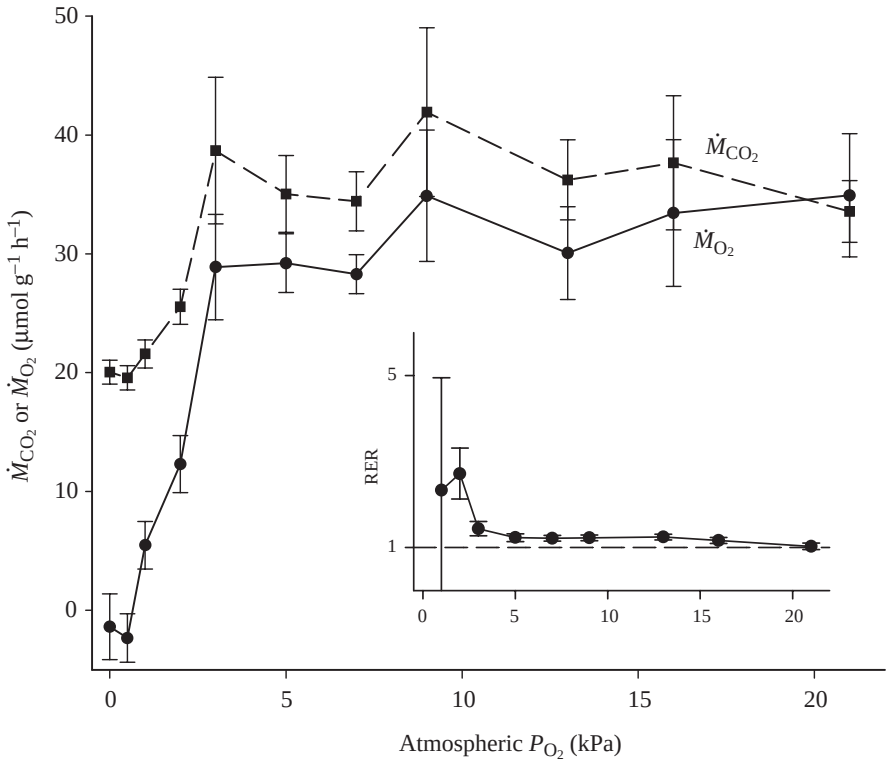

Fig. 4. Effect of decreasing atmospheric oxygen partial pressure (P_{O_2}) on mass-specific CO_2 emission ($\dot{M}_{CO_2}$) and mass-specific O_2 consumption ($\dot{M}_{O_2}$) for adult grasshoppers. Critical oxygen pressures (P_c) were similar for both indices of metabolic rate. The inset depicts the respiratory exchange ratio (RER) for these animals as atmospheric P_{O_2} decreases to the P_c. RERs at 0 and 0.6 kPa O_2 are off scale and are not shown; N=8 for each treatment group.

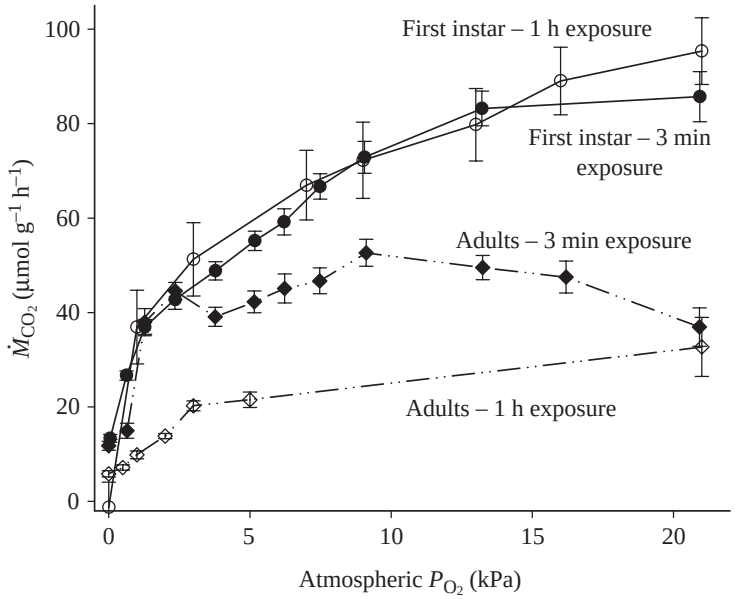

Fig. 5. Effect of exposure time on mass-specific CO_2 emission ($\dot{M}_{CO_2}$) responses to decreasing levels of atmospheric P_{O_2} in adult (diamonds) and first-instar (circles) grasshoppers. One hour exposures (open symbols) are compared with 3 min exposures (solid symbols). Values are means ± S.E.M.; N=8 for each treatment group.

only the first-instar grasshoppers did not increase breathing frequency in response to hypoxia, and the responsiveness of abdominal pumping frequency to hypoxia was similar from the third instar to the adult stage [abdominal pumping frequency=2.2(P_{O_2})+42.2; r^2=0.12; Fig. 8].

Across-instar effects on the responsiveness of tidal volume to hypoxia

Our index of tidal volume, percent change in abdominal height, was 35 times higher in normoxia for adults compared with first instars (Fig. 9). Juveniles compressed their abdomens less than 1% of maximal height, compared with a 20% abdominal compression in adults (Fig. 9). Because we measured tidal volume at different P_{O_2}s for each instar, we were able to statistically compare only the responses at common P_{O_2}s (21, 5 and 1 kPa). There was a significant interaction effect of instar and P_{O_2} on tidal volume (repeated-measures ANOVA, P_{O_2} × instar, $F_{2,28}$=64.8, P<0.001). Adults increased tidal volume almost 50% in response to hypoxia, while juveniles showed no significant change in tidal volume (repeated-measures ANOVA, adults – $F_{4,28}$=21.8, P<0.0001; first instar – $F_{5,35}$=1.4, P=0.24).

Discussion

Throughout development, the grasshopper respiratory system improved in its ability to respond to hypoxia, as indicated by the increased responsiveness of ventilation frequency (Fig. 8) and tidal volume (Fig. 9) to hypoxia; together, these lead to a fourfold increase in the mass-specific capacity of the tracheal system to conduct oxygen (G_{max}; Fig. 10). Older grasshoppers were also much more able to tolerate hypoxia, as the P_c for metabolic rate decreased sevenfold during ontogeny (Fig. 7). The increased tracheal system conductance with age may have been in preparation for a switch in the principal mode of locomotion from hopping to flight. In addition, the increased responsiveness to hypoxia of the tracheal system of older grasshoppers may be related to a general increased use of convective gas exchange in larger insects. Furthermore, our finding that development strongly affected the ability of grasshoppers to respond to ventilatory system challenges may mean that juveniles would be more sensitive to hypoxic environments encountered within restricted burrows, at high altitudes or during discontinuous gas exchange (Lighton, 1996).

Validation of methods

CO_2 emission as an index of aerobic metabolic rate

We used CO_2 emission as our index of metabolic rate in the across-instar comparisons because we could measure fractional CO_2 content of the excurrent air stream quickly and more accurately than if we measured O_2, especially for the smallest grasshoppers. This approach raises the concern that CO_2 emission might not reflect the P_c for aerobic metabolism. For example, the P_c for $\dot{M}_{CO_2}$ might be lower than the P_c for $\dot{M}_{O_2}$ if $\dot{M}_{CO_2}$ was elevated at low P_{O_2}s due to anaerobiosis or CO_2 washout from body tissues due to hyperventilation. In our study, despite persistent elevation of $\dot{M}_{CO_2}$ at very low P_{O_2}s, adult grasshoppers had identical P_c values for $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$ (Table 2; Fig. 4). The P_c for $\dot{M}_{CO_2}$ is probably also close to the P_c for $\dot{M}_{O_2}$ in juveniles, because juveniles exhibited much lower ratios of CO_2 emission in anoxia to CO_2 emission in normoxia (Fig. 11; ANOVA $F_{3,28}$=15.2, P<0.001). Therefore, we conclude that the P_c for $\dot{M}_{CO_2}$ provided a reasonable approximation of the P_c for aerobic metabolism.

Interestingly, the RERs that we measured (Fig. 4 inset) were higher than those measured for resting *Schistocerca gregaria* (0.82; Krogh and Weis-Fogh, 1951). One possible explanation for the discrepancy is that fuel use was different. Our grasshoppers were fed on green leaf lettuce, kale and dried wheat germ, whereas the grasshoppers in the prior study were fed green cabbage and dried grass. Alternatively, our grasshoppers could have been in a stressed state. However, the normoxic, acute metabolic rates we measured (after 20 min chamber acclimation time) were similar to those measured over 1 h, indicating that grasshoppers either were still stressed after 1 h or were settled within 20 min. Typically, grasshopper ventilation frequencies return to normal 20 min after a disturbance (Gulinson and Harrison, 1996). Perhaps the difference was due to inherent differences between the two species.

Acute exposure to hypoxia

We were also concerned that the use of short-term exposures to hypoxia would not reflect steady-state conditions. However, calculated P_c values for $\dot{M}_{CO_2}$ did not differ between first instar animals exposed for three minutes or one hour to the test P_{O_2}s (Table 2; Fig. 5). Adult grasshoppers exposed to hypoxia for 1 h did have much lower CO_2 emission rates and a slightly higher P_c compared with acutely exposed animals (Fig. 5; Table 2). We attribute the elevated $\dot{M}_{CO_2}$ for the 3 min exposures in adults to transient increases in CO_2 emission that occurred in response to each drop in atmospheric P_{O_2} (Fig. 1). The increased CO_2 emission may be attributed to two events. First, it has been noted that grasshoppers may physically struggle to escape when exposed to lower oxygen (Hochachka et al., 1993; Wegener, 1993). Secondly, exposure to hypoxia causes an increase in ventilation (enhancing CO_2 loss) relative to metabolic rate (CO_2 production), which secondarily causes a drop in hemolymph P_{CO_2} and total CO_2 content (Greenlee and Harrison, 1998). An alternative explanation for the transient increase in CO_2 emission is that acid metabolites,

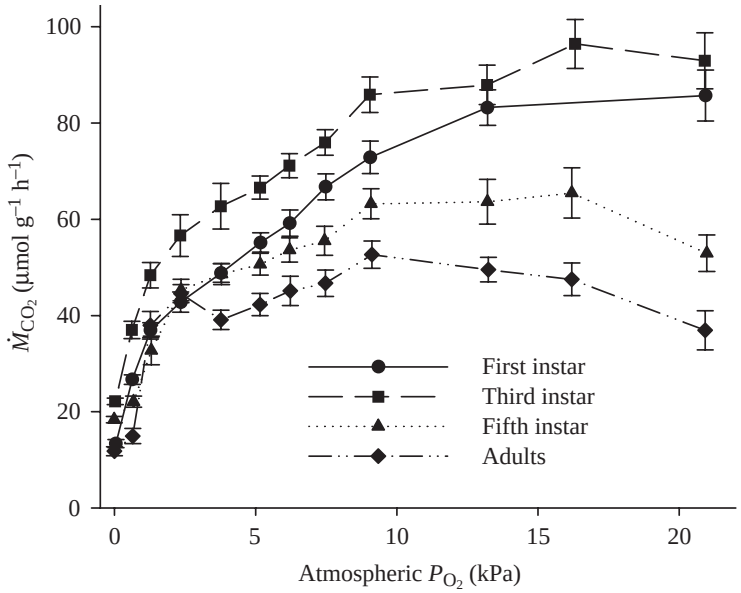

Fig. 6. Effect of decreasing atmospheric oxygen partial pressure (P_{O_2}) on mass-specific CO_2 emission ($\dot{M}_{CO_2}$) for first-instar (circle), third-instar (square), fifth-instar (triangle) and adult animals (diamond). Values are means ± S.E.M.; N=8 for each treatment group.

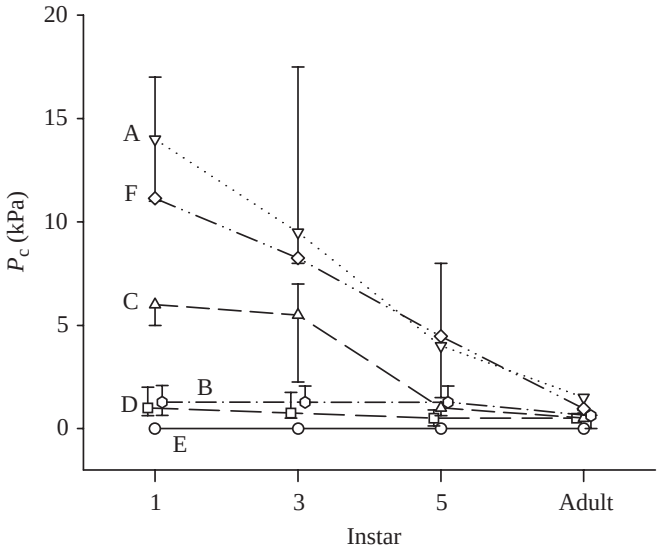

Fig. 7. Critical oxygen partial pressure (P_c) values for mass-specific CO_2 emission ($\dot{M}_{CO_2}$) determined in multiple ways and for abdominal pumping *versus* instar. Each symbol indicates the median; error bars mark the 25th and 75th percentiles. (A) P_c for $\dot{M}_{CO_2}$ determined using 95% confidence intervals (N=8 for each treatment group), (B) P_c for abdominal pumping (N=8 for each group), (C–E) P_c determined as the oxygen partial pressure (P_{O_2}) when $\dot{M}_{CO_2}$ decreases below 75%, 50% or 25% of normoxic $\dot{M}_{CO_2}$ (N=8 for each group) and (F) P_c determined using paired t-tests to identify the first P_{O_2} at which mean $\dot{M}_{CO_2}$ from Fig. 6 was significantly lower than normoxic $\dot{M}_{CO_2}$.

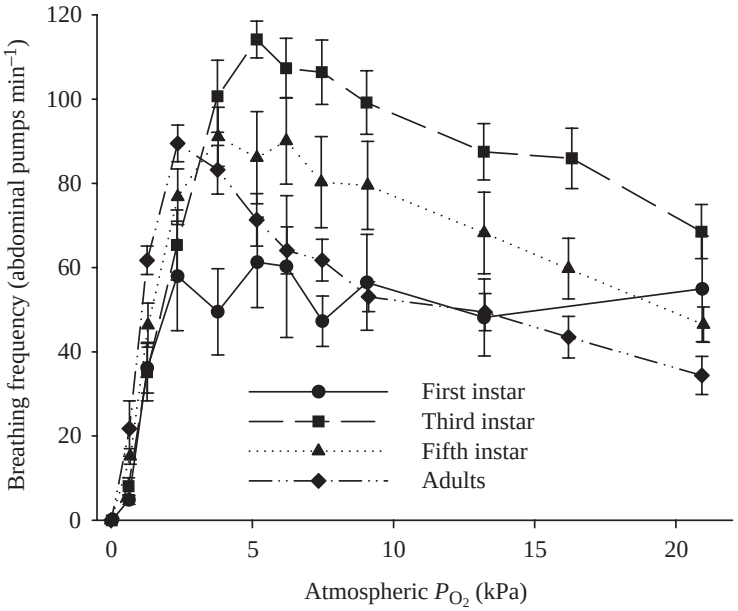

Fig. 8. Effect of decreasing atmospheric oxygen partial pressure (P_{O_2}) on abdominal pumping frequency for first-instar (circles), third-instar (squares), fifth-instar (triangles) and adult animals (diamonds). Values are means ± S.E.M.; N=8 for each treatment group.

such as lactate, accumulated in the tissues as P_{O_2} decreased (Hochachka et al., 1993). Acid metabolite accumulation would decrease hemolymph pH and hence increase internal P_{CO_2}, enhancing CO_2 elimination. However, it is unlikely that acid metabolites accumulated in the hemolymph because we have direct evidence that hemolymph pH increases during hypoxia and bicarbonate concentration is higher than expected at lower P_{O_2}s (Greenlee and Harrison, 1998). Thus, the washout of CO_2 from the hemolymph and tissues as the animal moved towards a new, lower, steady-state internal P_{CO_2} is likely to be the major cause of the transient increase in $\dot{M}_{CO_2}$ upon exposure to hypoxia. It is worth noting that juveniles showed no transient increase in $\dot{M}_{CO_2}$ upon exposure to hypoxia (Fig. 5), again consistent with our conclusion that they lack a ventilatory response to hypoxia.

Interestingly, a transient rise in CO_2 emission upon exposure to a lower P_{O_2} is observable even at relatively high P_{O_2}s where ventilation rates were unaffected by hypoxia (Fig. 1). One possibility is that ventilation increased transiently in response to hypoxia and then lowered in response to reduced internal P_{CO_2} levels by the time we measured it. Alternatively, the transient rise in $\dot{M}_{CO_2}$ could be due to transient changes in non-ventilatory mechanisms for increasing tracheal conductance (e.g. increasing spiracular opening or decreasing tracheolar fluid levels).

Method for determination of P_c

Critical points are commonly determined in physiological research, and a variety of methods have been proposed for statistical analysis of critical point data (Nickerson et al., 1989; Yeager and Ultsch, 1989). However, in our study, and many others, critical points were determined by exposing individuals to a range of environmental conditions, an experimental design that should be analyzed using repeated-measures approaches (Potvin et al., 1990). Unfortunately, published methods for determining P_cs have ignored the statistical problems with a repeated-measures design (Yeager and Ultsch, 1989). In the present study, we developed a method that allowed us to assign a P_c value to each animal and then use this as a data point for further statistical analysis. This method may be generally useful for investigators who wish to compare critical points across groups of animals

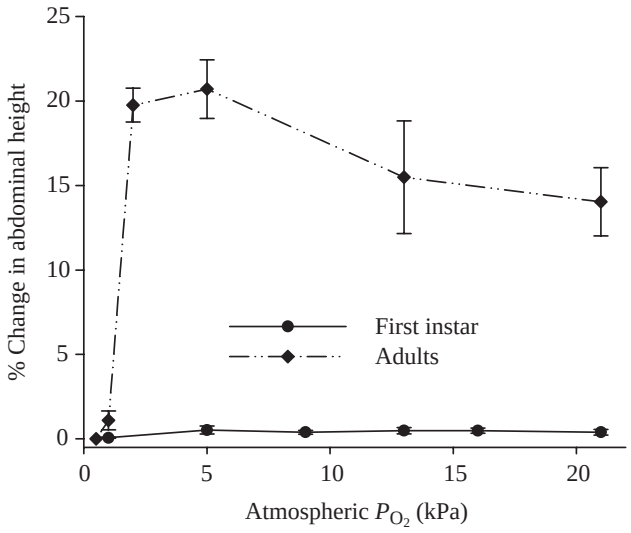

Fig. 9. Percent change in abdominal height during abdominal pumping at various oxygen partial pressures (P_{O_2}s) for adults (diamonds) and first instars (circles). Values are means ± S.E.M.; N=8 for each treatment group.

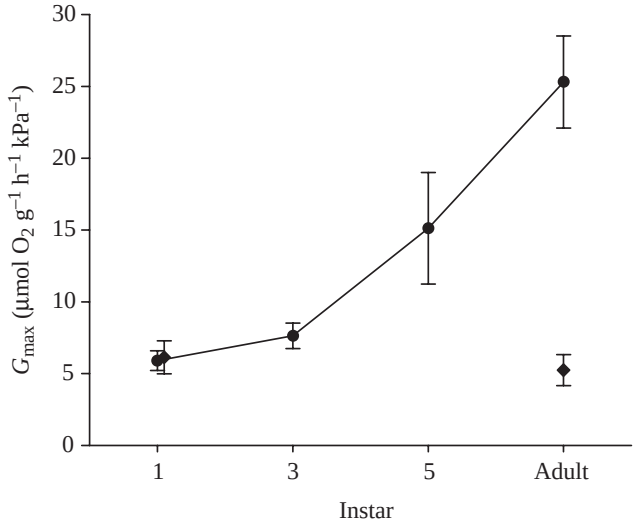

Fig. 10. Maximal tracheal system conductance (G_{max}) *versus* instar. Circles are from short-term exposures; diamonds represent long-term exposures. Values are means ± S.E.M.; N=8 for each treatment group.

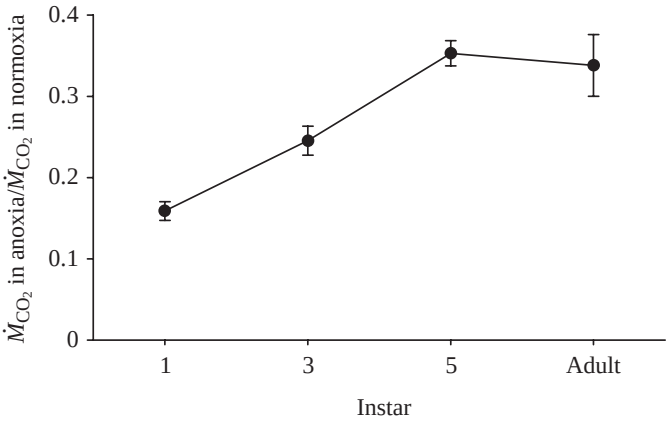

Fig. 11. Mass-specific CO_2 emission ($\dot{M}_{CO_2}$) in anoxia divided by $\dot{M}_{CO_2}$ in normoxia as a function of instar. $\dot{M}_{CO_2}$ in anoxia was measured over 3 min after exposure to progressively more hypoxic gases (Fig. 6). Values are means ± S.E.M.; N=8 for each treatment group.

in studies where the parameters are repeatedly measured for each animal.

Our technique could be criticized because the animal's resting $\dot{M}_{CO_2}$ value has a strong effect on the P_c, and visual inspection of Fig. 6 shows a very large drop in $\dot{M}_{CO_2}$ for all instars at ~2–3 kPa. To address this issue and compare several methods of determining P_c, we first analyzed the individual data files and identified the P_{O_2} at which $\dot{M}_{CO_2}$ dropped below the 75th, 50th or 25th percentile of normoxic $\dot{M}_{CO_2}$ (Fig. 7, lines C, D and E, respectively). Of these lines, the 75th and 50th percentiles result in a significant decrease of P_c with instar (Kendall's rank correlation coefficient, 75th – τ_{32}=–0.65, P<0.01; 50th – τ_{31}=–0.32, P<0.01). Only 14 animals had $\dot{M}_{CO_2}$ that dropped below 25% of normoxic metabolic rate and, of these, eight were first-instar juveniles. There was no effect of instar on these P_c values (median=0 kPa). We also used paired t-tests to determine the first P_{O_2} at which mean $\dot{M}_{CO_2}$ decreased significantly below the mean $\dot{M}_{CO_2}$ in normoxia (Fig. 7, line F). P_c determined in this way decreased approximately sixfold with instar. The fact that most of these methods yield linear decreases of P_c with age supports our conclusion that P_c falls with age/size in *S. americana*. However, the fact that there is no age effect on the P_{O_2} at which $\dot{M}_{CO_2}$ drops to 25% of its normoxic rate suggests that even the youngest grasshoppers may be able to sustain some aerobic metabolism at very low atmospheric P_{O_2}. Alternatively, the very low $\dot{M}_{CO_2}$ measured at low air P_{O_2} may represent anaerobic metabolism.

Ontogenetic variation in the ventilatory response to hypoxia

In general, as grasshoppers grew, their hypoxia tolerance increased, as shown by the decreased P_c for $\dot{M}_{CO_2}$ with age. This pattern is similar to that seen in the lobster *Nephrops norvegicus*, where adults have a lower P_c for O_2 consumption compared with larvae (Spicer, 1995). Adult and older juvenile crayfish, *Procambarus clarkii*, respond to hypoxia by increasing ventilation frequency, whereas larval ventilation frequency decreases in hypoxia (Reiber, 1997). To our knowledge, P_c has not been compared throughout ontogeny for any other insect species, so it is unclear whether this pattern will prove to be widespread throughout the arthropods.

Adult grasshoppers exposed to hypoxia were able to maintain constant CO_2 emission down to a P_{O_2} of 1.8 kPa (Figs 6, 7). Adult grasshoppers had such a low P_c because they increased both breathing frequency and abdominal pumping height in response to hypoxia (Figs 6–9). Together, the increases in abdominal pumping frequency and tidal volume resulted in a fourfold increase in G_{max} from first-instar to adult grasshoppers (Fig. 10; ANOVA effect of instar, $F_{1,30}$=31.6, P<0.001). Interestingly, while G_{max} increased strongly with age when using $\dot{M}_{CO_2}$ measures and P_c values from the 3 min exposures to hypoxia, G_{max} was similar for first instars and adults when analyzed using 1 h exposures (Fig. 10). This pattern occurred because the 1 h exposure to hypoxia substantially decreased $\dot{M}_{CO_2}$ in adults but not first instars (Fig. 5) and P_c increased more dramatically in adults (Table 2). The physiological bases to the disparity between the 1 h and 3 min patterns for G_{max} are unknown without information on internal gas level variation during ontogeny. Based on our present data, the most likely explanation seems to be that $\dot{M}_{CO_2}$ in adults exposed to short-term hypoxia was elevated by changes in internal P_{CO_2} that did not occur in juveniles (Figs 5 and 11 suggest greater CO_2 washout occurs during hypoxia for adults). When CO_2 emission occurs at rates equivalent to metabolic CO_2 production (as presumably happens when $\dot{M}_{CO_2}$ is measured over 1 h), G_{max} is size invariant. This suggests that first instars were able to achieve similar gas exchange rates despite lower tidal volumes and ventilatory frequencies, presumably because their smaller size enhances diffusive gas exchange.

The increased percent change in abdominal height (Fig. 8) is in contrast to our previous work (Greenlee and Harrison, 1998), which showed that tidal volume did not change in response to hypoxia in *S. americana*. The difference between the two studies may be due to population differences or the fact that we previously measured three dimensions of the abdomen; in the present study, we measured only the dorso-ventral dimension for a quicker index of tidal volume. However, prior research on *Locusta migratoria*, a closely related species, did show increases in both breathing frequency and tidal volume with decreased P_{O_2} (Arieli and Lehrer, 1988). To further address this discrepancy, we calculated tidal volumes for adults at 2, 5, 10 and 21 kPa O_2 using the equation:

$$\text{Tidal volume} = \frac{\dot{M}_{CO_2} \times 22.4}{\text{Breathing frequency} \times F_{CO_2}}, \quad (6)$$

where $\dot{M}_{CO_2}$ is the whole animal CO_2 emission rate in μmol min^{-1}, 22.4 (μl μmol^{-1}) is the ratio for converting $\dot{M}_{CO_2}$ to a volume, and F_{CO_2} is the fraction of CO_2 in the tracheal air for adult *S. americana* from Greenlee and Harrison (1998). According to this calculation, tidal volume did not vary with P_{O_2} (mean=29±1.9 μl breath^{-1}), supporting our prior

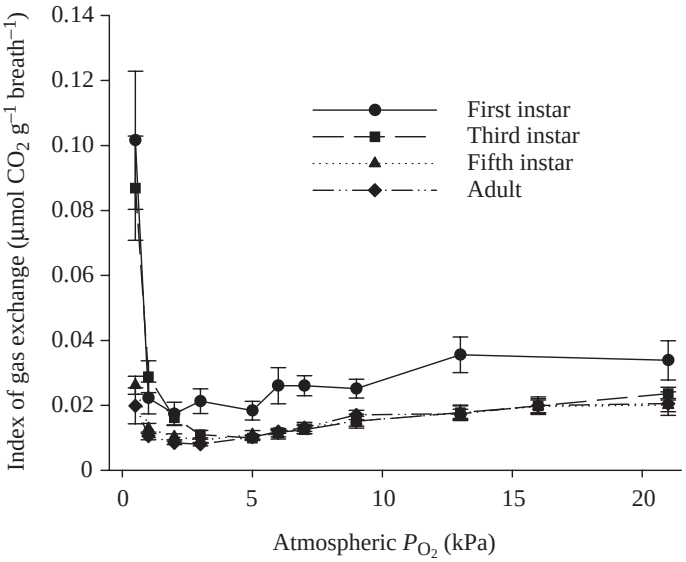

Fig. 12. Index of gas exchange per breath ($\dot{M}_{CO_2}$ in μmol g^{-1} min^{-1} divided by abdominal pumps min^{-1}) as a function of oxygen partial pressure (P_{O_2}) for first-instar (circle), third-instar (square), fifth-instar (triangle) and adult animals (diamond). Values are means ± S.E.M.; N=8 for each treatment group.

conclusion that tidal volume does not change during hypoxia exposure (Greenlee and Harrison, 1998). However, these calculated values were lower than those we measured optically (mean=44±3.7 μl $breath^{-1}$), possibly due to approximately 40% lower ventilation frequencies measured in that study (Greenlee and Harrison, 1998).

In contrast to the pattern seen in older grasshoppers, first-instar animals had no apparent ventilatory response to hypoxia. They showed no change in ventilation frequency (Fig. 8) or tidal volume (Fig. 9), and $\dot{M}_{CO_2}$ dropped, though not always significantly, with every decrease in atmospheric P_{O_2} (Fig. 6). Conceivably, first instars could have responded to hypoxia by increasing diffusive gas exchange (e.g. opening spiracles or removing tracheolar fluid). To test this idea, we calculated the ratio of $\dot{M}_{CO_2}$ (μmol min^{-1}) to abdominal pumping frequency. If younger grasshoppers were using more diffusive gas exchange, then the μmol CO_2 g^{-1} $breath^{-1}$ should be higher in first instars and should increase during hypoxia. Indeed, in first-instar grasshoppers, this index of gas exchange per breath was higher than that in older instars (Fig. 12), supporting the hypothesis that first instars were more reliant on diffusion than older grasshoppers. Alternatively, first instars could simply have had higher internal and expired P_{CO_2} levels. However, there is no evidence that first instars responded to hypoxia by opening spiracles or removing tracheolar fluid, as the μmol CO_2 g^{-1} $breath^{-1}$ was constant as air P_{O_2} decreased until P_{O_2}s dropped below the P_c for abdominal pumping (Fig. 12).

Why do first-instar grasshoppers lack a ventilatory response? One hypothesis is that younger grasshoppers have not developed the complete neural circuitry for responding to hypoxia. This idea is supported by the findings of Miller and Mills (1976), who noted that first- and second-instar *S. gregaria* also lack synchronization between spiracular valve activity and abdominal pumping. The adult pattern of synchronization between abdominal pumping and spiracular valve activity appears sporadically in the third instars (Miller and Mills, 1976). In our study, third-instar animals demonstrated an adult-like ventilatory response to hypoxia (Fig. 8). Alternatively, first-instar grasshoppers may have not yet developed the capacity to sense oxygen. This hypothesis could be tested by looking for members of the HIF-1α oxygen-sensing cascade (Lavista-Llanos et al., 2002) and comparing the expression patterns between instars. Interestingly, expression of HIF-1α and HIF-1β has been found to decrease with juvenile development in mice, the opposite of what we would predict for grasshoppers (Madan et al., 2002). Finally, first-instar grasshoppers may lack the large air sacs found in older animals, reducing the capacity for convective gas exchange.

What causes P_c *to decline throughout development?*

Rearranging equation 2 to solve the P_c for $\dot{M}_{CO_2}$, we can see that the P_c depends positively on $\dot{M}_{CO_2}$ and negatively on conductance:

$$P_c = \frac{\dot{M}_{CO_2}}{G_{max}} . \quad (7)$$

From first instar to adult, the P_c for $\dot{M}_{CO_2}$ decreased eightfold (Fig. 7). Much of the decrease was accounted for by the approximately fourfold increase in G_{max} (Fig. 10). However, the approximately threefold decrease in mass-specific $\dot{M}_{CO_2}$ at the P_c with age also contributed strongly to the trend towards decreasing P_c with age (Fig. 13).

The decrease in mass-specific metabolic rate with size is well known among animals (Schmidt-Nielsen, 1984; West et al., 1997). However, the increase in mass-specific gas-exchange capacity with age was less expected (Fig. 10). One possibility is that the increased tracheal system conductance with age in these grasshoppers facilitates flight, an adult activity that requires a 40–50-fold increase in O_2 consumption above basal rates (Krogh and Weis-Fogh, 1951). Additionally, larger insects may generally be more able to cope with hypoxia because they have evolved mechanisms, such as abdominal pumping, to facilitate convective gas exchange, as may be necessary for adequate oxygen delivery in large insects (Weis-Fogh, 1964).

P_c *for abdominal pumping*

In contrast to the decrease in P_c for $\dot{M}_{CO_2}$ throughout ontogeny, the P_c for abdominal pumping was very low across all instars. At each instar, abdominal pumping continued even after $\dot{M}_{CO_2}$ began to drop. The large disparity between the P_c for $\dot{M}_{CO_2}$ and the P_c for ventilation frequency strongly suggests that insects are able to preferentially shut down certain body systems in response to hypoxia. Additionally, O_2 delivery to

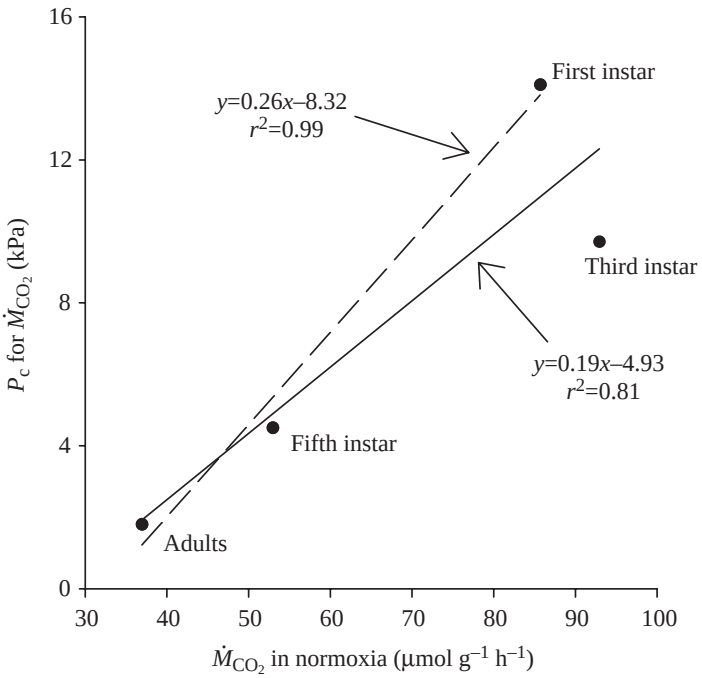

Fig. 13. Critical pressure (P_c) *versus* normoxic mass-specific CO_2 emission ($\dot{M}_{CO_2}$). The broken line shows the relationship when third instars are not included in the regression. The solid line occurs when third instars are included in the regression; N=8 for each treatment group.

all parts of the respiratory system may be better than to other parts of the body, since the tracheal supply to the pacemaker neuron is substantial.

Interestingly, when we calculated P_c as the P_{O_2} at which $\dot{M}_{CO_2}$ was lower than 50% of the normoxic rate, this line overlapped the P_c for abdominal pumping (Fig. 7). Does this correlation mean that the P_c for abdominal pumping represents the 'true' critical point for oxygen delivery? This strongly depends on what is meant by 'true' critical point. Any significant drop in $\dot{M}_{CO_2}$ could be due to either a direct oxygen limitation of chemical reactions or to an oxygen-sensing mechanism that reduces oxygen-consuming behavioral or physiological functions (for example, hypoxia induces fatigue in humans under conditions in which oxygen is not believed to be limiting to muscle mitochondrial function). It is likely that animals have several P_c values that vary with the function being observed; an exciting test would be to determine the P_{O_2} at which other functions, such as feeding, protein synthesis or locomotion, are affected.

Insect gigantism during the Paleozoic era

In this study, we partially tested the idea that larger insects have more difficulty with gas exchange. A negative scaling of the safety margin for oxygen delivery is one possible prediction derived from the hypothesis that atmospheric hyperoxia in the late Paleozoic facilitated insect gigantism (Graham et al., 1995; Dudley, 1998). Clearly, the results from this study provide no support for this prediction. In fact, our finding that smaller grasshoppers had a much higher P_c for gas exchange (Figs 6, 7) was exactly the opposite of what would be predicted if larger insects had decreased safety margins for gas exchange. However, our experiments did not distinguish between the effects of body size and development; to do so, the safety margin for oxygen delivery must be compared across individuals of different species at the same developmental stage. Additionally, a better test of whether gas exchange becomes more challenging for larger insects may be to examine the scaling of P_c for gas exchange for insects at their maximal $\dot{M}_{O_2}$. Indeed, *Erythemis simplicicollis* dragonflies are oxygen limited during flight in normoxia, evidence that safety margins for oxygen delivery are small during activity (Harrison and Lighton, 1998).

It is generally thought that larger insects must use convective gas exchange to achieve adequate oxygen delivery (Weis-Fogh, 1964), and our data strongly suggest that this is true within the developmental stages of *S. americana* (Figs 8, 9, 13). Therefore, an alternative prediction derived from the hypothesis that larger insects have more difficulty with gas exchange is that larger insects may use compensatory mechanisms to overcome diffusion limitations. If so, we might expect the largest extant insects to exhibit maximal use of convective gas exchange. Increased use of convection might allow larger insects to have more responsive respiratory systems and control over diffusive water loss (Kestler, 1985). Examining the scaling of air sac volumes and use of convection across insects of various sizes would allow testing of this hypothesis.

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