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

II. Within-instar effects

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Summary

We hypothesized that oxygen delivery becomes more difficult for insects and tracheate arthropods as they progress throughout an intermolt period. During this time, body mass can more than double, yet the major tracheae and spiracles cannot be increased in size until molting. Also, tissue growth could compress air sacs used for convective gas exchange. To test these possibilities, we investigated the effect of within-instar growth on respiratory parameters, including CO_2 emission rate, ventilation frequency, tidal volume and critical oxygen partial pressure (P_{O_2}) for first-, third- and fifth-instar juveniles and adults of the American locust *Schistocerca americana*. We found that late-stage grasshoppers tended to have 40% higher total CO_2 emission rates but 15% lower mass-specific CO_2 emission rates and 35% higher ventilation frequencies than early-stage animals. Maximal tracheal system conductance decreased by 20–33% at the end of an instar, possibly due to compression of air sacs. In addition, animals nearing the end of an instar had higher critical P_{O_2} values for abdominal pumping, and late-stage adults had 50% lower tidal volumes, suggesting that increases in tissue mass throughout an instar may hinder the ability of animals to breathe deeply. Late-stage adults had lower critical P_{O_2} values for CO_2 emission, although this pattern was not found in any juvenile instars, indicating that late-stage juveniles compensate for decreased conductance by increasing ventilation frequency or the use of diffusive gas exchange. Our data suggest that late-stage arthropods are more vulnerable to hypoxia and may have reduced aerobic capacities and lower tissue P_{O_2}s than early-stage arthropods.

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

Introduction

Arthropod body mass can more than double during an intermolt period (insects – Nijhout, 1979; Davis et al., 1988; Stockhoff, 1992; Matsuki et al., 1994; crustaceans – Jacobi and Anger, 1985; Anger et al., 1989; Verhoef et al., 1998), requiring increased O_2 delivery to metabolically active tissues. Components of the gas exchange systems of many arthropods are composed largely of rigid chitinous/protein structures that cannot increase in size except during a molt. In insects, the new cuticle is essentially formed around the old tracheal lining and is typically bigger and more complex with each molt (Keister, 1948; Chapman, 1998). Some insects, such as the mycetophilid fly *Sciara coprophila*, replace their entire tracheal system during larval–larval molts; others with perhaps more complex branching replace only the larger tracheae. This means that the spiracles and major tracheae cannot increase in diameter within an instar until they are shed at the molt and replaced with larger structures. Although the smallest, unsclerotized respiratory structures, the tracheoles, can proliferate as tissues grow (Wigglesworth, 1984), the lack of within-molt flexibility in the dimensions of the more peripheral gas exchange structures (e.g. spiracles and tracheae) may constrain the oxygen delivery capacity of the entire tracheal system relative to tissue needs. Hence, these molting arthropods face a similar problem: within an intermolt period, tissue mass and oxygen consumption requirements increase while the sclerotized components of the gas exchange system cannot increase in size.

Oxygen delivery demands depend not only on body mass but also on mass-specific oxygen consumption ($\dot{M}_{O_2}$). Therefore, the within-instar variation in gas exchange requirements depends on how mass-specific metabolic rate changes during that period. In arthropods, this pattern can be complex. For example, in the crustacean *Nephrops norvegicus*, mass-specific metabolism does not vary within a molt cycle, so changes in total oxygen consumption parallel the increase in body mass (Alcaraz and Sarda, 1981). In grasshoppers (*Locusta migratoria*), shrimp (*Xiphonpenaeus kroyeri*) and spider crabs (*Hyas coarctatus* and *Libinia ferreirae*), variation in mass-specific metabolic rate within the instar depends on the

animal's developmental stage (Clarke, 1957b; Jacobi and Anger, 1985; Anger et al., 1989; Carvalho and Phan, 1998). Insects with air sacs may be particularly susceptible to developing a deficit in respiratory capacity relative to tissue oxygen needs because, as they grow and increase in mass within an intermolt period, the chitinous integument may constrain increases in animal volume, leading to compression of the larger, air-filled tracheae and unsclerotized air sacs. In fact, tracheal system volumes decrease by 90% during the fourth instar in *Locusta migratoria* (Clarke, 1957a).

In the present research, we tested the hypothesis that *Schistocerca americana* grasshoppers at the end of an instar (late-stage animals) have reduced capacity for oxygen delivery relative to oxygen demand. We predicted that the late-stage animals would have decreased safety margins for O_2 delivery compared with early-stage individuals due to reduced maximal tracheal conductances relative to tissue gas exchange needs. Specifically, if grasshopper body mass doubles during an instar, then (1) metabolic rate should increase by about 70% (Schmidt-Nielsen, 1984). If there is no ventilatory or morphological compensation, then (2) the safety margin for O_2 delivery should decrease by a similar percentage as animals develop through an instar. We also predicted that late-stage animals would have (3) reduced tidal volumes due to increased tissue mass and decreased air-space and (4) increased ventilatory frequencies to compensate for the reduced tidal volumes and greater gas exchange needs. Finally, we predicted that (5) tracheal or air sac compression should result in decreased maximal tracheal conductance during an instar and perhaps a greater decrease in the safety margin for oxygen delivery than predicted by the rise in metabolic rate.

Materials and methods

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 marked with paint 200 grasshoppers that hatched on the same day. We monitored the colony each day, and, when an animal molted, we repainted it. All animals that molted on a given day had the same paint color, so we knew an individual's total age in days and its age within an instar with a resolution of one day. To test effects of within-instar development, we used two groups: early-stage and late-stage. Animals that were one day past molting were treated as early-stage animals. To determine the end of an instar, we observed the colony and when the first animals began to molt, we noted their paint color and age. We then captured animals that were one day younger within that instar and designated them late-stage animals. Late-stage adults were taken for experiments when they were 30 days into the adult stage. We chose that time because by then body mass had stabilized and the grasshoppers were sexually mature. The experimental protocols and calculations for flow-through respirometry, tidal volume indices, critical P_{O_2} values (P_c) and tracheal system conductances were exactly as previously described (Greenlee and Harrison, 2004). All animals were exposed for 3 min to each O_2 concentration (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 measures of CO_2 emission and ventilation frequency; adults – 21, 13, 5, 2, 1 and 0.5 kPa O_2; juveniles – 21, 16, 13, 9, 5 and 1 kPa O_2 for measures of tidal volume).

Statistics

Statistical tests were performed with SYSTAT 10.2.01. We used one-tailed significance tests (P<0.1) for all the tests, since we predicted directions of change. To detect variation in normoxic ventilatory parameters and maximal tracheal system conductance, we used analysis of variance with instar and stage as independent variables. We also used *t*-tests to identify the effect of stage within specific instars. To determine how stage within an instar affected the response to hypoxia, we used repeated-measures analyses of variance (ANOVAs), since each animal was exposed to multiple levels of P_{O_2}. In these tests, instar and stage were independent variables and P_{O_2} was the repeated variable. We also used repeated-measures ANOVAs to test for significant differences within a particular instar. We used non-parametric tests for statistical tests of P_c values, because these were discrete variables. To test for the effect of stage within an instar on the P_c for mass-specific CO_2 emission rate ($\dot{M}_{CO_2}$) and for abdominal pumping, we used the Scheirer–Ray–Hare extension of the Kruskal–Wallis test, a non-parametric two-way ANOVA (Sokal and Rohlf, 1995). We also used Mann–Whitney *U* tests to detect differences within a specific instar.

Results

Body mass and normoxic ventilatory parameter changes within an instar

Body mass increased by an average of 74% from the beginning to the end of an instar (Fig. 1; Table 1). The increase

Table 1. *Percent changes in body mass and in normoxic breathing parameters from early to late stage within an instar*

Instar	Body mass	Breaths min^{-1}	Tidal volume	Whole-animal $\dot{M}_{CO_2}$	Mass-specific $\dot{M}_{CO_2}$
1	83*	–17	40	67*	–8.1
3	62*	53*		13	–31*
5	90*	34		54*	–19*
Adult	61*	72*	–49*	52*	–3.6

Asterisks indicate a significant change from early to late stage in an instar (P<0.1).

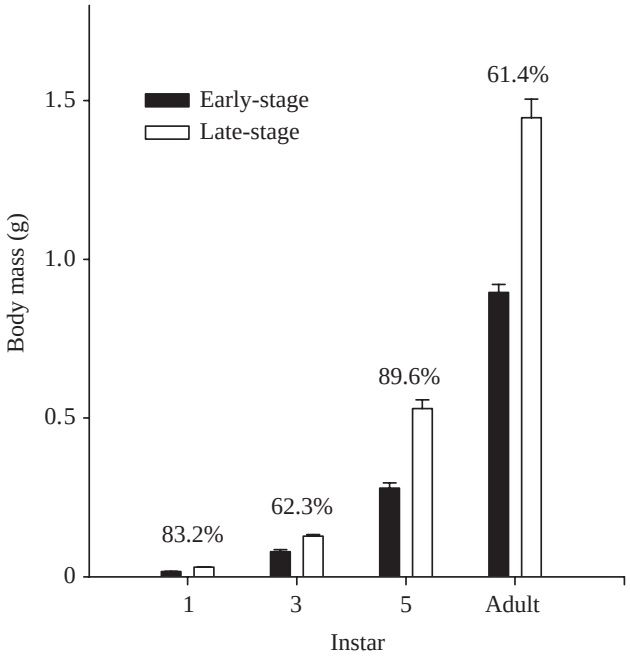

Fig. 1. Body mass for early-stage (open bars) and late-stage (filled bars) grasshoppers by instar. Numbers above pairs of bars indicate percentage increase within each instar.

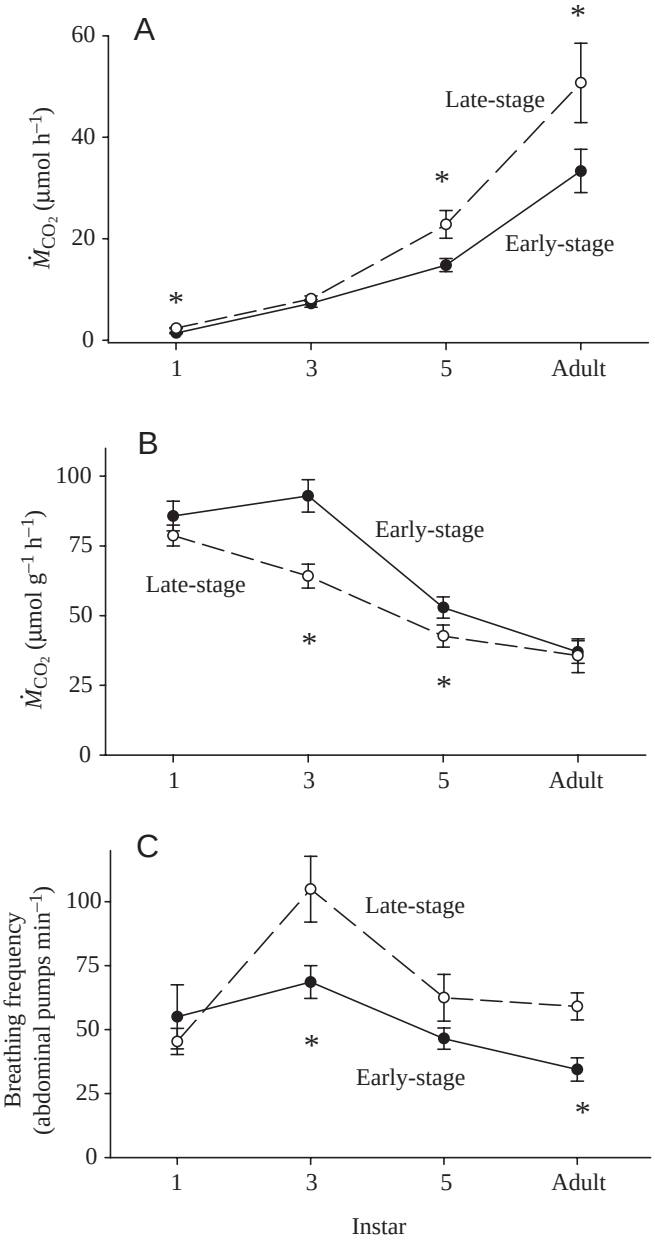

Fig. 2. (A) Whole-animal CO_2 emission rate ($\dot{M}_{CO_2}$), (B) mass-specific $\dot{M}_{CO_2}$ and (C) abdominal pumping frequency for early-stage (filled symbols) and late-stage (open symbols) animals in normoxia by instar. Asterisks indicate a significant difference between stages.

in whole-animal CO_2 emission rate ($\dot{M}_{CO_2}$) varied by instar (ANOVA, instar × stage interaction, $F_{1,60}$=6, P<0.02). Whole-animal $\dot{M}_{CO_2}$ increased by more than 50% throughout an instar in all but the third instar, during which time CO_2 emission rate only increased by 12% (Fig. 2A; Table 1). Mass-specific $\dot{M}_{CO_2}$ decreased significantly within an instar (Fig. 2B; Table 1; ANOVA, $F_{1,60}$=6.11, P=0.016). Normoxic breathing frequency significantly increased with stage in the third instar (t=–2.53, P=0.029) and in adults (t=–3.55, P=0.003; Fig. 2C; Table 1). Adults nearing the end of the instar had significantly reduced tidal volumes (as indicated by percentage change in abdominal height), but first instars showed no stage effect (ANOVA, instar × stage interaction, $F_{1,28}$=10.73, P=0.01; Table 1; Fig. 3).

Response of CO_2 emission to hypoxia

The effect of stage on mass-specific $\dot{M}_{CO_2}$ during progressive hypoxia depended on the animal's instar (repeated-measures ANOVA, P_{O_2} × instar × stage interaction, $F_{9,540}$=2.4, P<0.02; Fig. 4). When we tested each instar separately, there was a significant interaction between P_{O_2} and stage for each instar (repeated-measures ANOVA, P_{O_2} × stage interaction within each instar; instar 1 – $F_{10,140}$=1.65, P<0.1; instar 3 – $F_{11,154}$=5.32, P<0.001; instar 5 – $F_{11,154}$=3.71, P<0.001; adult – $F_{11,154}$=4.07, P<0.001), indicating that animals at different stages within the instar responded differently to hypoxia. However, much of the difference appeared to be due to lower mass-specific $\dot{M}_{CO_2}$s later in the stage, regardless of P_{O_2}. There was no overall effect of stage within an instar on P_c for $\dot{M}_{CO_2}$. For first, third and fifth instars, early- and late-stage animals did not differ significantly in the P_c for $\dot{M}_{CO_2}$; however, for adults, animals later in the stage had a higher P_c (Fig. 5; Mann–Whitney U test, U=12.5, P<0.02).

Tidal volume in hypoxia

We used percent change in abdominal height as an index of tidal volume and were able to statistically compare the responses of first-instar and adult grasshoppers at three common P_{O_2} levels (21, 5 and 1 kPa; Fig. 3). The response of abdominal pumping height to P_{O_2} varied with stage depending on the instar (repeated-measures ANOVA, P_{O_2} × stage × instar

interaction, $F_{2,56}$=3.3, P<0.05). When we analyzed each instar separately, we found a significant interaction between P_{O_2} and stage for adults (repeated-measures ANOVA, $F_{4,56}$=12.9, P<0.001; Fig. 3B) but not first instars (Fig. 3A). Late-stage adults had lower tidal volumes in normoxia but had similar maximal tidal volumes as early-stage adults in 5 kPa P_{O_2} (Fig. 3B).

Ventilation frequency

Abdominal pumping frequency in hypoxia varied with stage depending on the animal's instar (repeated-measures ANOVA, P_{O_2} × stage × instar interaction, $F_{10,600}$=4.6, P<0.001; Fig. 6). When we examined each instar separately, we found significant interactions between P_{O_2} and stage on ventilation frequency in every instar (repeated-measures ANOVA, instar 1 – $F_{10,140}$=2.5, P<0.01; instar 3 – $F_{10,140}$=9.7, P<0.001; instar 5 – $F_{10,140}$=8.5, P<0.001; adult – $F_{10,140}$=20.3, P<0.001), indicating that for all instars, stage within an instar significantly affected the ventilatory response to hypoxia. First-instar animals showed no variation in breathing frequency from the beginning to end of an instar except at P_{O_2} levels below 6 kPa (Fig. 6A). Below an air P_{O_2} of 6 kPa, the abdominal

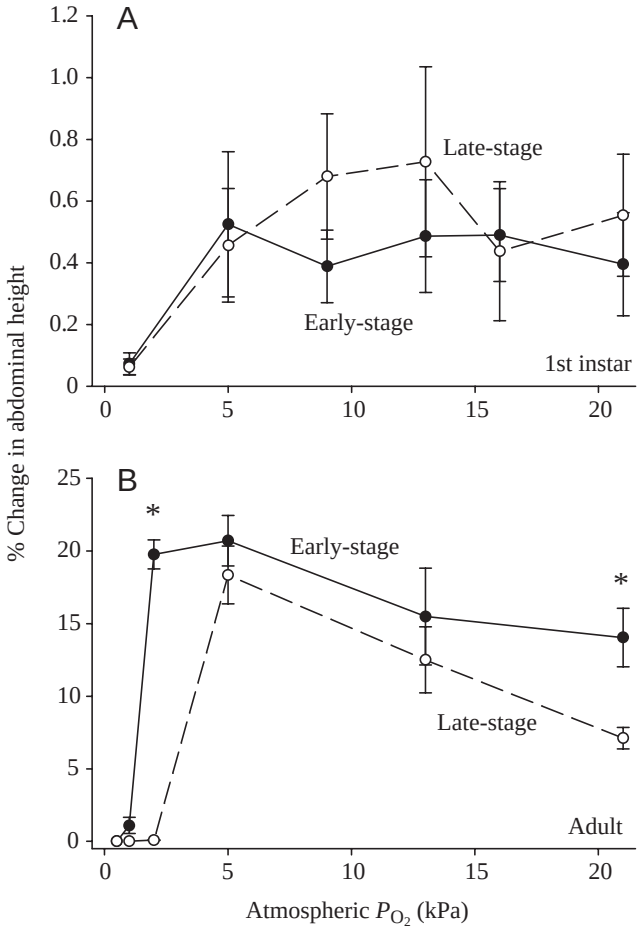

Fig. 3. Tidal volume, indexed by the percent change in abdominal height, in response to decreasing atmospheric oxygen partial pressure (P_{O_2}), for (A) first instars and (B) adults at the early-stage (filled symbols) and late-stage (open symbols) of an instar. Asterisks indicate a significant difference between stages.

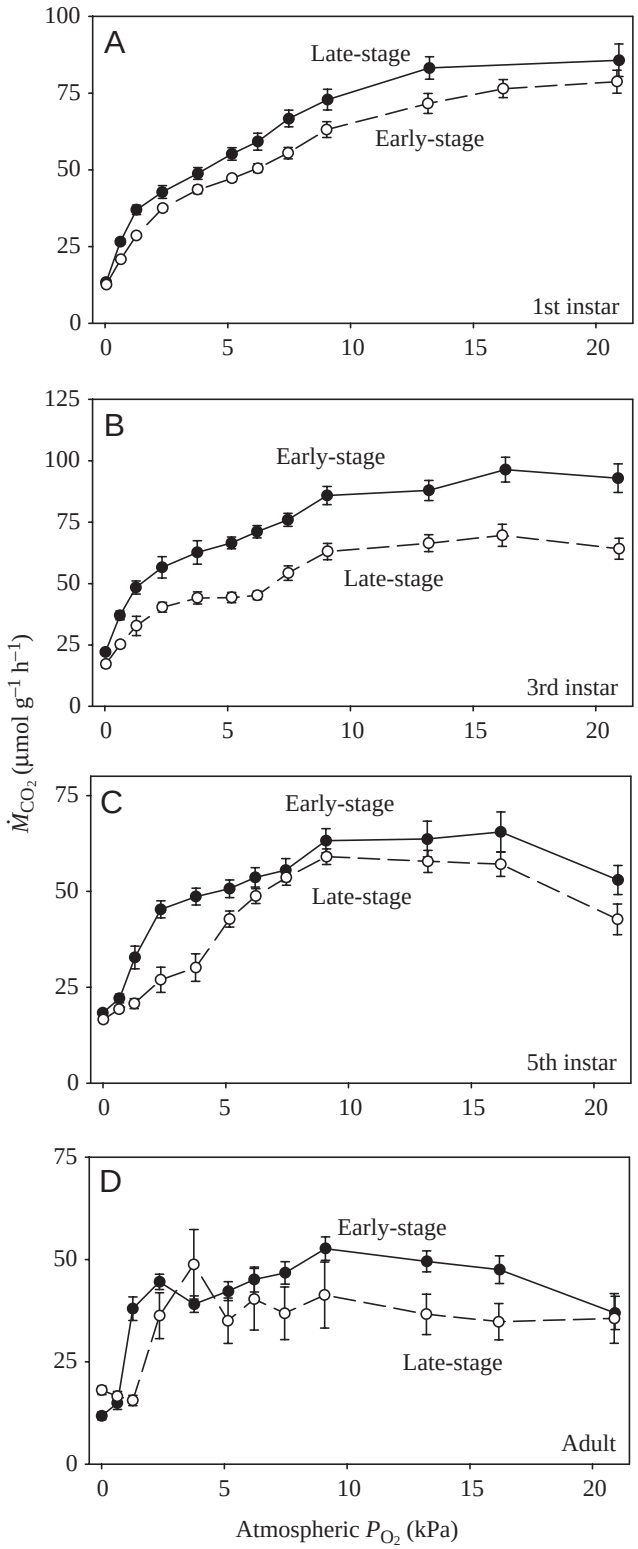

Fig. 4. Mass-specific CO_2 emission ($\dot{M}_{CO_2}$) as a function of atmospheric oxygen partial pressure (P_{O_2}) for early-stage (filled symbols) and late-stage (open symbols) animals; (A) first instar, (B) third instar, (C) fifth instar and (D) adult.

pumping rates of late-stage first instars dropped off more dramatically than those of early-stage first instars. In all of the other instars, breathing frequencies were 30–50% higher in normal air for late-stage animals compared with early-stage animals. Also, abdominal pumping frequencies of late-stage grasshoppers dropped at a higher ambient P_{O_2} compared with those of early-stage grasshoppers (Fig. 6B–D). All animals at the end of each instar had a significantly higher P_c for abdominal pumping frequency than animals at the beginning of an instar (Fig. 7; Scheirer–Ray–Hare extension of the Kruskal–Wallis test, H=14.58, P<0.05; for individual instars – Mann–Whitney U test, P<0.01).

Maximal total tracheal system conductance

Maximal total tracheal system conductance (μmol g^{-1} h^{-1} kPa^{-1}; calculated at the P_c) decreased by an average of 35% from the beginning to the end of an instar, depending upon the animal's instar (ANOVA, stage × instar interaction, $F_{1,60}$=3.25, P<0.08; Fig. 8). When we analyzed each instar separately, we found significant effects of stage in every instar (instar 1 – $F_{1,14}$=4.98, P<0.05; instar 3 – $F_{1,14}$=5.80, P<0.04; instar 5 – $F_{1,14}$=3.80, P<0.08; adult – $F_{1,14}$=4.07, P<0.08).

Discussion

A variety of evidence supports the hypothesis that oxygen delivery becomes more problematic as insects progress through an intermolt period. The maximal mass-specific ability of the tracheal system to deliver oxygen decreased (Fig. 8) and the P_c for abdominal pumping was higher at the end of each

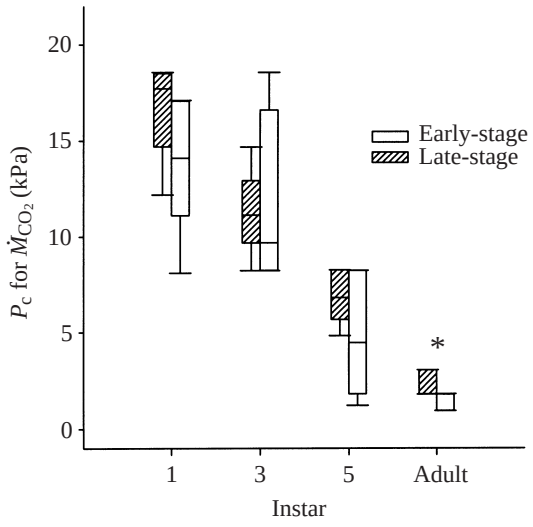

Fig. 5. Critical oxygen partial pressure (P_c) for mass-specific CO_2 emission ($\dot{M}_{CO_2}$) for early-stage (open boxes) and late-stage (hatched boxes) animals in each instar. The boundary of each box marks the 25th percentile and the 75th percentile. The line within each box indicates the median. Error bars mark the 10th and 90th percentiles. Asterisks indicate a significant difference between early- and late-stage animals.

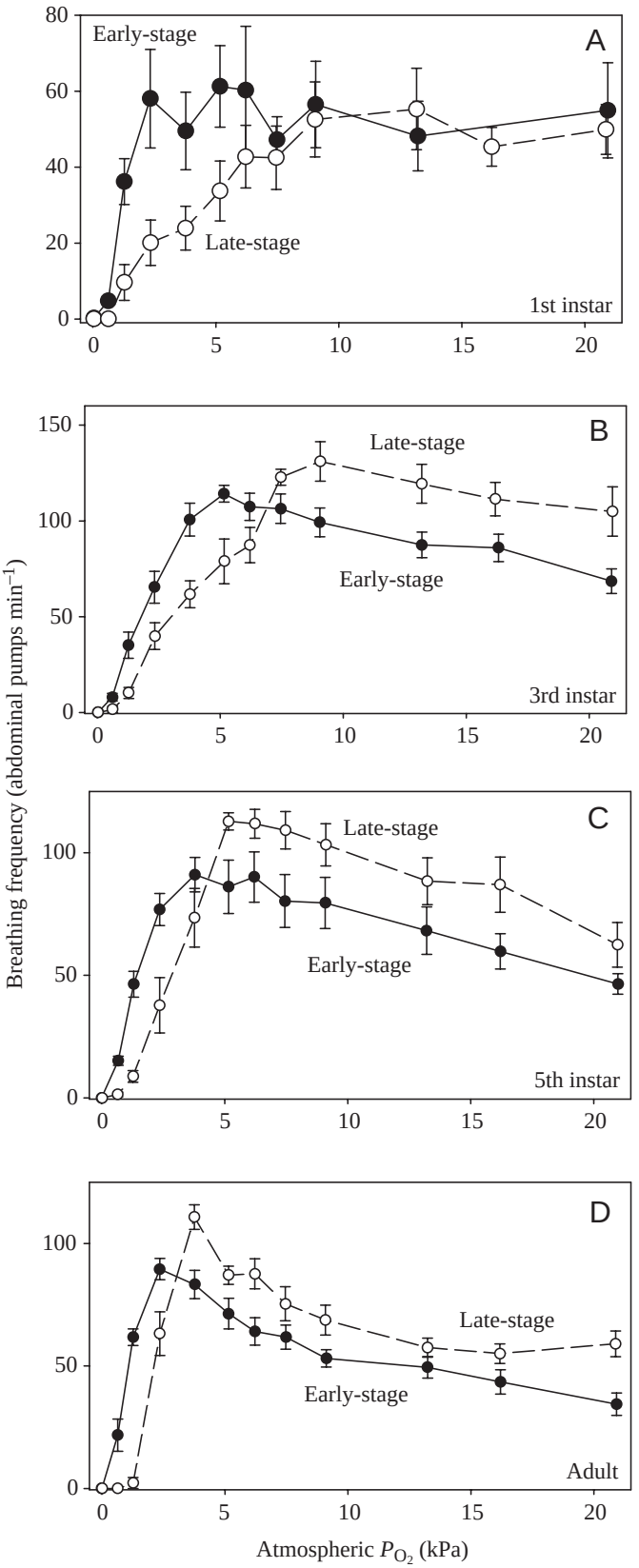

Fig. 6. Abdominal pumping as a function of atmospheric oxygen partial pressure (P_{O_2}) for early-stage (filled symbols) and late-stage (open symbols) animals; (A) first instar, (B) third instar, (C) fifth instar and (D) adult.

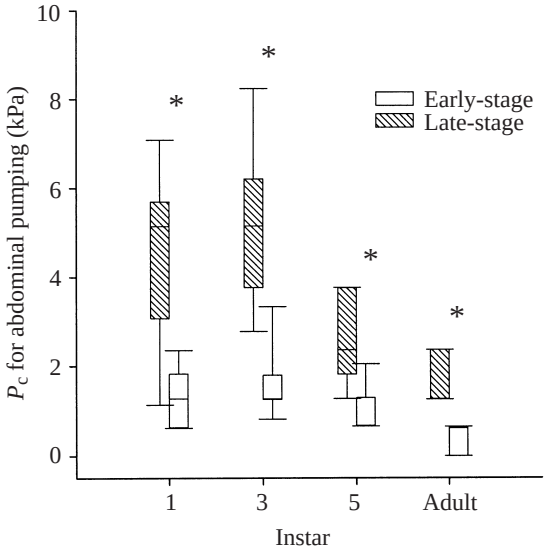

Fig. 7. Critical oxygen partial pressure (P_{O_2}) for abdominal pumping for early-stage (open boxes) and late-stage (hatched boxes) animals in an instar. Boxes and lines as in Fig. 5. Asterisks indicate a significant difference between stages within an instar.

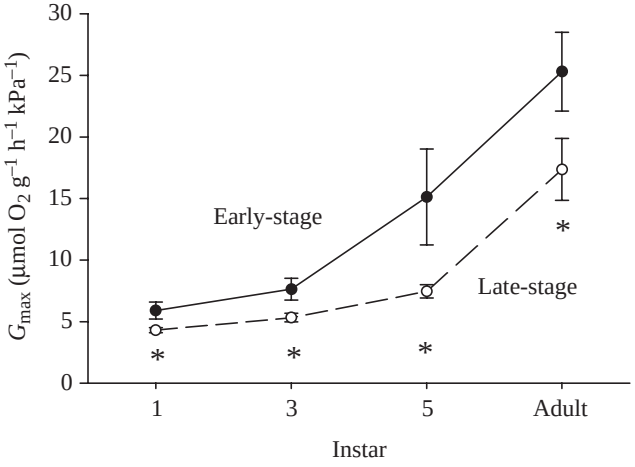

Fig. 8. Maximal tracheal system conductance (G_{max}) *versus* instar for early-stage (filled symbols) and late-stage (open symbols) grasshoppers. Asterisks indicate a significant difference between early- and late-stage animals.

instar (Fig. 7). The P_c for $\dot{M}_{CO_2}$ did not vary with stage for juveniles, suggesting that tracheal oxygen delivery matched tissue needs during the intermolt period for these animals. However, late-stage adult grasshoppers had a higher P_c for $\dot{M}_{CO_2}$ compared with early-stage animals. Late-stage adults were more susceptible to hypoxia than early-stage animals due to their lower tidal volumes (Fig. 3) and tracheal system conductances despite higher breathing frequencies (Fig. 6), suggesting that tissue growth within an instar impeded the ability of the insects to breathe deeply (Figs 3, 5).

Effects of developmental stage on ventilatory parameters

CO_2 emission rate

Total gas exchange rates increased within each instar by 13–67%. However, the percent increases in total CO_2 emission were not matched to percent increases in body mass, as mass-specific $\dot{M}_{CO_2}$ decreased from beginning to end of an instar (Table 1; Fig. 2B). The greatest decrease in mass-specific $\dot{M}_{CO_2}$ of 30% occurred during the third instar, indicating that a large part of the third instar mass increase may have been due to increases in non-metabolic tissue, such as cuticle or fat.

The relationship between metabolic rate and mass throughout development may be dependent upon species, as relationships reported in the literature are highly variable. For example, mass-specific oxygen consumption decreased strongly within most instars of *Potamophylax nigricornis* (caddisfly larvae) and *Galleria mellonella* (wax moth larvae; cited in Sehnal, 1985). The results we report here for *Schistocerca* grasshoppers differ from those of Clarke (1957b), who found that, in *Locusta migratoria* grasshoppers, oxygen uptake rate ($\dot{V}_{O_2}$) increases were matched exactly by increases in body mass for first- to third-instar animals. The parallel relationship between $\dot{V}_{O_2}$ and mass disintegrated by the fifth instar, when mass-specific $\dot{V}_{O_2}$ decreased by a third (Clarke, 1957b). According to our measurements, within-instar scaling of $\dot{M}_{CO_2}$ varied slightly with each instar in *S. americana* but did not differ much from across-instar patterns and the classic scaling of metabolic rate in animals (Fig. 9).

Index of tidal volume

We predicted that late-stage animals would have decreased tidal volumes due to increased tissue mass and smaller air sacs.

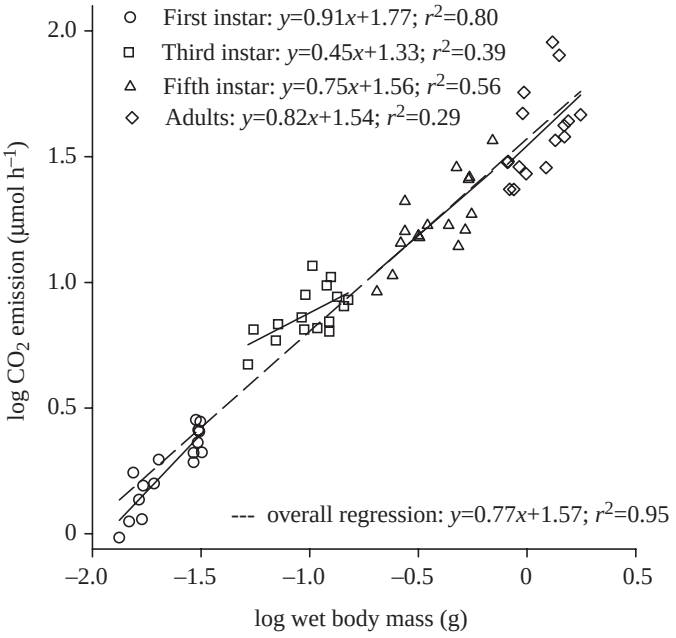

Fig. 9. Scaling of absolute CO_2 emission with wet body mass for each instar. The broken line represents the scaling relationship when all data points are regressed.

In normal air, abdominal pumping height was 50% lower in late-stage adults compared with early-stage adults (Fig. 5B). However, we found no difference in abdominal pumping height for the first instar (Fig. 5A). First-instar grasshoppers had such low tidal volumes that tissue growth may have little effect.

Interestingly, late-stage adults were able to increase abdominal pumping height to levels exhibited by early-stage adults. Perhaps the late-stage adults achieved maximal inhalation volumes similar to those of early-stage adults by using their inspiratory muscles. During most situations, grasshoppers use only the expiratory muscles (Hustert, 1975), and, if this were the case, reduced air sac volumes may have translated to reduced tidal volume. However, inspiratory muscles tend to be active during very heavy breathing (as would occur during hypoxia exposure) in grasshoppers (Hustert, 1975), and the consequent expansion of abdominal volume could allow the maintenance of maximal tidal volume.

CO_2 loss per breath and expired P_{CO_2}

Does ontogenetic development within an instar affect the use of diffusive *versus* convective gas exchange or result in varying internal gas tensions? We lack direct data to answer this question but we can indirectly address this issue by examining the effect of stage on CO_2 loss per breath (μmol breath^{-1}; calculated from $\dot{M}_{CO_2}$/ventilatory frequency). Across air P_{O_2} levels above 5 kPa, CO_2 emission per breath did not vary between early- and late-stage animals for third and fifth instars and adult grasshoppers (Fig. 10). For adults, convection can account for all trans-spiracular CO_2 transport (Greenlee and Harrison, 1998), therefore:

$$\text{Expired } P_{CO_2} = \frac{\dot{M}_{CO_2}}{\text{Breaths min}^{-1} \times \text{Tidal volume}} . \quad (1)$$

Tidal volume decreased by 50% in late-stage adults (Fig. 3B), while $\dot{M}_{CO_2}$/ventilatory frequency was constant. Thus, expired P_{CO_2} must have increased by 50% in late-stage adults. If convection is also predominant for first instars – since, for those animals, tidal volume did not change but CO_2 per abdominal pump increased (Fig. 10) – expired P_{CO_2} must have also increased. Alternatively, there could have been an increase in the use of diffusive gas exchange late in the first instar. CO_2 emission per breath increased strongly for all grasshoppers at air P_{O_2} levels below the P_c for ventilatory frequency. This increase could indicate that all animals increased diffusive gas exchange at very low P_{O_2} levels (e.g. opening of spiracles or removal of tracheolar fluid) or increased micro-ventilatory movements (Hustert, 1975).

Ventilation frequency

First-instar animals showed no difference in ventilation frequency between early and late stages, a pattern similar to that seen with tidal volume (Fig. 6A). The lack of a developmental effect in first instars could reflect a different strategy for responding to hypoxia. As noted above, late-stage

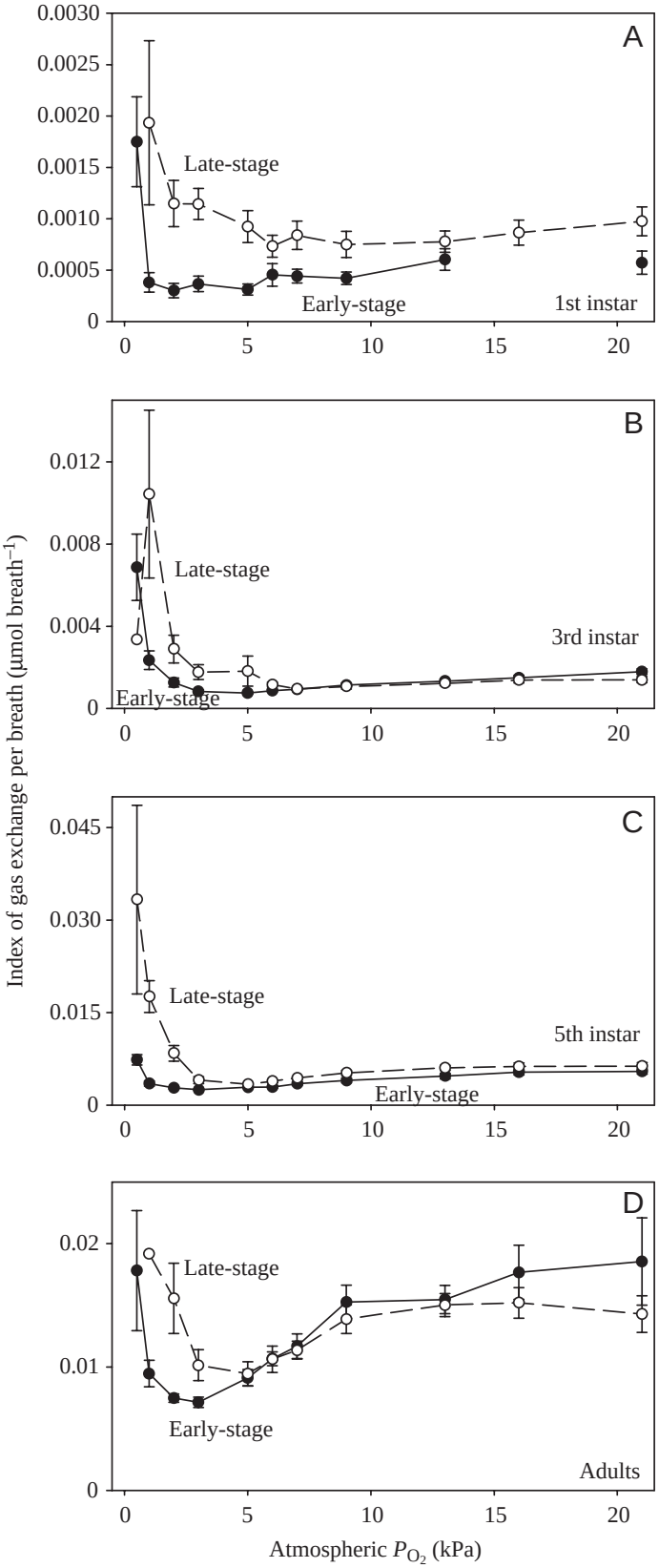

Fig. 10. Index of gas exchange per breath (μmol CO_2 breath^{-1}) as a function of atmospheric oxygen partial pressure (P_{O_2}) for early-stage (filled symbols) and late-stage (open symbols) animals; (A) first instar, (B) third instar, (C) fifth instar and (D) adult.

first-instar grasshoppers have a greater μmol CO_2 $breath^{-1}$, suggesting either increased use of diffusion or higher internal P_{CO_2}s (Fig. 10). Measurement of internal gases or quantification of the amount of diffusive gas exchange using anaesthetized animals would allow discrimination of these possibilities.

Late-stage animals (except first instars) increased ventilation frequency compared with early-stage animals (Fig. 6B–D), probably to compensate for decreased tidal volumes (Fig. 3). Maximal ventilation frequencies were significantly higher in late-stage fifth-instar animals and late-stage adults when compared with early-stage animals (t=–2.77 and –3.2, P=0.019 and 0.007, respectively; Fig. 6C,D). This ability to increase ventilation frequency partly ameliorates the effect of tracheal compression/fixed spiracle size on the maximal tracheal system conductance. It is unclear what mechanism would facilitate the increase in maximal breathing frequency at the end of an instar. Perhaps animals at the end of the instar have an increased chemical drive for ventilation (i.e. lower internal P_{O_2} or higher P_{CO_2}) or there may be changes in ventilatory muscle properties (such as increased mass or recruitment). Another possibility is that the sensitivity to neural excitation in response to hypoxia is increased at the end of an instar. This hypothesis could be tested by measuring the effect of hypoxia on the frequency of action potentials from ventilatory motor nerves (Bustami et al., 2002).

Maximal tracheal system conductance

Total mass-specific tracheal system conductance was lower in late-stage relative to early-stage grasshoppers at every instar (Fig. 8). The decrease in conductance could have been due to air sac or tracheal compression from tissue growth (Figs 3, 8). However, the lack of an effect of stage on tidal volume suggests air sac compression could not account for a decrease in maximal conductance in first instars. Perhaps tissue growth causes an increase in the length of secondary tracheae and possibly even a decrease in diameter if tracheae were stretched as a consequence of tissue growth. Another possibility is that tracheole density may decrease during the intermolt period.

P_c *within an instar*

In general, the P_c for abdominal pumping was lower than the P_c for CO_2 emission. For the first and third instars, the P_c values for abdominal pumping for late-stage animals were significantly and substantially higher than those of early-stage animals (fourfold; Fig. 7). One potential explanation for the larger stage effect on the P_c for abdominal pumping in younger animals is that the younger animals were generally less tolerant of hypoxia. In other words, the greater hypoxia sensitivity of metabolism in first instars relative to adults (Fig. 4) can be thought of as arising from the summed effects of a number of more hypoxia-sensitive tissues in the first instars. Also, animals may have preferentially shut down less critical tissues to respond to hypoxia. If first- and third-instar animals exchange gases primarily by diffusion, then the ventilatory muscles may be relatively non-critical and would be shut down at a higher P_{O_2}. By contrast, adults, which are highly dependent upon convection for gas exchange (Greenlee and Harrison, 1998, 2004), had relatively low P_c values for abdominal pumping and much less of a stage effect, perhaps because the ventilatory muscles are critical for surviving hypoxia.

Contrary to our prediction, the P_c for CO_2 emission did not differ between the early- and late-stage juvenile instars, although the predicted stage effect did occur in adults (Fig. 5). Perhaps our statistical power was insufficient to discriminate such an effect, since the median P_c was higher for all late-stage animals (Fig. 5). However, for the juvenile instars, the percentage decrease in maximal conductance in late-stage animals (Fig. 8) was similar to the decrease in mass-specific $\dot{M}_{CO_2}$ (Fig. 2B), suggesting that respiratory capacity did match gas exchange needs throughout those instars. For the first instars, the elevated CO_2 emission per breath in late-stage animals suggests that the matching may have occurred through increased diffusive gas exchange (e.g. by increased spiracular opening in late-stage animals). For the third and fifth instars, CO_2 emission per breath only increased in late-stage animals relative to early-stage animals below the P_c, suggesting that, for these instars, the increased ventilation frequencies were sufficient to allow matching of respiratory capacity and gas exchange.

Possible implications of decreasing safety margins for O_2 *delivery within an instar*

Our data suggest that young, late-stage insects may be particularly sensitive to high-altitude hypoxia. The P_c for CO_2 emission found for the late-stage first-instar grasshoppers was 17.5 kPa (Fig. 5), the P_{O_2} of air at an altitude of 1500 m. This calculation suggests that oxygen availability could limit gas exchange of late-stage first-instar grasshoppers of many species over large portions of their range (Branson and Redlin, 2001). However, if diffusion is important for oxygen delivery in first-instar grasshoppers, the increased oxygen diffusion coefficient at decreased barometric pressure may potentially offset the decrease in P_{O_2} (Joos et al., 1997). The reduced maximal tracheal system conductance of late-stage animals suggests that their maximal metabolic rates and locomotory performance may also be compromised late in the instar. Thus, late-stage insects may be more vulnerable to predators or less able to disperse or migrate.

An intriguing possibility is that decreases in tissue oxygen levels late in the intermolt period may be a signal that could trigger molting. Molting is initiated by a complex pathway, currently thought to begin with a decline in ecdysteroids. A declining level of ecdysteroids stimulates release of pre-ecdysis-triggering hormone (PETH; Zitnan and Adams, 2000) and ecdysis-triggering hormone (ETH) from epitracheal glands (Zitnan et al., 1996). Release of ETH initiates release of eclosion hormone (EH), and a positive feedback system occurs between EH and ETH (Ewer et al., 1997). However, the initial trigger of the cascade remains elusive. In the tobacco hornworm (*Manduca sexta*), molting can be initiated by

achievement of a critical body mass (Nijhout, 1975, 1979). In the milkweed bug (*Oncopeltus fasciatus*), abdominal stretch receptors stimulated by injecting the animals with fluid triggered molting (Nijhout, 1975, 1979). This finding supported the hypothesis that not only body mass but also increased body size could initiate molting.

An additional hypothesis is that decreased tissue oxygen levels late in the instar may be part of the mechanism for the initiation of molting. The fall in maximal tracheal conductance, the rise in the P_c for $\dot{M}_{CO_2}$ in adults and the indirect evidence for a rise in internal P_{CO_2} all suggest that internal P_{O_2} levels may fall late in the instar. This decreased tissue P_{O_2} could serve as a logical cue for triggering molting. The idea that decreased O_2 availability may trigger molting is supported by the findings of Greenberg and Ar (1996), whose studies of mealworms (*Tenebrio molitor*) reared in hypoxia, normoxia and hyperoxia showed that the duration of intermolt periods was directly proportional to P_{O_2}. In addition, since the epitracheal glands are located near each spiracle (Chapman, 1998), they are conveniently located for sensing changes in O_2 delivery. Direct measurements of the effect of stage on tracheal P_{O_2} and the effect of manipulation of tracheal P_{O_2} on EH and ETH will be necessary to test whether decreased P_{O_2} actually can serve as an initiator of molting in insects.

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