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Respiratory changes throughout ontogeny in the tobacco hornworm caterpillar, *Manduca sexta*

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Summary

The respiratory system of growing caterpillars is challenged in two distinct ways as they develop from hatchlings to fifth instars preparing for pupation. First, across instars, body sizes and tracheal lengths increase substantially. Second, within each instar, animal mass can more than double while major tracheal respiratory system structures, such as spiracles and large tracheae, are fixed in size until molting. To test whether these growth processes result in a decrease in O_2 delivery capacity relative to tissue oxygen needs, we exposed feeding *Manduca sexta* larvae of various ages to decreasing levels of atmospheric O_2 and measured their metabolic rate and ability to feed. We found that near the beginning of all instars, *M. sexta* were able to maintain gas exchange and feed down to approximately 5 kPa O_2, indicating that these insects are able to match tracheal O_2 delivery to increased metabolic rates across instars. However, gas exchange and feeding of caterpillars nearing the molt were limited at much higher O_2 levels (up to 15 kPa O_2), suggesting that caterpillars have limited capacities to increase tracheal O_2 delivery as O_2 consumption rates increase within instars. It seems possible that the safety margin for O_2 delivery may disappear completely in the last hours before ecdysis, providing an ultimate if not proximate explanation for the necessity of molting.

Key words: insect respiration, metabolic rate, gas exchange, development.

Introduction

The mechanisms and consequences of growth and its regulation are of primary importance to developmental biology and physiology. Growing arthropods gain substantial mass both within and across intermolt periods (instars), so the respiratory system of developing insects must cope with tremendous increases in O_2 consumption and CO_2 production, as well as increases in tracheal lengths that may challenge diffusive capacity. For example, in the tobacco hornworm caterpillar, *Manduca sexta*, body masses more than triple within each instar, body lengths increase more than 10-fold from first to fifth instar (K.J.G., unpublished) and total body mass increases 10,000-fold across the larval stages (Goodman et al., 1985).

How can the insect tracheal system compensate for growth and increased gas exchange needs across and within instars? The tracheal system may potentially compensate for increased lengths by either morphological or physiological mechanisms. Across instars, morphological mechanisms of compensation may include increasing tracheal diameters at each molt (Beitel and Krasnow, 2000). In addition, tracheal branching and sprouting are sensitive to P_{O_2} (Wigglesworth, 1954; Locke, 1958; Jarecki et al., 1999), and tracheole density increases with age in grasshoppers (Hartung et al., 2005). Thus if tissue P_{O_2} were to decrease due to inadequate O_2 delivery, tracheole proliferation could be stimulated, providing an opportunity for tracheal morphology to match O_2 delivery needs. Physiological changes in the mechanisms of gas exchange are also possible. Grasshoppers increase their mass-specific tracheal capacities as they grow across instars by increasing convective gas exchange through increases in the rate of abdominal pumping and tidal volume (Greenlee and Harrison, 2004a). Caterpillars have traditionally been thought to breath by diffusion (Krogh, 1920), but they may use hemolymph pulsations generated by micro-contractions of the intersegmental muscles of the abdomen to drive convection through the compressible tracheae (Slama, 1999; Smits et al., 2000).

In addition to dramatic body size increases across instars, within each instar, caterpillars increase in body mass at least 100% and up to 1000% in the fifth instar (Goodman et al., 1985). Do these size increases correlate with increasing challenges for oxygen delivery? In *Drosophila*, tracheal diameters do not increase during an intermolt period (Beitel and Krasnow, 2000). Similarly, spiracles are sclerotized and can only increase in size at the molt. Thus, the only likely morphological mechanism to allow increased O_2 delivery within an intermolt period is tracheole sprouting (Locke, 1958;

Jarecki et al., 1999). Physiological mechanisms, such as increasing convective gas exchange, could compensate for increased O_2 demands; however compression of flexible tracheae by growing tissues may impede the animal's ability to increase convection. For example, grasshoppers late in the intermolt period have decreased ability to respond to hypoxia (i.e. critical P_{O_2} increases throughout the instar, Greenlee and Harrison, 2004b). The decreased ability to respond to hypoxia may be caused by a decrease in the volume of the air sacs that become compressed as tissue grows within the sclerotized exoskeleton (Clarke, 1957; Greenlee and Harrison, 2004a). However, since the caterpillar exoskeleton is only sclerotized at the head, leaving the rest of the body free to expand throughout the instar (Eaton, 1988), tissue growth seems less likely to compress the tracheae.

If an insect exchanges gases by diffusion, then larger body sizes may lead to increasing problems with gas exchange due to the well-documented exponential decreases in diffusion rates with distance. This idea has contributed to the suggestion that atmospheric oxygen levels may be linked to insect body size (Graham et al., 1995; Dudley, 1998). In the present study, we first tested the hypothesis that larger caterpillars are unable to match tracheal O_2 delivery capacity to tissue O_2 needs during ontogeny. If larger insects have problems with oxygen delivery, then we would predict that larger caterpillars would have higher critical P_{O_2} values (P_c, the P_{O_2} below which metabolism and feeding can no longer be sustained). Dimensional differences are most prominent across instars, so to test this hypothesis we compared caterpillars at the beginning of different instars. In addition, we tested the hypothesis that as caterpillars grow throughout an instar, their oxygen delivery capacity does not match increases in tissue oxygen demand due to lack of plasticity in tracheal structure within an intermolt period. If animals nearing the molt have problems with oxygen delivery, we would predict that caterpillars near the end of an instar would have higher P_c values compared with animals that have recently molted.

Materials and methods

Caterpillars

Manduca sexta L. larvae were reared from eggs (Carolina Biological Supply, Burlington, NC, USA) on a wheat-germ-based artificial diet as previously described (Ojeda-Avila et al., 2003). We reared the caterpillars and conducted our experiments at room temperature (25°C). Animals were observed each day for signs of molting and the instar noted. We placed animals from each instar into one of two treatment groups: early- or late-stage animals; each treatment group had N=8 or 9. Early-stage animals were used the next day after the head capsule slipped and molting was complete. The first and third instars last approximately 3 days, therefore we considered them late-stage when they were 2 days past molting. The fifth instar lasts approximately 10 days, with peak body mass occurring between days 4 and 5. Therefore, to maximize difference in body mass, fifth instar animals were considered to be late-stage when they were 4 days past molting.

Respirometry

We measured CO_2 emission of caterpillars during feeding to maximize metabolic rates and to minimize struggling in our respirometry system. Food was placed in a small dish and weighed using an analytical balance to the nearest 0.0001 g (Mettler AE-240, Columbus, OH, USA). The dish and food were then placed in the respirometry chamber and flushed with CO_2-free air (approximately 100 ml min^{-1}) for 20 min to remove any CO_2. Food treated in this manner had no measurable CO_2 emission when placed in the chamber alone. Additionally, water loss from the food was negligible (<0.7% of the initial food mass) as estimated by placing food alone in the chamber for approximately 2 h, a period longer than any experimental trial. The experimental animal was then weighed in the same manner, placed on the food in the chamber, and allowed to acclimate to the chamber for 20 min. During each trial, caterpillars were exposed to 10 min each of five different gas mixes (21, 15, 10, 5, 3 and 0 kPa O_2) generated with a Brooks 5878 mass flow controller and Brooks mass flow meters (Brooks Instruments, Hatfield, PA, USA). Gas mixes were subsampled from a 60 ml syringe and pushed through the chamber by an Ametek R-1 flow controller (Ametek, Pittsburgh, PA, USA) at 18–160 ml min^{-1} STP. The excurrent air stream was directed through a CO_2 analyzer (Li-6252 Li-Cor, Lincoln, NE, USA) to measure CO_2 fraction in the air and then through an Ametek S3-A oxygen analyzer to verify the gas mixture. Signals from the CO_2 and O_2 analyzers were digitized and recorded using Sable Systems DataCan software and hardware (Salde Systems International, Las Vegas, NV, USA). We calculated $\dot{M}_{CO_2}$ (μmol g^{-1} h^{-1}) as:

$$\dot{M}_{CO_2} = \frac{\dot{V}_{in}\,(F_{ECO_2} - F_{ICO_2}) \times 2678.58}{M_b}\,, \qquad (1)$$

where $\dot{V}_{in}$ was the upstream flow rate, F_{ECO_2} was the fraction of CO_2 in the excurrent airstream, and F_{ICO_2}, the fraction of CO_2 in the incurrent airstream, was equal to 0, and M_b was body mass (g). Additionally, 2678.58 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}). We converted our measures of CO_2 emission (ml s^{-1}) to metabolic energy (Watts) using the Joule equivalence 23.7 kJ (per litre of CO_2)$^{-1}$ (Withers, 1992) for a respiratory exchange ratio (RER) of 0.88, that of *M. sexta* (Alleyne et al., 1997).

In addition to determining metabolic rates, we calculated critical P_{O_2} values ($P_{c\text{-}CO_2}$) (Greenlee and Harrison, 2004a). Briefly, we compared 95% confidence intervals around the mean CO_2 emission rates at each P_{O_2}. A P_{O_2} was considered to be the $P_{c\text{-}CO_2}$ if the mean $\dot{M}_{CO_2}$ at the next higher and across all higher P_{O_2}s were significantly higher.

We then calculated maximal mass-specific tracheal system conductance, G_{max} (μmol g^{-1} h^{-1} kPa^{-1}, Greenlee and Harrison, 2004a). Briefly, we assumed that at the P_{O_2} just higher than the $P_{c\text{-}CO_2}$ for $\dot{M}_{CO_2}$, the animals were maximally conducting gases through wide-open spiracles and a fluid-free tracheal system,

and that mitochondrial P_{O_2} at the $P_{c\text{-}CO_2}$ is near zero. We converted $\dot{M}_{CO_2}$ to $\dot{M}_{O_2}$ assuming a RER of 0.88 and calculated:

$$G_{max} = \frac{\dot{M}_{O_2}}{\text{atmospheric } P_{O_2}}, \quad (2)$$

where atmospheric P_{O_2} is the P_{O_2} just higher than the $P_{c\text{-}CO_2}$.

Feeding behavior

Animals began feeding as soon as they were placed on food in the respirometry chamber. We observed them during each hypoxic exposure to determine whether they were continuing to feed, and considered total feeding time to be the acclimation time in the chamber plus the time until the animal stopped feeding. The P_{O_2} at which animals ceased feeding was identified as critical point for feeding ($P_{c\text{-feeding}}$). Food was weighed after the last hypoxic exposure, and total feeding rate (g h^{-1}) was calculated as the difference in food mass from the beginning to the end of the trial divided by the time that the animals were observed feeding. Animals ate on average 25% of the food offered (range 2–71%).

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%. We analyzed $\dot{M}_{CO_2}$ data using repeated measures analysis of variance (ANOVA), since individual caterpillars were exposed to multiple P_{O_2} values. Feeding rates were compared using ANOVAs with the independent variables being instar and stage. P_c and $P_{c\text{-feeding}}$ 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).

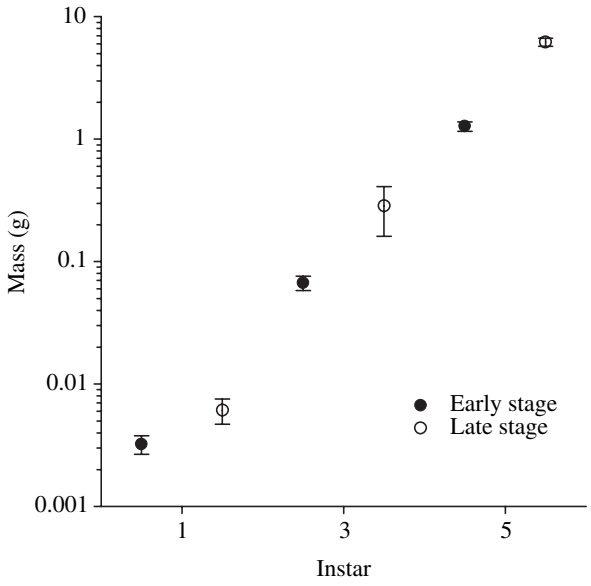

Fig. 1. Body mass of first, third and fifth instar caterpillars at early (filled symbols) and late (open symbols) stages within an instar.

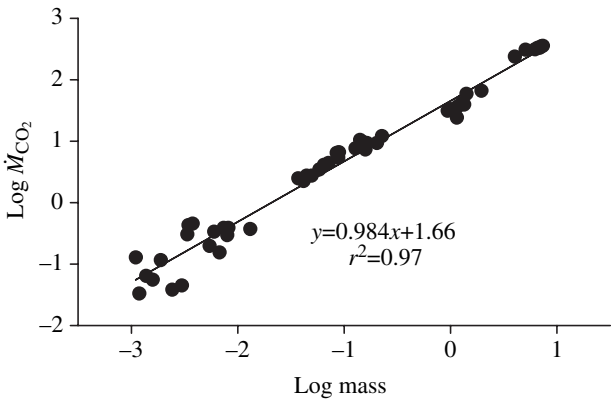

Fig. 2. Scaling of normoxic CO_2 emission rate with body mass (g).

Results

Body mass and normoxic CO_2 emission rate

As animals grew, body mass increased over 2000 times from 1 day to 17 days of age (0.003±0.0005 g and 6.2±0.5 g, respectively; Fig. 1). The increase in body mass from early- to late-stage depended upon the animal's instar (ANOVA, instar × stage interaction: $F_{1,46}$=24.7, P<0.001; Fig. 1).

Whole-animal, normoxic CO_2 emission rates increased four orders of magnitude from the youngest to the oldest animals in our study (Fig. 2; 0.229±0.05 to 317±14.9 μmol h^{-1}; ANOVA, $F_{1,46}$=52.4, P<0.001). CO_2 emission scaled with body mass (M_b) to the 0.98 power (log $\dot{M}_{CO_2}$ μmol h^{-1}=0.98 [log M_b] + 1.66, r^2=0.97, P<0.001; Fig. 2), and metabolic energy scaled with body mass to the 0.96 power [Watts=0.0071(mass)$^{0.96}$].

Mass-specific, normoxic $\dot{M}_{CO_2}$ varied differently with stage depending upon the instar (ANOVA, stage × instar interaction, $F_{1,46}$=17.7, P<0.0001). For instance, first and third instar caterpillars' CO_2 emission rates decreased from early to late stage, while late-stage fifth instar caterpillars increased mass-specific $\dot{M}_{CO_2}$ to 60% higher than the early-stage value (Fig. 3).

Hypoxic CO_2 emission, P_c, maximal tracheal system conductance

In response to decreasing levels of atmospheric O_2, caterpillars generally maintained CO_2 emission rates at their normoxic levels, until a critical point was reached. However, depending on the instar, early- and late-stage animals responded differently to the decreased P_{O_2} (Fig. 3; repeated measures ANOVA, instar × stage × P_{O_2} interaction, $F_{5,230}$=7.9, P<0.001). Analyzing each instar separately, we found that the CO_2 emission rate response to hypoxia depended strongly on stage (Fig. 3; repeated measures ANOVA, P_{O_2} × stage interaction; first instar: $F_{5,75}$=4.7, P<0.001; third instar: $F_{5,75}$=5.8, P<0.0001; fifth instar: $F_{5,70}$=21.4, P<0.0001).

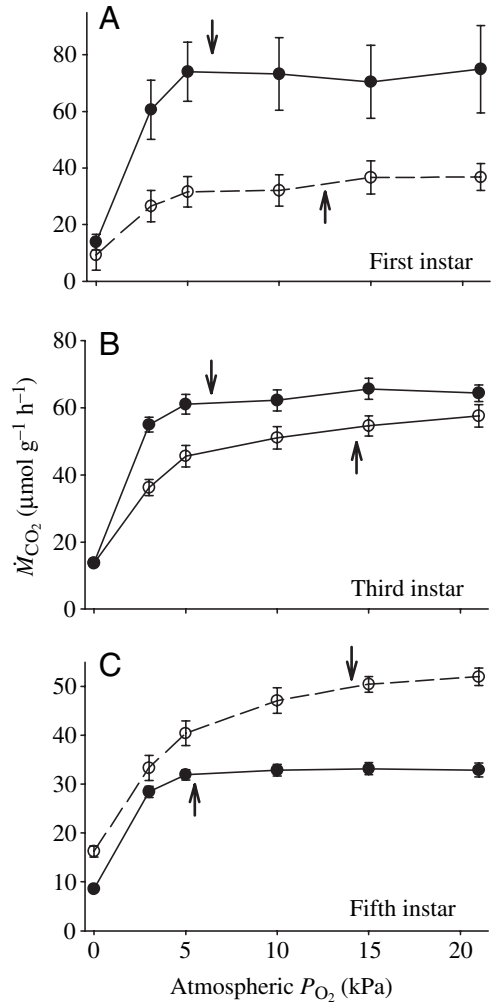

Fig. 3. CO_2 emission rates in response to decreasing atmospheric P_{O_2} for each instar and stage. Arrows indicate critical P_{O_2} values ($P_{c\text{-}CO_2}$), filled symbols represent early-stage animals, and open symbols represent late-stage animals.

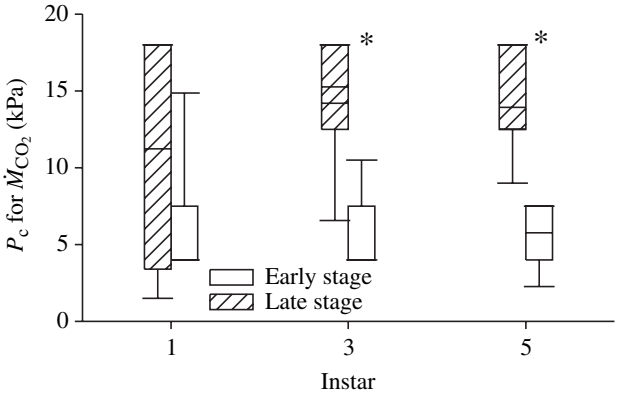

Fig. 4. $P_{c\text{-}CO_2}$ as a function of instar. The boundaries of each box indicate the 25th and the 75th percentiles, respectively. The line within the each box indicates the median. Error bars mark the 10th and 90th percentiles. Open boxes are early-stage and hatched boxes are late-stage animals. Asterisks denote significant differences between early and late stages.

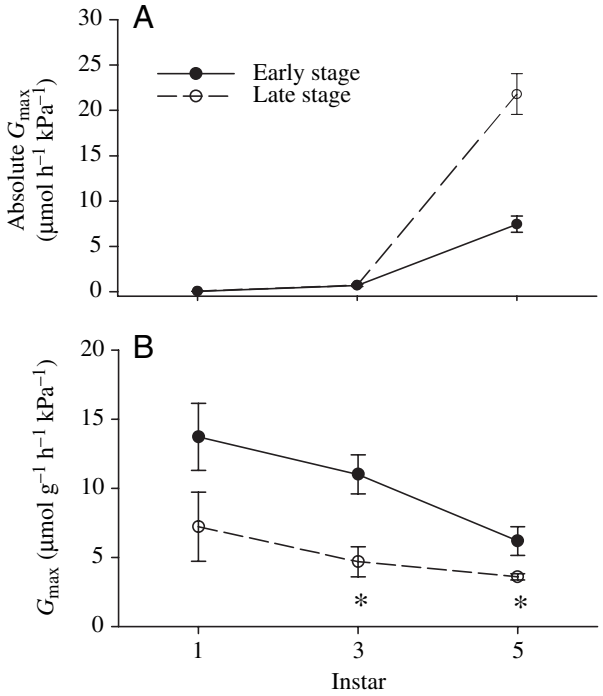

Fig. 5. Absolute G_{max} (A) and mass-specific G_{max} (B) *versus* instar. Filled symbols are early-stage caterpillars; open symbols are late-stage caterpillars. Asterisks denote significant differences between early and late stages.

$P_{c\text{-}CO_2}$ also varied with stage depending on the caterpillar's instar (Fig. 4). When we analyzed each instar separately, we found that late-stage third and fifth instar caterpillars had significantly higher P_c values than early stages (Fig. 4, Mann-Whitney U test, third instar: U=8, P<0.01; fifth instar: U=2, P=0.001), while there was no stage effect in the first instars (Fig. 4).

Mass-specific maximal tracheal system conductance, G_{max}, decreased with instar (Fig. 5; ANOVA, effect of instar, $F_{1,46}$=11.1, P<0.01). Within each instar, mass-specific G_{max} decreased on average 49% from early to late stage (Fig. 5; ANOVA, effect of stage, $F_{1,46}$=8.1, P<0.01).

Feeding behavior

Three animals did not eat during the experiment and were therefore excluded from these analyses. The remaining 47 animals ate continuously during the experiment until the P_{O_2} became limiting, at which point they raised their heads off the food. Some animals even moved away from their food. Food eaten (g h^{-1}) varied with instar (Fig. 6, ANOVA, effect of instar, $F_{1,43}$=14.2, P<0.001).

When we corrected the amount of food eaten for caterpillar body mass, [food eaten (g) g caterpillar^{-1} h^{-1}], we found that the amount of food eaten varied with instar depending on the animal's stage (Fig. 6, ANOVA, instar × stage interaction, $F_{1,43}$=5.6, P<0.03). All late-stage animals ate less food

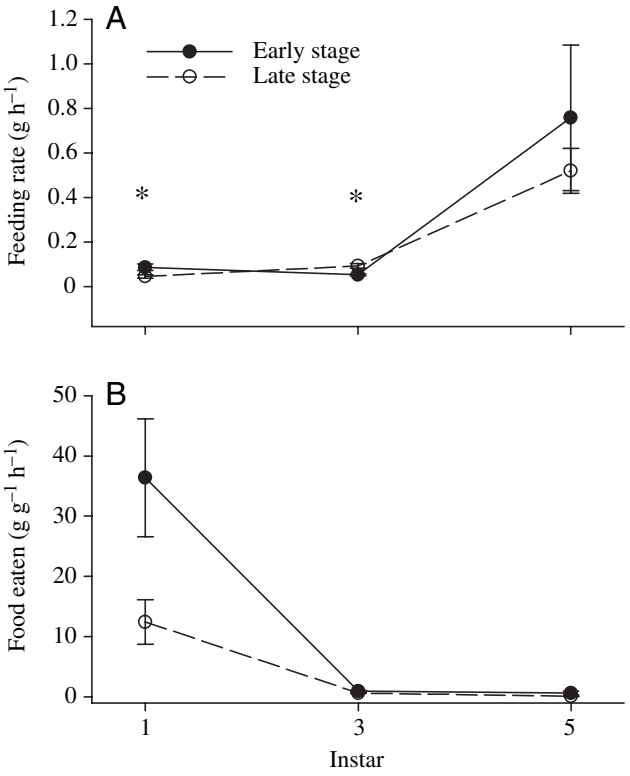

Fig. 6. Feeding rates (A; food eaten, g h^{-1}) and mass-specific feeding rates (B; food eaten, g g^{-1} h^{-1}) of caterpillars *versus* instar for early and late-stage animals. Asterisks denote significant differences between early and late stages.

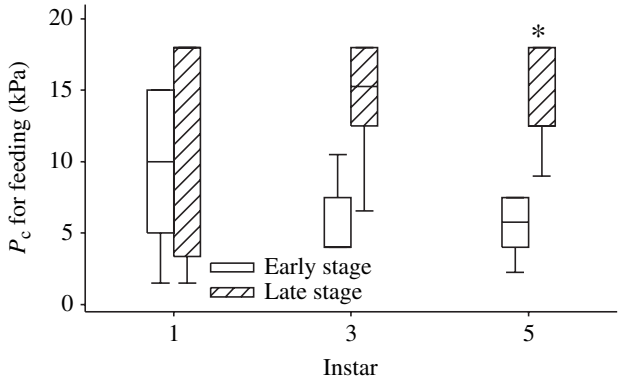

Fig. 7. $P_{c\text{-feeding}}$ *versus* instar. The boundaries of each box indicate the 25th and the 75th percentiles respectively. The line within the each box indicates the median. Error bars mark the 10th and 90th percentiles. Open boxes are early-stage and hatched boxes are late stage animals. Asterisks denote significant differences between early and late stages.

(g g caterpillar^{-1} h^{-1}) compared with early-stage-animals; however these decreases were not statistically significant (ANOVAs, effect of stage, first instar: $F_{1,13}$=4.7, P=0.05; third instar: $F_{1,15}$=4.3, P=0.056; fifth instar: $F_{1,13}$=3.2, P=0.099).

$P_{c\text{-feeding}}$ varied with instar (Fig. 7; Scheirer-Ray-Hare extension of the Kruskal-Wallis test, effect of instar, $H_{1,43}$=11.0, P<0.05). In addition, $P_{c\text{-feeding}}$ decreased across instar for early-stage animals (Kruskal-Wallis test statistic=13.9, P<0.01). When we analyzed each instar separately to test for stage effects, we found that early-stage fifth instars continued to feed at significantly lower P_{O_2} values than late-stage animals (Mann-Whitney U test, U=14, P=0.03).

Discussion

The changes in P_c within an instar were much more striking than the differences across instars. Within an instar, early-stage caterpillars had 2–3-fold lower $P_{c\text{-}CO_2}$ values compared with late-stage caterpillars, despite the lower mass-specific CO_2 emission rates for late-stage first and third instar animals (Figs 3 and 4). These data support the hypothesis that caterpillars within an instar experience a shrinking safety margin, as the increase in tissue oxygen needs is not matched by changes O_2 delivery capacity. The rise in P_c during an instar can be explained simply by increased tissue O_2 demands with an unchanging tracheal system capacity in the 1st and 3rd instars, since absolute, maximal G_{max} does not vary within these instars (Fig. 5). Interestingly, absolute, maximal G_{max} increases in the 5th instar, but by less than the increase in absolute gas exchange, leading to the decrease in P_c.

Across instars, larger caterpillars did not appear to have more difficulty than smaller caterpillars in responding to hypoxia. There was no significant effect of instar on $P_{c\text{-}CO_2}$, while $P_{c\text{-feeding}}$ actually decreased in the older/larger instars (Figs 4, 7). Thus tracheal conductances matched or exceeded changes in oxygen consumption rates across instars.

CO_2 emission rate in normoxia

CO_2 emission rates scaled with body mass to the 0.98 power (Fig. 2), a finding similar to that for some lepidopterans throughout ontogeny (cecropia moths, *Hyalophora cecropia*, 0.9, Schroeder and Dunlap, 1970; *M. sexta*, 1.0, Dahlman and Herald, 1971; and brown-tailed moths, *Euproctis chrysorrhoea*, 1.0, Migula, 1974) but not others (corn earworms, *Heliothis zea*, 0.7, Edwards, 1970; tent caterpillars, *Malacosoma neustria*, 0.8, Migula, 1974; and *M. sexta*, 0.8, Alleyne et al., 1997). Our finding that $\dot{M}_{CO_2}$ varies strongly within an instar suggests that the variation among scaling coefficients reported for caterpillars could be due to variation in the age within an instar of the caterpillars studied.

Variation in age within an instar may contribute strongly to the variation in mass scaling of metabolic rate during ontogeny across insect species. For example, mass-specific metabolic rate was higher for late-stage fifth instar caterpillars than for early stage caterpillars of the same instar, while an opposite pattern was observed for the first and third instars (Fig. 3). Late-stage fifth instar caterpillars had increases in absolute CO_2 emission (630% from early-stage) that were double the increases in body mass (360% from early-stage). A possible explanation is that preparation for wandering and pupation increase metabolic rate in fifth instars relative to other instars.

The high late-instar metabolic rates of fifth instars contribute to the reduced safety margin for O_2 delivery observed for this group. Our results indicate that future studies of the ontogenetic scaling of gas exchange and, probably physiological processes in general, must carefully control for age within instar.

Hypoxia response and P_c *for* $\dot{M}_{CO_2}$

Across-instar effects

Newly molted caterpillars were able to maintain CO_2 emission down to low P_{O_2} levels (as low as 5 kPa). These results are comparable to those found for other insects (Keister and Buck, 1974; Wegener and Moratzky, 1995; Greenlee and Harrison, 1998; Emekci et al., 2002; Greenlee and Harrison, 2004a). The constant $P_{c\text{-}CO_2}$ across instars results from a matching of maximal tracheal conductance to metabolic rate changes, as both mass-specific G_{max} and CO_2 emission rate decrease by about 50% from first to fifth instar (Fig. 5B). These patterns are not a general phenomenon in insects, as P_c in grasshoppers decreases and absolute G_{max} increases during ontogeny (Greenlee and Harrison, 2004a).

CO_2 emission rates at low P_{O_2} levels were (for a short time at least) maintained at relatively high levels. In anoxia, all animals were able to maintain CO_2 emission rates at 30% of their normoxic values for the 10 min trial (Fig. 8). These high ratios of anoxic $\dot{M}_{CO_2}$/normoxic $\dot{M}_{CO_2}$ suggest possible use of anaerobic metabolism during extreme hypoxia/anoxia. In support of this hypothesis, caterpillar intersegmental muscles contain considerable quantities of lactate dehydrogenase (Gade, 1975). The high ratios of anoxic $\dot{M}_{CO_2}$/normoxic $\dot{M}_{CO_2}$ (Fig. 8) are also likely due to washout of CO_2 from the tissues as hypoxia/anoxia promote maximal spiracular opening (Case, 1956), evacuation of fluid from the tracheoles (Wigglesworth, 1931) and reduced tissue P_{CO_2} values (Greenlee and Harrison 1998).

Within-instar effects

CO_2 emission rates and feeding rates of caterpillars late within each instar were clearly limited at higher P_{O_2} values than their earlier counterparts as evidenced by the much higher $P_{c\text{-}CO_2}$ and $P_{c\text{-feeding}}$ in late-stage caterpillars. Early-stage caterpillars had lower $P_{c\text{-}CO_2}$ values compared with late-stage caterpillars, despite the generally lower mass-specific CO_2 emission rates for late-stage animals (Figs 3 and 4). These data support the hypothesis that caterpillars within an instar experience a shrinking safety margin, as changes in O_2 delivery capacity do not parallel changes in tissue oxygen needs. In fact, mass-specific G_{max} decreased by nearly 50% from early to late-stage within each instar (Fig. 5B), whereas within-instar variation in mass-specific CO_2 emission rates was different for each instar (Fig. 3). First instar animals showed a similar decrease in both mass-specific $\dot{M}_{CO_2}$ and G_{max}, while third instar animals only had a 10% decrease in mass-specific $\dot{M}_{CO_2}$, and fifth instar animals actually had a 60% increase in mass-specific $\dot{M}_{CO_2}$ from the beginning to the end of an instar.

Why does mass-specific G_{max} decrease within an instar? If the tracheal system was fixed within an instar (same spiracle and main tracheal dimensions, no tracheole sprouting), then absolute G_{max} should be constant, but mass-specific G_{max} would fall due to the rise in tissue mass with constant tracheal morphology. This pattern is observed for the first and third instars (Fig. 5), suggesting that the within-instar rise in $P_{c\text{-}CO_2}$ for the third instars can be explained by rising animal oxygen needs without an increase in tracheal system oxygen delivery capacity. In the fifth instars, however, caterpillars increase absolute G_{max} 192% (Fig. 5A) from early to late-stage, indicating a within-instar enhancement of oxygen delivery capacity. This increase in G_{max} could be due to an increased use of convection or tracheole sprouting. The increase in absolute G_{max} is far less than the 630% rise in absolute CO_2 emission rate, leading to the rise in $P_{c\text{-}CO_2}$ within the fifth instar.

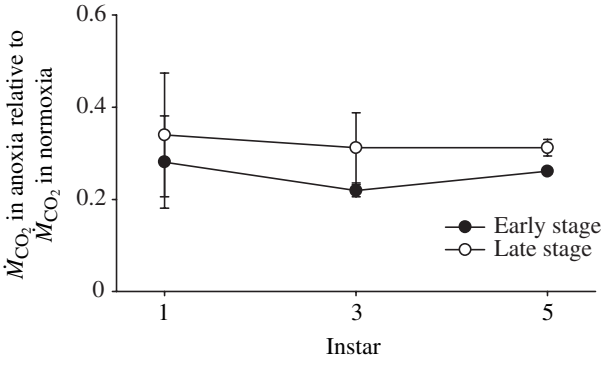

Fig. 8. $\dot{M}_{CO_2}$ in anoxia divided by $\dot{M}_{CO_2}$ in normoxia as a function of instar. Filled symbols are early-stage caterpillars; open symbols are late-stage caterpillars.

Feeding behavior

Caterpillars were able to continue feeding, in most cases without interruption, to very low P_{O_2} values (Fig. 7). The oldest caterpillars in our study ate slightly more food (g g^{-1} h^{-1}, Fig. 6) compared to fifth instar *M. sexta* in another study using the same diet and temperature (approximately 0.06 g g^{-1} h^{-1} over a 4 h period, Kingsolver and Woods, 1997). However, the ontogenetic pattern of increasing feeding rates with age that we observed follow those of previous researchers (Fig. 6). For example, zebra caterpillars (*Melancha picta*) feeding on sugarbeet leaves increased feeding rates (leaf area/day) with age more than 70-fold (Capinera, 1979). Growing armyworms (Mamestra configurata, Bailey, 1976), saltmarsh caterpillars (Estigmene acrea, Capinera, 1978), and cotton bollworms (Helicoverpa zea, Huffman and Smith, 1979) also increased leaf feeding area per day with age. In addition, the mass-specific feeding rates of our caterpillars decreased 60-fold from hatching to fifth instar (Fig. 6), a finding similar to that observed in other lepidopteran larvae (Slansky Jr, 1993).

Possible mechanisms of hypoxia tolerance

The low and similar values of $P_{c\text{-}CO_2}$ and $P_{c\text{-feeding}}$ suggest that caterpillars early in the intermolt periods have very large

safety margins for O_2 delivery. The existence of these large safety margins for O_2 delivery in the absence of any outward signs of increasing ventilation begs the question, what is the mechanism for the large safety margin for O_2 delivery? One possible explanation for their hypoxia tolerance is that these insects have 'overbuilt" tracheal systems (larger diameters than needed) and simply tolerate substantial variation in tissue P_{O_2} during exposure to hypoxia. Another potential explanation is that the head movements during feeding are also used for ventilation, possibly creating convective gas exchange by compressing tracheae as the hydrostatic skeleton changes shape with each rhythmic movement (Westneat et al., 2003). Hemolymph pressure changes could also be driven by contractions of intersegmental (Slama, 1999) or heart (Smits et al., 2000) muscles that would generate convective gas flow and increase during hypoxia. Lastly, caterpillars may increase spiracular opening and/or remove tracheolar fluid to increase gas exchange (Wigglesworth, 1981).

Implications

The decreased P_c values and G_{max} within an instar support the hypothesis that O_2 may play a role in triggering larval to larval molting in insects (Figs 4 and 5). Also, since the late-stage animals we measured were at least one day away from molting, the effect of within-instar development on P_c and G_{max} may be even more striking as animals more closely approach ecdysis. We hypothesize that as animals near the end of an instar, internal P_{O_2} decreases, providing a signal for the initiation of the molting pathway. The signaling cascade is currently thought to begin with a decline in ecdysteroids, which stimulates release of pre-ecdysis-triggering hormone (PETH) (Zitnan and Adams, 2000) and ecdysis-triggering hormone (ETH) from epitracheal glands (Zitnan et al., 1996). Eclosion hormone (EH) is then released by ETH and a positive feedback system occurs between EH and ETH (Ewer et al., 1997). However, it is unclear what the initial trigger of the pathway is. In one investigation, Nijhout (1975) found that in *M. sexta* molting is initiated by achievement of a critical body size, a finding potentially consistent with internal gas tensions being important as a trigger. The involvement of O_2 in the signal transduction pathway to molting is supported by the findings of Greenberg and Ar (1996) who found that in mealworms the number of molts was inversely proportional to P_{O_2}. The $P_{c\text{-}CO_2}$ in late-stage animals was approximately 15 kPa, indicating that a small safety margin for O_2 delivery still exists at this time. However, the late-stage animals we measured were at least one day away from molting, with approximately 50% more mass to be gained (Goodman et al., 1985). Thus, it seems possible that the safety margin for O_2 delivery may disappear completely in the last hours before ecdysis, providing an ultimate if not proximate explanation for the necessity of molting.

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