

Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis

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Edited* by Mary Jane West-Eberhard, Smithsonian Tropical Research Institute, Ciudad Universitaria, Costa Rica, and approved July 28, 2011 (received for review April 27, 2011)

Body size profoundly affects many aspects of animal biology, including metamorphosis, allometry, size-dependent alternative pathways of gene expression, and the social and ecological roles of individuals. However, regulation of body size is one of the fundamental unsolved problems in developmental biology. The control of body size requires a mechanism that assesses size and stops growth within a characteristic range of sizes. Under normal growth conditions in *Manduca sexta*, the endocrine cascade that causes the brain to initiate metamorphosis starts when the larva reaches a critical weight. Metamorphosis is initiated by a size-sensing mechanism, but the nature of this mechanism has remained elusive. Here we show that this size-sensing mechanism depends on the limited ability of a fixed tracheal system to sustain the oxygen supply to a growing individual. As body mass increases, the demand for oxygen also increases, but the fixed tracheal system does not allow a corresponding increase in oxygen supply. We show that interinstar molting has the same size-related oxygen-dependent mechanism of regulation as metamorphosis. We show that low oxygen tension induces molting at smaller body size, consistent with the hypothesis that under normal growth conditions, body size is regulated by a mechanism that senses oxygen limitation. We also found that under poor growth conditions, larvae may never attain the critical weight but eventually molt regardless. We show that under these conditions, larvae do not use the critical weight mechanism, but instead use a size-independent mechanism that is independent of the brain.

Most species of animals experience determinate growth and stop growing within a characteristic range of body size (1–4). This raises the question of how body size is sensed such that it is regulated within this range. How body size is sensed remains one of the fundamental unsolved problems in developmental biology.

In insect larvae, the signal to stop growing and initiate a molt is the secretion of the steroid hormone ecdysone. Larval-larval molts are caused by pulses of ecdysone, and in the last larval stage of the tobacco hornworm, *Manduca sexta*, ecdysone causes the larva to stop feeding and initiate metamorphosis (5). The timing of ecdysone secretion is determined by the critical weight (6–9), a size threshold at which the endocrine events that eventually result in the cessation of feeding, entry into the wandering stage, and metamorphosis are initiated. The discovery of the critical weight demonstrated that initiation of the metamorphic molt depends not on instar duration or growth rate, but rather on size.

The time course of these endocrine events is independent of further nutrition and growth. Therefore, the critical weight can be operationally assessed as the weight at which nutrition is no longer necessary for a normal time course to the cessation of feeding, entry into the wandering stage, and the pupal molt (5, 6, 9). The weight that we measure is a proxy for a physiological variable that is correlated with mass, such as the volume of metabolizing tissues. Studies of genetic strains with different adult body sizes have shown that the critical weight in the last

(fifth) larval instar is proportional to the initial weight of that instar (6).

Results and Discussion

Here we report that under normal rearing conditions, larval-larval molts are also initiated based on a size-sensing mechanism. Regressing the critical weight of the instars on the initial size of the instar gives a regression coefficient of 4.82 ($r^2 = 0.996$), demonstrating that in each instar, the critical weight is 4.82 times the initial size of the instar (Fig. 1). This suggests that there is a common mechanism in every instar that triggers an ecdysone event (a larval-larval molt or cessation of feeding, followed by the pupal molt) once a particular size is reached, and that this critical size is measured relative to some property that is set at the beginning of each instar.

The most obvious candidate properties are those associated with the new external skeleton that is deposited at each molt. In caterpillars, the soft exoskeleton of the body wall is not fixed in size but rather grows throughout the instar; thus, it is not a good candidate for limiting growth (10). Although the head capsule does not grow and thus could constrain growth by limiting the food ingestion rate, absorption in the gut, not the ingestion rate, is the rate-limiting step for growth in *M. sexta* (11). The spiracles and tracheal system deposited at each molt could constrain growth by limiting oxygen delivery. A large body of work shows that oxygen levels influence body size in insects (12–19). Hypoxia not only reduces body size (18), but also constrains the evolution of increased body size by limiting the variation available to selection (16).

In the late stage of each larval instar, the respiration rates of *M. sexta* caterpillars come close to the maximum oxygen delivery rate allowed by the tracheal system of that instar (20). Similar constraints have been observed in grasshoppers (21). Given that the tracheae could be limiting oxygen supply, and that insects seem to be nearing the maximum delivery rate of oxygen, we hypothesized that critical weight in each instar could be the size at which oxygen delivery becomes compromised.

We measured the volume of the tracheal system and found that it does not increase during the course of each instar, but instead increases discretely at each molt (Fig. 2). The methods that we used did not permit assessment of the tracheoles, which might proliferate during the intermolt period; however, the tracheoles do not become functional until the following molt. We found that the tracheal volume at the beginning of each instar scaled with an exponent of 1.04 with respect to the initial mass of the instar (Fig. 2). Thus, the tracheal system scales essentially

Author contributions: V.C. and H.F.N. designed research; V.C. performed research; V.C. and H.F.N. analyzed data; and V.C. and H.F.N. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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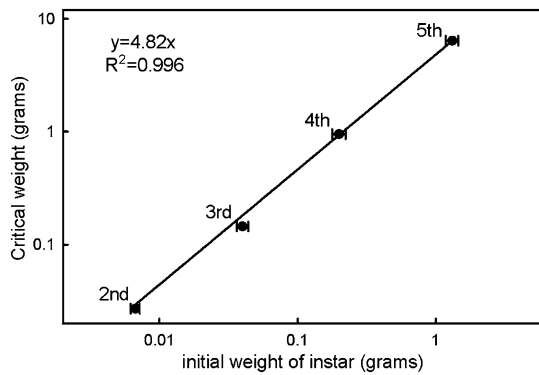


Fig. 1. Critical weights are proportional to the initial weight of the instar. Critical weights were estimated by means of starvation experiments. Bars are SDs.

isometrically with total body volume at the beginning of each successive instar.

Early in each instar, the tracheae are convoluted and thus have built-in slack to allow extension of the tracheal tubes as the body grows (Fig. 3). As the larval body grows, the tracheal system becomes more sparsely distributed (Fig. 3), because the same tracheal distribution network must supply a much larger volume of tissue. Tracheal size set at the beginning of the instar should limit respiration rates as body mass increases within instars.

We measured the respiration rate of third, fourth, and fifth instar larvae and found that it initially increased linearly with body mass up to when the larvae reached the critical weight, after which it remained constant (Fig. 4), possibly because it was close to the maximum oxygen delivery rate permitted by the dimensions of the tracheal systems. At the critical weight, oxygen delivery limits growth; indeed, the growth rate of *M. sexta* larvae was found to follow an sigmoid trajectory with the inflection point at the critical weight, after which the growth rate gradually declines (6). Moreover, Greenlee and Harrison (9) found that late-instar *Manduca* caterpillars were not able to maintain an adequate oxygen supply at lower oxygen tensions. Thus, late-instar caterpillars consume oxygen at the maximum oxygen delivery rate permitted by the dimensions of their tracheal systems. These findings suggest that in each instar, the larvae have an oxygen delivery ceiling imposed by the constant dimension of the

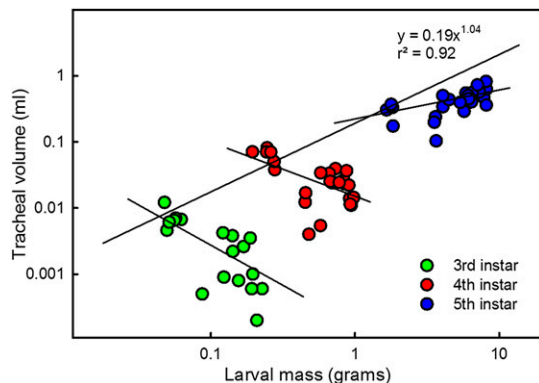


Fig. 2. The tracheal system does not grow within each instar, but increases in size discretely at each molt. There is an apparent slight decrease in tracheal volume during the third and fourth instars. Regression line is for the initial volumes in the instar for data points between 6 and 12 h after ecdysis. Each point represents a single individual.

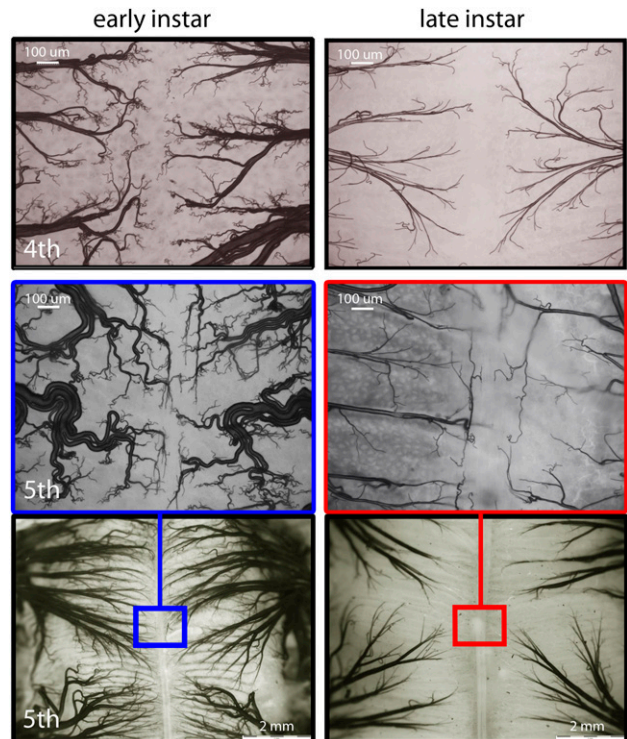


Fig. 3. The tracheal system does not grow within each instar, but the body does. Photos show the distribution of the tracheal network along the midline of the midgut at the beginning and end of the fourth (Top) and fifth (Middle and Bottom) larval instars.

tracheal system, and that the mechanism that senses this ceiling defines the critical weight.

If oxygen supply is limiting, then the constraint should be lifted when larvae are exposed to hyperoxia. To test this hypothesis, we measured the respiration rate of fourth instar larvae, above the critical weight, that were switched from normoxic to hyperoxic conditions and found that their rate of oxygen consumption increased by $36.9 \pm 6.2\%$ (SEM, $n = 6$; $P = 0.0019$, t test).

We reasoned that if the critical weight indeed corresponded to the size at which the oxygen supply becomes limiting, then it should be possible to alter the critical weight by changing the oxygen tension in the environment of the larva. We also found that fourth instar larvae kept under 5% hypoxia (5% oxygen and 95% nitrogen) from the beginning of the fourth instar molted to the next instar at a weight well below that of normoxic larvae (Fig. 5). Indeed, they molted at weight below the normoxic critical weight of 1.1 g. Likewise, fifth instar larvae in 5% hypoxia from the beginning of the fifth instar exposed their dorsal vessels at significantly lower weights than normoxic controls (Fig. 5), and close to the normoxic critical weight of ~ 7.0 g. Thus larval-larval molts, like the metamorphic molt, are initiated at a critical size, and the two kinds of molts appear to be controlled by the same mechanism.

Hyperoxic fourth instar larvae molted at modestly, but nonetheless statistically significantly, higher weights than normoxic larvae (Fig. 5; $P = 0.008$). In contrast, hyperoxic fifth instar larvae molted at approximately the same weight as normoxic larvae (Fig. 5; $P = 0.236$). Thus, hyperoxia had less effect on body size than did hypoxia. Our results are consistent with previous work on the effects of oxygen levels on body size showing that hypoxia has a stronger and more consistent effect on body size, whereas the response to hyperoxia is variable, weak, and sometimes nonlinear (16, 18, 19, 22). The inability of hyperoxic

reducing the length of the path over which oxygen needs to diffuse to reach internal tissues (33, 34). It has been suggested that the evolution of gigantic insects in the Paleozoic was associated with a high level of atmospheric oxygen (13, 17, 18).

The molting cycle in insects is generally assumed to have evolved as a mechanism to deal with a nongrowing external skeleton. Our results show that the nongrowing tracheal system also limits growth, by setting a ceiling on the rate at which oxygen can be supplied to growing tissues (Fig. 4).

Materials and Methods

Animals. Larvae of *M. sexta* were reared on an artificial diet in individual containers at 27 °C under a long-day (16-h light/8-h dark) photoperiod.

Tracheal Morphology. The gut tracheae were chosen because they are easily visible and consistently identifiable (thereby allowing comparisons between individuals). Tracheae were examined using two imaging methods. First, tracheae were stained, and then the larvae were dissected. The tracheae were filled under vacuum with a cobalt solution and then precipitated as black cobalt sulfide (35). Low-magnification images were obtained with an Olympus DP17 camera mounted on an Olympus SZX16 stereo microscope, using Olympus Microsuite Biological Suite software for Imaging Applications. Second, unstained tissue from the dorsal midline of the midgut mounted on a microscope slide was examined at high magnification. These preparations were photographed with a Hamamatsu Orca-ER C4742-95 digital camera mounted on a Leica DMRBE compound microscope, using Simple PCI imaging software (Hamamatsu). In all images, the brightness and contrast were adjusted using the "auto-levels" tool in Adobe Photoshop CS5.

Tracheal Volume Measurement. Tracheal measurements were done using the water displacement technique of Wigglesworth (35). Larvae were weighed, immersed in water containing 0.1% detergent (Tween), and then placed in a vacuum desiccator attached to a MaximaDry vacuum pump (Fisher Scientific). A vacuum was maintained for 15 min, after which the vessel was allowed to return to atmospheric pressure. As the pressure in the vessel increased, water entered the evacuated tracheal system. The larvae were then dried with a paper towel and reweighed. The difference from the initial weight was taken as the volume of the tracheal system. This method gives an accurate measure of the tracheal volume and gives the same results as the inert gas displacement method of Lease et al. (22); however, this method likely is not able to measure the volume of the finest tracheoles.

Respiration. Respiration rates for third, fourth, and fifth instar larvae were measured using a constant-pressure respirometer. Measurements were done in a temperature-controlled room at 25 °C. The respirometer was constructed from a 15-mL test tube with a 1-mL pipette inserted in a one-hole stopper. A drop of colored water served as the volumetric marker. CO₂ was absorbed by a wad of paper towel soaked in a 20% solution of potassium hydroxide in the test tube. A control tube, identical in all respects except for the presence of an experimental animal, served as a monitor for effects of variation in atmospheric pressure and vessel temperature. After inserting a larva in an experimental tube and sealing the control tube, we waited for 10 min until the volume of the control tube had stabilized, then measured the rate of oxygen consumption in the experimental tubes at 1 min intervals to the closest 0.01 mL. The respiration rate was measured for 1–2 h for the third instars, for 0.5–1 h for the fourth instars, and for 15–20 min for the fifth

instars. In all cases, there was no change in the volume of the control tubes during the measurement period.

Critical Weight Determination. Larvae of the fourth and fifth instars were weighed at body masses 0.4–1.4 g (fourth instar) and 4.0–9.0 g (fifth instar). Approximately half of the larvae were allowed to continue feeding, and the rest were starved by removing their food and replacing it with a block of 3% agar. Larvae were checked daily for slippage of the head capsule (fourth instars) or gut purge (fifth instars). Head capsule slippage occurs at 13–16 h after the secretion of ecdysone (24) in the fourth instar, and gut purge occurs at ~12 h after secretion of ecdysone in the fifth instar (36, 37). The mass at starvation, date of starvation, and date of head capsule slippage or gut purge were recorded for each larva.

Larvae were grouped by 0.1-g weight increments in the fourth instar and by 1.0-g weight increments in the fifth instar, and the average time to molt (or gut purge) was calculated. SEs were calculated as well. For each weight class, a *t* test was performed to determine whether the time to molt (or gut purge) was statistically different between starved and fed larvae. The critical weight was estimated as the smallest weight at which there was no statistical difference in timing between feeding and starved larvae. Approximately 100 animals in the fourth instar and 140 animals in the fifth instar were used for the critical weight experiments in normoxia (Fig. 6). In the earlier instars (Fig. 1), the critical weight experiment was done as described above, except that the larvae were examined for head capsule slippage (second instar, *n* = 72; third instar, *n* = 92; fourth instar, *n* = 146).

Hypoxia and Hyperoxia. Larvae were introduced into a Plexiglas glove box (Scienceware) when they were head capsule-slipped from the third to fourth instar (hypoxic fourth instar larvae) or head capsule-slipped from the fourth to fifth instar (hypoxic fifth instar larvae). After larvae were introduced, the glove box was sealed and flushed for 60 min with a mixture of either 10% oxygen:90% nitrogen, 5% oxygen:95% nitrogen, or 40% oxygen:60% nitrogen (National Welders). After the appropriate level of oxygen was achieved the flow of gas was reduced to 250 mL/min for the duration of the experiment. A ToxiRaell oxygen analyzer (RAE Systems) was used to continuously monitor oxygen levels in the glove box. A ScoutPro electronic balance (Ohaus) within the glove box was used to weigh larvae daily to the nearest 0.01 g.

Critical Weight in Hypoxia. Critical weight experiments in hypoxia were conducted in the same manner as in normoxia, except that larvae were in the glove box under various oxygen tensions. All larvae were introduced into the relevant atmosphere during head capsule slippage of the molt that led to the instar for which the critical weight was measured. For each graph, there were 55 larvae in fourth instar and 74 larvae in fifth instar under 10% oxygen and 70 larvae in fourth instar and 60 larvae in fifth instar under 5% oxygen.

Neck Ligations. Larvae to be neck ligated were deeply anesthetized with CO₂, and a blood-tight ligature was placed behind the head capsule using waxed dental floss. This ligature isolated both the brain and subesophageal ganglion from the rest of the body. Because the head contains no spiracles, it dies soon after this operation.

ACKNOWLEDGMENTS. We thank A. Moczek, K. Preuss, W. Smith, and Y. Suzuki for helpful suggestions and comments on the manuscript; R. Dilling and I. Jalli for discussions; and C. Shreve and L. Grunert for assistance throughout the project. This research was funded by National Science Foundation Grants IOS-0641144 and IOS-0744952 (to H.F.N.) and by Sigma Xi Grants in Aid of Research G2009150531 and G20101015155261 (to V.C.).

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