RESEARCH ARTICLE

The role of reduced oxygen in the developmental physiology of growth and metamorphosis initiation in Drosophila melanogaster

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SUMMARY

Rearing oxygen level is known to affect final body size in a variety of insects, but the physiological mechanisms by which oxygen affects size are incompletely understood. In Manduca sexta and Drosophila melanogaster, the larval size at which metamorphosis is initiated largely determines adult size, and metamorphosis is initiated when larvae attain a critical mass. We hypothesized that oxygen effects on final size might be mediated by oxygen effects on the critical weight and the ecdysone titers, which regulate growth rate and the timing of developmental transitions. Our results showed that oxygen affected critical weight, the basal ecdysone titers and the timing of the ecdysone peak, providing clear evidence that oxygen affected growth rate and developmental rate. Hypoxic third instar larvae (10% oxygen) exhibited a reduced critical weight, slower growth rate, delayed pupariation, elevated baseline ecdysone levels and a delayed ecdysone peak that occurred at a lower larval mass. Hyperoxic larvae exhibited increased basal ecdysone levels, but no change in critical weight compared with normoxic larvae and no significant change in timing of pupariation. Previous studies have shown that nutrition is crucial for regulating growth rate and the timing of developmental transitions. Here we show that oxygen level is one of multiple cues that together regulate adult size and the timing and dynamics of growth, developmental rate and ecdysone signaling.

INTRODUCTION

Body size impacts almost every aspect of organismal function, but the mechanism of size regulation remains one of the fundamental unsolved problems in developmental biology. Holometabolous insects, in particular Manduca sexta and Drosophila melanogaster, are respectively the physiological and genetic model organisms for the study of size regulation in animals. Because insects do not grow as adults, the time and size at which a larva ceases growing and initiates its metamorphic molt strongly impacts its adult size (Layalle et al., 2008; Nijhout et al., 2006). In M. sexta (Nijhout and Williams, 1974) and D. melanogaster (Mirth and Riddiford, 2007; Mirth et al., 2005; Shingleton, 2005; Shingleton, 2010), it has been hypothesized that a key parameter in insect size regulation is the critical weight. Critical weight is operationally defined as the size beyond which further nutrition is not necessary for a normal time course to pupariation (Davidowitz et al., 2003; Nijhout and Williams, 1974).

Many of the hormonal mechanisms that regulate molting and that occur after attainment of critical weight have been elucidated. Molting is a response to an increase in the level of circulating ecdysone, which is produced by the prothoracic glands (PGs) (Zitnan and Adams, 2005). The synthesis of ecdysone is regulated by a number of factors, including prothoracotrophic hormone (PTTH) and insulin-like peptides, both produced by the brain. Recent research has made great progress in identifying the signaling pathways that control ecdysone synthesis in the PGs of D. melanogaster (Colombani et al., 2005; Mirth et al., 2005). Although the molecular mechanisms that regulate ecdysone synthesis and secretion are fairly well elucidated, the physiological mechanism that triggers ecdysone at the right time and right size are not known. We hypothesize that decreasing internal oxygen partial pressure (P_{O_2}) might be the physiological mechanism for sensing size and triggering the ecdysone pulse at the right time.

Previous work suggests that oxygen availability changes through the instar, providing both a potential physiological cue and a functional reason to trigger molting in insects. As the insect’s body mass increases during the course of an instar, there is a corresponding increase in oxygen demand by new tissue. The tracheal system is part of the exoskeleton and delivers oxygen to all cells in the body. Some components of the tracheal system (e.g. diameters of the spiracles and major tracheal trunks) are fixed in size at the molt, and remain constant in size within an instar (Beitel and Krasnow, 2000; Callier and Nijhout, 2011). It has been hypothesized that the rising oxygen need combined with partial inflexibility in tracheal morphology could lead to oxygen demand outstripping supply, causing cellular P_{O_2} to decline with time within an instar and trigger molting (Peck and Maddrell, 2005). Consistent with this hypothesis is the observation that late-instar D. melanogaster, Schistocerca americana grasshoppers and M. sexta are more susceptible to hypoxia than early-instar animals (Callier and Nijhout, 2011;
Greenlee and Harrison, 2005; Heinrich et al., 2011). Also consistent with this hypothesis is the observation that metabolic rates rise linearly with age before the critical weight in *M. sexta*, but plateau after the critical weight, consistent with oxygen limitation of metabolism later in the instar (Callier and Nijhout, 2011). Finally, in mealworms, the duration of a juvenile instar is linearly correlated with atmospheric oxygen level (Greenberg and Ar, 1996). In addition, nitric oxide signaling (NOS) is known to be critical for metamorphosis initiation (Cáceres et al., 2011; Yamanaka and O’Connor, 2011). NO-mediated E75 inactivation in the prothoracic gland is necessary to promote ecdysteroidogenesis. NOS can be induced by low-oxygen conditions (Cáceres et al., 2011; Yamanaka and O’Connor, 2011). This provides a potential molecular mechanism by which declining internal oxygen levels might modulate ecdysteroidogenesis, and therefore growth rate and developmental transitions.

We hypothesized that falling internal $P_{O_2}$ could be a physiological cue by which a *D. melanogaster* larva senses its size and initiates the hormonal cascade for metamorphosis. If this hypothesis is correct, then lower rearing oxygen levels should decrease the critical weight and accelerate ecdysone secretion and development rate. In contrast, higher rearing oxygen levels should increase the critical weight and maximal larval mass, delay the ecdysone peak and pupariation, and cause the ecdysone peak to occur at heavier masses. To test this hypothesis, we assessed the effect of atmospheric oxygen level (normoxia: 21% $O_2$, hypoxia: 10% $O_2$, hyperoxia: 30% $O_2$) on critical weight, growth, the timing of pupariation and attainment of maximal larval mass, and the dynamics of the ecdysoid gland in third instar *D. melanogaster* larvae. By exposing the larvae to altered oxygen only during the third instar, we hoped to avoid compensatory changes in tracheation that might minimize effects of altered oxygen (Henry and Harrison, 2004; Jarecki et al., 1999). In this way we could determine (1) whether oxygen limitation affects the critical weight, and (2) whether a shift in critical weight corresponds to a shift in underlying hormonal physiology that regulates molting.

**MATERIALS AND METHODS**

**Flies**

All *D. melanogaster* larvae used in the study were from an isogenic stock of Samarkand (Sam) strain kept as a breeding colony of ~3000 individuals across ~20 vials. All flies were reared on standard cornmeal molasses diet (Stieper et al., 2008).

**Measurement of critical weight**

Critical weight was assayed at 10, 21 and 30% $O_2$, using the method described in Stieper et al. (Stieper et al., 2008). Larvae from eggs oviposited in normoxia over 24 h were reared at low density (<200 per dish) on standard cornmeal molasses in eight 50 mm Petri dishes at 27°C, continuous light and 60% humidity. After 60 h, all third instar (L3) larvae were cleared from each dish. The dishes with their remaining larvae were then placed in a clear polycarbonate glove box and aerated at one of the test oxygen levels (10, 21 or 30%). This ensured that all experimental larvae eclosed to the third instar at the test oxygen level, but that they spent the majority of the second instar in normoxia. Oxygen conditions were regulated with a Roxy-8 Oxygen Regulator (Sable Systems, Las Vegas, NV, USA).

Every 4 h after transfer to the test oxygen conditions, 40–50 L3 larvae were sampled from each dish, weighed and starved individually in a ventilated 1.5 ml Eppendorf tube containing ~0.5 ml of 1.5% agar to prevent larval dehydration. Thus we collected and starved ~300 larvae at each oxygen level across a full range of third instar sizes. Larvae were then checked every 4 h for pupariation and, if pupariated, re-weighed 48 h later. Consequently, for each pupating larva we had a mass prior to starvation, time to pupariation (TTP; ±2 h) after starvation, and resulting pupal mass.

We used the ‘breakpoint’ method to calculate critical size at each oxygen level, as described previously (Ghosh et al., 2013; Stieper et al., 2008). In *D. melanogaster*, starvation before attainment of critical weight leads to a much greater developmental delay than starvation after attainment of critical weight (Stieper et al., 2008). Consequently, a plot of larval mass at starvation and TTP shows a change in slope, or breakpoint, at the critical weight, which can be detected using a bi-segmented linear regression. Because of high levels of variation in wet mass among larvae of ostensibly the same age, the performance of the bi-segmented linear regression is improved if one uses the relationship between pupal mass and TTP to find the breakpoint, and then uses the relationship between pupal mass and larval mass at starvation to convert the breakpoint to a larval critical weight (Stieper et al., 2008). The performance of the bi-segmental linear regression is further improved if the plot of pupal mass versus TTP is rotated 5 rad around the origin prior to analysis (Stieper et al., 2008). Consequently, we: (1) plotted the relationship between pupal mass and TTP; (2) rotated the plot +5 rad around the origin; (3) detected the pupal mass and TTP at the breakpoint in the plot using the ‘segmented’ package in R (Muggeo, 2008); (4) back-rotated these values −5 rad; and (5) converted the pupal mass at the breakpoint to a larval critical weight using the linear relationship between (log) larval mass at starvation and (log) pupal mass. The significance of the breakpoint was tested using a Davies test and the position of the breakpoint was determined using the ‘segmented’ package in R (Muggeo, 2008). We generated 95% confidence intervals for the critical weight and the TTP at critical weight by repeating the analysis on 1000 bootstrap data sets. To test for pairwise differences in critical weight and TTP at critical weight between any two oxygen levels, we permuted the data 1000 times to generate a null distribution of the difference and used this to generate a two-directional P-value for the observed difference. Significance for the observed pairwise difference was Bonferroni-corrected to *P*<0.0167.

**Growth curves and collection of larvae for ecdysone assay**

Flies were allowed to lay eggs for a 2 h period as described above. At 36 h after egg collection, larvae were redistributed into 50 mm food plates at a density of 100 larvae per plate. Larvae were allowed to grow in normoxia until the L2–L3 molt. At ~60 h after oviposition, larvae that molted to the third instar within 1 h were grouped together and placed into the different oxygen treatments. From 0 to 62 h after ecysis to the third instar, 60–150 larvae were sampled and weighed at 4 h intervals. We weighed pupae 48 h after pupariation. Larvae were then stored in methanol at −80°C for further processing. In total, the sample sizes of larvae weighed and assayed for ecdysone are as follows: 1536 larvae in hyperoxia, 1947 larvae in hypoxia and 1417 larvae in normoxia.

**Analysis of growth curves**

All larval and pupal mass data were log-transformed prior to analysis to ensure homoscedasticity and normality. Peak larval mass was set as the average observed 32–44 h after eclosion, a time period when all of the oxygen treatments yielded cohorts with masses not significantly different from the cohort with the heaviest mass for that oxygen treatment. The median timing of pupariation was determined by constructing a logistic regression of pupariation state...
against age and using the regression equation to estimate the age at which 50% of larvae had pupated.

**Ecdysone assays**

Ecdysone assays were performed in the Brent laboratory at the Arid Land Agricultural Research Center (Maricopa, AZ, USA), with a method modified from a prior study (Brent and Dolezal, 2009). Larvae from the growth curve study were partitioned into groups of 40–60, then stored in microcentrifuge tubes containing 500 μl methanol. Larval tissue was homogenized and then centrifuged at 17,000 g for 10 min. After collecting the supernatant, residual ecdysteroids were collected from the pellet with a second methanol extraction. The pooled supernatant was lyophilized, resuspended in 200 μl methanol and stored at −80°C until analysis. Ecdysone was extracted from the storage methanol so that none was lost in storage.

Duplicate 10 μl aliquots of each sample were incubated overnight in borosilicate glass culture tubes (6×50 mm) with 100 μl of [3H]-20-hydroxyecdysone stock (1 mg ml⁻¹; Perkin-Elmer, Waltham, MA, USA) in borate buffer, and 100 μl of a polyclonal ecdysteroid antiserum (H-22 antibody, L. Gilbert, UNC-CH, Chapel Hill, NC, USA) at 4°C on an orbital shaker. To minimize intra- and inter-assay variability, new standard competition curves were generated for each set of samples run, using 20-hydroxyecdysone stock (Sigma-Aldrich, St Louis, MO, USA) in quantities from 15.6 to 250 pg, a range that was well within the detection limits. After 18 h, 20 μl of cleaned Protein A Solution (Pansorbin, CalBiochem, San Diego, CA, USA) was added to each tube to precipitate the complex during another hour of incubation at room temperature. Samples were then centrifuged at 5000 g and the remaining pellet was washed twice with 100 μl borate buffer. The pellet was resuspended in two 50 μl washes of double-distilled H₂O and transferred to a scintillation tube containing 3 ml of ScintiSafe 30% (Fisher Chemical, Waltham, MA, USA). The incorporation of microlabel was determined using a 2450 MicroBeta2 scintillation counter (Perkin-Elmer). The concentration of ecdysone was calculated in 20-hydroxyecdysone equivalents estimated by nonlinear regression of the standard curve (Brent et al., 2006) and adjusting for the cross-reactivity of the H-22 antibody (Warren and Gilbert, 1986). Log-transformed ecdysone titers were...
Rearing larvae in hypoxic conditions (10% O2) significantly reduced critical weight relative to normoxic conditions (21% O2) (permutation test, \(P < 0.001\)). Critical weight for larvae reared in hyperoxic conditions (30% O2) was intermediate between critical weights for larvae reared in hypoxic and normoxic conditions, but not significantly different from either (permutation test, \(P > 0.05\); Fig. 1A, B). The TTP of larvae for which starvation was initiated at the critical weight was extended in those exposed to both the hypoxic and hyperoxic conditions relative to those under normoxia, although only significantly in hypoxic larvae (permutation test, hypoxia vs normoxia, \(P=0.005\), hyperoxia vs normoxia, \(P=0.063\); Fig. 1C).

Oxygen level also significantly influenced growth during the final larval instar: growth was not linear, but larval masses did not have overlapping 95% confidence intervals. Growth tended to follow a characteristic increase to a peak larval mass with a subsequent plateau or decline in mass as larvae stopped feeding and searched for a place to pupariate (wandering larval phase) (Fig. 2). However, larval age and oxygen treatment showed strong and nonlinear interaction effects on growth patterns. Larvae reared under hyperoxia had similar masses as the hypoxic larvae early in the instar, but reached masses similar to the normoxic larvae later in the instar (Fig. 2). Hypoxic larvae grew more slowly and reached a lower peak larval mass (Figs 2, 3).

Oxygen level also significantly affected pupal mass (ANOVA, \(P<0.001\); Fig. 3). The specific effects of oxygen level on pupal mass paralleled the effects on larval mass: compared with larvae and pupae reared under normoxia, those reared under hypoxia weighed less while those reared under hyperoxia showed no difference. Oxygen level also affected the timing of pupariation (logistic regression, likelihood ratio test, \(P<0.001\)), with hypoxic larvae pupariating later and hyperoxic larvae pupariating sooner relative to normoxic larvae (Fig. 4, Table 1).

**RESULTS**

**Oxygen effects on growth and critical weight**

Rearing larvae in hypoxic conditions (10% O2) significantly reduced critical weight relative to normoxic conditions (21% O2) (permutation test, \(P<0.001\)). Critical weight for larvae reared in hyperoxic conditions (30% O2) was intermediate between critical weights for larvae reared in hypoxic and normoxic conditions, but not significantly different from either (permutation test, \(P>0.2\) for both; Fig. 1A, B). The TTP of larvae for which starvation was initiated at the critical weight was extended in those exposed to both the hypoxic and hyperoxic conditions relative to those under normoxia, although only significantly in hypoxic larvae (permutation test, hypoxia vs normoxia, \(P=0.005\), hyperoxia vs normoxia, \(P=0.063\); Fig. 1C).

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**Oxygen effects on ecdysone titer**

Oxygen had a significant effect on the ecdysone titer, and this effect depended on the age and mass of the larvae (ANOVA, oxygen level x age, \(P<0.001\); Fig. 5). Normoxic larvae showed the characteristic developmental pattern for ecdysone: low concentrations at the beginning of the instar followed by a strong and linear increase in concentration with time during the wandering stage, when masses of the cohorts decrease (Warren et al., 2006).
In hypoxic conditions, basal ecdysone levels were elevated (Fig. 5B). The peak in ecdysone concentration at the end of the instar was clearly shifted to occur later and in smaller larvae (Fig. 5A,C, supplementary material Fig. S1).

In hyperoxic conditions, basal ecdysone levels were also elevated relative to normoxic conditions, but not as strongly as for hypoxic larvae (Fig. 5B). The peak in ecdysone levels could not be distinguished statistically from that of the normoxic larvae (Fig. 5A,C, supplementary material Fig. S1).

Baseline ecdysone levels (values averaged before the ecdysone peak) were also strongly affected by rearing oxygen, with baseline levels highest in hypoxic larvae and intermediate in hyperoxic larvae (ANOVA, oxygen level, \( P < 0.001 \); Fig. 5B).

**DISCUSSION**

**Oxygen modulates developmental rate**

Our data provide clear evidence that reduced oxygen modulates growth and the timing of developmental transitions in *D. melanogaster*. Larvae reared in low oxygen exhibited a reduced critical weight, suggesting that hypoxia shifted the set point for initiation of the neuroendocrine cascade that leads to cessation of feeding and wandering. Hypoxia also reduced the mass at which the ecdysone peak occurred. While hyperoxia did not affect the critical weight or the timing of the ecdysone peak, it did decrease the time from attainment of maximal larval mass to pupariation, suggesting that hyperoxia benefits processes occurring during wandering or pupariation.

The lack of an effect of hyperoxia on critical weight, maximal larval mass and the timing of the ecdysone peak can be interpreted in multiple ways. One interpretation is that, normally, there is a sufficient safety margin for oxygen delivery, so hyperoxia provides no benefit. Another possible interpretation is that the 30% oxygen treatment is sufficiently high to induce stress responses that would cancel any growth-stimulating effect of hyperoxia.

Hypoxia strongly suppressed growth rates, and this likely explains the extended development time in hypoxia. Our data suggest that oxygen lowers the critical weight but also influences the duration of the terminal growth period. Thus, despite hypoxic larvae attaining the critical weight at a smaller size, their slow growth rate and extended terminal growth period still results in

<table>
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<tr>
<th>Parameter</th>
<th>Hypoxia Predicted</th>
<th>Hypoxia Observed</th>
<th>Hyperoxia Predicted</th>
<th>Hyperoxia Observed</th>
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<td>critical weight</td>
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<tr>
<td>Growth rate</td>
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<td>Increased</td>
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<tr>
<td>Time to pupariation</td>
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<td>Delayed</td>
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<tr>
<td>Ecdysone titer</td>
<td>No change in basal titer; earlier pupariation peak</td>
<td>Increased basal titer; delayed and subdued pupariation peak</td>
<td>No change in basal titer; delayed pupariation peak</td>
<td>Increased basal titer; pupariation peak not significantly different from normoxia</td>
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Table 1. Comparison of observed and predicted responses based on the hypothesis that declining internal oxygen later in the instar limits growth/metabolism and causes molting

![Graph](image-url)
Oxygen affects growth and metamorphosis

Diversity in the mechanisms that underlie cessation of growth in insects

*Drosophila* and *Manduca* have been the major model organisms for the study of size control, but differences in underlying physiology between critical weight in these two organisms are becoming increasingly apparent. In *D. melanogaster*, insulin signaling in the prothoracic gland regulates ecdysone secretion and critical weight, but in *M. sexta*, insulin signaling is insufficient to stimulate ecdysone secretion from the prothoracic gland (Walsh and Smith, 2011) and low nutrition causes a disappearance of the critical weight phenomenon (Davidowitz et al., 2003) rather than a reduction in critical weight. Juvenile hormone plays no role in the critical weight of *D. melanogaster* (Riddiford and Ashburner, 1991; Riddiford et al., 2003; Zhou and Riddiford, 2002), whereas it is central to the disinhibition of the hormonal cascade that elicits metamorphosis in *Manduca* (Nijhout, 1994). This suggests that although phenomenologically similar, the critical weight in *M. sexta* and *D. melanogaster* may have different underlying physiological bases.

Broader comparisons also suggest that the mechanisms that determine adult size vary widely among species. Different insects use different cues for the regulation of ecdysone secretion, and these cues are usually ecologically relevant to a specific species: e.g. stretch receptors trigger ecdysone secretion in *Oncopeltus* (Nijhout, 1979), and starvation induces ecdysone secretion in *Onthophagus taurus* dung beetles, as long as they have passed a minimum viable size (Shafiei et al., 2001). *Drosophila melanogaster* also accelerate the timing of pupariation in response to starvation after the critical weight (Stieper et al., 2008), so like dung beetles, they may be exhibiting a bail-out response that is adaptive in their specific ecological context, where food resources are ephemeral. The variety of cues utilized to stimulate ecdysone secretion should come as no surprise because each species exists in a different ecological context. Because different insects respond to different ecologically relevant cues, it seems likely that different insects exhibit developmental checkpoints whose underlying mechanisms are actually quite different. Model insects are probably only models of themselves. Rather than searching for a single size-sensing and determination mechanism in insects, future research should attempt to link size-determination mechanisms with the particular species’ ecological and evolutionary context.

Although the mechanisms underlying growth cessation and ecdysone release in normal conditions are diverse, the effect of low-oxygen rearing conditions is consistent across species (Callier and Nijhout, 2011; Greenberg and Ar, 1996; Harrison and Haddad, 2011; Harrison et al., 2010). This suggests that the physiological mechanisms by which low oxygen decreases growth rate and final size may be shared across species. Our finding that hypoxia shifts the basal ecdysone titer as well as the timing of ecdysone peaks provides a novel and unexpected physiological mechanism for oxygen’s effects on growth and size.
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AUTHOR CONTRIBUTIONS


COMPETING INTERESTS

No competing interests declared.

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