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RESEARCH ARTICLE

HIF signaling in the prothoracic gland regulates growth and development in hypoxia but not normoxia in *Drosophila*

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ABSTRACT

The developmental regulation of body size is a fundamental life-history characteristic that in most animals is tied to the transition from juvenile to adult form. In holometabolous insects, this transition is ostensibly initiated at the attainment of a critical weight in the final larval instar. It has been hypothesized that the size-sensing mechanism used to determine attainment of critical weight exploits oxygen limitation as a larvae grows beyond the oxygen-delivery capacity of its fixed tracheal system; that is, developmentally induced cellular hypoxia initiates the synthesis of the molting hormone ecdysone by the prothoracic gland. We tested this hypothesis in *Drosophila* by assaying cellular hypoxia throughout the third larval instar at 21 and 10 kPa O_2, using the activity of the HIF (hypoxia inducible factor)-signaling pathway as a measure of hypoxia. While HIF signaling was elevated at low levels of environmental O_2, it did not markedly increase during development at either oxygen level, and was only suppressed by hyperoxia after feeding had ceased. Further, changes in HIF signaling in the prothoracic gland alone did not alter body size or developmental time in a way that would be expected if cellular hypoxia in the prothoracic gland was part of the critical weight mechanism. Our data do show, however, that reduced HIF signaling in the prothoracic gland decreases survival and retards development at 10 kPa O_2, suggesting that prothoracic HIF signaling is a necessary part of the beneficial plasticity mechanism that controls growth and development in response to low oxygen level.

KEY WORDS: Hypoxia inducible factor, Hypoxia, Developmental regulation, Body size regulation, Oxygen

INTRODUCTION

Adult body size is a key characteristic of all animal species, and yet within a species, body size can show considerable, sometimes dramatic, variation. Much of this variation is a reflection of the environmental conditions in which an individual grows and develops, a phenomenon called phenotypic plasticity. For example, animals often mature at smaller body sizes in response to poor nutrition, low oxygen level, and – for ectotherms at least – high temperature (Atkinson and Sibly, 1997; Harrison et al., 2015; Koyama and Mirth, 2018; Kutz et al., 2019). Understanding the mechanisms that regulate the effect of the environmental variation on body size is a major goal for studies of growth regulation, with implications for understanding plastic and adaptive responses to anthropogenic environmental change, including climate change (Callier and Nijhout, 2011; Daufresne et al., 2009; Gardner et al., 2011; Mirth and Shingleton, 2019). It is generally thought that stressors, such as high temperature, poor nutrition or hypoxia, mediate development to a smaller body size via stress-sensing mechanisms that interact with the hormonal control of development. Although we have made substantial progress in understanding the mechanisms by which low nutrition affects growth and final body size, the same is not true for the regulation of growth and final body size by temperature or oxygen level (Callier and Nijhout, 2011; Harrison et al., 2015; Koyama and Mirth, 2018). The effect of oxygen level on adult body size is of particular interest, because low oxygen (hypoxia) reduces adult body size in almost all animals, including humans (Harrison et al., 2015; Schols and Westerterp, 2002).

In contrast to our relatively poor understanding of the systemic regulation of body size by oxygen level, the cell-autonomous effects of low oxygen are much better elucidated. HIF (hypoxia inducible factor) signaling is a highly conserved mechanism among eukaryotes by which cells sense and respond to hypoxia (Wang and Semenza, 1993). At the core of HIF signaling is the heterodimeric transcription factor Hypoxia-Inducible Factor 1 (HIF-1), which comprises two subunits: HIF-1α and HIF-1β. At normal oxygen levels, HIF-1α is hydroxylated by HIF-prolyl hydroxylase (Hph) and targeted for degradation. When oxygen levels fall, prolyl hydroxylation is suppressed, HIF-1α binds to HIF-1β, and collectively they drive the expression of genes associated with the cellular response to hypoxia. HIF signaling is well known to stimulate the growth of oxygen-delivery tissues (capillaries and red blood cells in vertebrates, tracheae in insects) and to cause up-regulation of proteins involved in anaerobic ATP production, such as pyruvate dehydrogenase and lactate dehydrogenase (Kim et al., 2006; Semenza, 2011). Further, low oxygen can also act on cells more directly, by limiting cellular respiration and the production of ATP.

In principle, the effects of oxygen on body size can be mediated through the cellular response to oxygen: low oxygen levels activate HIF signaling and reduce cellular respiration and ATP production, slow cell proliferation, decrease organismal growth rate and reduce adult body size. However, several studies suggest that this simple model of the regulation of adult body size by oxygen is not sufficient, at least for the fruit fly *Drosophila melanogaster*. First, the P_{O_2} that reduces *Drosophila* growth rate and adult body size is much higher than the P_{O_2} that reduces aerobic metabolic rate and stimulates lactic acid fermentation, indicating that the reduction in body size is not simply a consequence of suppression of aerobic metabolism and stimulation of anaerobiosis (Kapali et al., 2022). Second, the growth rates of different organs in *Drosophila* show different sensitivities to changes in oxygen level, also suggestive of

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oxygen regulating growth through systemic mechanisms rather than mechanisms that rely on a cellular response to physiological oxygen levels (Kapali et al., 2022).

The hypothesis that the effects of oxygen on growth rate are regulated systemically via hormones is supported by two recent studies. The first study demonstrated that, in *Drosophila*, stimulating a hypoxic response in the fat body alone (by activating HIF signaling organ autonomously) is sufficient to reduce growth by decreasing the release of insulin-like peptides (dILPs) from the brain (Texada et al., 2019). Under normoxic (21% O_2) conditions, circulating dILPs bind to the insulin receptor (InR) of dividing cells, activate insulin/insulin-like growth factor signaling (IIS) and promote cell proliferation. When dILP levels are low as a result of HIF signaling in the fat body, there is a reduction in IIS signaling, slowing growth and reducing adult body size. The second study demonstrated that hypoxic growth suppression also requires the steroid hormone ecdysone (Kapali et al., 2022). Low oxygen (10 kPa O_2) increases the levels of circulating ecdysone, which stimulates the production of the insulin-binding protein ImpL2. This in turn binds dILPs, suppresses IIS in dividing cells and reduces adult body size. Blocking HIF signaling in the fat body, the synthesis of ecdysone or the production of ImpL2 prevents growth suppression in hypoxic conditions and increases growth rate and body size toward levels observed in normoxia (Kapali et al., 2022; Texada et al., 2019). Therefore, in low oxygen, *Drosophila* larvae grow at a slower rate than can be supported metabolically. Thus, the effect of oxygen on growth and final body size is an example of adaptive phenotypic plasticity – the result of selection for the ability of a genotype to express phenotypes that maximize fitness under different environmental condition. This is in contrast to uncontrolled, uncoordinated pathological responses to environmental change that reduce fitness.

It has been suggested that oxygen limitation also plays a role in regulating growth in normoxic conditions. The volume of the major trunks of the tracheal system is thought to be fixed within each instar in holometabolous insects (Callier and Nijhout, 2011; Lundquist et al., 2018) and there is evidence that oxygen supply capacities do not match the rise in oxygen demand later in each larval instar in some but not all insects (Harrison et al., 2018). Several authors have suggested that the resulting functional hypoxia, which we refer to as developmentally-induced hypoxia, is a signal for molting (Callier and Nijhout, 2011; Greenberg and Ar, 1996; Greenlee and Harrison, 2004; Kivelä et al., 2016; Nijhout and Callier, 2015). Specifically, it has been hypothesized developmentally induced hypoxia is the size-sensing mechanism that determines critical weight, the size at which the synthesis of the ecdysone by the prothoracic gland (PG) is ostensibly initiated. Ecdysone levels subsequently rise, which leads to the cessation of growth and metamorphosis, and fixes adult body size. This hypothesis is supported by data showing that attainment of critical weight coincides with the point in development when the tracheal system starts to become limiting for respiration in the tobacco hornworm *Manduca sexta* (Callier and Nijhout, 2011). Additionally, critical weight and larval, pupal and adult body size are reduced by hypoxia and increased by hyperoxia in *M. sexta*, also consistent with the hypothesis (Callier and Nijhout, 2011; Wilmsen and Dzialowski, 2023). However, while hypoxia also reduces critical weight and adult size in *Drosophila*, hyperoxia does not increase critical weight, larval, pupal or adult mass (Callier et al., 2013; Klok et al., 2009). Further, the observation that the tracheal system becomes limiting after attainment of critical weight in *M. sexta* suggests that the critical weight mechanism anticipates hypoxia, but is not a response to it. Thus, the role that limited oxygen delivery plays in regulating developmental timing and growth regulation under normoxic conditions is equivocal and may vary across species.

Here, we tested the hypothesis that limited oxygen delivery as a larva grows – and the resulting functional hypoxia – is used to regulate developmental timing in *Drosophila*, using two approaches. First, we assessed whether there is evidence for an increase in functional hypoxia as the final instar progresses under normoxic conditions by looking at HIF signaling. While it is plausible that moderate hypoxia might occur without HIF signaling being activated, given the strong association between HIF and hypoxic signaling in *Drosophila* (Centanin et al., 2010), a rise in whole-body HIF signaling during ontogeny would provide evidence for increasing functional hypoxia within the instar. The expression of *HIF proly hydroxylase* (*Hph*: *fatiga* in *Drosophila*) is positively regulated by HIF-1α as a negative feedback loop in *Drosophila* and other animals, providing a reporter of HIF signaling (Hardy et al., 2012; Lavista-Llanos et al., 2002). Additionally, in other invertebrates, hypoxia can either positively or negatively regulate the transcription of *HIF-1α* (*sima* in *Drosophila*) (Hardy et al., 2012; Lavista-Llanos et al., 2002; Lundquist et al., 2018; Soñanez-Organis et al., 2009; Sun et al., 2016). We therefore reared *D. melanogaster* in 21 kPa O_2 and measured whole-body expression of *Hph* and *HIF-1α* mRNA and HIF-1α protein, to test

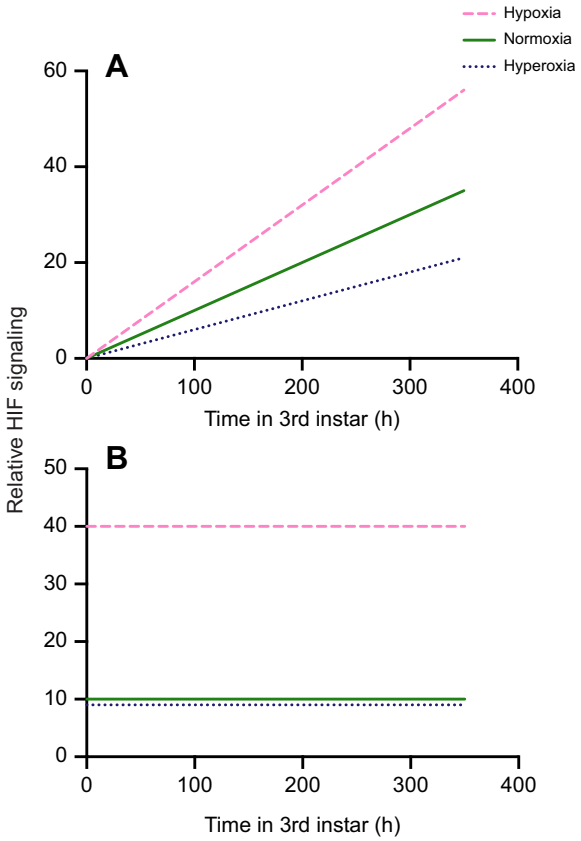

Fig. 1. Predicted HIF signaling under the two scenarios. (A) Prediction for how age during the third instar will affect HIF signaling if functional hypoxia develops through the instar, triggering molting at some threshold. An additional plausible pattern would be that HIF signaling is constant and low across early ages, and then rises above some threshold age/size. (B) Prediction for how age during the third instar will affect HIF signaling if HIF signaling is age independent and there is minimal functional hypoxia in normoxic larvae.

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whether functional hypoxia increased during ontogeny (Fig. 1A). Further, if larvae do use functional hypoxia to regulate developmental timing, then rearing larvae at low oxygen levels (10 kPa O_2) or high oxygen levels (40 kPa O_2) should accelerate and delay HIF signaling, respectively (Fig. 1A). We therefore measured *Hph* and *HIF-1α* expression in larvae reared at 10 kPa O_2 and 40 kPa O_2. Finally, we assayed the expression of *HIF-1β* (*tango* in *Drosophila*), which is a critical component of the transcriptional response to hypoxia and HIF signaling, to determine whether its expression was also developmentally regulated. Conversely, *Drosophila* larvae reared in normoxia may not experience significant functional hypoxia at any point in the instar; this hypothesis predicts that HIF signaling may be increased by hypoxic rearing, but unaffected by age or hyperoxia (Fig. 1B).

Second, we functionally tested whether HIF signaling in the PG specifically regulates developmental timing in response to oxygen level. Even if developmentally induced hypoxia might not occur at the whole -body level, it is possible that hypoxia in specific locations might serve as a developmental trigger. As an example, hypoxia in the fat body can reduce insulin secretion and growth, through the fat body-specific activation of HIF signaling (Texada et al., 2019). While it has been shown that hypoxic rearing increases ecdysone levels, which then slows growth by increasing concentrations of insulin-binding protein (Kapali et al., 2022), the mechanisms by which hypoxia induces a rise in ecdysone secretion and whether this occurs during normal development are unclear. We therefore activated or inhibited HIF signaling in the PG alone to determine whether it affected developmental timing and body size. If reduced oxygen delivery is the size-sensing mechanism that determines critical weight and this is sensed by the PG via HIF signaling, then increasing HIF signaling in the PG alone should cause larvae to overestimate their size. Larvae should consequently accelerate development and eclose early at a reduced body size (Fig. 2). In contrast, reducing HIF signaling in the PG should have the opposite effect (Fig. 2). Additionally, larvae with reduced HIF signaling in the PG may have reduced survival as they grow beyond the capacity of their tracheal system to deliver oxygen to support growth and metabolism.

It is also possible that oxygen sensing by the PG might not be important for regulating normoxic development, but is critical to enable larvae to develop more rapidly and transition to adulthood at a smaller size to escape dangerous hypoxic conditions. If so, down-regulating HIF signaling in the PG during hypoxic rearing should extend development time, increase final body size and (if this plasticity is beneficial) reduce survival.

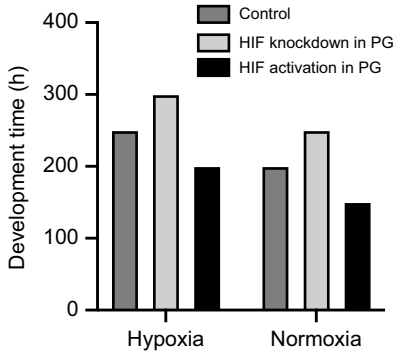

Fig. 2. Predicted effects on developmental time if HIF signaling in the prothoracic gland (PG) stimulates molting in *Drosophila*. Developing in hypoxia is known to slow development. Darkness of bar indicates relative HIF signaling.

MATERIALS AND METHODS

Fly stocks and culture conditions

The following flies were used in this study: *phm-GAL4* (enabling gene manipulation in the PG, a gift of Christen Mirth; Mirth et al., 2005); *UAS-HIF-1α.RNAi* (BDSC_33894, enabling knockdown of *HIF-1α*); *UAS-Hph.RNAi* (BDSC_34717, enabling knockdown of *Hph*); and Samarkand wild-type (BDSC_4270). All constructs were backcrossed into *yw* (BDSC_1495, yellow body, white eyes) for 5 generations to generate coisogenic lines, while a double balancer (BDSC_8204) was used for long-term maintenance of lines. Flies were maintained on standard malt-based cornmeal diet (Fly Food B, LabExpress, Ann Arbor, MI, USA) at 25°C and 21% O_2 in 300 ml bottles.

Staging of larvae for qPCR and western blot analysis

All larvae used for gene and protein expression experiments were from the same population of the Samarkand strain of *D. melanogaster*. Under normoxic conditions, females were allowed to oviposit on Petri dishes containing standard malt-based fly food and larvae were allowed to hatch and develop through the first two larval instars in normoxia. Larvae were then staged at ecdysis to the third larval instar and either immediately frozen or transferred to a new Petri dish to continue development in an oxygen-regulated chamber under one of three oxic conditions: hypoxia (10 kPa O_2), normoxia (21 kPa O_2) or hyperoxia (40 kPa O_2). Oxygen levels were regulated by a ROXY-8 system (Sable Systems International, Las Vegas, NV, USA). Larvae were maintained at these oxygen levels and sampled every 4 h until 48 h after ecdysis to the third larvae instar (AEL3) for qPCR and at 24 h and 48 h for western blot analysis. Sampled larvae were frozen and stored at −80°C. Larvae were collected at Arizona State University and shipped frozen to North Dakota State University.

Gene expression quantification

To quantify gene expression, we pooled 4–5 larvae per age sample, mRNA was extracted using Trizol (Invitrogen, Fisher Scientific, Waltham, MA, USA) and cDNA was generated using an RT-cDNA synthesis kit (Quanta Bioscience, Gaithersburg, MD, USA). To check for genomic DNA contamination, we ran negative control reactions with no reverse transcriptase. We used Perfecta SYBR Super mix (Quanta Biosciences, Gaithersburg, MD, USA) to conduct qPCR using a Stratagene Mx3000p detection system (Stratagene, La Jolla, CA, USA). Primer sequences are provided in Table 1. mRNA copy number was calculated using the standard curve method as previously described (Biga et al., 2013). Standards were generated using 10-fold dilutions of gene-specific targets produced by standard PCR and cloning techniques (Table 2). Experimental samples, standards and negative control reactions were all run in duplicate for each sample.

Western blot analysis

To quantify protein levels, larvae were collected and pooled into three cohorts at 0 h AEL3 (30 larvae pooled for each sample) and three cohorts at each oxygen level at 24 h and 48 h AEL3 (10 larvae pooled for each sample). Each sample was homogenized using an electric tissue homogenizer (IKA, Wilmington, NC, USA) in 100–500 µl of cell lysis buffer (Cell Signaling, Danvers, MA, USA), and centrifuged at 12,000 ***g*** at 4°C for 10 min. The supernatant was tested for total protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

For western blotting, proteins were separated by molecular weight using Bio-Rad 10% pre-cast TGX gels (Bio-Rad

Table 1. *Drosophila melanogaster* qPCR primers

Oligonucleotide name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
Hph (*fatiga*)	GGCACCAATTGGTTTAC	GGATGTCCCTTGAGTCTCATTT
HIF-1α (*sima*)	CGAGTTTAGCGGCAACCAGT	TGTGCGGGGTCCTACTTTCA
HIF-1β (*tango*)	TCGGGCAGGGTGATCTATGT	TCGGGATGGATGTGCTCATAC
Actin	GGACTCGTACGTGGGTGATGA	TCTCCATATCGTCCCAGTTGGT

Laboratories). Separated proteins were transferred to nitrocellulose membranes (TransBlot Turbo Kit, Bio-Rad Laboratories), and blocked in 40 ml of 5% dry milk (HyVee, Des Moines, IA, USA) in TBS with 0.1% Tween 20 (TBS-T) for 1 h on a rocking shaker. After washing 3 times in TBS-T, the membrane was incubated at 4°C overnight with the primary antibody (rabbit anti-*Drosophila* HIF-1α; a gift from Drs Jim Marden and Pablo Wappner) at 1:1000 dilution in 5% BSA (40 µl of anti-HIF-1α in 40 ml of 5% bovine serum albumin) at 4°C overnight on a shaker. The membrane was then washed 3 times in TBS-T and incubated for 1 h with goat anti-rabbit IgG secondary antibody HRP conjugate (Thermo Scientific, Rockford, IL, USA) at 1:5000 dilution (8 µl of secondary antibody in 40 ml of 5% TBS-T and 2 g of dry milk). After washing the membrane again, 3 times in TBS-T, antibodies were detected using a West-Femto kit (ThermoFisher, Grand Island, NY, USA) with a 30 min exposure time on a chemi-imager (Alpha-Innotech, Miami, FL, USA).

Manipulation of HIF signaling in the PG

We up- and down-regulated HIF signaling in the PG by knockdown of *Hph* and *HIF-1α* expression, respectively, using *phm-GAL4* to drive expression of *UAS-Hph.RNAi* and *UAS-HIF-1α.RNAi*. Eggs were collected every 4 h and split evenly between two oxygen treatments – 10 kPa or 21 kPa. For each treatment, larvae were reared on standard malt-based diet at 50–150 larvae per vial and left to complete development. Vials were checked for adult eclosion every 12 h and adults were sexed and genotyped as either experimental genotypes (*phm>Hph.RNAi* or *phm>HIF-1α.RNAi*) or control genotypes (+/+, +/*phm-GAL4* and +/*UAS-Hph.RNAi* or +/*UAS- HIF-1α.RNAi*) (the presence of *phm-GAL4* was marked by rescue of w^-, while the presence of *UAS-Hph.RNAi* or *UAS-HIF-1α.RNAi* was marked by rescue of y^-). Adults were then left in normoxia for 24 h to complete cuticle sclerotization before being weighed using a Mettler Toledo Mx5 balance (±0.001 mg). Developmental time was calculated as hours from oviposition to adult eclosion for each fly, while larval survival was calculated as the total number of eclosing adults as a proportion of the total number of eggs oviposited.

Statistical analysis

All the data and the R scripts used to analyze them are available from Dryad (https://doi.org/10.5061/dryad.gf1vhhmxm). In no cases was the person making a measurement blind to the experimental treatment group, as usually it was necessary to determine fly morphology individually to determine genotype. We also did not choose sample sizes based on a power analysis because we did not have preliminary data on the variance in the data or likely effect sizes. We checked all data for the relevant assumptions of our statistical tests. Gene expression data were square root transformed to uphold the assumptions of normality and homoscedasticity and pooled into four 12 h time cohorts (0–12 h AEL3, 13–24 h AEL3, 25–36 h AEL3 and 37–48 h AEL3) before analysis. No samples were excluded from the analyses.

To test whether gene expression changed through time in normoxia (21 kPa O_2), hypoxia (10 kPa O_2) and hyperoxia (40 kPa O_2), we fitted the model $E_{ij}=A_j+\varepsilon_{ij}$, where E is gene expression level, A is pooled age AEL3 in hours (categorical factor), ε is error and the subscripts refer to the number of levels for each parameter in the model. We subsequently conducted Bonferroni-corrected *post hoc* pairwise-comparisons across successive 12 h cohorts using the *contrasts* function in the R stats package (http://www.R-project.org/).

To test whether gene expression changes with age and/or oxygen level, we fitted the model: $E_{ijk}=O_i+A_j+O{\cdot}A_{ij}+\varepsilon_{ijk}$ (model 1), where O is oxygen level and A is age. If any main effect or interaction was found to be non-significant, it was removed from the model, the analysis re-run and contrasts between levels of the significant effects were conducted using the *emmeans* package in R (https://CRAN.R-project.org/package=emmeans).

To test whether oxygen level affected gene expression independent of age, we used an F-test to compare model 1 (above) with a model that included the main effects of age, the interaction between age and oxygen but not the main effect of oxygen (model 0: $G_{ij}=C_1+O_i{\cdot}C_1+C_2+O_i{\cdot}C_2+C_3+O_i{\cdot}C_3+\varepsilon_{ij}$, where C_1 is the contrast between 0–12 h and 37–48 h, C_2 is the contrast between 13–24 h and 37–48 h, etc.) (see Levy, 2014 preprint).

To test the effect of *Hph* and *HIF-1α* knockdown in the PG on body size, we fitted the model: $B_{ijkm}=O_i+S_j+T_k+O_i{\cdot}S_j+O_i{\cdot}T_k+S_j{\cdot}T_k+O_i{\cdot}S_j{\cdot}T_k+G_m+\varepsilon_{ijkm}$, where B is body size, T is fly type (experimental versus control, fixed factor), S is sex and G is genotype (random factor). This allowed us to compare a single experimental genotype (*phm>Hph.RNAi* or *phm>HIF-1α.RNAi*) with three control genotypes (+/+, +/*phm-GAL4* and +/*UAS-Hph.RNAi* or +/*UAS- HIF-1α.RNAi*) in a single test. In this model, $O{\cdot}T$ captures the differential effect of oxygen on body size between experimental and control flies; that is, whether oxic plasticity is affected *Hph* and *HIF-1α* knockdown in the PG. Sex was included as a factor because it affects body size in *Drosophila*. Again, if any main effect or interaction was found to be non-significant, it was removed from the model and the analysis re-run. We subsequently contrasted body size within each sex:gene-knockdown combination in a *post hoc* analysis using the *emmeans* package in R (https://CRAN.R-project.org/package=emmeans).

Table 2. Primers used to generate gene-specific targets for standard curves used in qPCR

Oligonucleotide name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
HIF-1α (*sima*)	ACGCGATGATGACGATTCCG	TGTGCGGGGTCCTACTTTCA
HIF-1β (*tango*)	GCGCATAAGACCAGCCGAAT	TCATATCGTTGGCTGCCGTG
Hph (*fatiga*)	GAGCGCCGCTATGAGGATCT	CAGTGACCGCGTTTGTCCAC

To explore the effect of *Hph* and *HIF-1α* knockdown in the PG on developmental time at both 10 and 21 kPa O_2, we first fitted the logistic mixed model: $\text{logit}(E_{ijk})=A_i+T_j+A_i{\cdot}T_j+G_k+\varepsilon_{ijk}$, where E is whether a fly has a eclosed or not (a dichotomous variable), A is the age since oviposition in hours, and $A{\cdot}T$ captures whether there is a difference in developmental time between experimental and control flies. We then used the parameters of the model to predict the average age at eclosion for the experimental and control flies – that is, the age at which the probability of eclosion was 50% (EC50) – along with their 95% confidence intervals (CIs).

To determine the effect *Hph* and *HIF-1α* knockdown in the PG in the oxic plasticity of developmental time, we fitted the model: $\text{logit}(E_{ijkm})=A_i+T_j+O_k+A_i{\cdot}T_j+A_i{\cdot}O_k+T_j{\cdot}O_k+A_i{\cdot}O_j{\cdot}T_k+G_m+\varepsilon_{ijkm}$, where $A{\cdot}O{\cdot}T$ captures the differential effect of oxygen on developmental time between experimental and control flies. We again used the parameters of the model to calculate the change in the average age of eclosion in control and experimental flies reared in hypoxia versus normoxia.

To test the effect of *Hph* and *HIF-1α* knockdown in the PG on survival at 10 and 21 kPa O_2, we fitted the logistic mixed model: $\text{logit}(V_{ijkm})=O_i+T_j+O_i{\cdot}T_j+G_k+\varepsilon_{ijkm}$, where V is whether a fly survives to adulthood or not (a dichotomous variable).

All linear models were implemented using the base package in R and the significance of the main effects and interactions was evaluated using type III ANOVA. All mixed models were implemented using the *lme4* package in R (Bates et al., 2015) and the significance of fixed effects was evaluated using type II Wald chi-square test, which compares models with and without the effect of interest.

RESULTS

HIF signaling during the third instar

HIF signaling, as indexed by *Hph* expression (Fig. 3A), indicates that *Drosophila* third instar reared in normoxia do not develop functional hypoxia later in the instar. We found that environmental hypoxia increased expression of *Hph* relative to normoxia, when controlling for larval age (two-way ANOVA, $F_{\text{oxygen}[1,59]}=16.603$, $P<0.001$), providing evidence for functional hypoxia at the whole-body level for larvae reared in 10 kPa oxygen. We did not, however, see any change in *Hph* expression with larval age in normoxia (one-way ANOVA, $F_{3,29}=0.688$, $P=0.5667$), suggesting that these larvae do not become functionally hypoxic later in development. There was, however, a significant interaction between the effects of oxygen level and age on the expression of *Hph* in the third larval instar (two-way ANOVA, $F_{\text{age:oxygen}[6,81]}=4.345$, $P<0.001$). Larval age significantly affected *Hph* expression in both environmental hypoxia (one-way ANOVA: $F_{3,30}=6.601$, $P=0.0014$) and hyperoxia (one-way ANOVA: $F_{3,22}=3.994$, $P=0.021$). In hypoxia, there was a significant increase in *Hph* expression from 0–12 h to 13–24 h AEL3 ($t=4.357$, $P<0.001$). In hyperoxia, in contrast, expression was stable from 0–12 h until 25–36 h AEL3, but significantly decreased at 37–48 h AEL3 ($t=-2.424$, $P=0.024$). Collectively, therefore, the pattern of *Hph* expression suggests that hypoxic but not normoxic reared larvae experience increased HIF signaling.

Whole-body *HIF-1α* expression was regulated developmentally and showed a significant age-by-oxygen treatment interaction (two-way ANOVA, $F_{\text{age:oxygen}[6,81]}=2.5901$, $P<0.024$) (Fig. 3B). There was, however, no significant main effect of oxygen treatment on *HIF-1α* expression when controlling for age (two-way ANOVA, $F_{\text{oxygen}[2,81]}=0.318$, $P=0.728$). The significant age-by-oxygen interactive effect was associated with greater increases in *HIF-1α* expression in lower oxygen levels. In hypoxia, *HIF-1α* expression levels increased significantly between 0–12 h and 13–24 h AEL3 ($t=2.076$, $P=0.0466$), and between 13–24 h and 25–36 h AEL3 ($t=2.166$, $P=0.0384$). In normoxia, *HIF-1α* expression increased significantly from 13–24 h to 25–36 h AEL3 ($t=2.5548$, $P=0.01664$). In hyperoxia, the only significant change in *HIF*-1α expression with age was a significant decrease between 25–36 h and 37–48 h AEL3 ($t=-2.425$, $P=0.0239$).

Measurement of HIF-1α protein by western blot also supported developmental but not oxygen regulation (Fig. 3D,E). There was no significant interaction between the effects of oxygen level and age on HIF-1α protein levels (two-way ANOVA: $F_{\text{age·oxygen}[2,15]}=0.2074$, $P=0.815$), and no significant effect of oxygen when the interaction was removed from the model (two-way ANOVA: $F_{\text{oxygen}[2,17]}=1.539$, $P=0.243$). There was, however, a significant effect of age when oxygen level was removed from the model (one-way ANOVA: $F_{\text{age}[2,19]}=15.657$, $P<0.001$), such that HIF-1α protein levels increased in the first half of L3 ($t=3.281$, $P=0.0118$), and increased further in the second half of L3 ($t=2.883$, $P=0.0286$).

Whole-body *HIF-1β* expression was also regulated developmentally and showed a significant age-by-oxygen treatment interaction (two-way ANOVA, $F_{\text{age:oxygen}[6,81]}=2.221$, $P=0.049$) (Fig. 3C). As for *HIF-1α* expression, there was no main effect of oxygen on *HIF-1β* expression when controlling for age (two-way ANOVA, $F_{\text{oxygen}[2,81]}=2.365$, $P=0.100$). In environmental normoxia and hyperoxia, *HIF-1β* expression increased in the second half of L3, between 13–24 h and 37–48 h AEL3 (normoxia: $t=2.996$, $P=0.0056$; hypoxia: $t=2.889$, $P=0.0071$), while in hypoxia, *HIF-1β* expression also increased between 0–12 h and 13–24 h AEL3 ($t=2.990$, $P=0.0055$). Again, these developmental trends were not significant in hyperoxia, although *HIF-1β* expression did fall between 25–36 h and 37–48 h AEL3 ($t=-2.930$, $P=0.0078$).

Effects of prothoracic HIF signaling on body size

If HIF-signaling in the PG is part of the critical weight mechanism used to regulate developmental timing and final body size in normoxia or hypoxia, then inhibiting HIF signaling by PG-specific knockdown of *HIF-1α* should increase adult body size, while activating HIF signaling by PG-specific knockdown of *Hph* should decrease body size. There was a significant sex-by-treatment interaction, such that inhibiting HIF signaling in the PG (*phm>HIF-1α.RNAi*) increased body size in females but did not affect body size in males (LMM: $\chi^2_{\text{type×sex}(1)}=14.386$, $P<0.001$, Fig. 4A). However, activating HIF signaling in the PG (*phm>Hph.RNAi*) also increased body size in females but not in males (LMM: $\chi^2_{\text{type×sex}(1)}=4.863$, $P=0.027$, Fig. 4B). These effects were not significantly different in normoxia versus hypoxia (LMM: *phm>Hph.RNAi*, $\chi^2_{\text{type×sex×O}_2(1)}=0.0002$, $P=0.990$; *phm>HIF-1α.RNAi*, $\chi^2_{\text{type×sex×O}_2(1)}=0.528$, $P=0.468$). Thus, these data do not support the hypothesis that HIF signaling in the PG regulates developmental timing and final body size as part of the critical weight mechanism.

The data also do not support a role for HIF signaling in the PG in the regulation of body size in response to oxygen level. Low oxygen reduced body size in all flies (LMM: $\chi^2_{\text{O}_2(1)}>186.414$, $P<0.001$ for both *phm>Hph.RNAi* and *phm>HIF-1α.RNAi*; Fig. 4). If HIF signaling in the PG regulates the oxic plasticity of body size, then we would expect the effects of activating or suppressing HIF signaling in the PG to have different effects in hypoxia versus normoxia. Specifically, we would expect the suppression of HIF signaling in the PG (*phm>HIF-1α.RNAi*) to increase body size in hypoxia but not normoxia, and the activation of HIF signaling (*phm>Hph.RNAi*) to decrease body size in normoxia but not hypoxia. There was, however, no interaction between oxygen level

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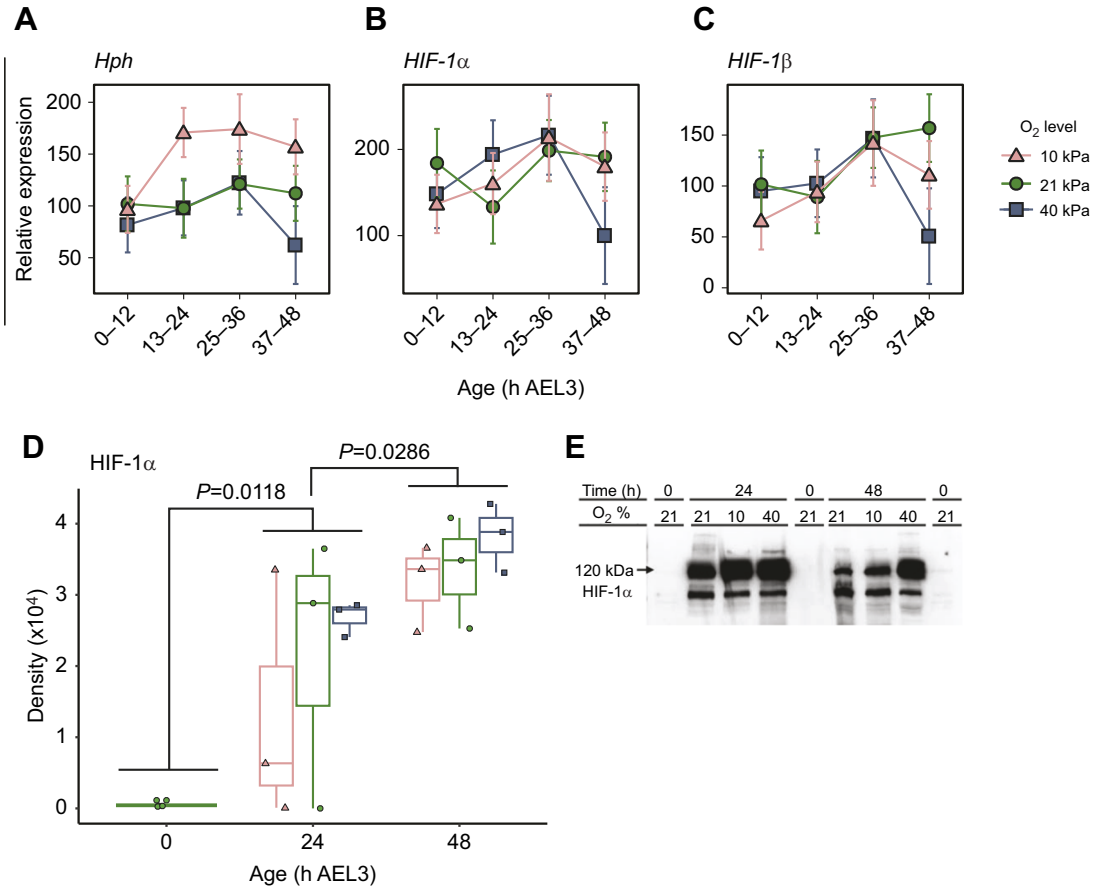

Fig. 3. Gene and protein expression patterns across development time in third instar larvae. (A) *Hph* expression (square root of relative copy number) increased significantly after transfer to hypoxia at the beginning of the third larval instar (t=4.357, P<0.001) and stayed elevated (one-way ANOVA, $F_{oxygen[1,59]}$=16.603, P<0.001). *Hph* expression did not change with larval age in normoxia (one-way ANOVA, $F_{3,29}$=0.688, P=0.5667), but expression declined at the end of the third instar in hyperoxia (*post hoc* contrast, t=−2.424, P=0.024). (B,C) *HIF-1α* and *HIF-1β* expression were not oxygen sensitive (one-way ANOVA, $F_{oxygen\ [2,81]}$<2.365, P>0.1 for both) but, across oxygen levels, both changed with age (two-way ANOVA, $F_{age:oxygen[6,81]}$>2.221, P<0.049 for both). (D) HIF-1α protein levels increased with age (one-way ANOVA, $F_{age[2,19]}$=15.657, P<0.001), but there was no effect of oxygen level on this increase (two-way ANOVA, $F_{age·oxygen[2,15]}$=0.2074, P=0.815). Box plots show median, upper and lower quartile, smallest and largest value within 1.5× the lower and upper quartile, respectively, and any outside values more than 1.5× and less than 3× the interquartile range. (E) Example western blot of HIF-1α expression. For A, B and C, error bars are 95% confidence intervals (CIs) of the mean. Sample sizes for gene expression measurements are shown in Table S1; samples size is 3 for each measure of HIF-1α protein. Each sample is a pooled measure of 2–5 larvae, with more larvae pooled at younger ages.

and either *Hph* or *HIF-1α* knockdown when controlling for sex (LMM: $\chi^2_{type\times O_2(1)}$<0.943, P>0.332 for both *phm>Hph.RNAi* and *phm>HIF-1α.RNAi*), indicating that changing HIF signaling in the PG did not affect the oxic plasticity of body size.

Effects of prothoracic HIF signaling on developmental timing

If HIF signaling in the PG is part of the critical weight mechanism used to regulate developmental timing during normoxia or hypoxia, then activating HIF signaling by PG-specific knockdown of *Hph* should accelerate development, while inhibiting HIF signaling by PG-specific knockdown of *HIF-1α* should retard development (Fig. 2). Our results partially supported these predictions, most strongly for the essentiality of HIF signaling in the PG for reducing developmental time in hypoxia. In normoxia, we found that activating HIF signaling in the PG (*phm>Hph.RNAi*) did not affect the time to adult eclosion relative to the control genotypes (Fig. 5A; GLMM: $\chi^2_{age\times type[1]}$=0.526, P=0.4682). In contrast, suppressing HIF signaling in the PG (*phm>HIF-1α.RNAi*) caused a slight but significant delay in adult eclosion of 12 h (GLMM: $\chi^2_{age\times type[1]}$=17.659, P<0.001) (Fig. 5B).

In all genotypes, environmental hypoxia delayed adult eclosion (EC50, age at which 50% of *Drosophila* eclosed) relative to normoxia (Fig. 5): that is, all genotypes showed oxic plasticity of developmental time. However, activating HIF signaling in the PG had no effect on the extent of this oxic plasticity: hypoxia delayed EC50 by 13.77 h (95% CI: 11.62–15.92 h) in *Hph* knockdown flies and by 11.58 h (95% CI: −0.05–23.21 h) in control flies (Fig. 5Ai,ii). In contrast, suppressing HIF signaling in the PG made developmental time much more sensitive to reduced oxygen: hypoxia delayed EC50 by 51.33 h (95% CI: 47.14–55.52 h) in *HIF-1α* knockdown flies but by only 11.20 h (95% CI: 1.62–20.78 h) in control flies (Fig. 5Bi,ii). Correspondingly, PG-specific knockdown of *Hph* did not affect developmental timing in hypoxia (Fig. 5Aii; GLMM: $\chi^2_{age\times type[1]}$=1.137, P=0.2862), while PG-specific knockdown of *HIF-1α* did (Fig. 5Bii; GLMM: $\chi^2_{age\times type[1]}$=43.60, P<0.001). Collectively, these data suggest that HIF-1α in the PG, but not Hph, is necessary for normal developmental timing in both normoxia and hypoxia and is necessary and important for regulating the plastic response of developmental timing to changes in oxygen level.

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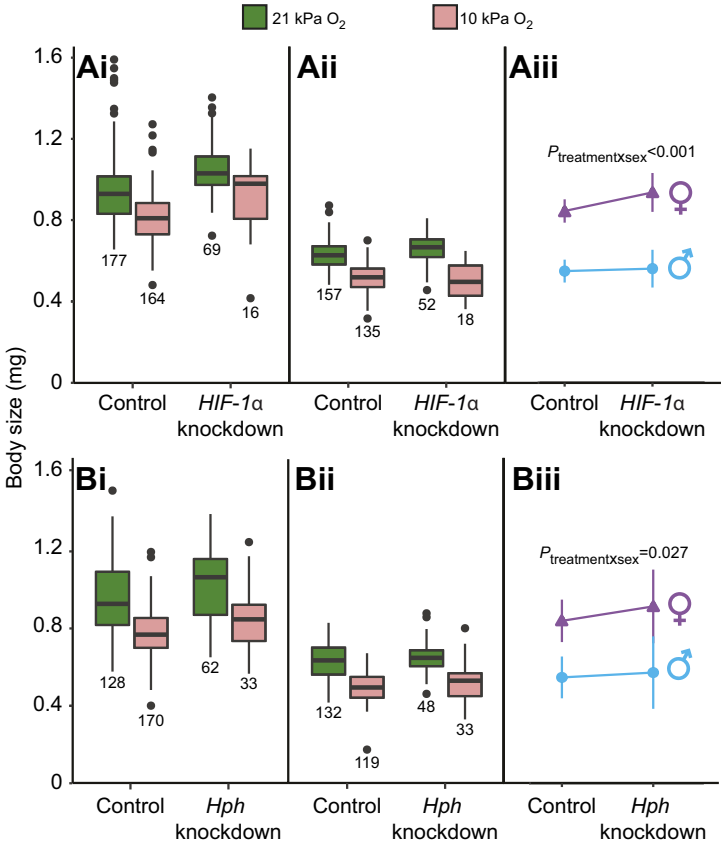

Fig. 4. The effect of changing HIF signaling in the PG on male and female body size in normoxia and hypoxia. (A) Suppressing HIF signaling by knocking down *HIF-1α* expression with *phm>HIF-1α.RNAi* increased body size in (i) females but not in (ii) males, independent of oxygen level (iii) (LMM: $\chi^2_{type\times sex[1]}$=14.386, P<0.001). In all genotypes, low oxygen reduced body size ($\chi^2_{O_2[1]}$>186.414, P<0.001 for both *phm>Hph.RNAi* and *phm>HIF-1α.RNAi*). (B) Activating HIF-signaling by knocking down *Hph* expression with *phm>Hph.RNAi* increased body size in (i) females but not in (ii) males, independent of oxygen level (iii) (LMM: $\chi^2_{type\times sex[1]}$=4.863, P=0.027). Means and 95% CIs shown. Numbers below plots are sample sizes.

Effects of prothoracic HIF signaling on survival

If HIF-signaling in the PG is a major developmental cue in normoxia, then activating HIF signaling by PG-specific knockdown of *Hph* may reduce larval survival to levels seen in hypoxia. This was not observed: survival was the same in control and *Hph*-knockdown larvae in both hypoxia and normoxia (Fig. 6). In contrast, suppressing HIF signaling by PG-specific knockdown of *HIF-1α* reduced survival in hypoxia (χ^2=74.38, P<0.001) but not in normoxia, relative to controls (χ^2=0.87, P=0.832). Thus, *HIF-1α* expression in the PG is necessary to enhance larval survival in hypoxic conditions.

DISCUSSION

Overall, our results strongly support a beneficial role for HIF signaling in the PG to shorten developmental timing in hypoxic reared *D. melanogaster*. In contrast, our findings do not support the hypothesis that developing functional hypoxia is a trigger for molting in normoxic reared larval *D. melanogaster*, or that HIF signaling in the PG plays a significant role in the regulation of body size or developmental timing of normoxic *Drosophila*.

HIF signaling increases in larvae reared in 10% oxygen and HIF signaling in the PG plays a beneficial role in speeding development in hypoxic *Drosophila*

HIF signaling as indexed by whole-body *Hph* gene expression is strongly up-regulated throughout the larval third instar in hypoxia (Fig. 3A). Manipulation of HIF signaling in the PG suggests that HIF signaling is necessary for regulating the plastic response of developmental timing but not body size to low oxygen level. Knockdown of *HIF-1α* expression strongly extended developmental time for *D. melanogaster* reared in 10 kPa oxygen. In *Drosophila*, the growth response to hypoxia is, in part, mediated by an increase in ecdysone synthesis by the PG (Kapali et al., 2022). The observation that knockdown of HIF signaling in the PG substantially retards developmental time in hypoxia relative to controls suggests that the increase in ecdysteroidogenesis in hypoxia may be due to increased HIF signaling in the PG. Counterintuitively, however, even though we found that PG-specific knockdown of HIF-signaling affected the oxic plasticity of developmental time, it did not affect the oxic plasticity of body size: control and *phm>HIF-1α.RNAi* flies were the same size at 10 kPa O_2. This is surprising given the extended developmental time of *phm>HIF-1α.RNAi* flies. We measured developmental time as oviposition to adult eclosion, however, and adult body size is largely fixed at the end of the larval feeding phase. It is possible, therefore, that the extension of developmental time in *phm>HIF-1α.RNAi* flies reared at low oxygen is due to an increase in the duration of the wandering phase or pupation, or to slower growth rates. Additional studies exploring the growth trajectories and developmental timing of *phm>HIF-1α.RNAi* larvae at 10 kPa O_2 are therefore necessary. The effects of hypoxia on body size seem most likely to be mediated by effects on insulin signaling, and/or FOXO signaling in a variety of tissues (Ding et al., 2022; Texada et al., 2019).

Activation of HIF signaling by knockdown of *Hph* for *Drosophila* rearing in hypoxia did not significantly affect body size, development time or survival. This may indicate that rearing in 10 kPa O_2 maximally stimulates HIF signaling in the PG. Additionally, it is possible that we only induced a weak knockdown of *Hph* expression, insufficient to affect phenotype. Using PG-specific knockout of *Hph* using CRISPR-Cas9 could test for this possibility. Finally, chronic activation of PG HIF signaling in *phm>Hph.RNAi* larvae throughout development may lead to

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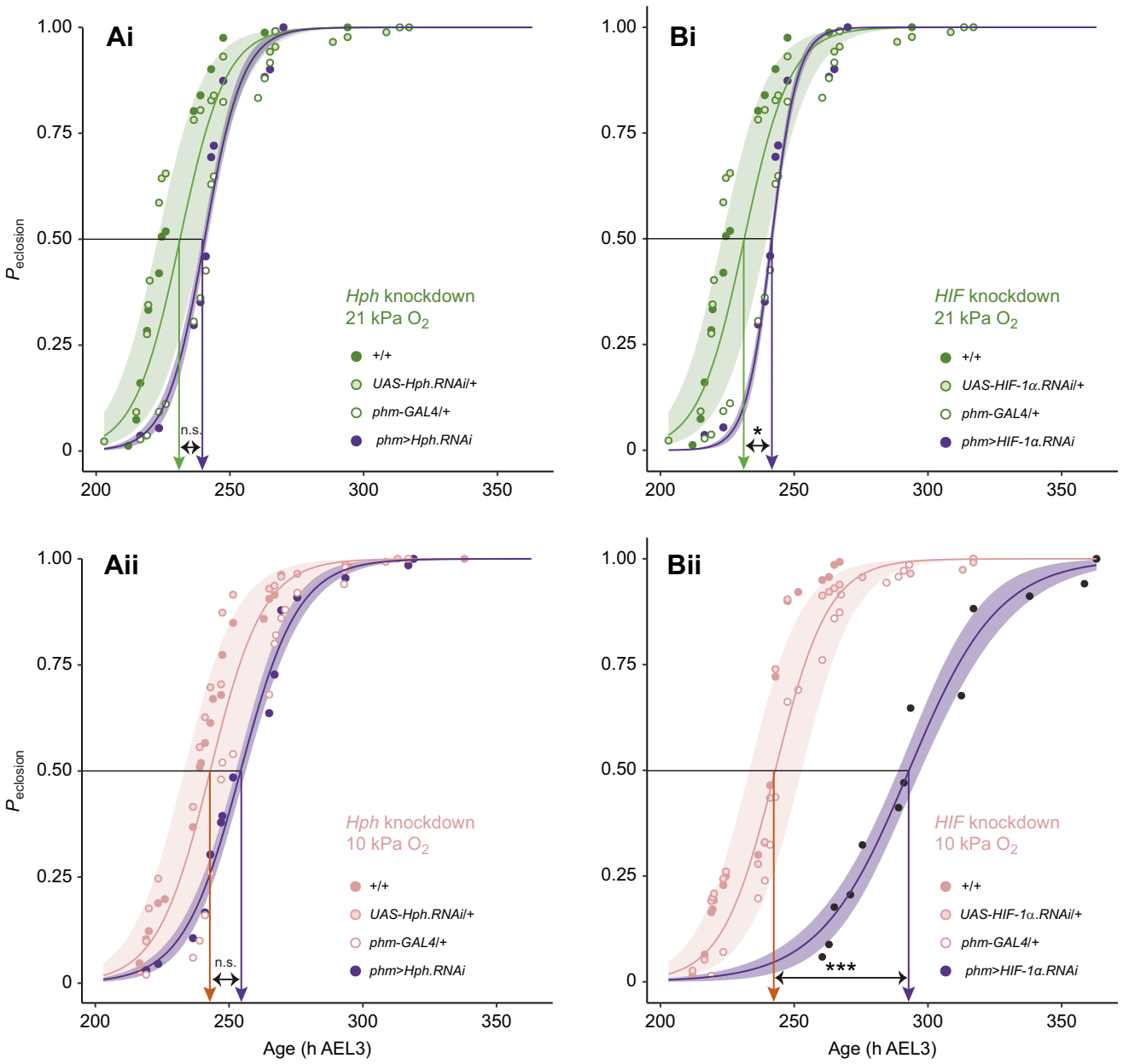

Fig. 5. The effect of changing HIF signaling in the PG on male and female developmental time in normoxia and hypoxia. (A) Activating HIF signaling by knockdown of *Hph* expression with *phm>Hph.RNAi* did not affect developmental time relative to control genotypes at either 21 kPa (i) or 10 kPa O_2 (ii) (GLMM: $\chi^2_{age\times type[1]}<1.374$, $P>0.2682$ for both). (B) Suppressing HIF signaling by knockdown of *HIF-1α* expression with *phm>HIF-1α.RNAi* marginally retarded development at 21 kPa O_2 (i) (GLMM: $\chi^2_{age\times type[1]}=17.689$, $P<0.001$), but substantially reduced it at 10 kPa O_2 (ii) (GLMM: $\chi^2_{age\times type[1]}=43.60$, $P<0.001$). In all genotypes, low oxygen retarded development (GLMM: $\chi^2_{age\times O_2[1]}>18.3675$, $P<0.001$ for both *phm>Hph.RNAi* and *phm>HIF-1α.RNAi*). Arrows show EC50: the age at which 50% of flies are predicted to have eclosed. n.s., not significant, *$P<0.05$, ***$P<0.001$. Shading indicates 95% CIs of the fit. Sample sizes are shown in Table S2. + indicates wild-type; *UAS.xxx.RNAi* indicates the upstream activator sequence gene that produces double-stranded RNA for the indicated gene if paired with GAL4; *phm-GAL4* indicates the activator gene driving expression of the UAS gene in the PG.

downregulation of downstream receptors or pathways, mitigating the effects on growth and development.

Suppression of *HIF-1α* in the PG by RNAi significantly reduced survival of flies, supporting the hypothesis that HIF signaling in the PG is part of the adaptive plasticity mechanism that controls growth and development in response to low oxygen levels. These data suggest that shortening development time and eclosing at a smaller body size allows *Drosophila* to avoid potentially lethal conditions, improving fitness. This mechanism includes an increase in circulating ecdysone, presumably due to an increase in ecdysteroid synthesis and/or release from the PG (Callier et al., 2013). Future studies should focus on testing the hypothesis that HIF signaling in the PG is responsible for the increase in ecdysone levels in hypoxia.

HIF signaling in the PG does not play a role in controlling developmental timing in normoxic *Drosophila*

Our data do not support the hypothesis that HIF signaling in the PG is the size-sensing mechanism underlying the critical weight phenomenon. Although a PG-autonomous decrease in HIF signaling increased body size – which is consistent with the hypothesis – it does

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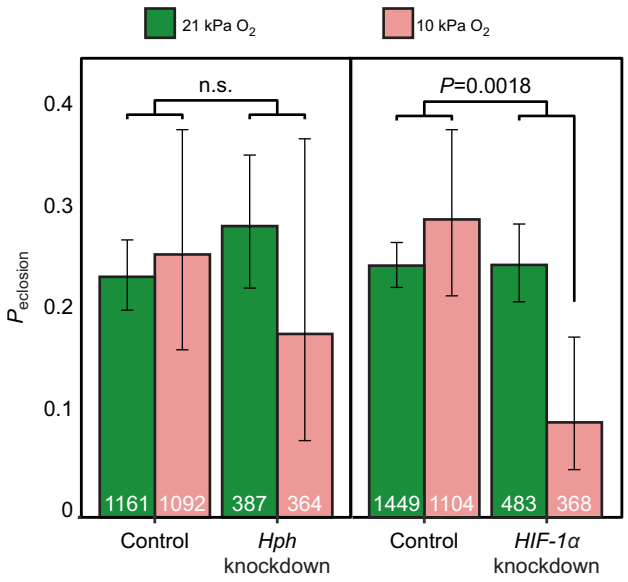

Fig. 6. *HIF-1α* expression in the PG is necessary to maintain survival at low oxygen levels. Knockdown of *HIF-1α* in the PG reduced survival at 10 kPa O_2 but not at 21 kPa O_2 (GLMM: $\chi^2_{type\times O_2[1]}$=9.7820, P=0.0018). There was no effect of *Hph* knockdown on survival regardless of oxygen level (GLMM: $\chi^2_{type\times O_2[1]}$=0.9729, P=0.3240). Means and 95% CIs are shown. Numbers within bars are sample sizes.

so in females only, while a PG-autonomous increase in HIF signaling has the same effect. Finally, while a PG-autonomous decrease in HIF signaling also slightly delays development – also consistent with the hypothesis – a PG-autonomous increase in HIF signaling has no detectible effect on developmental timing. Together, these data suggest that while HIF signaling in the PG can influence development in *Drosophila*, this does not occur in the systematic manner required for a size-determining mechanism.

Developmentally induced functional hypoxia does not appear to be an important trigger for the timing of maturation and molting in *Drosophila*

It has been hypothesized that functional hypoxia from excessive growth relative to respiratory supply is used by holometabolous insects as a cue to initiate metamorphosis in the final larval instar, and that this is the size-sensing mechanism underlying the critical weight phenomenon. We tested this hypothesis in *D. melanogaster* larvae by: (1) assaying the expression of *Hph* to determine whether third instar larvae experience increasing functional hypoxia as the instar progresses; and (2) testing whether increasing atmospheric oxygen by 1.43 times (from 21% to 30%) ameliorates any increase in HIF signaling as the instar progresses. Our results do not support the hypothesis that critical weight reflects progressively increasing whole-body functional hypoxia in *Drosophila*: HIF signaling as indexed by *Hph* expression does not increase as development progresses at normal oxygen levels (Fig. 3), a key prediction of the hypothesis (Fig. 1A). While HIF-1α protein increased with age, its level was unaffected by rearing oxygen level, suggesting that the age-related increase is driven by a developmental factor other than hypoxia. For the effects of hyperoxia, our results did not match either prediction shown in Fig. 1. Hyperoxia suppresses HIF signaling only in the last 12 h of the instar (Fig. 3A). One plausible conclusion from this pattern is that some functional hypoxia occurs at a relatively constant level throughout the third instar, and that after feeding ceases, hyperoxia can reduce functional hypoxia and HIF signaling. If so, this could explain the weak to minimal effect of hyperoxia on the critical weight and body size of *Drosophila* (Callier et al., 2013; Frazier et al., 2001; Klok et al., 2009). While our findings seem to exclude a progressive rise in whole-body HIF signaling during normoxic development, it remains possible that functional hypoxia in a specific tissue, such as the fat body, might be an important trigger for the initiation of molting. The fat body is known to sense hypoxia via HIF and TORC signaling, improving hypoxic viability, and transmitting neuroendocrine factors that inhibit insulin signaling from the brain, reducing body size (Lee et al., 2019; Texada et al., 2019).

The indices of HIF signaling that we used showed somewhat disparate results, but overall suggest that HIF signaling occurs throughout the third instar, independently of oxygen level except for in the final quarter of the instar. Expression of *Hph* and *HIF-1α* in normoxic larvae appeared steady throughout the instar, whereas, *HIF-1β* gene expression and HIF-1α protein increased with age. Hyperoxia did suppress gene expression of *Hph*, *HIF-1α* and *HIF-1β*, but only during the last 12 h of the instar, and even at this time, hyperoxia did not affect levels of HIF-1α protein. HIF signaling is well known to be activated hormonally in addition to activation by hypoxia. In *Drosophila*, activation of HIF signaling occurs in response to insulin-target of rapamycin signaling (Dekanty et al., 2005) and to the estrogen-related receptor (Li et al., 2013), and in mammals, oxygen-independent regulation of HIF signaling has been documented in response to diverse hormones (Alam et al., 2009; Wong et al., 2015). Thus, it seems likely that HIF signaling occurs throughout development of the *Drosophila* third instar, likely driven by neuroendocrine developmental signals unrelated to functional hypoxia. While HIF signaling does become hyperoxia sensitive at the end of the instar, hyperoxia lowered HIF signaling below the levels in younger larvae, rather than preventing the predicted rise in HIF signaling if oxygen sensing is an important size cue for molting (Fig. 1A versus Fig. 3A).

While our data do not support the hypothesis that developmentally induced functional hypoxia is an important cue for molting in *Drosophila*, plausibly, this may be true in other insects. Unlike in *Drosophila*, mild hyperoxia increases the duration of larval growth in *Tenebrio molitor* (Greenberg and Ar, 1996; Loudon, 1988), *M. sexta* (Harrison et al., 2013) and *Blatella germanica* (VandenBrooks et al., 2020). The mass gained during an instar also increases with hyperoxia and decreases in hypoxia in the penultimate larval instar of *Orthosia gothica* (Kivelä et al., 2018). There is a decline in the safety margin for oxygen delivery as the terminal juvenile instar progresses in *M. sexta* and *Schistocerca americana* (Greenlee and Harrison, 2004, 2005); this may be due to the decline in the volume of the tracheal system toward the end of the instar in these species (Callier and Nijhout, 2011; Greenlee et al., 2009; Lease et al., 2006), though there is some evidence for tracheal system growth during the instar, at least in *M. sexta* (Helm and Davidowitz, 2013). Perhaps correspondingly, late in the instar, respiration rates level off (Callier and Nijhout, 2011) and growth decelerates late in the instar in many larvae (Grunert et al., 2015; Kivelä et al., 2020). In *M. sexta* specifically, the leveling off of respiration rate begins at attainment of critical weight (Callier and Nijhout, 2011). In *M. sexta*, *HIF-1α* and *HIF-1β* gene expression and HIF-1α protein levels are higher at the end than at the beginning of most instars (but not the terminal instar), consistent with the hypothesis that functional hypoxia occurs and could signal molting in this caterpillar (Lundquist et al., 2018). Insects are notoriously variable and it would not be surprising if the mechanisms determining size vary among orders.

An alternative view is that tracheal systems become generally increasingly mismatched to the need for oxygen delivery as insects grow, but that the size-determining mechanism of insects somehow anticipates and prevents serious functional hypoxia. This is supported by the observation that, in *M. sexta*, respiration rates only level off after attainment of critical weight and the commitment to metamorphosis. This could occur by sensing of functional hypoxia at a particular location, or by some other size-sensing mechanism. Alternatively, the critical weight phenomenon may not require a size-sensing mechanism, and rather could be a threshold response to increasing ecdysone levels, whereby ecdysteroidogenesis switches from being regulated by factors exogenous to the PG, for example insulin signaling, to autogenous self-activation (Tyson et al., 2023). The resulting positive feedback loop leads to a rapid increase in ecdysone synthesis that triggers metamorphosis. Under this model, insulin signaling increases as a larva grows and pushes ecdysone levels across this threshold at a particular body size.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.J.G., A.W.S., V.J.C., J.F.H.; Methodology: J.B.C., K.J.G., A.W.S., J.F.H.; Formal analysis: J.B.C., K.J.G., A.W.S.; Investigation: J.B.C., K.J.G., A.W.S., A.E.G., H.C.S., T.L., J.F.H.; Resources: K.J.G., A.W.S., J.F.H.; Data curation: A.W.S., J.F.H.; Writing - original draft: J.B.C.; Writing - review & editing: J.B.C., K.J.G., A.W.S., V.J.C., J.F.H.; Supervision: K.J.G., A.W.S., J.F.H.; Project administration: A.W.S., J.F.H.; Funding acquisition: A.W.S., J.F.H.

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Data availability
All the data and the R scripts used to analyze them are available from Dryad (Campbell et al., 2024): https://doi.org/10.5061/dryad.gf1vhhmxm

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Table S1. Sample sizes for gene expression studies shown in Fig. 3.

Time point Hours in 3rd instar	Oxygen %	*Hph* Fatiga	HIF-1α Sima	HIF-1β Tango
0-12	10	11	11	11
0-12	21	8	8	8
0-12	40	8	8	8
13-24	10	10	10	10
13-24	21	7	7	7
13-24	40	8	8	8
25-36	10	5	5	5
25-36	21	10	10	10
25-36	40	6	6	6
37-48	10	8	8	8
37-48	21	8	8	8
37-48	40	4	4	4

Table S2. Sample sizes for developmental data shown in Fig. 5.+ indicates wildtype; UAS.xxx.RNAi indicates a parent with the gene to produce double-stranded RNA for the indicated gene, Phm-GAL4 indicates a parent with the gene to drive gene expression in the prothoracic gland.

RNAi gene	Oxygen, %	Genotype	Sample size
Hph	10	+/+	106
	10	UAS.Hhp.RNAi/+	142
	10	Phm-GAL4/UAS.Hph.RNAi	66
	10	Phm-GAL4/+	50
Hph	21	+/+	81
	21	UAS.Hhp.RNAi/+	87
	21	Phm-GAL4/UAS.Hph.RNAi	111
	21	Phm-GAL4/+	108
HIF-1α	10	+/+	140
	10	UAS.HIF-1α.RNAi/+	115
	10	Phm-GAL4/ UAS.HIF-1α.RNAi	34
	10	Phm-GAL4/+	71
HIF-1α	21	+/+	114
	21	UAS.HIF-1α.RNAi/+	129
	21	Phm-GAL4/ UAS.HIF-1α.RNAi	121
	21	Phm-GAL4/+	119