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Hypoxia-induced Suppression of EGF/MAPK Signaling Delays Steroid-Dependent Maturation in Drosophila

Turingan, Michael Jacobi

UNIVERSITY OF CALGARY

Hypoxia-induced Suppression of EGF/MAPK Signaling Delays Steroid-Dependent Maturation in *Drosophila*

by

Michael Turingan

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ABSTRACT

For proper animal development, tissues and organs require sufficient oxygen; defects in oxygen supply (hypoxia) can cause developmental disorders[1]. In humans, disrupted oxygen supply underlies many diseases[2]. Although tissue-culture studies have revealed much about adaptation to hypoxia at the cellular level[3] [4], less is known of what mediates whole-body responses. My research employs *Drosophila* to study how hypoxia affects development at the organismal level. *Drosophila* larvae have evolved to grow on decaying food – an environment of low ambient oxygen[5–7] [8]. Hence, they provide a good genetic model to study how hypoxia influences physiology and development. In the lab, larvae exposed to hypoxia (5% O2) adapt by reducing their growth and delaying development to the pupal stage. However, the molecular bases for these adaptations remain unclear. The larval-pupal developmental transition is controlled by a neuroendocrine pathway involving the prothoracic gland (PG), an endocrine organ that produces the maturation steroid hormone ecdysone [9,10]. At the end of the larval period, neuronal input to the PG triggers autocrine signaling through the conserved Epidermal Growth Factor Receptor (Egfr)/MAP kinase (ERK) pathway, which induces the PG to synthesize and release ecdysone[11]. This ecdysone acts on all tissues to initiate maturation[11]. My data suggest this autocrine Egf/ERK signaling is blunted in hypoxia, thus delaying the developmental transition. Since signaling and key aspects of steroid hormone regulation are conserved between *Drosophila* and humans [9,10,12], my work provides insights into how the program of development can adapt to fluctuating environmental conditions.

PREFACE

This thesis is original, unpublished, independent work by the author, M. Turingan.

ACKNOWLEDGMENTS

Thank you to the Grewal lab members, past and present, who have welcomed me, taught me, and shown me kindness. Thank you, Savraj, for your thoughtful mentorship. In these four years, you have fostered, by example, an academic and professional environment that has encouraged and allowed me to challenge myself, make mistakes, and grow from them. I could not ask for a better space to learn in and a better guide through all that grad school has to offer - good and bad.

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Thank you to my friends, inside the lab and out. You have motivated me to do my best and helped me to realize what my best is.

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Thank you, Geeta, for helping me rehearse my presentations on late weeknights, for all the pep talks and for being my constant inspiration to continue to go forward and to be fearless. I owe so much of this to you.

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INTRODUCTION

1.1 Hypoxia in Normal Physiology and Disease

The air we breathe contains around 20% oxygen. However, the oxygen levels our tissues and organs actually receive are much less than this[13]. The human brain and liver, for instance, receive under 4% oxygen and even within the lung tissue, levels reach only as high as ~6%[14]. Reduced oxygen availability is thus a feature of our normal physiology, and this state can be referred to as 'physoxia'[13]. Many pathologies, however, further reduce these levels below physiological limits such that our organs and tissues are subject to aberrantly low oxygen availability - a state known as 'hypoxia'[13]. Hypoxia may be localized to a specific tissue, as is seen in conditions such as ischemia[15], or it can be systemic as is the case in diseases like chronic obstructive pulmonary disease (COPD) and asthma, owing to impaired lung function [16,17]. Moreover, many cancers - particularly solid tumors - have been shown to dramatically reduce oxygen availability in their associated tissue[14]. Hence, low oxygen is both a feature of normal physiology and many pathologies.

Proper animal development requires sufficient oxygen; hypoxia during development necessitates adaptation by the body to ensure survival[1]. In humans, fetal development is particularly hypoxia-prone[18–21]. Normally, fetal oxygen tensions are significantly lower than those of the mother[20]. However, several clinical conditions such as preeclampsia can further reduce these below the physiological range, thus inflicting systemic hypoxic stress on the fetus[18–21]. This early-life hypoxia is associated with a host of postnatal responses including reduced growth, changes in brain structure and delayed maturation[20,22]. Postnatal hypoxia exposure is also known to disrupt development. Paediatric incidence of conditions which reduce systemic oxygen can hinder key developmental outcomes, such as sexual maturation[23]. One study, for instance, found that girls with cystic fibrosis exhibited a ~2-year delay in pubertal onset[24]. Moreover, delays in the development of secondary sexual characteristics have been observed in children with asthma[25], and obstructive sleep apnea[26]. The mechanisms underlying these whole-body responses to hypoxia are still poorly understood, constituting an important open question in the field of developmental biology.

Tissue culture studies have told us much about cellular responses to hypoxia[3,4]. However, much less is known about the physiological response at the whole-body level. Such research is important since adaptive responses to hypoxia often rely on communication between tissues and organs [27,28,29,30]. One review article outlines three requirements for hypoxia tolerance: i) reduction of oxygen demands, ii) prevention of cellular damage, and iii) maintenance of functional integrity[31]. As the authors emphasize, the issue of how to achieve this three-part goal concerns the whole animal, not just any given cell type or tissue[31]. Investigation into these three areas therefore requires an approach that looks at whole tissues and animals, rather than cell culture.

Many animals have evolved to withstand extreme oxygen deprivation in the wild. For example, some reptiles and fish can survive for weeks completely starved of oxygen (a state called anoxia)[32], whereas certain diving birds and burrowing mammals, like the naked mole rat, can tolerate extreme hypoxia for many hours[31]. How such animals adapt their physiology and development to environmental hypoxia is an interesting question and addressing it can importantly reveal biology underlying human responses to pathological hypoxia. Dissecting such responses by these specialized animals, however, is not feasible given ethical considerations, a lack of molecular tools precluding their utility as tractable model systems and difficulty maintaining them in a lab setting.

The fruit fly, *Drosophila*, can survive up to a few hours of anoxia and many more of extreme hypoxia[33]. In their natural ecology, *Drosophila* larvae burrow into and feed on decaying food rich in microorganisms – an environment characterized by low ambient oxygen – and hence they have evolved to grow in these hypoxic conditions[5–8]. Moreover, unlike most other hypoxia-tolerant animals, *Drosophila* is an established model for investigating the molecular basis for adaptation to various stresses, such as nutrient deprivation, during development[34]. This is owing in large part to i) highly modifiable genetic tools such as the *GAL4/UAS* binary expression system, and ii) conservation in key factors through which mammals and flies adapt their growth to their environment, including insulin and steroid hormones[34]. *Drosophila* thus presents a useful model for mechanistic insight regarding developmental adaptation to hypoxia. In the following sections, I first outline the normal course of *Drosophila* development and the signaling pathways which regulate the process. I then discuss what is currently known and what remains unclear about how *Drosophila* adapt this process to low oxygen.

1.2 *Drosophila Development: Growth and Maturation*

Drosophilae are holometabolous insects whose larvae undergo whole-body metamorphosis. The lifecycle can be divided into four stages: embryo, larva (consisting of three instar stages), pupa, and adult[35]. Embryogenesis lasts 24 hours, after which the larvae hatch and immediately begin feeding[35]. Over the course of four days, larvae in nutrient-rich conditions can increase their mass by ~200-fold before entering the pupal stage and metamorphosis also lasting four days[35]. At this point, a sexually mature adult fly is formed and emerges from the pupal case (a process called eclosion).

As for many animals which exhibit determinate growth, *Drosophila* only grow during the juvenile (larval) period[10]. Afterwards, maturation to the adult stage occurs and subsequent growth stops, resulting in a more-or-less fixed adult body size[10]. For these animals, then, final body size is a function of both the growth rate (rate of mass increase) and the duration of the growth period[10]. As such, both of these parameters are tightly regulated during development according to the animal's environment[10]. In the following sections, I will discuss this regulation, firstly of the larval growth rate, and then that of the timing of maturation (which in turn defines the duration of the growth period).

1.2.1 *Regulation of Larval Growth (mass increase)*

Insulin / PI3 kinase signaling

The insulin signaling pathway is the main endocrine regulator of body growth in larvae. *Drosophila* have 7 insulin-like peptides (Dilps) that each can circulate throughout the larval hemolymph and stimulate growth in all cells and tissues by binding to a cell surface receptor tyrosine kinase and activating the conserved phosphoinositide-3-kinase (PI3K) and AKT kinase signaling pathway[36] (**Figure 1**). One key source of Dilp expression is a cluster of neurosecretory cells in the brain termed the insulin-producing cells (IPCs)[37]. These express and release three Dilps (2,3 and 5) that have been shown to be particularly important for coupling dietary nutrients to the control of body growth[37]. When larvae are growing in rich nutrient conditions, expression and secretion of these IPC-derived Dilps is upregulated leading to stimulation of PI3K/Akt signaling and growth in all cells and tissues[37 38]. In contrast, in low nutrient conditions, the secretion of Dilps from the IPCs is reduced leading to lower levels of systemic PI3K/AKT signaling and reduced cell-, tissue- and body growth[36].

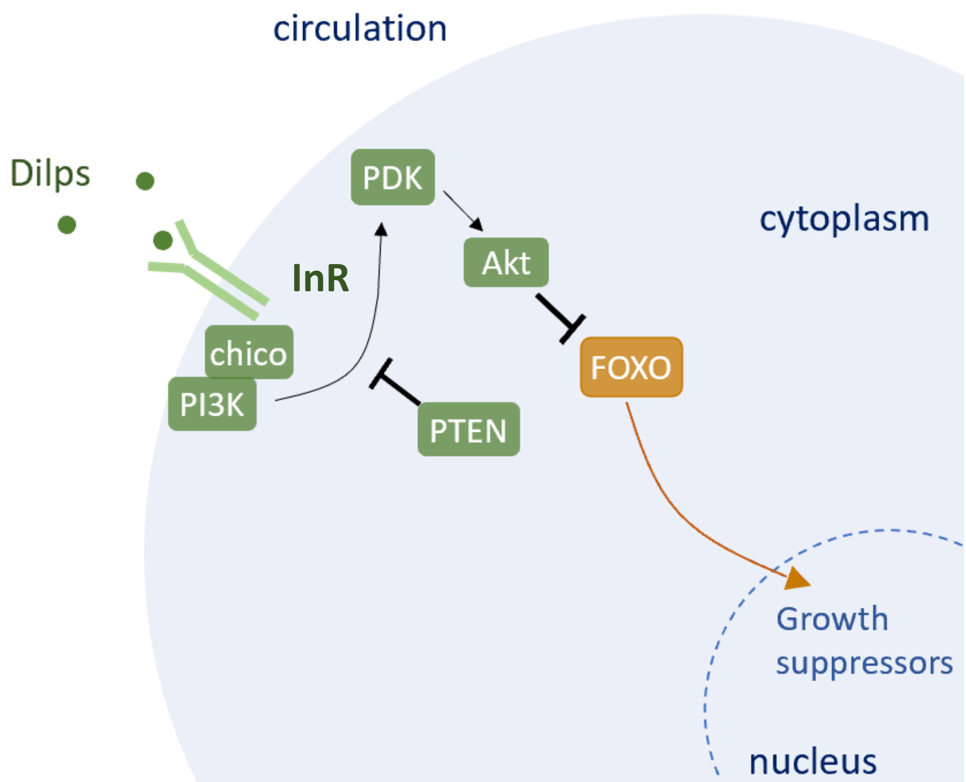

Figure 1. The *Drosophila* insulin/PI3K signaling pathway. Growth-promoting *Drosophila* insulin-like peptides (Dilps) are released into the circulating hemolymph by the insulin-producing cells (IPCs) in the brain and bind their cognate receptor tyrosine kinase, insulin receptor (InR) on peripheral tissues. InR phosphorylates the fly IRS1/IRS2 homolog, chico which in turn leads to PI3K activation and subsequent kinase cascade involving phosphoinositide-dependent protein kinase (PDK) and Akt which phosphorylates FOXO and excludes it from the nucleus.

TOR kinase signaling

TOR is a conserved serine/threonine kinase that functions in the mTOR complex 1 (mTORC1) to promote anabolic processes such as lipid and protein biosynthesis and simultaneously limit catabolic ones such as autophagy (**Figure 2**)[39]. Extensive work in yeast, mammalian cell culture and different model organisms including *Drosophila* has described how cells activate TOR in response to availability of extracellular nutrients such as amino acids and glucose[40]. In this way, TOR functions as a master regulator of nutrient-dependent cell growth and metabolism[39]. TOR has been best studied in *Drosophila* as a factor that couples dietary nutrients to cell, tissue and body growth[41]. In addition to promoting cell-autonomous growth, nutrient-dependent stimulation of TOR signaling in specific larval tissues such as the fat and muscle can also promote non-autonomous stimulation of whole body growth[42,43]. These effects occur in large part through stimulation of TOR-regulated humoral factors that promote production and release of IPC-derived Dilps and stimulation of systemic insulin signaling [42,43].

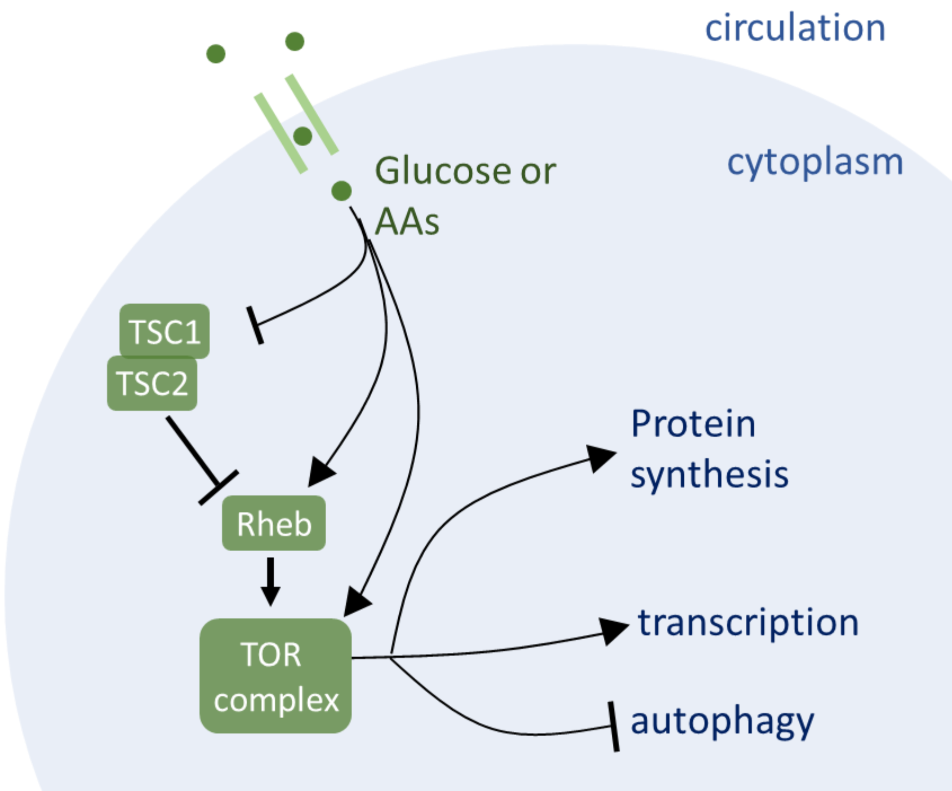

Figure 2. The TOR kinase (TORC1) pathway. Glucose and amino acids are uptaken by cells through transporters. TOR activity is upregulated in the presence of these, resulting in activation of anabolic processes like transcription and translation while catabolic processes like autophagy are suppressed. TOR=Target of Rapamycin; Rheb=Ras homolog enriched in brain, TSC=Tuberous Sclerosis Complex.

1.2.2 *Regulation of Larval Maturation*

The Steroid Hormone Ecdysone Controls Developmental Timing

Larval maturation to the pupal stage is triggered by a neuroendocrine circuit that leads to production of the steroid hormone ecdysone in the prothoracic gland (PG) (**Figure 3**)[9]. Small pulses of ecdysone control progression through molts as the larva grows, however it is one large pulse at the end of the larval period that promotes the larva-to-pupa transition (**Figure 4**)[9]. The production of ecdysone is controlled by expression of the Halloween genes in the PG which encode enzymes that mediate its biosynthesis[9]. The ecdysone that is produced then circulates throughout the animal and binds to its nuclear receptor to control transcription of genes that bring about metamorphosis[44]. The receptor is a heterodimer of ecdysone receptor (EcR) and ultraspiracle (USP) and its target genes mediate degradation of larva-specific tissues, pupal case formation, and differentiation of presumptive adult tissues[10,45,44]. It is therefore the timing of the final, large pulse of ecdysone at the end of the larval period that determines when a larva matures and hence controls the duration of the larval period (**Figure 4**)[9]. In this way, ecdysone is similar to the mammalian sex steroid hormones which also mediate the developmental transition from the juvenile growth stage to the adult stage[46].

Decades of research into this neuroendocrine circuit has described the signaling pathways that control ecdysone production, and moreover how these are able to integrate developmental and environmental cues to ensure proper timing of ecdysone pulses[9,10]. Below, I discuss the major signals that control ecdysone production by the PG. I then describe two checkpoints that use signaling through these pathways to ensure that maturation is timed correctly according to nutrient and developmental cues.

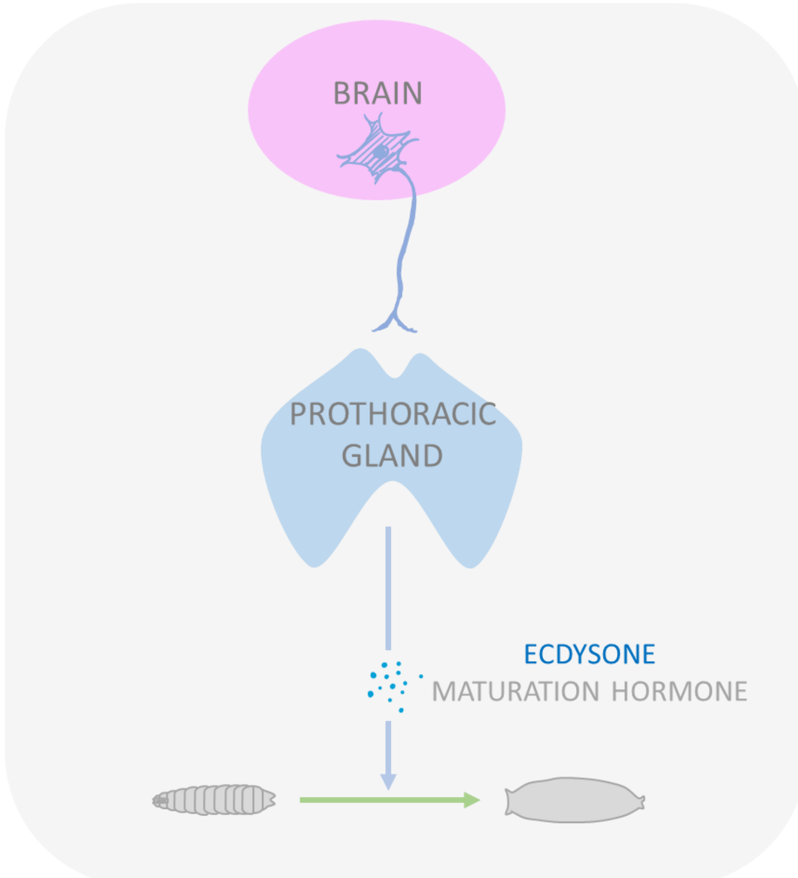

Figure 3. The neuroendocrine control of ecdysone production regulates larval maturation. At the end of the larval growth period, the steroidogenic prothoracic gland receives signaling input from brain-derived neuropeptides that stimulate ecdysone production from the gland. Ecdysone is secreted into circulation and acts on all larval tissues to mediate the developmental transition from larva to pupa.

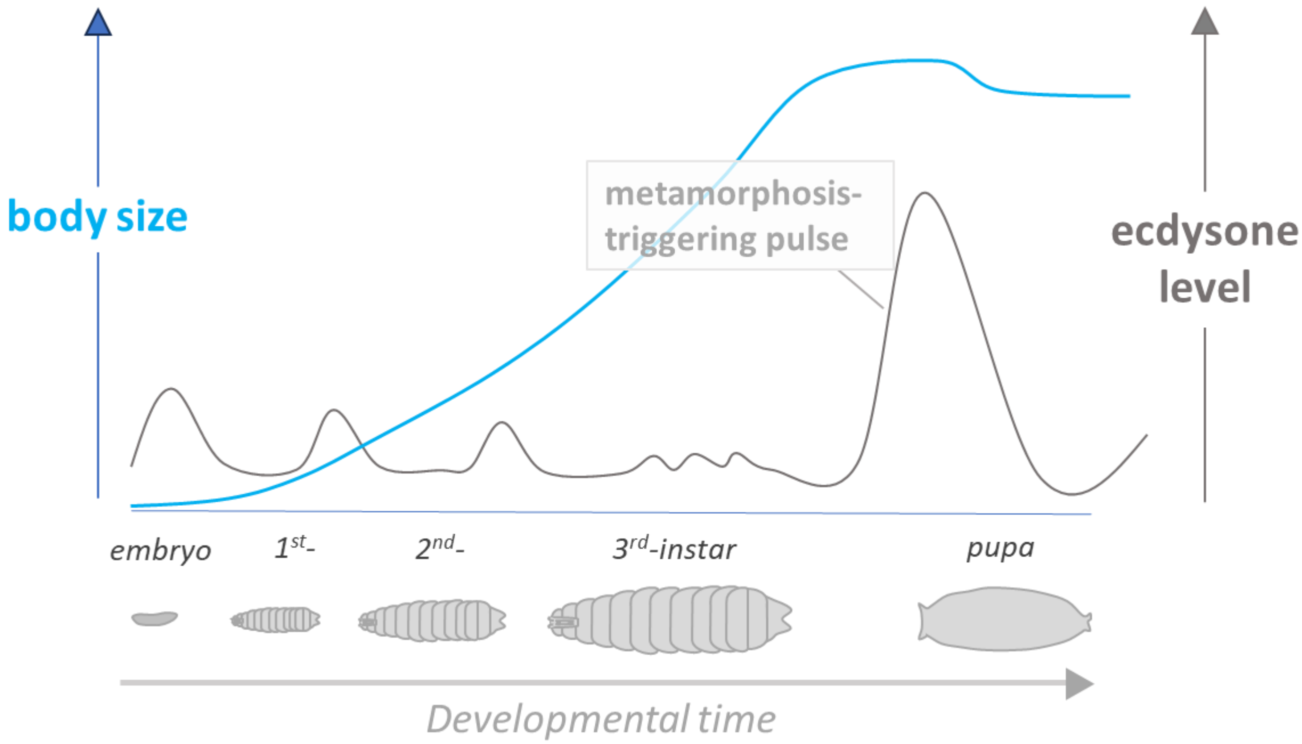

Figure 4. Duration of the *Drosophila* growth period, and thus final body size, is regulated by the steroid hormone ecdysone. Pulses of the steroid hormone ecdysone (grey) mediate the larval molts as body size (blue) increases. Upon reaching the final larval instar, substantial feeding and growth occurs before the maturation-inducing pulse of ecdysone triggers pupation and thus termination of body growth (plateau of blue line).

Signaling Pathways That Control Ecdysone Synthesis

Ptth/Torso

Prothoracicotropic hormone (Ptth) was one of the first-identified factors shown to stimulate ecdysone production in the PG. It is a neuropeptide produced by two pairs of neurosecretory cells in the brain that innervate the PG and stimulate ecdysone synthesis when activated[9]. Ptth signals through Torso, its cognate receptor tyrosine kinase (RTK) on the surface of PG cells[9,47]. Ptth/Torso activates the ERK pathway leading to ERK phosphorylation[9,47]. This signaling cascade promotes the expression of several Halloween genes, and thus ecdysone production (**Figure 5**)[9]. The Ptth$^+$ neurons themselves are regulated by several signals.

Ptth release is positively regulated by the Kisspeptin1 (Kiss1) homolog, Allatostatin A (AstA), a neuropeptide expressed by the AstA neurons[48]. Upon release, it binds its receptor AstAR1, expressed on the Ptth neurons - promoting Ptth release, ecdysone production and the larval-pupal transition[48]. In mammals, Kiss1 promotes the pubertal transition by activating the hypothalamic-pituitary-gonadal (HPG) axis[49]. The Kiss1 receptor, GPR54, is homologous to AstAR1, highlighting conserved mechanisms in the control of hormone production in both fly metamorphosis and mammalian puberty[49].

Ptth release is antagonized by the sesquiterpenoid called Juvenile Hormone (JH)[50]. Upon attainment of CW, there is a drop in JH titers - allowing Ptth to stimulate ecdysone production in the PG and the onset of metamorphosis[50]. This JH-Ptth axis is believed to be more important for development in the tobacco hornworm (*Manduca sexta*) than in *Drosophila*[50], although *Drosophila* Ptth neurons express the JH receptor[51]. In *Drosophila*, JH has been shown to directly act on the PG to antagonize ecdysone[52]. Nonetheless, Ptth represents a key juncture for ecdysone regulation by various signals.

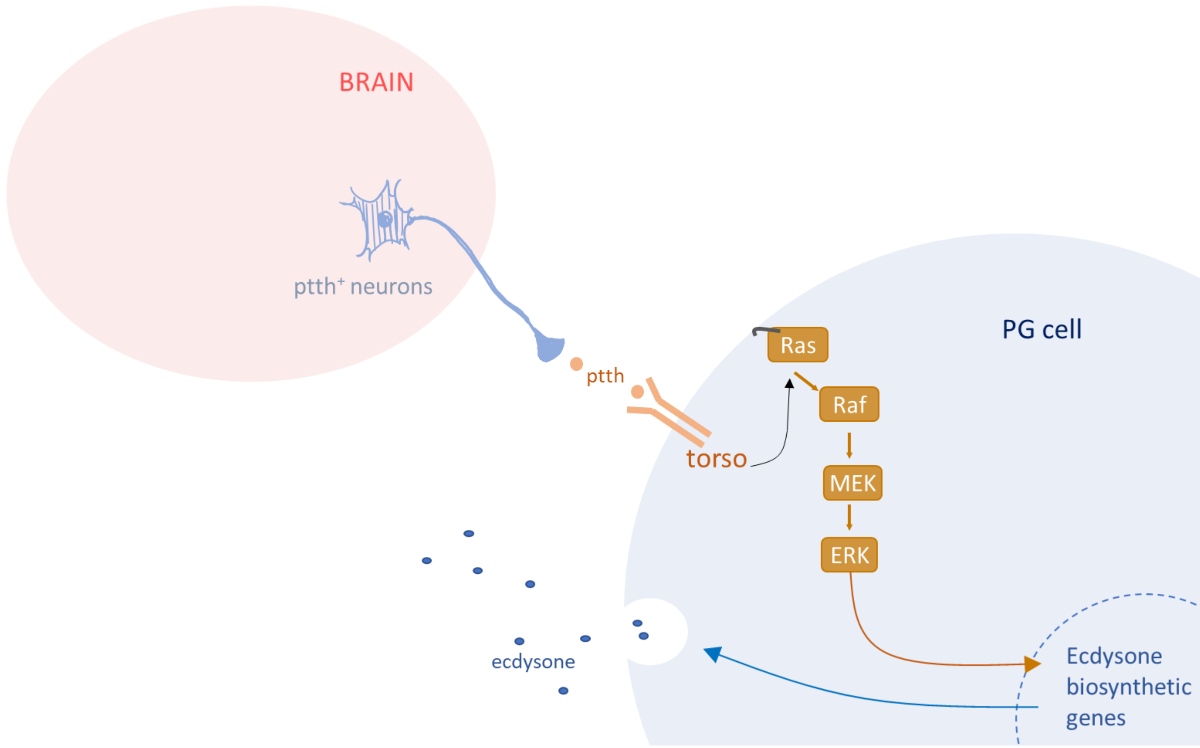

Figure 5. Ptth/Torso mediates Ras/ERK-dependent ecdysone production at the end of the larval period. Upon CW attainment, the neuropeptide Ptth is released and binds its receptor tyrosine kinase Torso on the PG cells. This triggers a kinase cascade ending in the phosphorylation of ERK. ERK then enters the PG cell nucleus to transcriptionally activate the ecdysone-biosynthetic Halloween genes whose encoded enzymes synthesize ecdysone that is then released into circulation.

Egfr/ERK signaling

Ptth/Torso/ERK induces the expression of ecdysone biosynthetic genes in the PG [47]. This elicits a small pulse of ecdysone which signals back to the gland in an autocrine manner via its nuclear receptor, EcR/Usp[11]. In the PG cells, activated EcR/Usp then transcriptionally activates the Egf ligands, spitz and vein[11]. These Egf ligands in turn signal in an autocrine manner to the PG via Egfr/ERK to produce more ecdysone[11]. This ecdysone then mediates further Egf ligand production by the PG, causing a feedforward circuit of ecdysone synthesis[11]. The resulting rapid surge in ecdysone acts on all larval tissues to induce metamorphosis[11]. This autocrine signal mediated by Egfr is thus required for amplifying ecdysone titers to levels sufficient for maturation[11]. Indeed, PG-specific genetic ablation of Egfr or the spitz and vein ligands arrests development at the L3 stage[11]. This is in contrast with Ptth loss-of-function which does not arrest development prior to pupation but merely elicits a marked delay in pupation[47]. This observation implicates Egf/Egfr signaling in the PG as the main controller of metamorphosis. Its role in the maturation-inducing neuroendocrine circuit is illustrated in **Figure 6**.

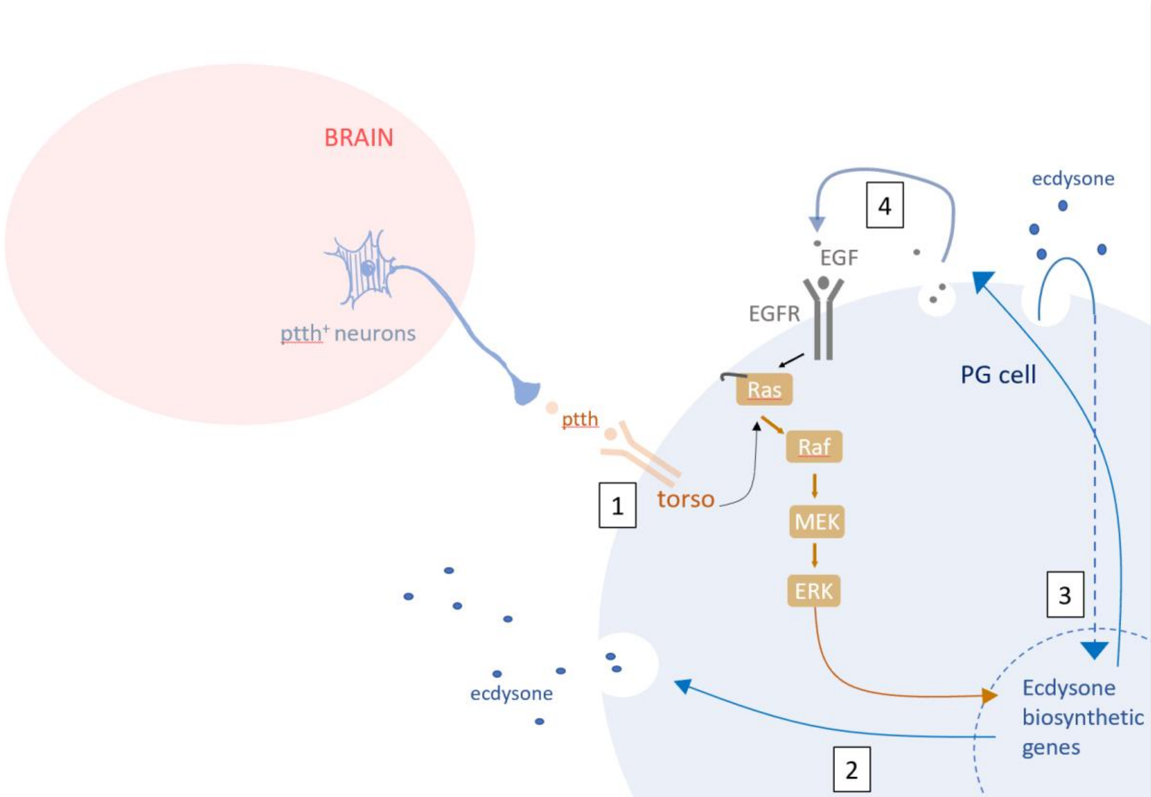

Figure 6. Autocrine Egf/Egfr signaling amplifies ecdysone production at the end of the larval period. Signaling by Ptth through Torso (1) first triggers Halloween gene expression, eliciting an initial pulse of ecdysone production and release by the PG (2). This ecdysone re-enters the PG cell and signals in autocrine fashion through the heterodimeric nuclear receptor EcR/Usp. The EcR/Usp bound to ecdysone enters the nucleus to affect gene expression (3). Among the positively-regulated target genes are Egf ligands *spitz* and *vein.* These are expressed and signal in an autocrine manner through Egfr on the PG cell (4), in turn further activating the Ras/ERK pathway and leading to greater Halloween gene expression to amplify ecdysone production to promote the larva-pupa transition. Adapted from Cruz et al. (2020).

Insulin/PI3K

Insulin signaling is a major pathway shown to couple nutrient availability and the timing of the juvenile-adult transition[53]. Extensive work has demonstrated that insulin signaling in the PG promotes ecdysone synthesis and the larval-pupal transition[54–58]. Genetic upregulation of PG-specific insulin signaling induces early metamorphosis and hence a lower final body size, whereas genetic downregulation causes the opposite[53]. More recent work has shown that control of ecdysone biosynthesis by insulin signaling in the PG is at least in part through interaction of FOXO with the EcR co-receptor, Usp[59]. Nuclear FOXO complexes with Usp and transcriptionally represses key ecdysone biosynthetic genes[59]. In the model proposed by this group, Dilp secretion by the IPCs increases as growing larvae feed[59]. As a result of increased circulating Dilps, there is more frequent signaling through InR in PG cells[59]. This leads to exclusion of FOXO from the PG, and thus de-repression of the ecdysone biosynthetic genes to promote metamorphosis[59]. This way, larval nutritional status is linked to the timing of maturation by PG-specific insulin activity.

mTORC1

Prior to maturation the cells of the PG undergo endocycling to massively increase their ploidy to a value of 64C[60]. This increase in ploidy in the steroidogenic PG cells is required for sufficient ecdysone synthesis at the larval-pupal transition[60]. Blocking PG endocycling results in developmental arrest at the larval stage due to blunted ecdysone production[60]. Recent work has implicated the mTORC1 pathway in this endocycling[60,61]. mTORC1 activity promotes endocycling by upregulating Cyclin E which in turn promotes S-phase entry[60]. As mTORC1 is a major integrator of nutritional status, this is thought to be a mechanism whereby nutrition informs the timing of the developmental transition from larva to pupa[60].

The Larval-Pupal Transition is Gated by Developmental and Nutritional Checkpoints

Attainment of a 'Critical Weight' Constitutes the Nutritional Checkpoint

As larvae feed and grow, they need to reach a point of having obtained sufficient nutrient stores that can be mobilized to support metamorphosis - a non-feeding period entailing the energy-expensive process of whole organism remodeling[62,10]. Thus, it is essential that larvae don't commit to maturation to the pupal stage before reaching this point - a point known as Critical Weight (CW)[10]. However, once they do reach CW, then even when starved of further nutrients they initiate metamorphosis as normal[10]. Hence, CW can be defined as the point where starvation does not prevent maturation (**Figure 7**)[63]. Growing in rich nutrient conditions, larvae reach this point in a few days, however in poor nutrients it is delayed[63]. This adaptation requires that larvae sense their nutritional status and couple it to the production of ecdysone by the PG[63]. Recent work suggest that this is achieved through both insulin/PI3K and TOR kinase signaling in the PG[53,59,64,60]. Genetic activation of insulin signaling in the PG induces precocious CW attainment and hence maturation in larvae whereas ablation of the pathway strongly delays it[53]. Moreover, genetic TOR activation specifically in the PG has been shown to significantly reverse developmental delay in nutrient-deprived larvae[64].

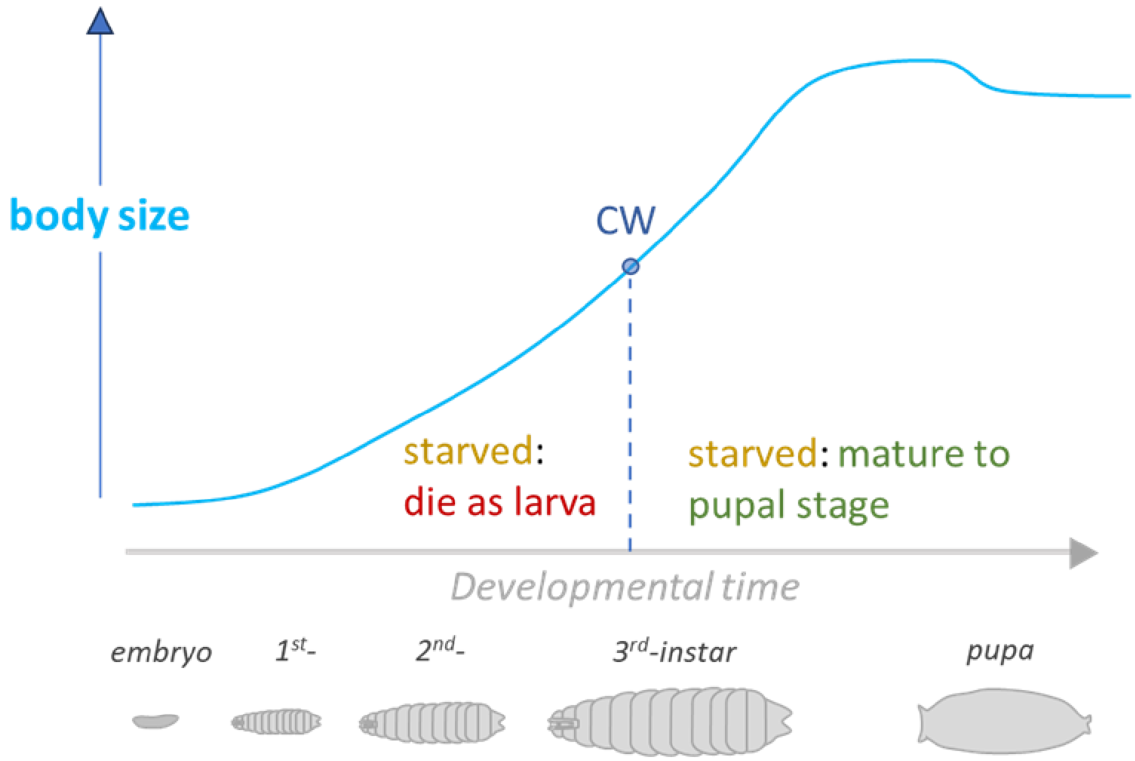

Figure 7. The Critical Weight (CW) checkpoint gates the metamorphic transition based on larval mass. As larvae feed, their body mass (blue) increases. They must reach a certain size in order to undergo maturation, a size called CW. Starvation before reaching CW leads to death but starvation after leads larvae to pupate on time with fed controls. This checkpoint occurs in the early third instar (intersection of dotted line and X-axis). Nutrient reduction applied pre-CW delays maturation, but does not delay timing if applied post-CW. Adapted from Mirth and Riddiford (2007).

Proper Tissue Growth Constitutes the Developmental Checkpoint

In the larva, the presumptive adult structures develop as discrete tissues called imaginal discs. These give rise to the eyes, wings, and legs of the adult fly, among other structures. The end of the larval period marks the end of imaginal tissue growth. Thus, an important prerequisite for maturation to the adult stage is proper growth and patterning of the imaginal discs[10,45]. The onset of pupariation is delayed if imaginal discs suffer lesions[65]. This delay allows for the damaged tissues to regenerate before metamorphosis occurs[65]. Moreover, tumor growth in any of the imaginal discs delays or completely blocks the onset of metamorphosis[65]. How is imaginal disc growth coupled to maturation? Damaged or tumor-bearing discs secrete *Drosophila* insulin-like peptide 8 (Dilp8) into the hemolymph[65]. This signal binds its receptor, Lgr3, a relaxin-family peptide receptor, on a subset of neurons, thereby activating them[66]. The $Lgr3^+$ neurons in turn inhibit Ptth release by the $Ptth^+$ neurons, thus suppressing ecdysone production in the PG and delaying maturation[45,66].

1.3 How does Hypoxia Affect Larval Growth and Development?

In the lab, when exposed to moderate hypoxia (5% oxygen) larvae are able to survive and develop into pupae, however they adapt to these low oxygen conditions by reducing their growth rate and delaying development to the pupal stage. The few studies which have probed the mechanistic bases of these adaptations have chiefly suggested mechanisms for the growth reduction. However, the delay in larval maturation remains unexplored.

In this section, I describe what is currently known about how larvae adapt their development to hypoxia. Only a handful of papers have explored the mechanisms by which hypoxia suppresses larval growth and development. However, these papers point to regulation of HIF-1α, insulin and TOR as the main mechanisms by which larvae adapt their growth and survive in low oxygen.

Induction of HIF-1 alpha:

The Hypoxia-inducible factor-1α (HIF-1α) is a conserved, classical mediator of cellular adaptation to low oxygen[67]. Under normal oxygen conditions, HIF-1α is hydroxylated by the enzyme HIF prolyl hydroxylase (Hph, known as Fatiga in *Drosophila*) and subsequently targeted for degradation[67]. Upon reduction of oxygen, Hph is inhibited and HIF-1α is thereby stabilized and can enter the nucleus to mediate transcriptional responses to hypoxia (**Figure 8**)[67]. Extensive work in cell culture has explored how HIF-1α mediates cellular responses to hypoxia by regulating expression of genes related to glucose metabolism, angiogenesis, cell proliferation and survival[68].

Drosophila have a single HIF-1α homolog known as sima. Animals which are mutant for sima display reduced larval survival in hypoxia, but are viable in normal oxygen conditions – indicating a requirement for sima in developmental progression in hypoxia[69]. These effects depend on tissue-specific roles of sima. One example is sima function in the tracheae of larvae[70]. The tracheal system is a branched network of tubular epithelia which supplies oxygen to all organs and tissues[71]. Similar to mammalian blood vessels, the terminal branches of the tracheae are able to sprout new projections toward hypoxic areas[71]. This hypoxia-induced tracheation depends on sima in the tracheal terminal cells (TTCs) which induces the fibroblast growth factor receptor (FGFR) homolog, *breathless* (*btl*)[70]. This sensitizes tracheae to the FGF homolog, branchless (bnl), which is induced and secreted by hypoxic cells in a sima-dependent manner[70]. The FGF signal thereby promotes tracheation toward hypoxic areas. Thus, sima - specifically in the tracheal cells - mediates compensatory oxygenation of hypoxic tissues and organs. Another tissue-specific role of sima is evident in the *Drosophila* fat body (analogous to the mammalian liver)[27]. Here, sima was shown to reduce systemic growth in hypoxia through non-autonomous suppression of insulin signaling, as discussed below.

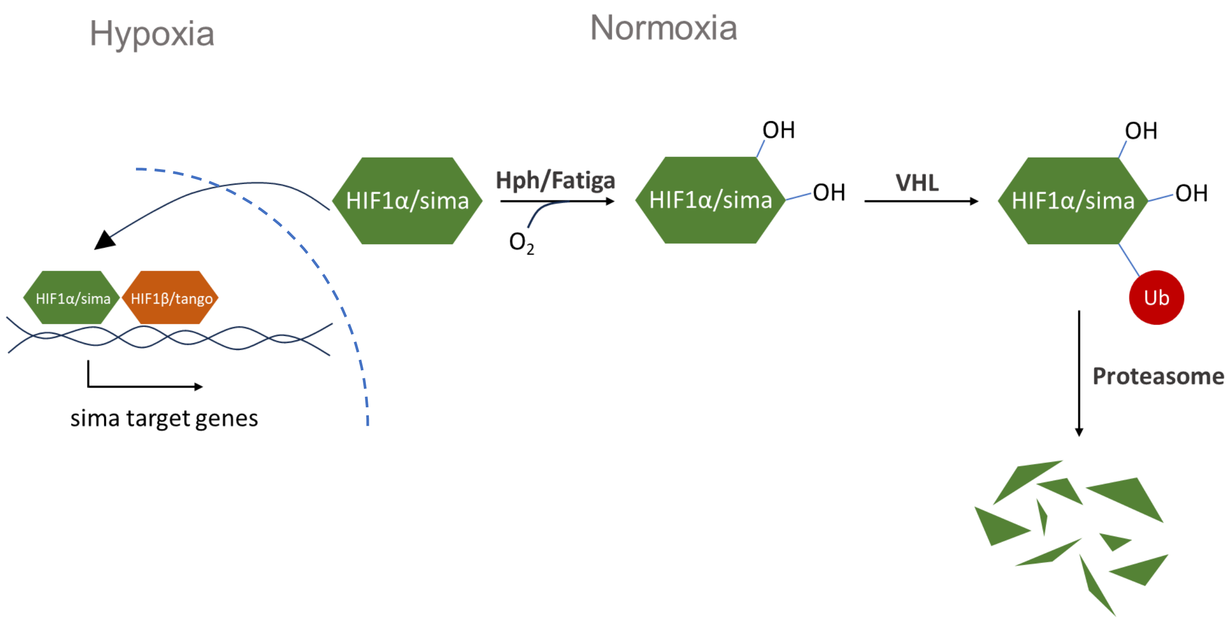

Figure 8. Regulation of HIF-1α/sima in *Drosophila.* When oxygen is present, the prolyl hydroxylase Hph (mammals)/ Fatiga (flies) and VHL mark HIF-1α for proteasome-mediated degradation. Reduced oxygen shifts the flux of HIF-1α leftward in the diagram, where it enters the nucleus of the hypoxic cell to mediate transcriptional changes with its partner HIF-1β/Tango. HIF=hypoxia-inducible factor; Hph=HIF prolyl hydroxylase; VHL=von Hippel Lindau tumor suppressor.

Suppression of Insulin signaling

Recent studies have described how moderate hypoxia can suppress larval growth by reducing systemic insulin signaling[27,72,30]. One way this occurs is through hypoxia suppressing the ability of the IPCs to secrete Dilps. One potential mechanism involves sima/HIF-1α in the larval fat body[27]. The authors proposed that the non-autonomous growth-restriction by the larval fat is mediated by as-yet-unidentified adipokines[27]. By this model, adipokines are upregulated in the fat in a sima-dependent manner and target the insulin-producing cells to blunt their secretory function[27]. Sima function in the larval fat body therefore modulates systemic growth according to oxygen availability[27].

Recent work from the Grewal lab also demonstrates that insulin suppression in hypoxia promotes larval survival by upregulating innate immunity[72]. This response is mediated by FOXO, which translocates to the nucleus upon reduced PI3K/Akt signaling to induce *Relish*, the *Drosophila* homolog of NFκB[72]. FOXO-dependent Relish induction was required for hypoxia tolerance, as Relish null mutants showed markedly reduced hypoxia survival, but remained viable in normoxia[72].

Suppression of TOR kinase signaling

Although mainly regarded as a regulator of growth in response to fluctuations in nutrient levels, studies in both *Drosophila* and cell culture reveal that mTORC1 activity is also suppressed by hypoxia[73]. Recent work from the Grewal lab - done in larvae grown in modest hypoxia (5% O_2) – demonstrate that larvae rewire their fat metabolism to accumulate lipid stores[29]. This accumulation of lipid stores was required for development of viable adults during the pupal stage following larval growth in hypoxia[29]. Interestingly, this lipid remodeling was independent of HIF-1α and due instead to reduced mTORC1 activity in the larval fat body[29]. When this mTORC1 suppression was prevented, by overexpression of the TOR activator, Rheb during larval development in hypoxia, animals did not develop past the pupal stage[29]. Hence, organismal lipid remodeling - by mTORC1 suppression in the fat body– is required for viable development in hypoxia.

1.4 Thesis Objectives and Aims

Drosophila larvae have provided a powerful model system to understand how growth and maturation are regulated during the juvenile stages of animal development. As discussed in the previous sections, a few studies have provided some insights into how hypoxia suppresses body growth during development [30,27,29,28]. **However, the mechanism(s) by which hypoxia delays larval maturation remain poorly understood.** For instance, it is not known whether hypoxia delays development by impinging on one or more of the several different signaling pathways that control ecdysone production or whether hypoxia may function to modulate the developmental and/or nutrient checkpoints that govern maturation. The central focus of my thesis research has been to elucidate the mechanism(s) by which hypoxia delays steroid-dependent maturation and thereby address this gap in knowledge. For this purpose, my research had two specific objectives:

1. Identify the signaling pathway(s) involved in the hypoxia-dependent developmental delay.

2. Identify the tissues wherein these signaling changes are important for the hypoxia-induced delay.

Chapter Two: **MATERIALS AND METHODS**

2.1 *Drosophila*

2.1.1 Drosophila Stocks

Fly Stock	**Source**
spok-GAL4;UAS-dicer	BDSC #80578
phm-GAL4	BDSC #80577
esg-GAL4	BDSC #84303
elav-GAL4	BDSC #458
UAS-Egfr-RNAi	BDSC #25781
P[lacW]spi^{s3547}/CyO	BDSC #10462
UAS-sSpi	BDSC #63134
UAS-spi-RNAi	VDRC #3920
UAS-vn-RNAi	VDRC #50358
UAS-Rheb	BDSC #9688
ywhsflp122;+;+	Prober and Edgar, 2002
yv	BDSC #36303
w^{1118}	BDSC #5905
GD control line	VDRC #60000
UAS-RafGOF	Prober and Edgar, 2002
UAS-sima RNAi	VDRC #106187
UAS-sima-RNAi (TRiP)	BDSC #33895
KK control line	VDRC #60100
w[*]; TI[RFP[3xP3.cUa]=TI]Ptth[attP]	BDSC #84568
r4-GAL4	BDSC #33832
rn-GAL4	BDSC #78345
Usp-IR	BDSC #27258
FOXO-RNAi	VDRC #107786
P0206-GAL4	gift from Mike O'Connor
phm-GAL4;UAS-dicer	gift from Mike O'Connor
ptth^{120F2A}, UAS-λTOP	gift from Mike O'Connor

Table 1. *Drosophila* stocks used in this study.

Note: 'BDSC' refers to the Bloomington *Drosophila* Stock Center, 'VDRC' refers to the Vienna *Drosophila* Resource Centre.

2.1.2 Control Strains

Control larvae were obtained by crossing the Gal4 line with flies of the same genetic background as the relevant UAS transgene line. For instance, the control cross for w^{1118}*; P0206-GAL4* x *yv; Egfr-RNAi* is w^{1118}*; P0206-GAL4* x *yv*.

2.1.3 Rearing Conditions

Flies were raised on standard medium of 150 g agar, 1600 g cornmeal, 770 g Torula yeast, 657 g sucrose, 2340 g D-glucose, 240 mL acid mixture (propionic acid/phosphoric acid) per 34 L water and maintained at room temperature or in 18°C.

2.1.4 Egg Collections

Adult flies were allowed to lay eggs on grape juice-agar plates supplemented with yeast paste at 25°C for a 4-hour period. 24 hours following the egg collection period, larvae were transferred in groups of 30 to vials containing food (30 larvae per vial) and left to develop. Grape juice agar plates were made according to the following recipe: 250 mL "Minute Maid" brand grape juice, 25 g table sugar, 1.5 g nipagin (methyl 4-hydroxy benzoate, Sigma), 22.5 g agar, 750 mL water.

2.1.5. Drosophila developmental timing

For each cross, virgin female *GAL-4* flies were crossed with males of the *UAS* line (or appropriate control line) of interest by placing both into an embryo collection cage containing a standard grape plate smeared with yeast paste to promote egg laying. Four hours later, the plates containing the laid eggs were removed and 24 hours following egg laying, newly hatched larvae were transferred to vials of standard fly medium. To each vial of food, 30 larvae were transferred unless otherwise noted. The number of organisms that pupated in each vial was counted and recorded each subsequent day at 8am and 3pm. For each treatment group, a minimum of 3 vials were used to calculate the average time to pupation.

2.1.6. Drosophila pupal volume

Pupae were first positioned ventral side-down on filter paper. Images of pupae were captured with the ZEISS SteREO Discovery.V8 microscope and ZEN imaging software (blue edition) at 1.25x magnification. From these images, the ZEN software was used to measure the width and length of each pupa (in µm), which were then inserted into a mathematical formula for the volume of an ellipsoid ($4/3\pi \times L/2 \times W$). Each value resulting from this calculation constituted the pupal volume of one animal.

2.1.7. Gene Expression System

GAL4/UAS

The Gal4/UAS system, as used in *Drosophila* genetics, is a tool for regulating the expression of a gene of interest (GOI) in a tissue- or developmental stage-specific manner. Expression of the yeast transcription factor, Gal4, is coupled to the activity of an enhancer of choice. The Gal4 protein (expressed in the tissue-/stage-specific manner dictated by the enhancer's activity) targets its cognate *Upstream Activating Sequence* (*UAS*), which is upstream of the coding sequence for the GOI (Duffy, 2002). To obtain experimental progeny, virgin female flies bearing the *enhancer-GAL4* construct in their germline are mated to male flies bearing the *UAS-GOI* in their germline. The *GAL4/UAS* system may also be used to knock down genes by RNA-interference by the following. Instead of an allele for the GOI, a sequence encoding a short hairpin (sh)RNA against the GOI is cloned downstream of the *UAS*. Once expressed, the shRNA is processed into small interfering (si)RNA by the Dicer enzyme, which in turn target the specified transcript for degradation by endogenous RNAi machinery (Perrimon et al., 2010). The knockdown efficacy of *UAS-RNAi* lines in this study have been verified in previous work in the lab using qRT-PCR for respective target genes.

2.2 Hypoxia Experiments

2.2.1 Hypoxia Exposure and Hypoxia Developmental Timing

Vials containing larvae were placed in an airtight chamber into which a gas mixture of 5% O_2 and 95% O_2 was continuously flowed. The rate of flow was controlled using an Aalborg model P gas flow meter. The time of exposure was either immediately after larval transfer to food (24 hours after egg-laying (h AEL)) or 120 h AEL (post-critical weight attainment). Larvae were left in hypoxia to pupate until no further pupation was observed for 24 hours after the initial onset of pupation was observed in all vials. During this time, developmental timing was performed on hypoxic larvae by counting pupae through the transparent walls of the hypoxia chamber.

2.2.2 Starvation treatment for Critical Weight Determination

w^{1118} larvae were collected at 24 h AEL and transferred into regular food vials (30 larvae per vial). At 72, 96 and 120 h AEL, larvae were removed from these vials by suspending them in 20% sucrose and transferred to vials containing either regular food or an equal volume of 0.8% agar.

2.2.3 Determination of Age at Critical Weight Attainment in Hypoxia and Normoxia

w^{1118} larvae were collected and transferred into regular food vials (30 larvae per vial). These vials were either placed into hypoxia to develop or left at ambient oxygen. At 96, 100, 112, 116, 120, 124 and 136 h AEL, larvae were transferred to 0.8% agar as in the above protocol and left to develop in ambient oxygen. The resulting percent pupation was calculated and plotted as a function of the age at starvation for each vial. A minimum of 3 vials of 30 larvae were used to calculate the mean percent pupation for each condition at each starvation time point. The point on the x-axis (age of starvation in h AEL) on the graph where >50% mean pupation was achieved was defined as the age at critical weight attainment for each treatment group (normoxia or hypoxia).

2.2.4 20-hydroxyecdysone feeding

w^{1118} larvae were collected at 24 h AEL and transferred into vials containing food supplemented either with 20-hydroxyecdysone (Sigma-Aldrich CAS number 5289-74-7) dissolved in 95% ethanol (final 20E concentration of 0.3 mg/mL) or an equal volume of 95% ethanol alone.

2.2.5 Immunostaining and Microscopy

Larvae were inverted and fixed in 8% formaldehyde (Electron Microscopy Science, Hatfield, U.S.A.) in PBS at room temperature for 30 minutes, then blocked for 2 hours in 1% BSA in PBS/0.1% Triton-X 100 at room temperature. Blocked samples were then incubated at 4°C overnight in primary antibody (rabbit anti-beta-galactosidase, Jackson ImmunoResearch Laboratories) dissolved at a 1:200 dilution in 1% BSA in PBS/0.1% Triton-X 100. Tissues were then incubated in secondary antibody (Alexa Fluor 568 (Molecular Probes) goat anti-rabbit secondary antibody (1:2000). DNA was visualized using Hoechst 33258 (Invitrogen, 1:10 000).

Brain-Ring gland complexes were then dissected out and mounted on glass slides using Vectashield mounting media. Confocal micrographs were captured using a ZEISS confocal microscope LSM 880.

2.2.6 Quantification of Spitz expression in PG cells

Confocal slices of ring glands from *spitz-LacZ* larvae stained for β-galactosidase were used for analysis in Fiji ImageJ2 (Version 2.3.0). β-galactosidase staining intensity was measured by taking four PG cell nuclei (as discerned by Hoechst staining) at random per PG and measuring the RFP fluorescence intensity in a box of fixed area which fit inside the PG cell nucleus. This way, 4 measurements were taken per animal.

2.2.7 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

A) Sample Collection

Whole larvae were used for qRT-PCR. Larvae were collected in groups of 8 in Eppendorf tubes and snap frozen on dry ice. They were kept at -80°C until immediately before RNA isolation

B) RNA isolation

RNA was extracted using TRIzol (Invitrogen, 15596-018) by the following procedure: TRIzol (250 µl) was added to tubes containing frozen samples, prior to homogenization using a motorized pestle. Another aliquot of TRIzol (250 µl) was added to the homogenized samples which were then left to incubate at room temperature for 5 minutes. Chloroform (100 µl) was added to the sample tubes, which were then shaken vigorously for 15 seconds and left to incubate at room temperature for 2 minutes before centrifugation for 15 minutes at 12000 relative centrifugal force (rcf) at 4°C. A portion of the upper aqueous phase (220 µl) was transferred into a new set of Eppendorf tubes by twice pipetting 110 µl. To these new tubes, isopropanol (250 µl) was added before shaking them for 15 seconds to mix the solution. They were then centrifuged for 10 minutes at 12000 rcf at 4°C. The resultant supernatant was then removed (pipetted out), leaving the RNA pellet, to which 75% ethanol (500 µl) was added before centrifuging at 7500 rcf for 5 minutes at 4°C. The supernatant was then removed and the pellet left to air dry. To the pellet, DNAse-RNAse free water (200 µl) was added. Finally, the tubes were heated for 5 minutes at 55°C to dissolve the RNA pellet and kept at -80°C until immediately before DNase treatment.

C) DNase Treatment

RNA samples were DNase-treated with Ambion Turbo DNase according to manufacturer's instructions (Ambion; 2238 G), as is described here. For each sample, 44 µl of RNA, 5 µl of 10X TURBO DNase buffer (Ambion, AM1907), and 1 µl of DNase (AM1907) were added to a PCR tube. These were incubated for 20 minutes at 37°C before adding 5 µl of DNase inactivation reagent to the tubes. Samples were then allowed to incubate at room temperature for two minutes before centrifugation at 10 000 revolutions per minute (rpm) at room temperature. 25 µl of supernatant were collected for each sample and transferred to new PCR tubes which were then stored at -80°C until immediately before cDNA preparation.

D) cDNA Preparation

The following reagents were combined in a 1.5 mL Eppendorf tube, constituting Master Mix 1: 1 µl random primer and 6 µl water, per sample. 8 µl of this mix was then added to PCR tubes each containing 4 µl of an RNA sample. Each of these tubes were then centrifuged to mix the contents and heated for 5 minutes at 65°C. Then, the following reagents were combined in a second 1.5

mL Eppendorf tube, constituting Master Mix 2: 4 µl 5X first-strand buffer, 2 µl DTT, 1µl RNAse-Out and 1 µl Superscript II, per sample. 8 µl of this mix was then added to each PCR tube (already containing RNA sample + Master Mix 1). The PCR tubes were then placed into a thermocycler for reverse transcription, using the following steps: 25°C for 10 min, 42°C for 50 min, 70°C for 15 min and 20°C for 30 min. The resultant cDNA samples were then diluted by a factor of 5 in DNase/RNase-free water and stored at 4°C.

E) Quantitative real-time PCR (qRT-PCR)

Resultant cDNA was used as a template for qRT–PCR (ABI 7500 real time PCR system using SyBr Green PCR mix) with the below primer pairs. PCR data were normalized to levels of Rpl32.

- spookier forward: TATCTCTTGGGCACACTCGCTG
- spookier reverse: GCCGAGCTAAATTTCTCCGCTT
- phantom forward: GGATTTCTTTCGGCGCGATGTG
- phantom reverse: TGCCTCAGTATCGAAAAGCCGT
- spitz forward: CGCCCAAGAATGAAAGAGAG
- spitz reverse: AGGTATGCTGCTGGTGGAAC
- rpl32 forward: ATGCTAAGCTGTCGCACAAA
- rpl32 reverse: GTTCGATCCGTAACCGATGT

2.3 Statistical Analyses

The mean times to pupation and pupal volume measurements were analyzed by two-way ANOVA followed by unpaired Student's t-test with Welch's correction for unequal variance, using an alpha value of 0.05. For qRT-PCR and immunostaining quantification, unpaired

Student's t-test with Welch's correction for unequal variance was employed for pairwise comparisons. Graphs and statistical analyses were produced using GraphPad Prism 9.0.0 for Windows by GraphPad Software (San Diego, California USA).

Chapter Three: **RESULTS**

3.1. Hypoxia Delays Larval Maturation

Larvae were reared either in ambient oxygen or hypoxia (5% oxygen) from hatching and their time to pupation was measured. Hypoxic larvae exhibited roughly a 2-day delay in development to the pupal stage (Figure 9A). Although pupating at a smaller final size, as described previously[27 29], the hypoxic animals showed no impairment in survival to the pupal stage, relative to their normoxic counterparts (Figure 9B and 9C). The onset of pupation is regulated by a neuroendocrine circuit that elicits a pulse of ecdysone production from the prothoracic gland (PG) at the end of the larval period[74]. As a readout for this pulse, we measured the expression of the Halloween genes *spookier* (*spok*) and *phantom* (*phm*), which encode enzymes required for ecdysone production. In normoxic control larvae, the expression of these genes peaked by 144 hours after egg-laying (h AEL) (Figure 9D), shortly after which the larvae pupated. In hypoxia however, the induction of both genes was blunted and delayed, with hypoxic larvae exhibiting peak induction of these Halloween genes only by 168 h AEL (Figure 9D).

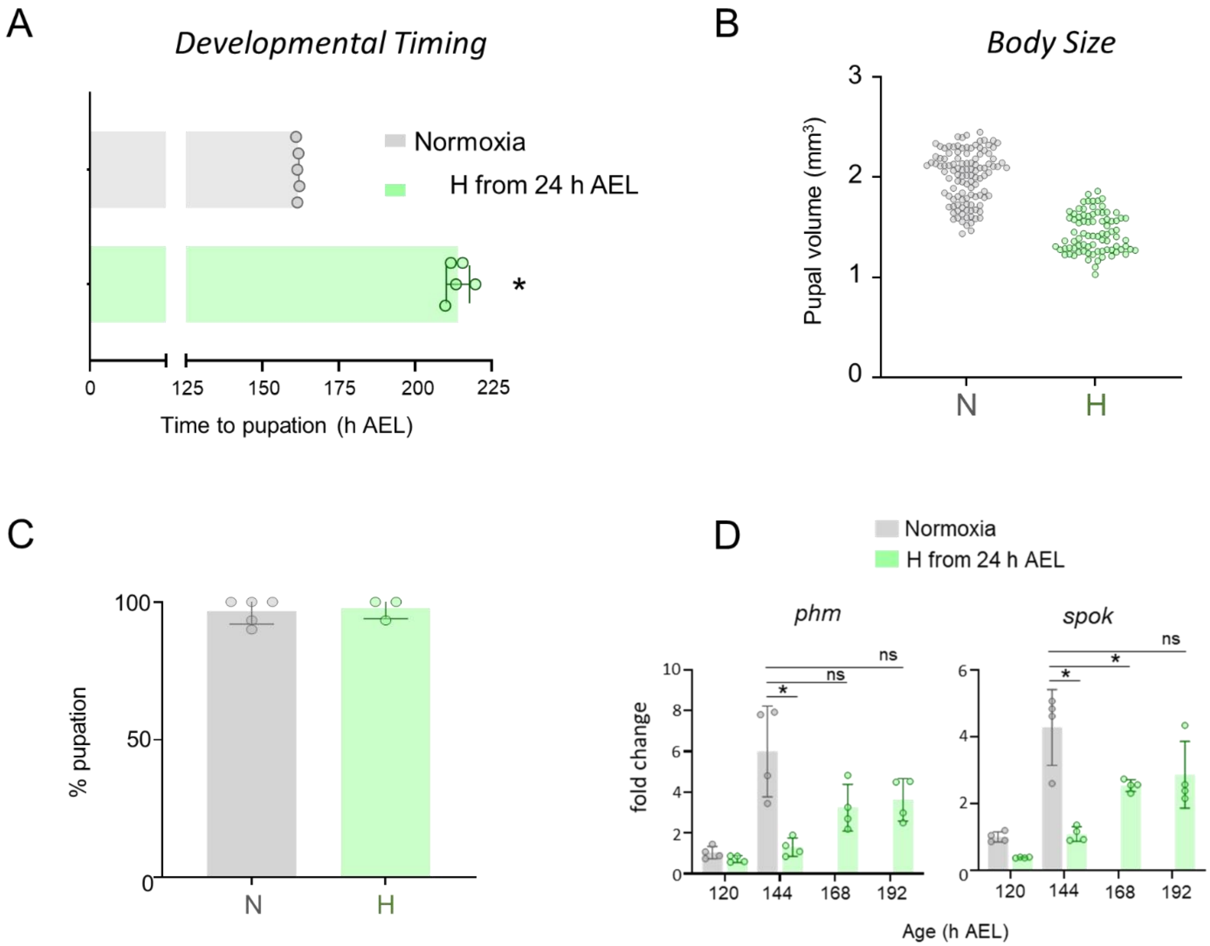

Figure 9. Hypoxia delays larval maturation and suppresses ecdysone production. (A) Average time to pupation of w1118 larvae reared in ambient oxygen or 5% O2 (from hatching), in hours after egg laying (h AEL). Each data point represents a vial of 30 larvae. n (# of vials of 30) ≥ 3 per condition. **(B)** Pupal volume of w1118 larvae reared in normoxia throughout development ('N') or in hypoxia from 24 h AEL ('H'). n (# of pupae) = 112 (normoxia) and 83 (hypoxia). **(C)** % survival to the pupal stage of w1118 larvae reared in normoxia throughout development or in hypoxia from 24 h AEL. Each data point represents the average calculated from a vial of 30 larvae. n (# of vials of 30 larvae) ≥ 3 per condition. **(D)** Relative mRNA levels (normalized to Rpl32) of ecdysone biosynthetic genes phantom (phm) and spookier (spok) from whole-larvae qRT-PCR of w1118 larvae reared in ambient oxygen or in 5% O2 from hatching (24 h AEL). n (# of independent samples) ≥ 3 per condition. * denotes $p < 0.05$.

3.2. Hypoxia Acts Post-Critical Weight to Suppress Ecdysone Signaling

The critical weight (CW) checkpoint is considered the point in development when the animal has amassed sufficient nutrient stores to undergo the energy-expensive process of metamorphosis[63]. CW attainment is defined experimentally as the point in larval development before which starvation blocks pupation and after which starvation does not block nor delay pupation[63]. The timing of this checkpoint is largely determined by the animal's growth rate which is in turn a function of insulin and TOR signaling[63 53 59]. As hypoxia is known to attenuate growth by impinging on these pathways[29 27], it was possible that the hypoxia-dependent delay in development solely reflects a lowered growth rate and subsequent delay in CW attainment. To test this, I firstly determined the developmental age at CW attainment for larvae reared in normoxia or hypoxia from hatching. This was done by shifting wild-type (w^{1118}) larvae to starvation media (agar and water) at increasing developmental ages and measuring the proportion of larvae which survived to the pupal stage. The developmental age at which >50% of larvae pupated when starved was denoted the age of CW attainment. In normoxia, this point was achieved by ~114 h AEL (Fig 10A). For animals reared in hypoxia, however, it was achieved at a markedly later age - by ~132 h AEL (Fig 10A), suggesting that developmental hypoxia does indeed delay the attainment of CW.

This ~18 hour delay in CW attainment, however, only accounted for less than half of the hypoxia-induced 2-day delay in pupation (Fig 10A vs Fig 9A), suggesting that the hypoxia-induced delay is not simply due to slow larval growth and a delay in reaching CW. Hypoxia could act post-CW to affect the late larval mechanisms that control maturation. To test this, I assessed the effect of hypoxia exposure applied either before or following CW attainment. Larvae were reared conventionally (on normal food and at ambient oxygen levels) and then at

72, 96 and 120 h AEL, they were either shifted to hypoxia or transferred to starvation media. As expected, larvae starved at 72 and 96 h AEL (pre-CW) died, whereas those starved at 120 h AEL (post-CW) survived to the pupal stage and exhibited no developmental delay (Figure 10B and 10C). Hypoxia exposure, in contrast, produced no survival defect but a strong delay when applied from the pre-CW time points of 72 h AEL (yielding a 43 hr delay) and 96 h AEL (yielding a 42 hr delay) (Figure 10C and 10B). Interestingly, hypoxia exposure from the post-CW time point of 120 h AEL also produced a strong developmental delay of ~34 hrs, which was only slightly shorter than the pre-CW-hypoxia-induced delay (Figure 10B). A reduction in final body size relative to controls was observed for all hypoxia-exposed larvae (Figure 10D). However, whereas the reduction elicited by both pre-CW treatments were comparable in magnitude, the reduction was significantly less pronounced for those exposed to hypoxia post-CW (Figure 10D). Taken together, these data suggest that although hypoxia acts early in development to reduce the larval growth rate and subsequently delay CW attainment, it can also specifically act late in larval development to suppress the mechanisms that promote maturation.

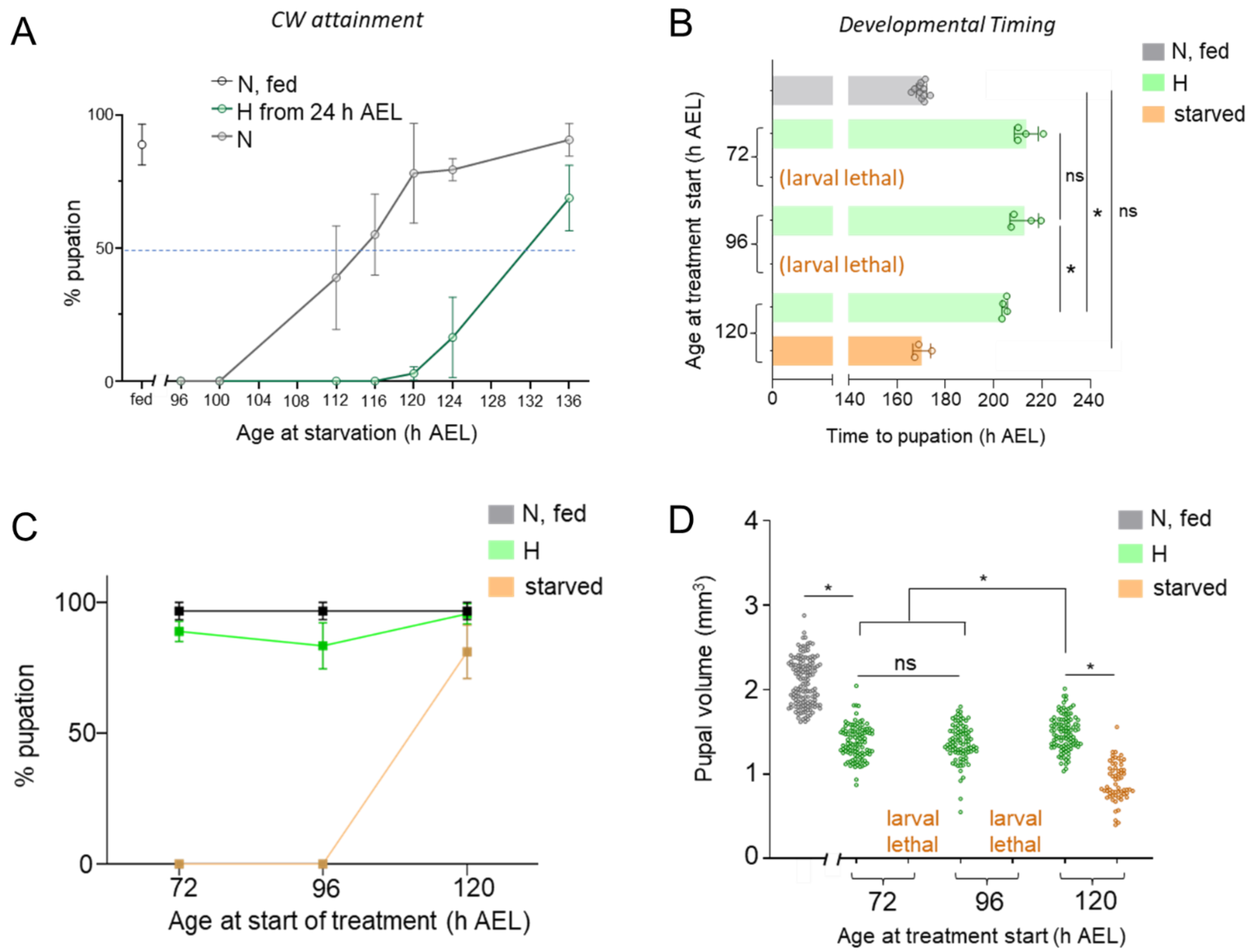

Figure 10. Hypoxia acts late in larval development to suppress maturation. (A) Determination of age at Critical weight (CW) attainment for animals reared in ambient or 5% O2 (Hypoxia, 'H') conditions. The age at CW attainment (in h AEL) was defined as the age which yielded 50% pupation upon starvation. Each data point represents the average of three vials of 30 larvae. n (# of vials of 30) ≥ 3 per condition. **(B)** Developmental timing of w1118 larvae either starved or exposed to 5% O2 at the indicated larval age. n (# of vials of 30) ≥ 3 per condition **(C)** % survival to the pupal stage of w1118 larvae either starved or exposed to 5% O2 at the indicated larval age. Each data point represents the average calculated from a vial of 30 larvae. n (# of vials of 30) ≥ 3 per condition **(D)** Pupal volume of w1118 larvae either starved or exposed to 5% O2 ('H') at the indicated larval age. n (# of pupae) = 148 (normoxia), 107 (H @ 72 h AEL), 91 (H @ 96 h AEL), 107 (H @ 120 h AEL), 62 (starvation @ 120 h AEL). * denotes $p < 0.05$.

In line with this, I found that post-CW hypoxia exposure (from 120 h AEL) blunted the expression of *phm* and *spok* to the same degree as hypoxia from hatching (24 h AEL) (Figure 11A), suggesting that the delayed ecdysone production observed in larvae exposed from early on in development may be majorly a consequence of hypoxia at the late rather than early larval stages. Moreover, feeding of 20-hydroxyecdysone (20E), the active form of ecdysone, did not affect the developmental timing of normoxic larvae but significantly rescued the timing of those reared in hypoxia from 120 h AEL (Figure 11B). Consistent with a shorter larval (growth) period, pupae of the hypoxic 20E-fed animals were significantly smaller than hypoxic controls (Figure 11C). The data presented thus far suggest, (i) hypoxia somehow suppresses the late-larval pulse of ecdysone to delay development, and (ii) larvae may employ distinct mechanisms in adapting their developmental time to oxygen-deprivation and nutrient-deprivation.

Moreover, to this second point, one way that *Drosophila* larvae have been shown to couple nutrient levels to the timing of maturation is through TOR signaling in the prothoracic gland (PG)[64]. In the model proposed by Layalle et al. (2008), low-nutrient growth conditions reduce TOR signaling activity in the PG and this in turn results in lower ecdysone production[64]. This group found that PG-specific activation of the TOR pathway by overexpressing the TOR activator, Rheb, did not affect developmental timing of well-fed controls, but strongly rescued the two-day delay seen in nutrient-deprived larvae[64]. It was possible that both nutrient-poor food and hypoxia exposure converge on TOR in the PG to delay timing. I therefore tested whether activation of TOR in the PG could similarly rescue the hypoxia-induced developmental delay. However, I saw no rescue of the hypoxia-induced delay by Rheb overexpression in the PG (Figure 12A), further suggesting that larvae use different mechanisms to adapt the timing of their maturation to low oxygen vs low nutrients.

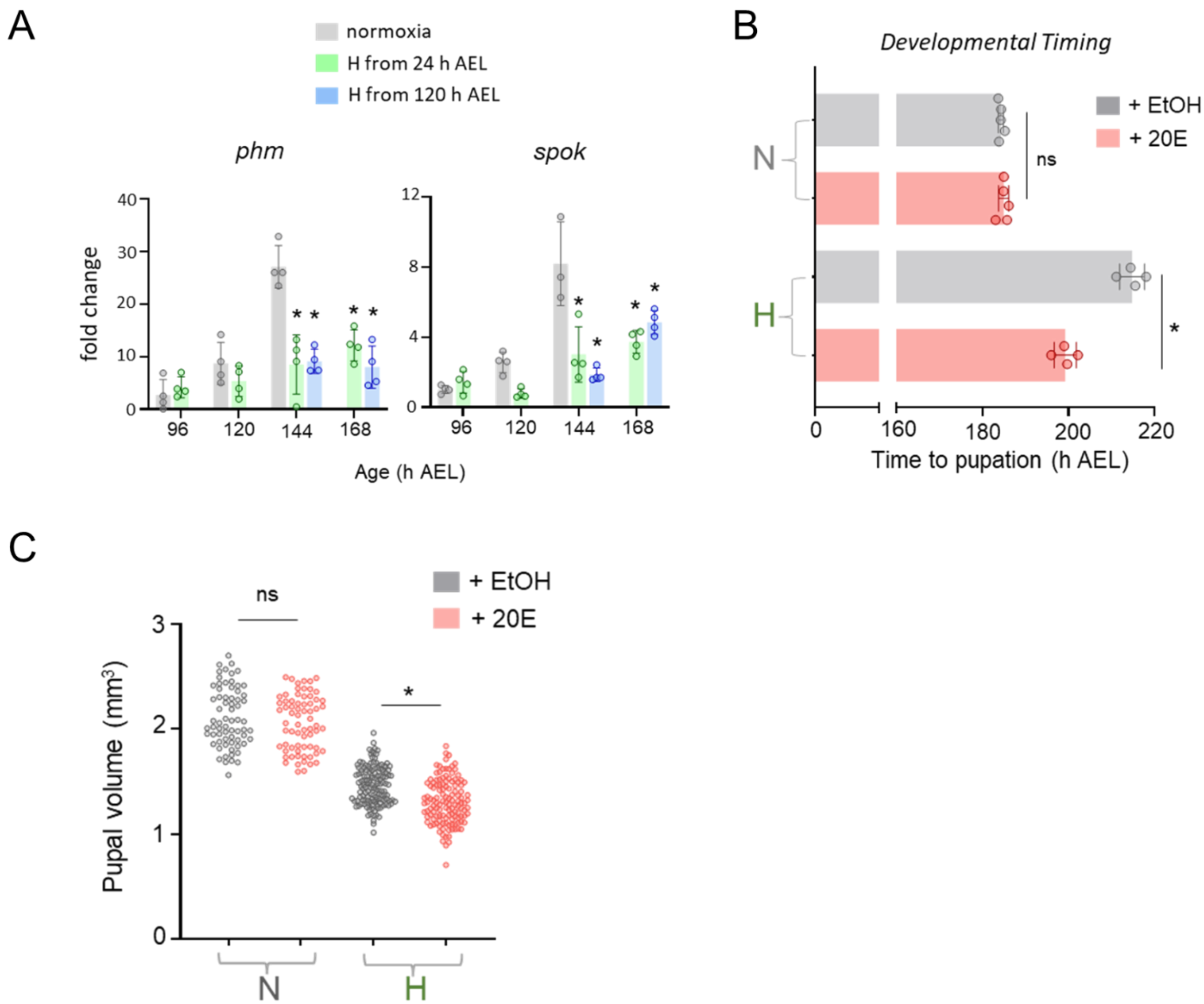

Figure 11. Post-CW hypoxia exposure delays maturation by suppressing ecdysone production. (A) Relative mRNA levels (normalized to Rpl32) of ecdysone biosynthetic genes phantom (phm) and spookier (spok) from whole-larvae qRT-PCR of w1118 larvae reared in ambient oxygen or in 5% O2 from either 24 h AEL or 120 h AEL. n (# of independent samples) ≥ 3 per condition. **(B)** Developmental timing of w1118 larvae fed either 20-hydroxyecdysone (20E) or ethanol (EtOH) vehicle, reared in either ambient oxygen or 5% oxygen from 120 h AEL (i.e., post-CW). n (# of vials of 30) ≥ 3 per condition. **(C)** Pupal volume of w1118 larvae fed either 20-hydroxyecdysone (20E) or ethanol (EtOH) vehicle, reared in either ambient oxygen or 5% oxygen from 120 h AEL (i.e. post-CW). n (# of pupae) = 68 (N, EtOH), 69 (N, 20E), 137 (H, EtOH), 127 (H, 20E). * denotes $p < 0.05$.

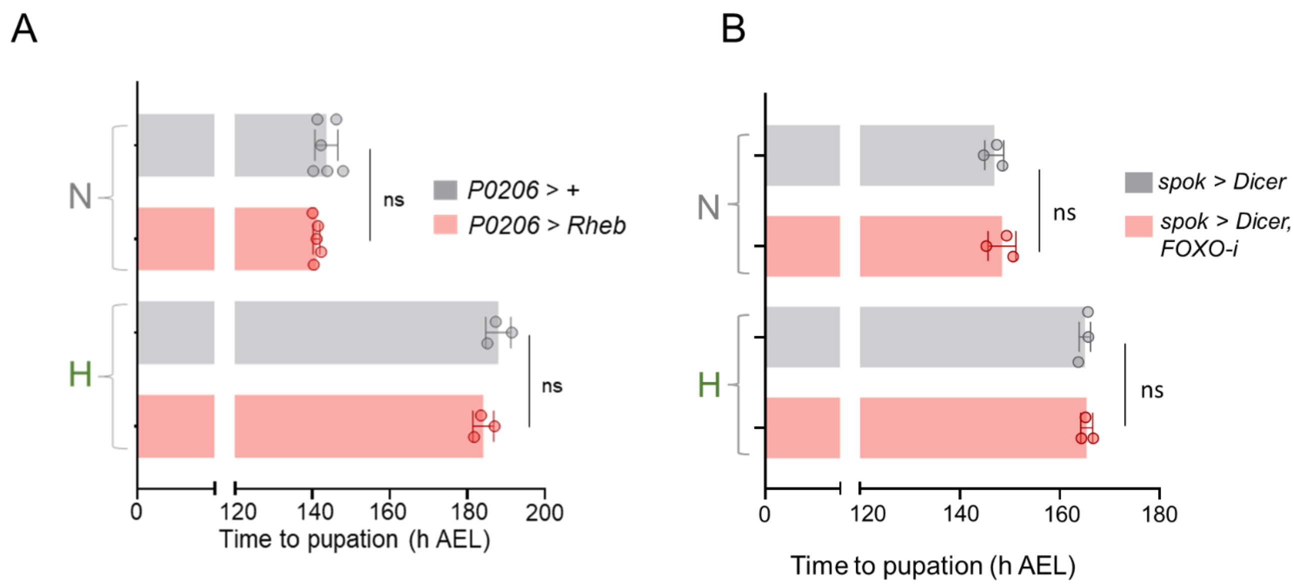

Figure 12. Hypoxia does not co-opt nutrient-responsive mechanisms to delay development. (A) Average time to pupation of larvae, either P0206 > + or P0206 > Rheb, reared in either normal oxygen conditions throughout development or shifted to 5% O2 at 120 h AEL. n (# of vials of 30 larvae) ≥ 3 per condition. **(B)** Average time to pupation of larvae, either spok > dicer or spok > dicer, FOXO-RNAi, reared in either normal oxygen conditions throughout development or shifted to 5% O2 at 120 h AEL. n (# of vials of 30 larvae) ≥ 3 per condition. * denotes $p < 0.05$.

3.3. The Hypoxia-induced Maturation Delay is Independent of HIF1-alpha/sima Function

I next assessed a role for the classic regulator of hypoxic responses, *HIF-1α* (called *sima* in *Drosophila*), in mediating the developmental delay. *HIF-1α* is continuously expressed but is rapidly degraded in the presence of oxygen[67]. In low-oxygen conditions, however, the HIF-1α protein is stabilized and subsequently translocates to the nucleus to alter the transcriptional profile of the hypoxic cell[67]. We and others have shown in *Drosophila* that it is required to modulate growth and promote survival under hypoxic stress[27 29 29 70]. I thus assessed its requirement for delaying ecdysone-dependent maturation in response to hypoxia exposure. I hypothesized that if hypoxia delays maturation by inducing sima, then depletion of sima would lead to acceleration of developmental timing in hypoxic larvae. To test this, I knocked down *sima* in the main tissues that regulate ecdysone production.

I expressed an RNAi construct against *sima* using two independent GAL4 drivers which express in the PG, *P0206-GAL4* and *phm-GAL4.* PG-specific sima knockdown with either driver did not attenuate the hypoxia-induced developmental delay (Figure 13A and 13B), suggesting the delay does not require sima function in the PG. The same was observed when *sima-RNAi* was expressed alongside *dicer* to improve RNAi efficiency (Figure 13C). Production of ecdysone by the PG, however, also relies on input from neuronal subsets such as the Ptth neurons which innervate the gland[10]. To assess a requirement for sima in this neuroendocrine circuit, I knocked down sima in all neurons using the pan-neuronal *elav-GAL4* driver and two independent *UAS-sima-RNAi* lines. In either case, I observed no reversal of the hypoxia-induced delay (Figure 14A and Figure 14B). Taken together, these data suggest the delay is independent of *sima/HIF-1α* function in the neuroendocrine circuit upstream of ecdysone production.

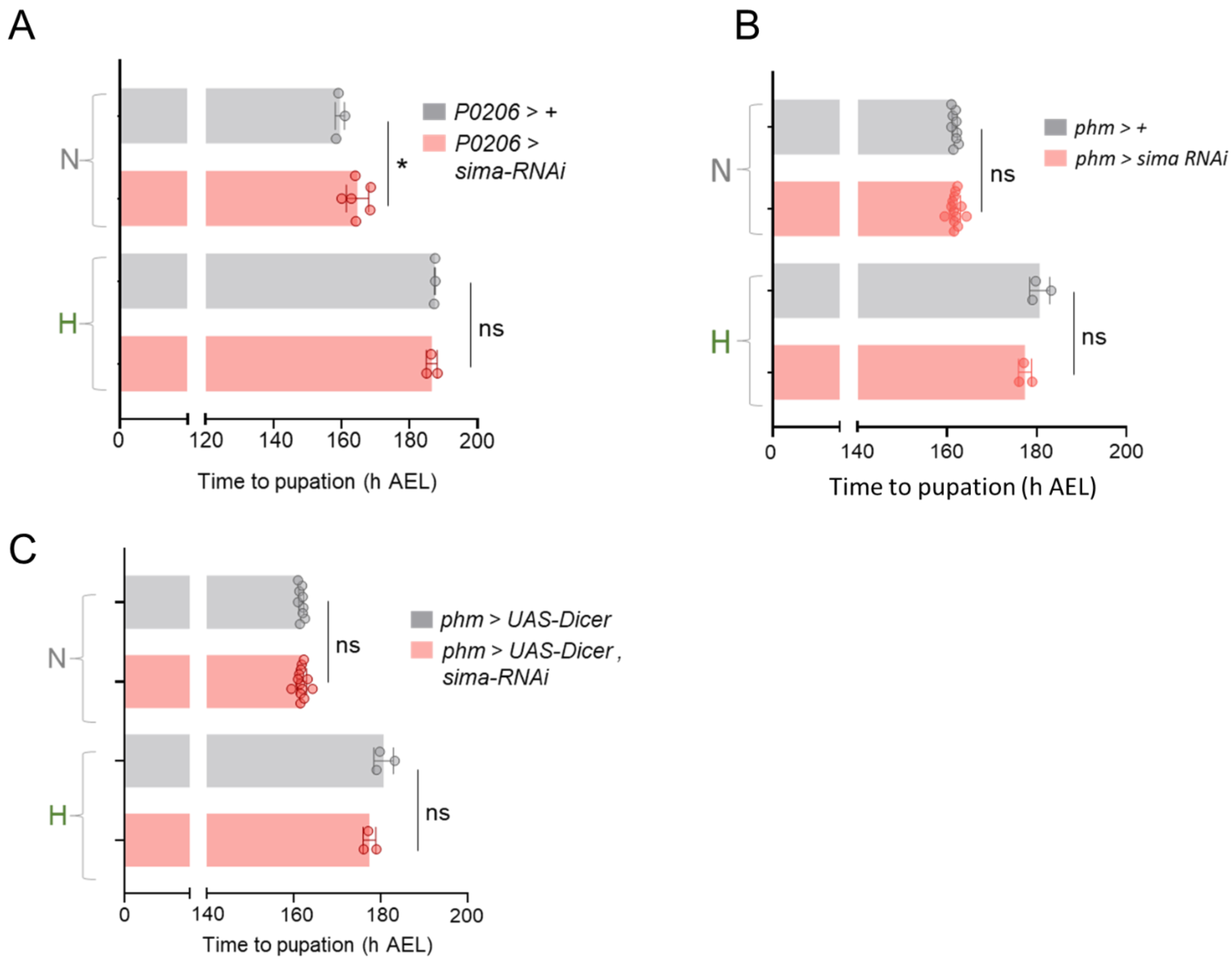

Figure 13. The hypoxia-induced maturation delay is independent of sima/HIF-1α in the PG. (A-C) Average time to pupation of larvae of the indicated genotype reared in either normal oxygen conditions throughout development ('N') or shifted to 5% O2 at 120 h AEL ('H'). n (# of vials of 30 larvae) ≥ 3 per condition. * denotes $p < 0.05$.

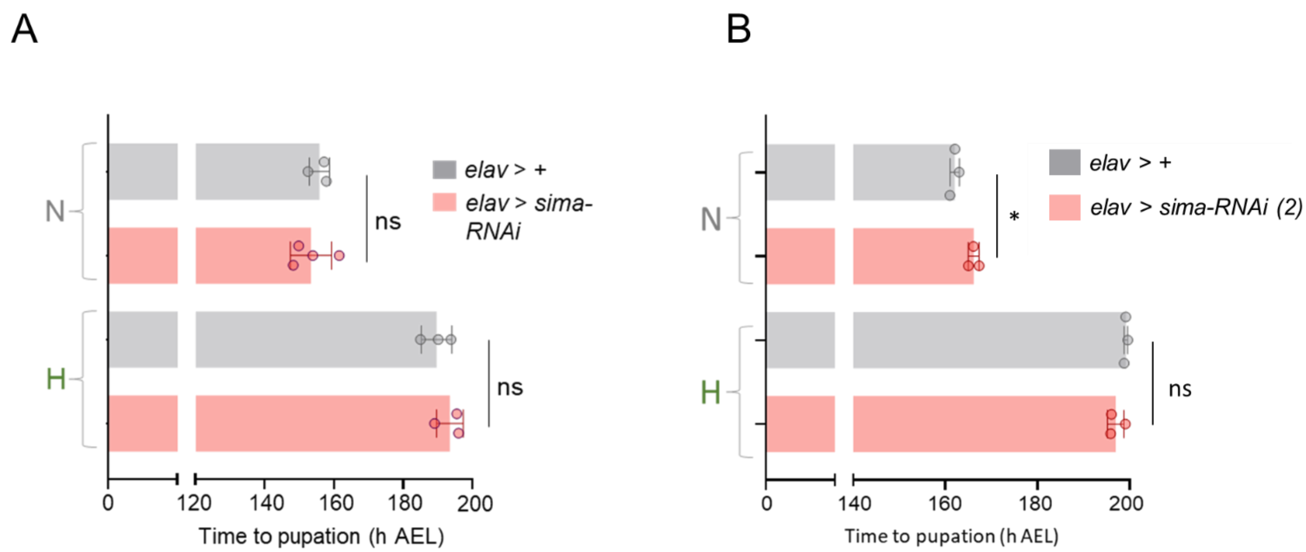

Figure 14. The hypoxia-induced maturation delay is independent of sima/HIF-1α in the neurons. (A,B) Average time to pupation of larvae of the indicated genotype reared in either normal oxygen conditions throughout development ('N') or shifted to 5% O2 at 120 h AEL ('H'). n (# of vials of 30 larvae) ≥ 3 per condition. * denotes $p < 0.05$.

3.4. The Hypoxia-induced Maturation Delay is Independent of PTTH/Torso Signaling

One of the major inputs to the PG that promotes ecdysone production at the larval-pupal transition is the neuropeptide, PTTH[75]. At the end of the larval period, it is released by a pair of neurons in each lobe of the brain which innervate the PG, and signals through its cognate receptor tyrosine kinase (RTK) Torso on PG cells, thus activating the MAP kinase cascade and transcription of Halloween genes by ERK[9 47]. Previous studies have shown PTTH to be important for adapting developmental time to other environmental cues, namely nutrient stress and crowded growth conditions, to promote subsequent adult fitness[76]. Thus, I assessed a role for PTTH/Torso in mediating the hypoxia-induced developmental delay. My hypothesis was that hypoxia delays development by suppressing Ptth/Torso signaling, and so if I genetically ablated this pathway, hypoxia exposure would then not delay development any further than the genetic ablation. To test this, I first examined developmental timing in *Ptth* null mutants. As shown by others[75], normoxic *Ptth* null mutants exhibited a strong delay and larger body size (13% increase), relative to controls (Figure 15A and 15B). A similar result was observed for a second, independent *Ptth* null mutant line (Figure 15C and 15D). These results are consistent with the role of Ptth in initiating the production of ecdysone in the PG at the end of the larval period[47]. Interestingly, in larvae exposed to hypoxia from 120 h AEL, loss of Ptth exacerbated the hypoxia-induced developmental delay, and the resultant pupae displayed a much larger size increase of 24% (Figure 15A and 15B). This same trend was seen with the second independent *Ptth* null mutant line (Figure 15A and 15B). Moreover, PG-specific depletion of the receptor for Ptth, Torso, delayed development as expected in normoxia, and exacerbated the hypoxia-induced delay (Figure 16A). Hypoxic Torso-RNAi animals displayed a 31% increase in body size, whereas the same manipulation in normoxia elicited just a 24% increase (Figure 16B). Together, these additive effects of Ptth loss-of-function and hypoxia suggest the hypoxia-induced developmental delay occurs independently of Ptth/Torso signaling.

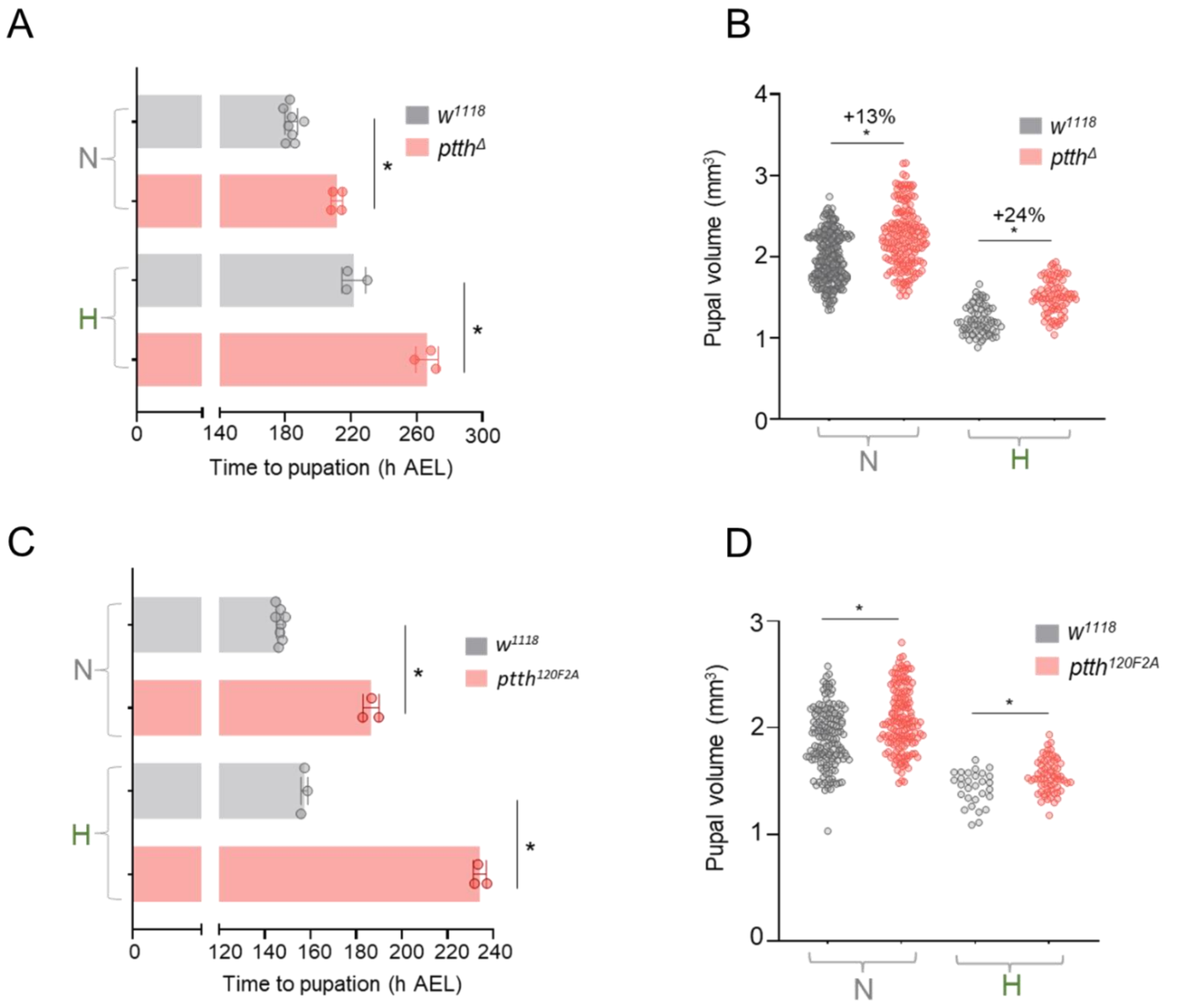

Figure 15. The hypoxia-induced maturation delay is independent of PTTH/Torso signaling. (A) Average time to pupation of larvae, either w1118 or mutant for ptth, reared in either normal oxygen conditions throughout development ('N') or shifted to 5% O2 at 120 h AEL ('H') n (# of vials of 30 larvae) ≥ 3 per condition. **(B)** Pupal size of animals, either w1118 or mutant for ptth, reared in normoxia or hypoxia from 120 h AEL. Each data point represents body size measured for one animal. n (# of pupae) = 254 (N w1118), 172 (N ptthΔ), 70 (H w1118), 79 (H ptthΔ). **(C)** Average time to pupation of larvae, either w1118 or ptth120F2A (an alternative ptth null mutant) reared in either normal oxygen conditions throughout development ('N') or shifted to 5% O2 at 120 h AEL ('H'). n (# of vials of 30 larvae) ≥ 3 per condition. **(D)** Pupal size of animals, either w1118 or mutant for ptth, reared in normoxia or hypoxia from 120 h AEL. Each data point represents body size measured for one animal. n (# of pupae) = 150 (N w1118), 150 (N ptth120F2A), 29 (H w1118), 67 (H ptth120F2A). * denotes $p < 0.05$.

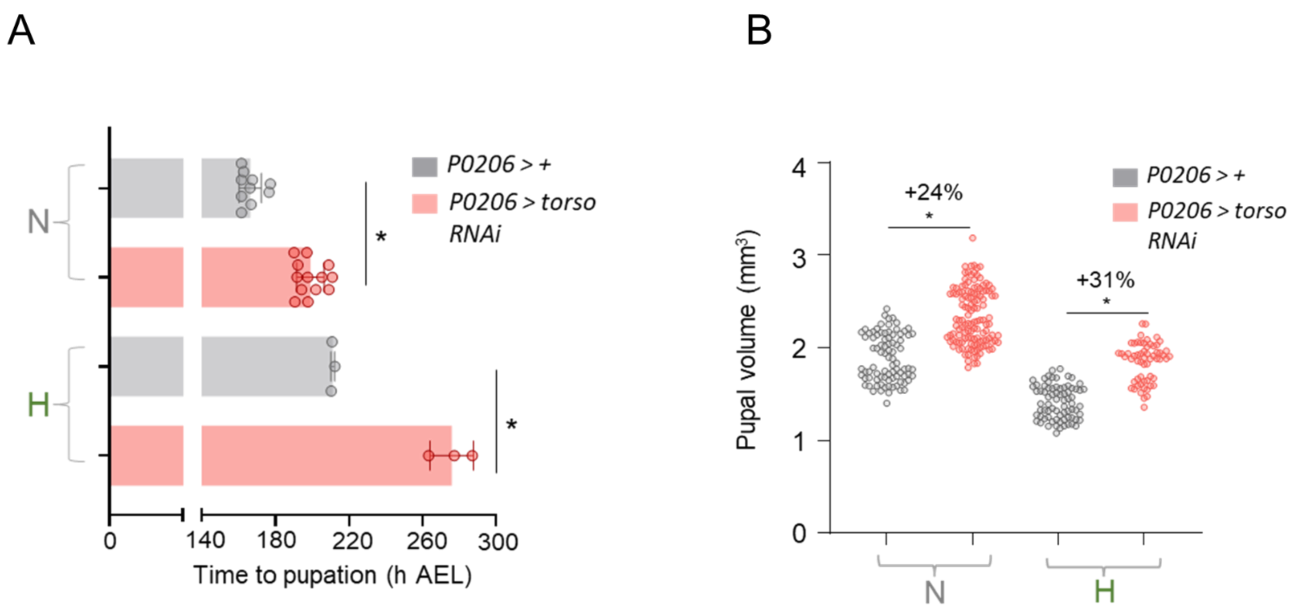

Figure 16. The hypoxia-induced maturation delay is independent of Torso signaling. (A) Average time to pupation of larvae, either P0206 > + or P0206 > torso RNAi, reared in either normal oxygen conditions throughout development or shifted to 5% O2 at 120 h AEL. n (# of vials of 30 larvae) ≥ 3 per condition. **(B)** Pupal size of animals, either P0206 > + or P0206 > torso RNAi, reared in normoxia or hypoxia from 120 h AEL. Each data point represents body size measured for one animal. n (# of pupae) = 86 (N P0206 > +), 127 (N P0206 > torso RNAi), 75 (H P0206 > +), 59 (H P0206 > torso RNAi). * denotes $p < 0.05$.

3.5. Hypoxia Delays Maturation by Suppressing Spitz/Egfr Signaling in the PG

Ecdysone synthesis by the PG at metamorphic onset is in large part stimulated by the Ras/ERK pathway[9]. One possibility therefore is that hypoxia delays larval maturation by inhibiting Ras/ERK signaling in the PG. To test this, I examined the effects of genetically activating the pathway to see if this was sufficient to reverse the hypoxia-mediated delay. I first expressed a constitutively active allele of Raf (Raf^{GOF}) with *P0206-GAL4* and observed dramatically reduced time to pupation under normal oxygen conditions (Figure 17A). This is consistent with the role of Ras/ERK in ecdysone production at metamorphic onset, as well as a previous report of accelerated development with PG-specific expression of constitutively active Ras, *Ras*^{V12}[47].

Interestingly, *Raf*^{GOF} expressed with *P0206-GAL4* also accelerated the development of larvae reared in hypoxia from 120 h AEL (Figure 17A). Expression with an alternate PG-specific driver, *spok-GAL4*, produced an even stronger acceleration (Figure 17B). Here, the hypoxic *Raf*^{GOF} animals pupated only slightly later than their normoxic *Raf*^{GOF} counterparts, representing a near-complete abolition of the hypoxia-induced delay (Figure 17B). Together, these results suggested that activation of the Ras/ERK pathway in the PG is sufficient to reverse the hypoxia-induced delay in development, and thus activity of the pathway may be reduced in hypoxia to delay development.

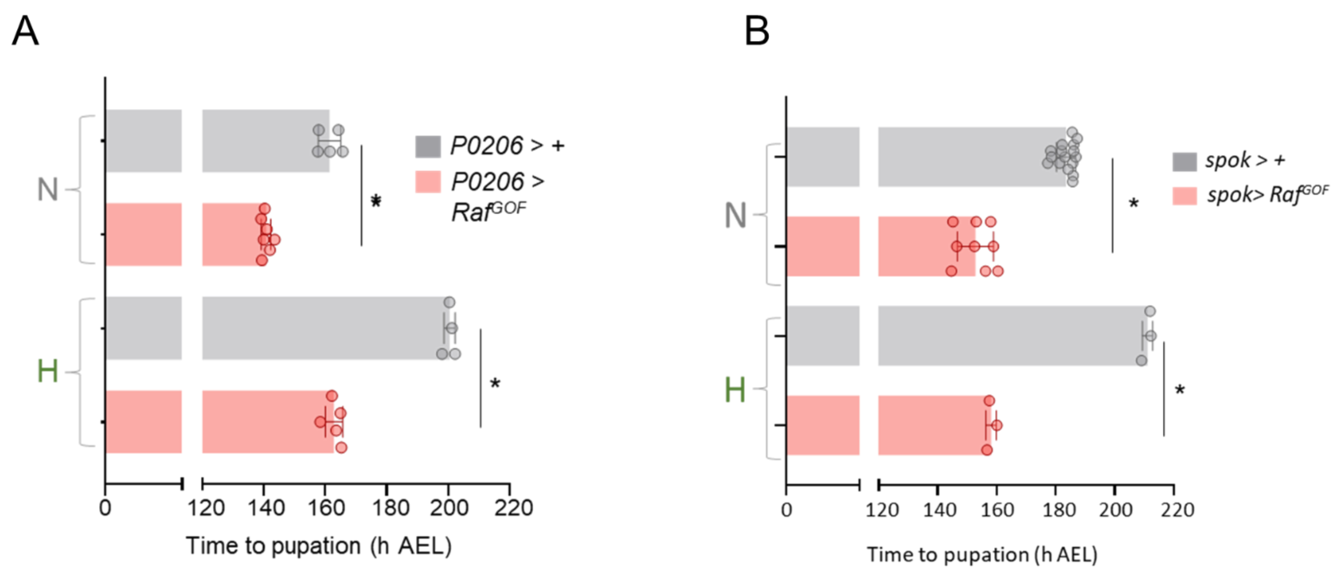

Figure 17. Activation of Ras/ERK in the PG is sufficient to reverse the hypoxia-induced delay in development. (A) Average time to pupation of larvae, either P0206 > + or P0206 > RafGOF, reared in either normal oxygen conditions throughout development (‘N’) or shifted to 5% O2 at 120 h AEL (‘H’). n (# of vials of 30 larvae) ≥ 3 per condition. **(B)** Average time to pupation of larvae, either spok > + or spok > RafGOF, reared in either normal oxygen conditions throughout development (‘N’) or shifted to 5% O2 at 120 h AEL (‘H’). n (# of vials of 30 larvae) ≥ 3 per condition. * denotes $p < 0.05$.

Recently, Epidermal Growth Factor Receptor (Egfr) was shown to be the main inducer of the Ras/ERK signal required in the PG for metamorphic onset[11]. It is possible that hypoxia functions by suppressing signaling through Egfr. Hypothesizing that this was the case, I predicted that with genetic depletion of the receptor, hypoxia would not produce an additive delay. RNAi-mediated Egfr knockdown with the strong PG-specific *phm-GAL4* driver led to developmental arrest at the larval stage (Figure 18A), precluding any assessment of the effect of hypoxia on developmental timing in these animals. To circumvent this, I used an alternate weaker PG driver, *P0206-GAL4*. In this case, I found that RNAi-mediated knockdown of Egfr did not arrest development but did cause a significant delay in pupation under normal oxygen conditions (Figure 18B), consistent with the essential role for Egfr signaling in the PG for controlling ecdysone production and larval maturation. Hypoxia, however, strikingly did not exacerbate the Egfr knockdown-dependent delay, with both control and *Egfr-RNAi* animals pupating at similar times in hypoxia (Figure 18B), and at similar final body sizes (Figure 18C). The lack of additive effects on developmental timing when both hypoxia and PG-specific Egfr depletion are applied suggest hypoxia might delay development through suppressed Egfr signaling in the PG. To further test this, I expressed constitutively active Egfr (λ*TOP*) in the PG using *P0206-GAL4* and found that this partially reversed the hypoxia-induced delay (Figure 18D). Taken together, these results suggest that attenuation of Egfr signaling in the PG might be one way hypoxia delays larval maturation. One recent study showed that in addition to Torso and Egfr, two other RTKs expressed on the PG - Atypical Lymphoma Kinase (Alk) and PDGF/VEGF-related (Pvr) - have been shown to stimulate ecdysone production and promote larval maturation[77]. Thus, hypoxia could potentially work by suppressing these two pathways. However, no significant delay in pupation was observed upon PG-specific RNAi against either receptor in normoxia (Figure 19A and 19B), suggesting that hypoxia-dependent suppression of either pathway was unlikely to underlie the hypoxia-mediated delay

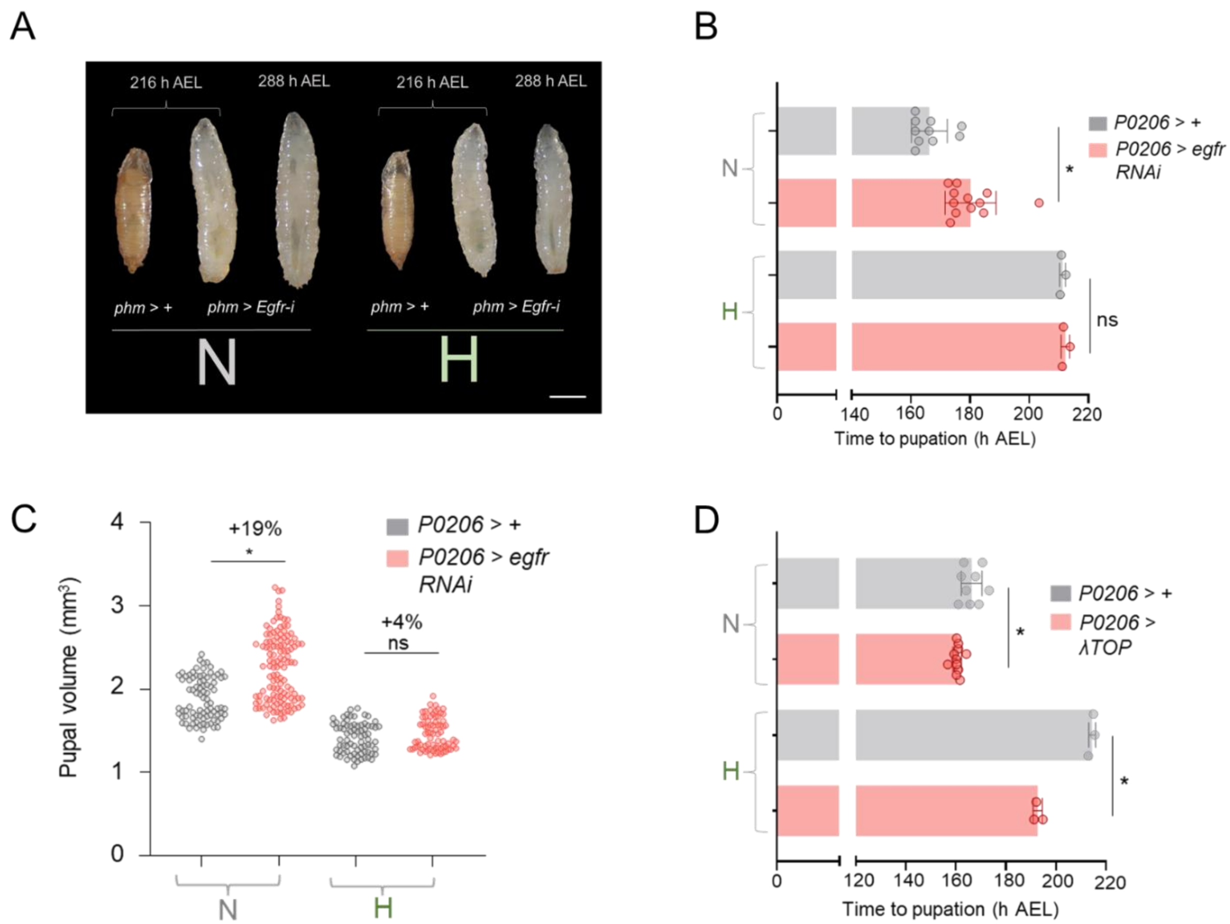

Figure 18. Hypoxia induces a maturation delay by suppressing Egfr/ERK signaling.
(A) Representative images of animals, either phm > + or phm > Egfr-RNAi¸ reared in normoxia throughout development or shifted to 5% O2 at 120 h AEL and imaged at the indicated larval age. In both treatments, phm > Egfr-RNAi arrests development prior to pupation. Scale bar = 1 mm. **(B)** Average time to pupation of larvae, either P0206 > + or P0206 > Egfr-RNAi, reared in either normal oxygen conditions throughout development or shifted to 5% O2 at 120 h AEL. n (# of vials of 30 larvae) ≥ 3 per condition. **(C)** Pupal size of animals, either P0206 > + or P0206 > Egfr-RNAi, reared in normoxia or hypoxia from 120 h AEL. Each data point represents body size measured for one animal. n (# of pupae) = 86 (N P0206 > +), 123 (N P0206 > Egfr-RNAi), 75 (H P0206 > +), 74 (H P0206 > Egfr-RNAi). **(D)** Average time to pupation of larvae, either P0206 > + or P0206 > λTOP, reared in either normal oxygen conditions throughout development or shifted to 5% O2 at 120 h AEL. n (# of vials of 30 larvae) ≥ 3 per condition. * denotes $p < 0.05$.

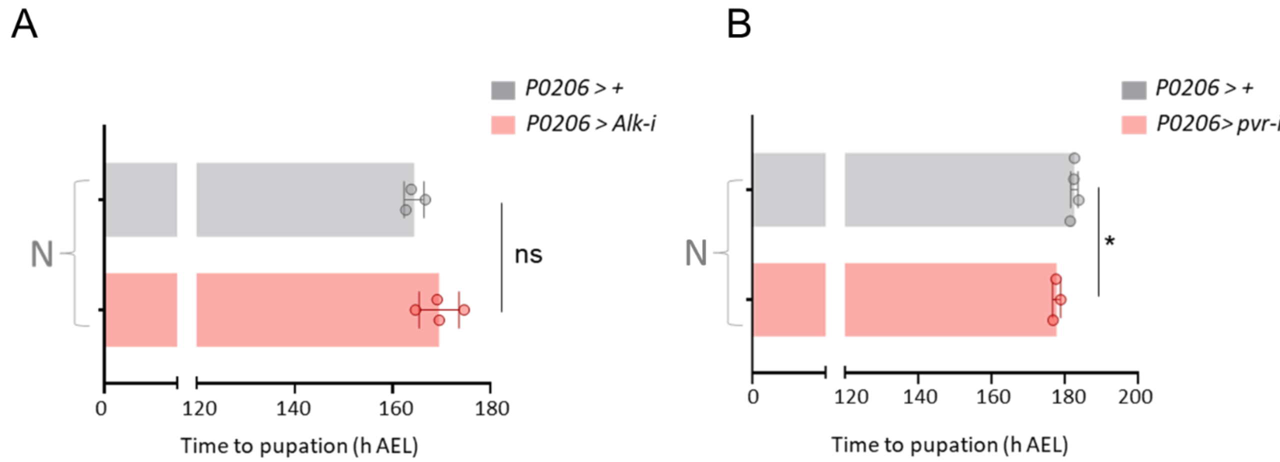

Figure 19. Developmental timing is not delayed upon knockdown of other PG RTKs upstream of Ras/ERK, Alk and Pvr. (A) Average time to pupation of larvae, either P0206 > + or P0206 > Alk-RNAi, reared in normal oxygen conditions. n (# of vials of 30 larvae) ≥ 3 per condition. **(B)** Average time to pupation of larvae, either P0206 > + or P0206 > Pvr-RNAi, reared in normal oxygen conditions. n (# of vials of 30 larvae) ≥ 3 per condition. * denotes $p < 0.05$.

The stimulation of Egfr in the PG at the end of the larval period is mediated by autocrine signaling by two Egf ligands, spitz and vein[11]. I found by qRT-PCR that one of these - *spitz* - was expressed in normoxic larvae at progressively higher levels with increasing larval age, peaking by 144 h AEL, shortly after which pupation occurred (Figure 20A). Interestingly, *spitz* induction was strongly blunted in larvae exposed to hypoxia from hatching and from post-CW (120 h AEL) (Figure 20A), mirroring the trend observed for the ecdysone-biosynthetic genes *phantom* and *spookier* (Figure 11A). This suggested that perhaps hypoxia blunts ecdysone production and delays development by suppressing the autocrine Egf signal required for metamorphic onset. To promote ecdysone synthesis and subsequent larval maturation, spitz is expressed and released by PG cells and signals back to these cells in an autocrine fashion[11]. To assess levels of *spitz* expression specifically in the PG, I employed an enhancer trap line which expresses *lacZ* under the control of the endogenous *spitz* promoter, and used beta-galactosidase levels as a proxy for *spitz* mRNA expression. PGs of larvae reared in hypoxia from 120 h AEL exhibited reduced beta-galactosidase signal intensity as compared to normoxic PG cells (Figure 20B). These results suggest that one way hypoxia may delay the onset of maturation is through suppression of the Egf ligand, and subsequent signaling through Egfr, in the PG. If this were the case, hypoxia exposure would not exacerbate the relative maturation delay caused by genetic depletion of *spitz* in the PG. To test this, I expressed a *UAS-RNAi* construct against *spitz* specifically in the PG using *spok-GAL4*. In normoxia, PG-specific knockdown of *spitz* delayed pupation relative to controls (Figure 20C), consistent with its late-larval role in promoting maturation. Interestingly, hypoxia exposure did not exacerbate the *spitz* knockdown-dependent developmental delay, as *spitz-RNAi* and control animals did not differ significantly in their time to pupation when reared in hypoxia from 120 h AEL (Figure 20C). This was consistent with the PG-specific *Egfr-RNAi* result which similarly showed a delay in normoxia but no further delay of hypoxic larvae (Figure 18B). Conversely, overexpression of the cleaved and secreted form of *spitz* (*sSpi*) using *spok-GAL4* did not affect developmental timing of normoxic larvae but markedly accelerated the development of larvae reared in hypoxia from 120 h AEL (Figure 20D), suggesting that restoring spitz production and secretion by the PG is sufficient to partially reverse the hypoxia-induced delay. Here, the extent of the reversal was comparable to that observed for 20E feeding (Figure 11B), indicating that restoring PG spitz is just as effective at ameliorating the hypoxia-induced delay as feeding animals the ultimate agent of metamorphic onset - 20E.

The mediator of this transcriptional change in *spitz* is yet to be determined. However, I found that knockdown of the nuclear receptor and EcR binding partner, USP, significantly accelerated larval development in hypoxia (Figure 21), warranting further investigation of its potential role in hypoxia-induced *spitz* repression. Cumulatively, my data suggest a model for the delay of maturation by hypoxia whereby the final larval pulse of ecdysone production is suppressed, at least partially through blunted autocrine spitz/Egfr/ERK signaling in the PG (Figure 22).

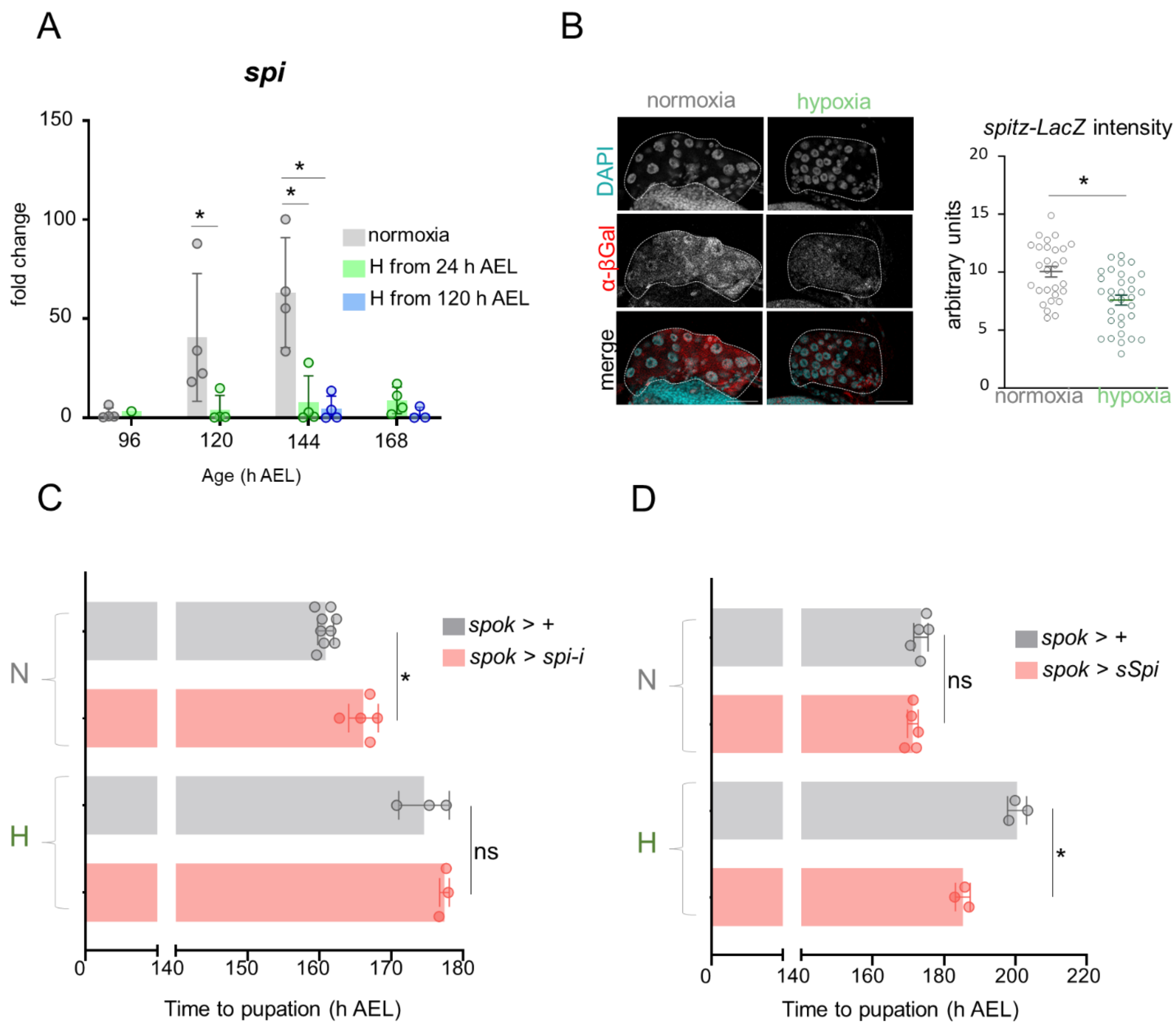

Figure 20. Hypoxia suppresses spitz expression to delay Egfr/MAPK-dependent maturation. (A) Relative mRNA levels (normalized to Rpl32) of Egf ligand spitz from whole-larvae qRT-PCR of w1118 larvae reared in ambient oxygen or in 5% O2 from either 24 h AEL or 120 h AEL. n (# of independent samples) ≥ 3 per condition, except for hypoxic larvae at 96 h AEL. **(B)** Representative confocal micrographs and immunofluorescence quantification for anti-beta-galactosidase immunostaining of PGs (indicated with dashed outline) from 140 h AEL spitz-LacZ larvae, either reared in normoxia throughout development or in hypoxia from 120 h AEL. Scale bar indicates 50 µm. **(C)** Average time to pupation of larvae, either spok > + or spok > spitz-RNAi, reared in either normal oxygen conditions throughout development ('N') or shifted to 5% O2 at 120 h AEL ('H'). n (# of vials of 30 larvae) ≥ 3 per condition.). **(D)** Average time to pupation of larvae, either spok > + or spok > sSpi (expressing cleaved/secreted spitz), reared in either normal oxygen conditions throughout development ('N') or shifted to 5% O2 at 120 h AEL ('H'). n (# of vials of 30 larvae) ≥ 3 per condition. * denotes $p < 0.05$.

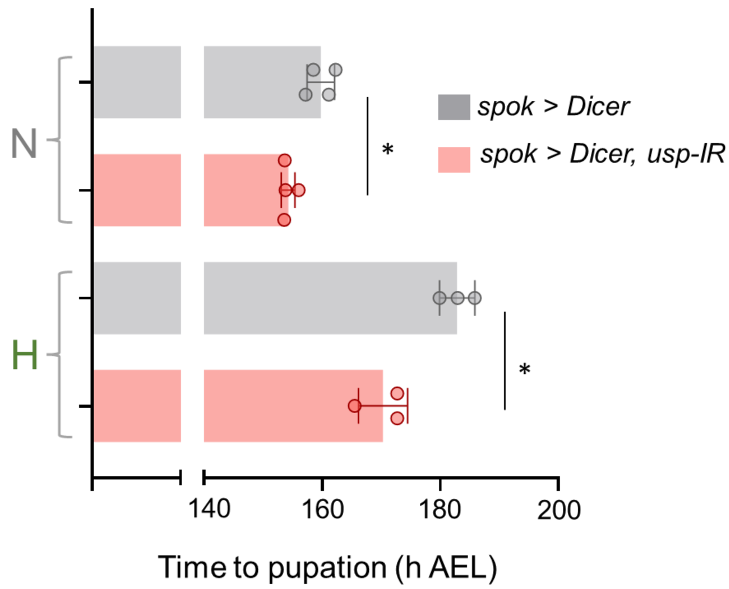

Figure 21. The Nuclear Hormone Receptor, USP partially reverses the hypoxia-induced delay. Average time to pupation of larvae, either spok > dicer or spok > dicer, usp-IR, reared in either normal oxygen conditions throughout development ('N') or shifted to 5% O2 at 120 h AEL ('H'). n (# of vials of 30 larvae) ≥ 3 per condition.)

CHAPTER FOUR: *Discussion*

My work suggests a model whereby hypoxia delays larval maturation largely through suppression of autocrine Egf/Egfr signaling in the PG upstream of ecdysone production (**Figure 22**). This is supported by the fact that, although both hypoxia exposure and RNAi inactivation of the Egf/Egfr pathway delayed development when applied individually, the magnitude of the delay produced by concurrent hypoxia exposure and RNAi was only as great as that caused by hypoxia alone. These non-additive effects suggest that both treatments (hypoxia and genetic inactivation of Egf/Egfr) work in the same pathway to delay maturation. Consistent with this model, I found that PG *spitz* mRNA was depleted in hypoxia and PG-specific overexpression of *spitz* or *Egfr* significantly rescued the hypoxia-induced developmental delay. The developmental delay is largely due to hypoxia suppressing the late-larval mechanisms controlling maturation, rather than simply slowed growth and hence delay in attainment of CW. This is evidenced by the fact that applying hypoxia after the CW checkpoint blunted the expression of ecdysone-biosynthetic genes and strongly delayed the onset of maturation. Finally, I find that these effects occur independently of HIF-1α/sima, as RNAi-mediated *sima* depletion in the ecdysone-regulating neuroendocrine circuit did not lead to any rescue of the developmental delay.

The handful of *Drosophila* studies which probe the adaptation of development to low oxygen have focused majorly on growth suppression and survival[27,29] [70] [33,78] [28]. By and large, these adaptations have been shown to rely on TOR, insulin/PI3K and HIF-1α[29,79] [70] [28] [27] [30]. Although insulin and TOR also directly control maturation[63] [53] [59], my data suggest that hypoxia impinges not on these but Egfr/ERK in the PG and moreover independently of HIF-1α. Therefore, my work suggests that hypoxia uses distinct mechanisms to specifically adapt developmental timing to low oxygen.

In light of my thesis data, many salient open questions are raised. Here, I outline four.

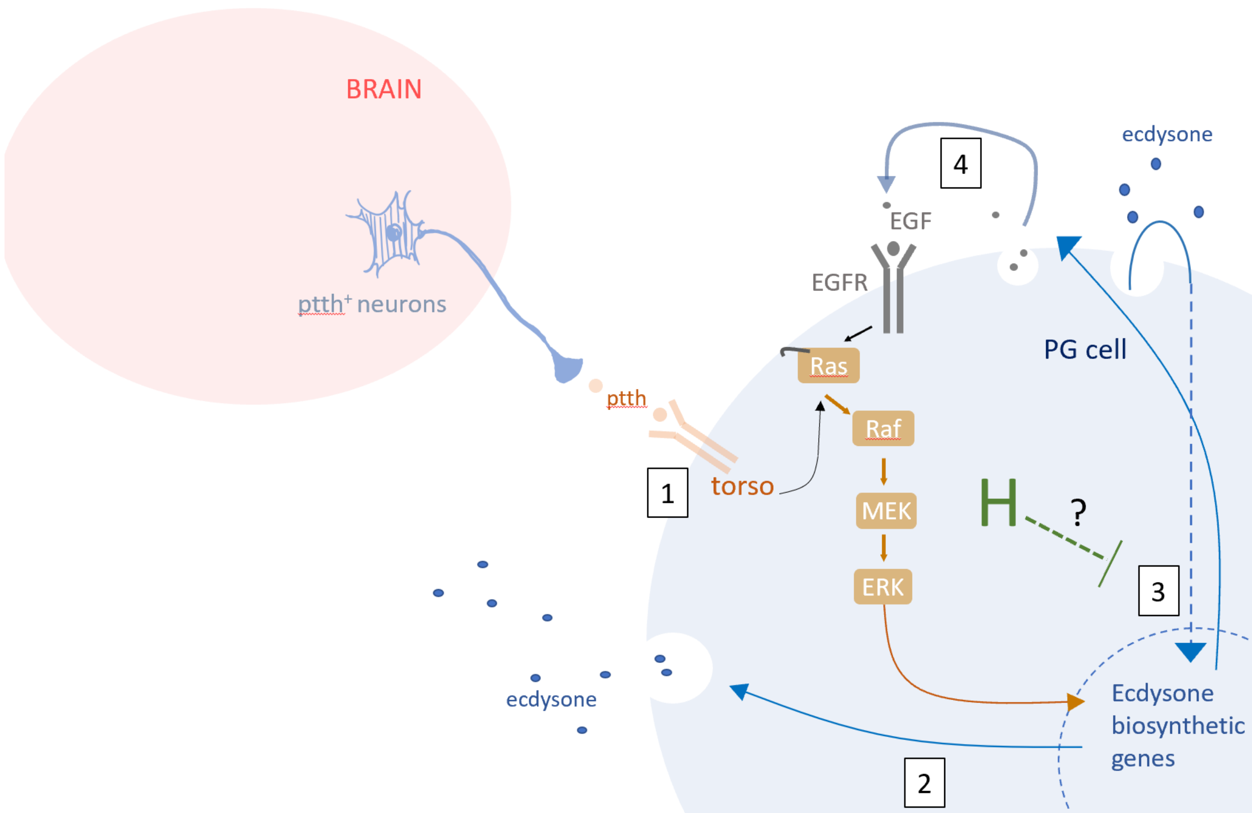

Figure 22. Model Figure. My data suggest that hypoxia acts specifically on the third step in the above sequence to delay larval maturation. This third step is the transcriptional upregulation of Egf ligand *spitz* in the PG.

4.1. What suppresses Egf ligand expression in response to hypoxia?

The upregulation of *spitz mRNA* in the PG at the end of the larval period is dependent on an initial pulse of ecdysone which signals in an autocrine fashion through the nuclear hormone receptor (NHR) complex, EcR/USP[11]. Thus, one possibility is that NHR-mediated signaling is blunted in hypoxia, leading to reduced *spitz* transcription. This would be consistent with literature which demonstrate a role for NHRs in mediating physiological responses to hypoxia[79,80]. For instance, the *Drosophila* NHR, estrogen-related receptor (dERR), was shown to transcriptionally reprogram hypoxia-exposed larvae to promote survival[79]. Interestingly, in *C.*

elegans, hypoxia has been shown to suppress EGF/ERK signaling by modulation of NHR activity[80]. In human breast cancer, EGFR/ERK pathway activity is subject to modulation by the NHR, estrogen receptor, which is in turn repressed in hypoxia[81 82]. Regulation of EGFR/ERK signaling by NHRs is thus a conserved scheme that may underlie hypoxia suppression of maturation. Several genetic tools are available to test this in *Drosophila*. I can first determine whether EcR-mediated transcription is suppressed in hypoxia by using the ecdysone response element (*EcRE*)-*LacZ* enhancer trap line which contains EcR target sites upstream of the *LacZ* coding region. This would allow me to use β-galactosidase levels as a readout for EcR-mediated transcription to thereby discern whether EcR activity is blunted by hypoxia specifically in the PG. If it is, I can follow up by using *GAL4/UAS* to overexpress EcR in the *spitz-lacZ* background in hypoxia to determine whether this rescues 1) PG expression of *spitz* and 2) developmental timing.

In response to another stress - nutrient deprivation - one group found modulation of PG NHR activity to underlie developmental delay[59]. In their proposed model, low insulin signaling causes FOXO to accumulate in the PG cell nuclei and outcompete EcR to form a heterodimer with USP; this complex then transcriptionally represses EcR/USP target genes, including the Halloween genes, to delay ecdysone production[59]. Our lab has shown FOXO-mediated transcriptional changes to underlie other hypoxia adaptations[72 72,83]. However, I found that PG-specific FOXO RNAi did not rescue hypoxia-induced developmental delay (unpublished data, Grewal Lab). Interestingly, though, PG-specific knockdown of USP yielded a moderate rescue, suggesting that perhaps USP complexes with a factor other than FOXO in hypoxia to repress EcR target genes, including *spitz*. To test this, I can perform PG-specific *USP* knockdown in the background of *spitz-lacZ* to determine whether PG *spitz* expression is rescued in hypoxia-exposed larvae upon depletion of USP.

Although the transcriptional regulation of ecdysone production is poorly understood, there are around 20 different transcription factors and chromatin modifiers expressed in the PG that have been suggested to play a role, reviewed in Kamiyama and Niwa (2022)[84]. This list might contain factors that serve to downregulate *spitz* expression in response to hypoxia. For instance, the transcriptional repressor Hairy (fly ortholog of HES1) is known to antagonize Halloween gene expression in the PG[85] and is also induced in low oxygen to promote hypoxia tolerance[78], thus warranting assessment of its role in the hypoxia-induced delay. A candidate RNAi screen can be readily performed on this and the other ecdysone-regulating factors by knocking them down in the PG and observing the effects on developmental timing in hypoxia. Moreover, other hypoxia-responsive factors can be added to the screen. Although my data rule out the classic hypoxia-responsive transcription factor, HIF-1α, there are several HIF-independent mediators of hypoxia adaptation that would be interesting to test, as discussed below. These needn't be transcription factors or chromatin modifiers because it is possible that low oxygen is sensed by enzymes that post-transcriptionally regulate one or more transcription factors to suppress *spitz*.

4.2 What is the proximal oxygen sensor required for the maturation delay?

Adapting larval maturation to hypoxia requires that the animal first assesses oxygen levels. This low-oxygen detection might happen directly in the PG to influence gene expression. One candidate for mediating this is KDM5, a chromatin modifier from the aforementioned list of PG-expressed factors[86]. It is a member of the alpha-ketoglutarate-dependent dioxygenases which use oxygen in their reaction cycle and are directly regulated by hypoxia[87 88]. As researchers found KDM5 to upregulate ecdysone production at the end of the larval period to promote maturation[86], it is possible that lowered KDM5 activity in response to hypoxia results in transcriptional repression of *spitz* to delay maturation. To test this, I can knock down KDM5 specifically in the PG and determine whether this produces an additive developmental delay in hypoxic larvae. If its suppressed activity in hypoxia mediates the suppression of ecdysone upstream of the maturation delay, one can predict that KDM5 depletion by RNAi in the PG would not delay development any further than hypoxia alone.

Prolyl hydroxylases (PHDs) represent another class of enzymes whose activity is directly regulated by oxygen[89]. Mammals have several of these enzymes which are best known for directly coupling the presence of oxygen to HIF-1α degradation[89]. Previously, the *Drosophila* genome was thought to have a single PHD, *Fatiga*, which primarily serves this canonical function of sima/HIF-1α degradation[90]. More recently, however, one other family member was identified - Sudestada 1 (Sud1)[91]. Researchers found that embryos depleted of *Sud1* mRNA failed to upregulate a HIF-dependent transcriptional reporter upon hypoxia exposure whereas *Fatiga*-depleted embryos showed strong reporter up-regulation[91], suggesting that Sud1 hydroxylates unique target proteins in response to hypoxia. Interestingly, the yeast homolog of Sud1 has been shown to be largely nuclear (as is *Drosophila* Sud1[91]) and regulate sterol response element binding protein (SREBP1) - involved in lipid metabolism - in an oxygen-dependent manner[92]. It would therefore be interesting to assess a PG-specific role for Sud1 in mediating the hypoxia-dependent developmental delay through regulation of one or more transcription factors.

4.3 What is the ecological significance of delaying maturation in hypoxia?

Larvae adapt the timing of their maturation to numerous environmental cues[10]. By far, the most extensively studied of these is nutrient availability. As represented by the Critical Weight paradigm, this adaptation is a way for larvae to ensure they have sufficient nutrient stores before committing to maturation; if they require more feeding and growth to meet this quota, they delay their maturation; if not, they pupate[10]. In this way, we can ascribe a logic to the maturation delay observed in nutrient-poor conditions. In rats, postnatal underfeeding has been shown to delay pubertal onset whereas a high fat diet accelerates it[93]. Recent work has even suggested the same relationship between juvenile nutrition and the timing of maturation to exist in humans[94 95]. Some of these studies find that childhood obesity correlates with precocious puberty[95 96]. Interestingly, a recent report in *Drosophila* demonstrated that lipid stores are the main proxy by which larvae assess their growth and then time their maturation accordingly[97]. This group found that the larval fat body (analogous to the mammalian liver and adipose tissue) secretes lipids that are carried by the lipid carrier, apolipophorin (Apolpp) and these lipids are directly sensed by the PG through a membrane protein called Semaphorin 1a (Sema1a)[97]. Larvae depleted of *Apolpp* in their fat body or *Sema1a* in their PG failed to undergo the larva-pupa transition despite growing on a high fat diet, suggesting that this fat-to-PG axis is essential for ecdysone production and larval maturation[97]. The link between larval adiposity and the onset of maturation could be a clue as to the adaptive reason for hypoxia-induced developmental delay.

Work from our lab has demonstrated that when grown in 5% O_2, larvae reduce TOR activity in their fat body to slow the breakdown and promote accrual of lipid stores[29]. In this study, aberrant activation of TOR in the fat body of hypoxic larvae led to depletion of lipid stores, which did not affect larval survival but instead caused lethality at the pupal stage[29]. This was thought to reflect insufficient energy to support metamorphosis, which is 90% dependent on energy from lipid stores[62]. Given these findings, one possibility is that larvae delay their maturation in hypoxia to accumulate sufficient fat stores for metamorphosis. The added metabolic expense of hypoxia tolerance might force larvae to reassess their relative nutrient stores in terms of the cost of metamorphosis in low oxygen as opposed to ambient oxygen. This would explain why larvae delay their maturation when exposed to hypoxia post-CW, but pupate on time when starved post-CW. To test the hypothesis that larvae delay their maturation to accrue sufficient lipid stores for metamorphosis in hypoxia, I can block the hypoxia-induced delay by PG activation of Ras/ERK signaling and use a standard triacylglycerol (TAG) assay to quantify larval lipids. I can then ask whether the precocious hypoxic pupae: 1) have reduced lipid stores compared to the delayed hypoxic controls, and 2) have reduced survival to the adult stage. Moreover, a recent report showed that larval fat stores are converted into long chain hydrocarbons in the adult to confer desiccation tolerance upon eclosion [98]. Precocious flies might lack such tolerance. I can also look at what else might be different about these animals. Perhaps their glycogen is depleted. Perhaps they display reduced fecundity. Any differences could potentially lend insight into the physiological and ecological significance of delayed development in response to hypoxia.

4.4 What is the clinical significance of this work?

The juvenile-to-adult transition in both flies and humans is controlled by the production of steroid hormones - ecdysone in flies, and sex hormones in humans[99]. The production of both of these is controlled by a neuroendocrine pathway (illustrated in **Figure 23**)[99]. In addition to these similarities in general function and design principles of their regulation, there is evidence for some conservation in the molecular control of ecdysone and sex hormones; for instance - in the neurohormones that control fly metamorphosis and human puberty. In flies, the neuropeptide allatostatin A (AstA) stimulates Ptth^{+} neurons at the end of the larval period through its receptor AstAR1 to properly time metamorphosis[49] [48]. In humans, the neuropeptide Kisspeptin (KISS1) stimulates the gonadotropin releasing hormone (GnRH)-positive neurons through its receptor KISS1R to properly time pubertal onset[100]. Interestingly, the receptors AstAR1 and KISS1R are orthologous, highlighting molecular conservation in addition to their functional similarity[48]. Moreover, there is apparent conservation in the transcriptional control of steroidogenesis in the fly and human. For instance, one of the few identified ecdysone-controlling transcription factors, FTZ-F1, is the fly ortholog of the human nuclear receptor Steroidogenic Factor 1 (SF-1) which regulates at least eight steroidogenic genes[12]. Together, these findings illustrate the parallels between fly metamorphosis and human puberty. Given these parallels, fly research can provide clinically relevant insights into the events that control steroid-mediated maturation and how these might be altered in the face of various stresses like hypoxia.

Hypoxia exposure during *Drosophila* development leads to both reduced body growth and a delay in the steroid-dependent juvenile-adult transition[101 28 27 29]. The same has been reported for human development at high altitude or with oxygen-limiting diseases[102 103 104]. Delayed puberty itself has many causes and is understandably associated with psychological difficulties in children and young adults[105]. Hormone therapy, such as administering androgens, has been a common treatment for pubertal delay[105]. From a physiological standpoint, a delay in puberty is not inherently deleterious but is often associated with health complications due to the diseases and conditions which often co-present with it such as anemia, cystic fibrosis and many others[106]. Clinical insights of my thesis and impactful future directions are more likely to be obtained when viewing the maturation delay as a purposeful adaptation to stress rather than a developmental defect caused by stress. For instance, what if someone were to have some genetic aberration that prevented them from delaying their maturation in the face of juvenile hypoxia? Precocious puberty is a human phenomenon with an array of genetic causes[107,108]. In contrast to pubertal delay, early pubertal onset can have serious negative health consequences[108]. For instance, advanced bone age is observed in precocious adults likely due to a truncated growth period[108].

One can speculate that, with the added stress of developmental hypoxia, failing to delay maturation could exacerbate these deleterious effects and lead to others later in adulthood. My work furthers our understanding of the biology that underpins the coupling of oxygen levels to maturation. Follow up research should probe the clinically relevant outcomes of when these adaptive mechanisms fail.

4.5 Limitations and Future Directions

My thesis has several important caveats to consider and later address. First, my model relies heavily on the lack of additive effects with respect to *spitz* and *Egfr* RNAi. As RNAi is variable in its knockdown efficacy, it can't be said with full confidence that the lack of additive delay in hypoxia upon Egfr-RNAi is due to both hypoxia and genetic depletion working in the same pathway to delay maturation or whether RNAi-mediated knockdown is perhaps weaker in hypoxia due to reduced *GAL4* expression. To circumvent this, I can employ the inducible CRISPR/Cas9 system[109] to achieve PG-specific knockout of *Egfr* or *spitz*. Secondly, I use *spitz-lacZ* expression as a readout for *spitz mRNA* levels in the PG. Discrepancies might exist in the stability of *lacZ* mRNA and *spitz* mRNA in hypoxia, leading to a lack of confidence in the observed differences in PG *spitz* expression. To measure *spitz* mRNA more directly, I can perform an *in-situ* hybridization for *spitz* in the PGs of hypoxic and normoxic larvae and see whether the results here agree with those seen with the lacZ reporter. Finally, I use the expression of the Halloween genes as a proxy for ecdysone production. Ecdysone secretion is a separate process that might be independently regulated[9]. So, it is possible that hypoxia additionally suppresses secretion of ecdysone, or that secretory function is upregulated to compensate for reduced ecdysone production. I should thus test whether circulating ecdysone is reduced in hypoxia, mirroring the trend of Halloween gene expression. This can be accomplished with a standard ELISA to quantify ecdysone levels in the hemolymph, as others have done[110].

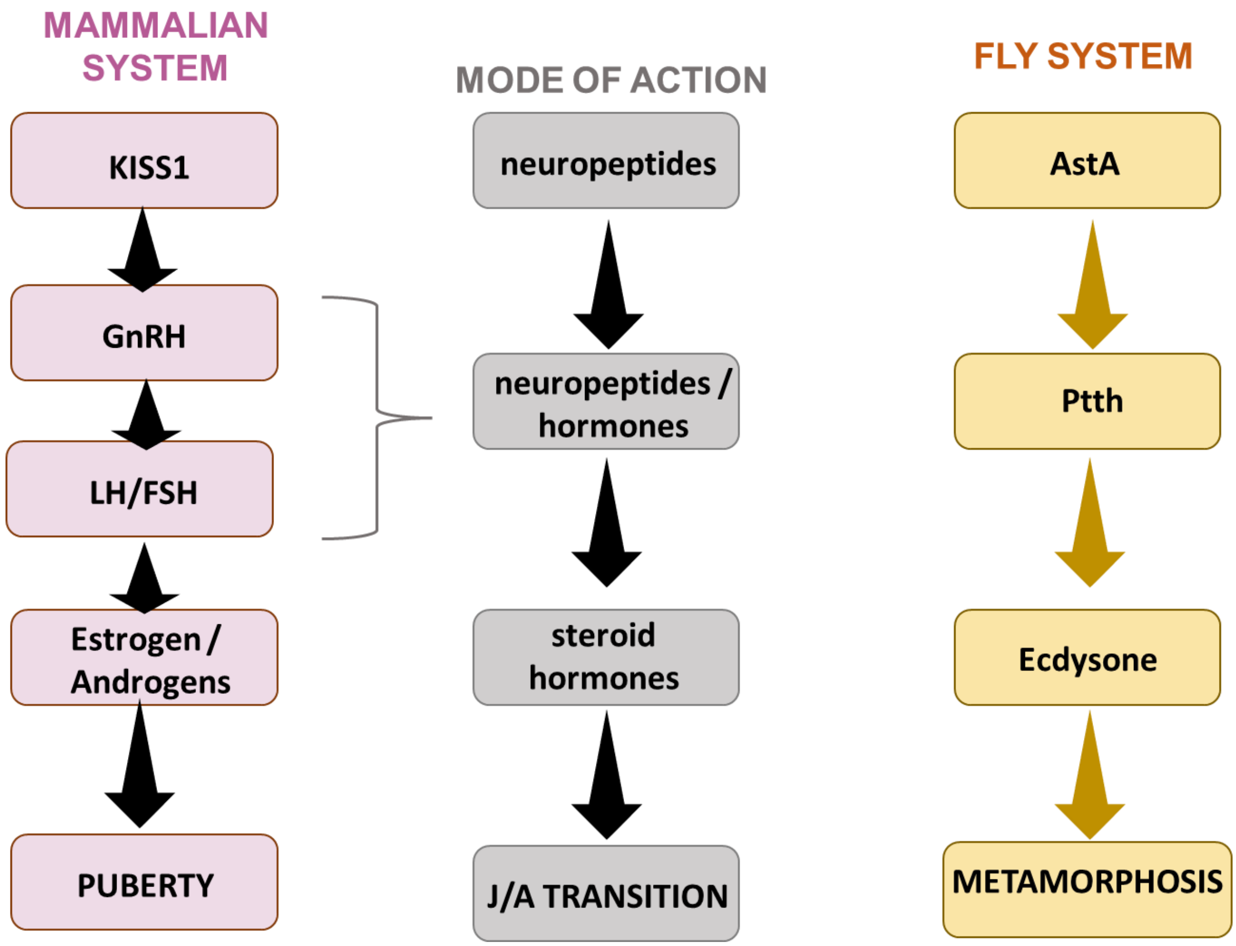

Figure 23. Similarities in the control of mammalian puberty and fly metamorphosis. Both the mammalian and fly system use neuroendocrine circuits and the cascade of signaling events follow the same general theme in the center (grey). After Barredo et al (2021).

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