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UNIVERSITY OF CALIFORNIA SAN DIEGO

Candidate Gene Identified in Humans Adapted to High-Altitude Depicts Hypoxia Tolerance in *Drosophila melanogaster*

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Madlina Babakhanlou

Committee in charge:

Professor Gabriel Haddad, Chair
Professor James Posakony, Co-Chair
Professor Joseph Pogliano

2018

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The Thesis of Madlina Babakhanlou is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2018

DEDICATION

To my family,

Alice, my mom, who always cared for me while I was away from home;

Hirman, my dad, who made sure I was awake each morning;

Emmanuel, my amazing brother, who took care of mom and dad while I was gone;

Anet, my aunt, who always encouraged me to keep going;

Bianca, my sunflower, who stayed awake with me until the sun rose to make edits;

and

Hrach, my beloved husband,

who always supported me in my endeavors.

EPIGRAPH

There are far, far better things ahead
than any we leave behind.

C.S. Lewis

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LIST OF ABBREVIATIONS

da-GAL4	Daughterless-GAL4
dHsl	*Drosophila* ortholog of *Hsl*
DNA	Deoxyribonucleic acid
EH	Eclosion hormone
Eh-GAL4	Eclosion hormone-expressing neuron-specific-GAL4
FFA	Free fatty acid
GAL4	Galactose-responsive gene 4
He-GAL4	Hemocyte-specific-GAL4
HIF-1	Hypoxia-inducible factor 1
Hsl	Hormone-sensitive lipase
Lsp2-GAL4	Third instar larval fat body-specific-GAL4
O_2	Oxygen
OK72-GAL4	Oenocyte-specific-GAL4
PKA	Protein kinase A
RNAi	RNA interference
ROS	Reactive oxygen species
TG	Triglycerides (synonymous for triacylglycerol)
UAS/GAL4	Upstream activating sequence-galactose-responsive gene 4
W^{1118}	White-1118

LIST OF SUPPLEMENTAL FILES

Supplemental File 1: Babakhanlou_Eclosion_Raw_Data_1.xlsx

Supplemental File 2: Babakhanlou_Eclosion_Raw_Data_2.xlsx

Supplemental File 3: Babakhanlou_Main_Eclosion_Plots.xlsx

Supplemental File 4: Babakhanlou_Triglyceride_Pilot.xlsx

Supplemental File 5: Babakhanlou_Triglyceride_Assay_Data.xlsx

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This Thesis, in full is co-authored by Madlina Babakhanlou, Tsering Stobdan, and Gabriel G. Haddad. The Thesis author was the primary investigator and author of this material.

VITA

Education

2016	Biochemistry and Cell Biology B.S. University of California San Diego
2018	Biology M.S. University of California San Diego

Publications

Cardiac Specific Knockdown of *Hsl*, a Candidate Gene from Human Studies at High Altitude, Improves Hypoxia Tolerance in *Drosophila*.
Babakhanlou M, Stobdan T, Haddad GG.
American Heart Association Scientific Sessions 2017

Synthesis of Benzoic Acid Derivative-Based Dendrimers as Potential Anti-burn Agents.
Avanes A, Le T, Peev T, Cruz E, Davidian A, Saboury J, Hakobyan H, Babakhanlou M, Macklin L, Kazaryan G, Nazaryan R, Sandman M.
Southern California Conference of Undergraduate Research 2013

ABSTRACT OF THE THESIS

Candidate Gene Identified in Humans Adapted to High-Altitude Depicts Hypoxia Tolerance in *Drosophila melanogaster*

by

Madlina Babakhanlou

Master of Science in Biology

University of California San Diego, 2018

Professor Gabriel Haddad, Chair
Professor James Posakony, Co-Chair

High altitude studies have been insightful in revealing putative hypoxia tolerant genes to improve pathological and physiological conditions that involve hypoxia or ischemia. Whole-genome sequencing of Ethiopian highlanders identified LIPE (Lipase E, Hormone Sensitive Type), as an important gene that allows them to live in chronic low oxygen conditions (Udpa *et al.*, 2014). Using *Drosophila melanogaster*, ubiquitous knockdown of the ortholog of this gene, hormone-sensitive lipase (*Hsl*), results in increased tolerance to chronic hypoxia. To elucidate the tissue specificity of the adaptive phenotype, UAS-*RNAi(Hsl)* line was crossed with different cell- or tissue-specific GAL4 drivers to systematically knockdown this gene, without altering its expression in the rest of the organism. There was a remarkable difference in eclosion rates when

Hsl was knocked down in eclosion hormone-expressing neurons, third instar larval fat body, oenocytes, and hemocytes, constituting a four-fold increase over controls (all knockdown crosses were >87%, $P<0.005$). Since *Hsl* plays a prominent role in lipid metabolism, its inhibition in the cell(s) or tissue(s) that produced the most profound response to hypoxia tolerance were quantified to provide evidence of any changes in triglyceride levels. An augmented triglyceride concentration was observed in *Hsl* knockdown crosses, specifically in, UAS-*RNAi(Hsl) x da*-GAL4 and UAS-*RNAi(Hsl) x Eh*-GAL4 with a 1.5- to 2-fold increase in hypoxia when compared with controls. This data suggests that lipid accumulation as a result of *Hsl* knockdown may be biochemically significant in promoting hypoxia tolerance.

INTRODUCTION

General Background

Hypoxia, a condition of a deficit in oxygen supply, presents a challenge for cells and tissues that require threshold oxygen levels to be met for their survival. The duration and severity of low oxygen tension can make vulnerable cells more inclined to injury. Consequently, this may result in organ failure, if sufficient oxygen levels are not met. Oxygen-sensing mechanisms, such as, the HIF-1 pathway, enable an organism to maximize oxygenation to cells and tissues in order to better tolerate dangerous fluctuations in oxygen levels. Hypoxia is pervasive in numerous pathological and physiological conditions, such as, myocardial infarction, stroke, cancerous tumors, and during fetal development (Vaupel, 2004; Giordano, 2005; Ginsberg, 2008; Dunwoodie, 2009). Adaptation to hypoxia involves various genetic and physiological defenses that trigger transcriptional and signaling mechanisms for an organism to thrive. Recent developments have broadened our understanding of hypoxia, allowing us to explore adaptability or tolerance to hypoxia across a wide range of model organisms. These scientific milestones have revealed key aspects of hypoxia survival strategies. However, there still remains a knowledge gap to be addressed in identifying and interfering with genes that facilitate hypoxia tolerance.

To understand the consequences of low oxygen at molecular and cellular levels, it has been insightful to study biological instances where tolerance to hypoxia is observed. For instance, human adaptation to high altitude can enlighten us to improve therapeutic modalities for sea level diseases that involve hypoxia in their pathogenesis (Zhou and Haddad, 2013; Stobdan *et al.*, 2015; Azad *et al.*, 2017; Stobdan *et al.*, 2017). The unique variations in their genome allow for tolerance and survival in chronic low oxygen conditions and reveal the possibility of conserved hypoxia

tolerant genes (Bigham *et al.*, 2009; Simonson *et al.*, 2010; Jha *et al.*, 2015; Stobdan *et al.*, 2018). These putative hypoxia tolerant genes encoded in their DNA suggest a strong selective pressure for genes associated with an organism's ability to ameliorate the damaging effects of hypoxia. Similarly, another biological instance occurs in the fetal heart, in which a specific gene expression programme is expressed in cardiomyocytes. These set of genes allow for their function at extremely low oxygen levels (Kuwahara *et al.*, 2012; Breckenridge *et al.*, 2013; Iruretagoyena *et al.*, 2014). Naturally occurring biological instances inherently tolerant to hypoxia may reveal important genes that orchestrate this response. In turn, researchers can apply these findings to human pathologies by targeting these genes to mitigate or prevent the damaging effects of hypoxia.

Drosophila as a Model Organism

Drosophila melanogaster is a versatile model organism that has been used to study a broad range of phenomena in biomedical research for over a century. There are many technical advantages of using *Drosophila* over vertebrate models. For instance, *Drosophila* are easy and inexpensive to culture in laboratory conditions. *Drosophila* can be genetically modified in numerous ways, produce large numbers of externally laid embryos, and have a much shorter life cycle (refer to **Figure 1**). Research using *Drosophila* has profoundly advanced our understanding of regenerative biology and will continue to contribute in the future of regenerative medicine (Jennings, 2011).

Many genes associated with human diseases are biologically conserved in *Drosophila melanogaster* (Bier, 2005; Gilbert, 2008; Pandey and Nichols, 2011). As such, *Drosophila* have been extensively used as a powerful *in vivo* model organism to dissect genetic mechanisms that

contribute to human diseases, including, aging, neurological- and cardiac-related diseases, cancer, and mechanisms underlying hypoxia tolerance or susceptibility (Michno *et al.*, 2005; Grotewiel *et al.*, 2005; Vidal *et al.*, 2006; Zhou *et al.*, 2009; Lu, 2009; Lessing *et al.*, 2009; Polesello *et al.*, 2011; Diop and Bodmer, 2012).

In the current study, we took advantage of the UAS/GAL4 system, a resourceful tool applied in *Drosophila* to reduce the expression of the *Hsl* gene in specific cells (refer to **Figure 2**). This is achieved by expressing double-stranded RNA corresponding to the *Hsl* gene sequence, known as RNA interference (*RNAi*) (Fischer *et al.*, 1988; Brand *et al.*, 1993; Dietzl *et al.*, 2007). The expression of transgenic *RNAi* constructs, such as, UAS-*RNAi(Hsl)* can be driven in a cell- or tissue-specific manner using the "GAL4 system." Thus, the UAS/GAL4 system provides a simple, yet powerful strategy to study the role of individual genes in specific cell(s) and tissues(s). In order to test for hypoxia tolerance, we have previously identified stages in a fly's life cycle that are sensitive to hypoxic stress (refer to **Figure 1**). This was necessary to develop a strong hypoxia tolerance assay as described in **MATERIALS AND METHODS** to successfully validate human ortholog candidate genes (Zhou *et al.*, 2013; Udpa *et al.*, 2014).

Rationale

Recently, our group performed whole-genome sequencing of Ethiopian highlanders (Udpa *et al.*, 2014). Statistical analysis of their DNA identified regions with a significant reduction of genetic variation. These distinct regions with a loss of diversity alluded to conserved hypoxia tolerant genes. Further narrowing it down, a 208 kbp gene-rich region located on chromosome 19 was of particular significance in both of the Ethiopian populations studied. The genes located in this prioritized region of their DNA were investigated in *Drosophila melanogaster*. We used RNA

interference (*RNAi*) of the orthologs of these genes to determine their role in the adaptive response to hypoxia, if any.

Global knockdown of *cic*, an ortholog of human CIC, *Hsl*, an ortholog of human LIPE, and *Paf-AHα*, an ortholog of human PAFAH1B3, unveiled important genes responsible for the adaptive phenotype (Udpa *et al.*, 2014). Of these three genes, *Hsl*, hormone-sensitive lipase, led to markedly improved tolerance to hypoxia and remarkable survival rates. When *Hsl* was knocked down ubiquitously (UAS-*RNAi(Hsl) x da*-GAL4) the eclosion rate at 5% O_2 was significantly higher (80.0%; $P<0.05$) when compared to the background controls (0% for UAS-*RNAi(Hsl)*). To further demonstrate our hypothesis that regulation of *Hsl* confers tolerance to low oxygen, we tested the impact in various cell or tissue types. The experiments in this Thesis address the following fundamental questions:

1) *Does tissue-specific knockdown of Hsl render hypoxia sensitive cells and tissues resistant and yield the adaptive phenotype?*

2) *What is the proposed mechanism that involves Hsl to combat the compromised oxygen availability?*

3) *Does Hsl act through a cell-type specific mechanism in response to hypoxia?*

4) *How does Hsl provide astute information that parallels pathological and disease states in conditions of hypoxia?*

MATERIALS AND METHODS

Fly Rearing and Collection

The *D. melanogaster* stocks carrying the UAS-*RNAi(Hsl)* transgene were obtained from Vienna *Drosophila RNAi* Center (stock number 109336; Vienna, Austria). The tissue-specific-GAL4 drivers were obtained from Bloomington Stock Center (Bloomington, IN, USA). A list of the tissue-specific-GAL4 drivers with their FlyBase stock ID and expression pattern can be found in **Table 1**. The *NP2222*-GAL4 stock originated in Kyoto Stock Center (Kyoto, Japan) and was a gift from Dr. Marc Freeman (University of Massachusetts Amherst).

All stocks were propagated on a standard cornmeal *Drosophila* medium with an alternating 12-hour light and 12-hour dark cycle at 22 ± 1°C. Virgin adult females homozygous for each tissue-specific-GAL4 driver were collected in separate vials of standard cornmeal medium. Virgin females were selected based on the presence of the meconium, which from the ventral view appears as a dark spot in their abdomen. After aging for at least four days, the virgin adult females were transferred to new vials and crossed with age-matched adult males homozygous for UAS-*RNAi(Hsl)* for three days. Pooling the flies initially ensures mating of the virgin females and males. Each vial that the virgin females were collected in was kept for 10 days to confirm that no larvae or pupae were found in the vial, thus validating the female flies used in the experiment were true virgins. Then the flies were distributed into three vials, constituting a set with 10 females and 5 males in each experimental vial, to allow for egg-laying for two days. The flies were transferred to a control vial to repeat the egg-laying process for a subsequent two days. The experimental vials containing the eggs were placed in the hypoxia chamber with 5% oxygen for a period of 21-days with an alternating 12-hour light and 12-hour dark cycle at 22 ± 1°C. After two days of egg-laying,

the adult flies were removed and the control vials with the eggs were exposed to room air with 21% oxygen as a control for a period of 21-days with an alternating 12-hour light and 12-hour dark cycle at 22 ± 1°C. The GAL4 drivers and UAS-*RNAi(Hsl)* stocks were included in parallel as self-cross controls. A minimum of six vials for each cross were completed in two different experiments in both 5% and 21% oxygen. Please refer to **Figure 3** for a visual representation of the details contained herein.

Hypoxia Tolerance and Vulnerability Tests

After 21 days in 5% or 21% oxygen with alternating 12-hour light and 12-hour dark cycles at 22 ± 1°C, the pupal cases were scored to calculate the eclosion rate for each condition (refer to **Figure 4** for eclosed versus uneclosed pupal cases). Tolerance to hypoxia was evaluated by comparing the eclosion rate (%) of control flies to experimental *Hsl* knockdown flies. Adult survival during hypoxia was noted for each cross.

Triglyceride Assays

To determine the appropriate number of larvae necessary to elicit a sensitive response in detecting concentration levels we first collected one, two, four, eight, and sixteen third instar larvae in 2 mL ceramic bead tubes (2.8 mm, MoBio) containing 0.05% Tween. The larvae were homogenized using Precellys 24 homogenizer and prepared as described in Grönke *et al.*, 2003. Triglyceride levels were measured using Infinity Triglyceride Reagent (Cat No., TR22421, Thermo Fisher) with 200 mg/dL Triglyceride Standard from Pointe Scientific (Cat No., T7531-STD).

Protein Assays

Protein levels were quantified using Standard BCA Protein Assay Kit (Cat No., 23225, Pierce). From the pilot run, it was determined that for all samples in the triglyceride and protein concentration assays, two third instar larvae were required in each to produce a measurable value.

For the experimental crosses two third instar larvae per 2 mL ceramic bead tube (2.8 mm, MoBio) were collected randomly from control and experimental groups in both 21% O_2 and 5% O_2 from all the aforementioned crosses (except that of oenocyte- and hemocyte-related). All measurements were performed in three technical and three biological replicates. Triglyceride levels were normalized by protein levels (refer to **Supplemental File 4** and **Supplemental File 5** for calculations).

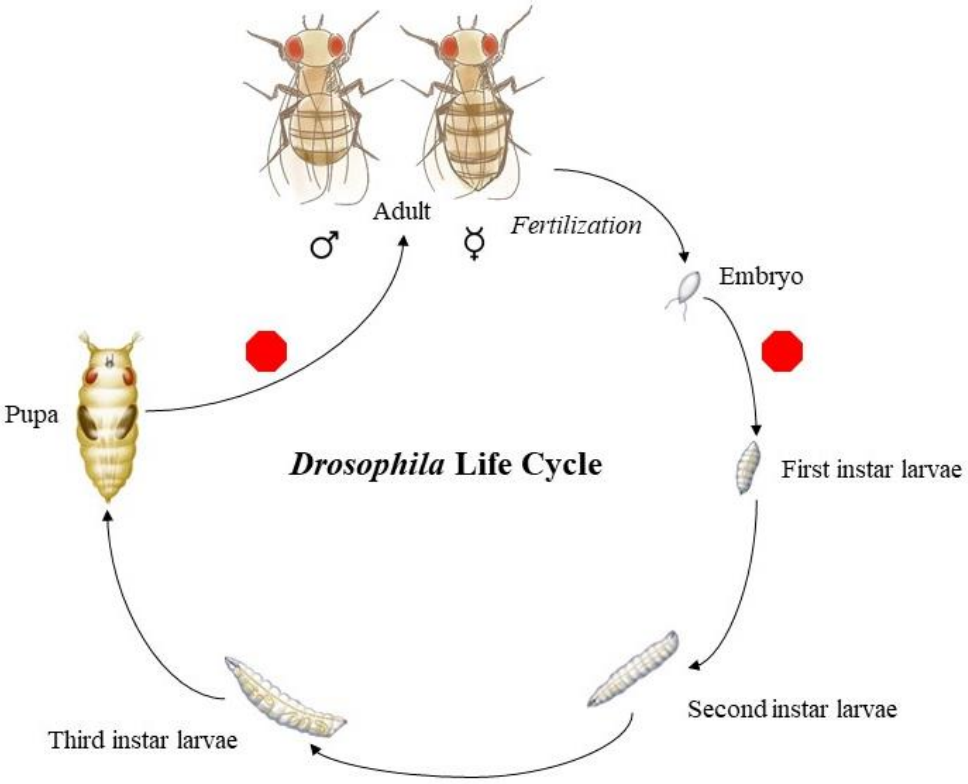

Figure 1 *Drosophila* Life Cycle

In the *Drosophila* life cycle there are two critical time points in development which hypoxia impedes: hatching of the embryo, and eclosion to a reproductive adult (emphasized by the red stop signs).

RESULTS

Experimental Evaluation of Tissue-Specific Hsl Knockdown Using Drosophila melanogaster

In order to understand the response to hypoxic stress, one can undertake two different approaches: a) to block the mechanism of damage or b) to evoke the mechanism of tolerance. In our previous study, we used the latter approach to decipher the role of *Hsl* in hypoxia tolerance in *D. melanogaster*. We saw that ubiquitous knockdown of *Hsl* in *Drosophila* was non-lethal in ambient conditions. Remarkably, in a hypoxic environment (5% O_2), knockdown of *Hsl* significantly improved hypoxia tolerance in *Drosophila*. By employing the UAS/GAL4 system, we wanted to test if down-regulation of *Hsl* in specific cell(s) or tissue(s) continues to foster the same hypoxia tolerance phenotype that we previously observed. Therefore, the main objective of the current experiment was to identify cell(s) or tissue(s) specificity of *Hsl* knockdown involved in the hypoxia tolerance phenotype. Thus, identifying in which cell(s) or tissue(s) does *Hsl* down-regulation evoke the mechanism of tolerance and promote successful development of the fly. We identified the spatiotemporal expression of *Hsl* and also referred to literature for its specificity (Kraemer and Shen, 2002; Holm, 2003; Yeaman 2004). A detailed list of all the GAL4 lines used in the current screening is provided in **Table 1**. The UAS-*RNAi(Hsl)* (stock number 109336; Vienna, Austria) was crossed with each of the GAL4 lines (refer to **MATERIALS AND METHODS**).

Table 1 Expression Pattern of *Drosophila* GAL4 Drivers

GAL4 Driver	FlyBase Stock ID	Expression Pattern
da	FBst0055851	Ubiquitous expression of GAL4 under the control of daughterless.
tin	FBst0066397	Heart-specific expression of GAL4.
hand	FBgn0032209	Cardioblasts, pericardial cells, lymph glands, garland cells.
DJ667	FBst0008171	Adult expression constant with age. Expressed in adult muscles.
Eh	FBst0006301	Expresses GAL4 in eclosion hormone-expressing neurons.
DJ626	FBst0008166	Adult expression decreases with age. Expressed in adult nervous system, cardia and oenocytes.
He	FBst0008699	Expresses GAL4 in hemocytes.
Eaat1	FBst0008849	Expresses GAL4 in the glial cells that produce the glutamate transporter EAAT1.
OK72	FBst0006486	GAL4 expressed in oenocytes.
elav	FBgn0260400	Expresses GAL4 in neurons under elav control. Expression begins in the embryonic nervous system at stage 12.
Lsp2	FBst0006357	GAL4 expressed in third instar fat body.
c825	FBst0006987	GAL4 expressed in amnioserosa, adult ovary, and adult male accessory glands and seminal vesicles.
DJ752	FBst0008182	Adult expression constant with age. Expressed in adult brain, muscles and cardia.
24B	FBst0001767	Expresses GAL4 in embryonic mesoderm.
DJ757	FBst0008184	Adult expression constant with age. Expressed in adult muscles.
GII5	FBst0007031	GAL4 expressed in embryonic peritracheal cells and pericardial cells.
NP2222	FBst0315565	GAL4 expressed in cortex glial cells. (CyO)

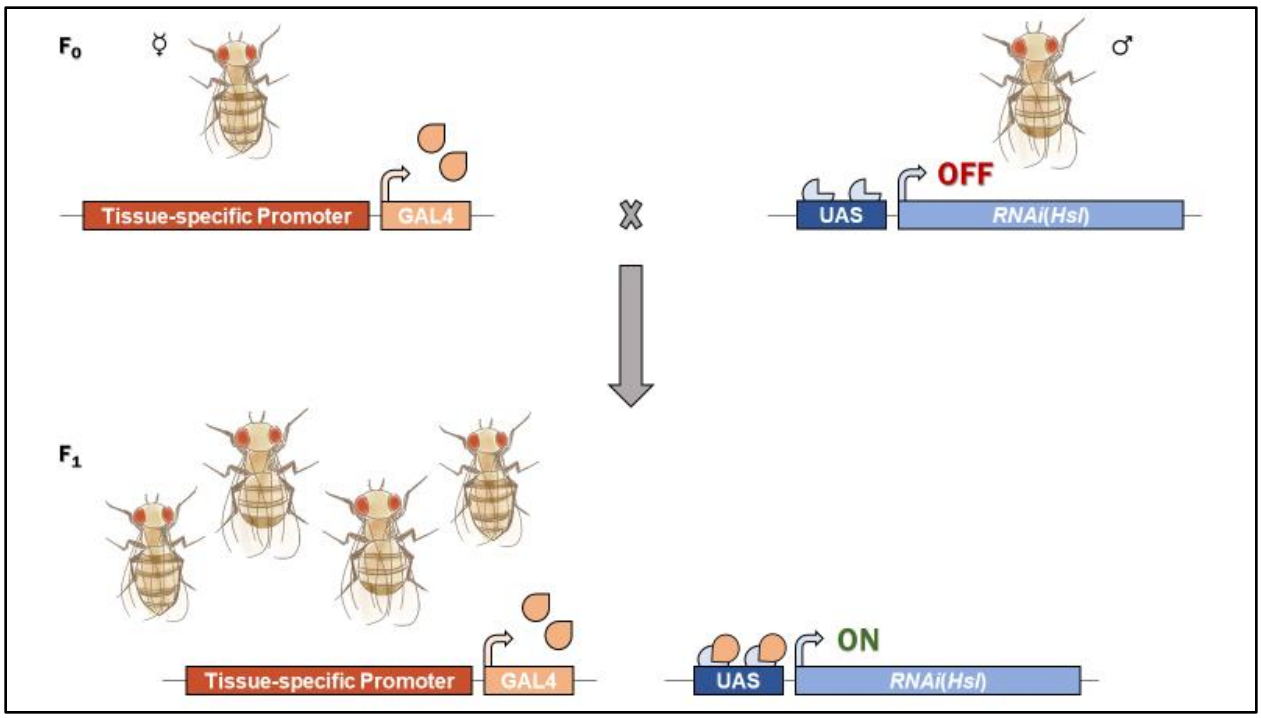

Figure 2 Using the UAS/GAL4 System in *Drosophila melanogaster*

Virgin females of each cell- or tissue-specific-GAL4 driver confer temporal and/or spatial specificity in expressing the GAL4 gene. Males contain an upstream activating sequence (UAS) for regulating *Hsl* through *RNAi*. Employing the UAS/GAL4 system by using these transgenic flies produces offspring that contain both constructs and will express *RNAi* which targets *Hsl* in that specific cell or tissue.

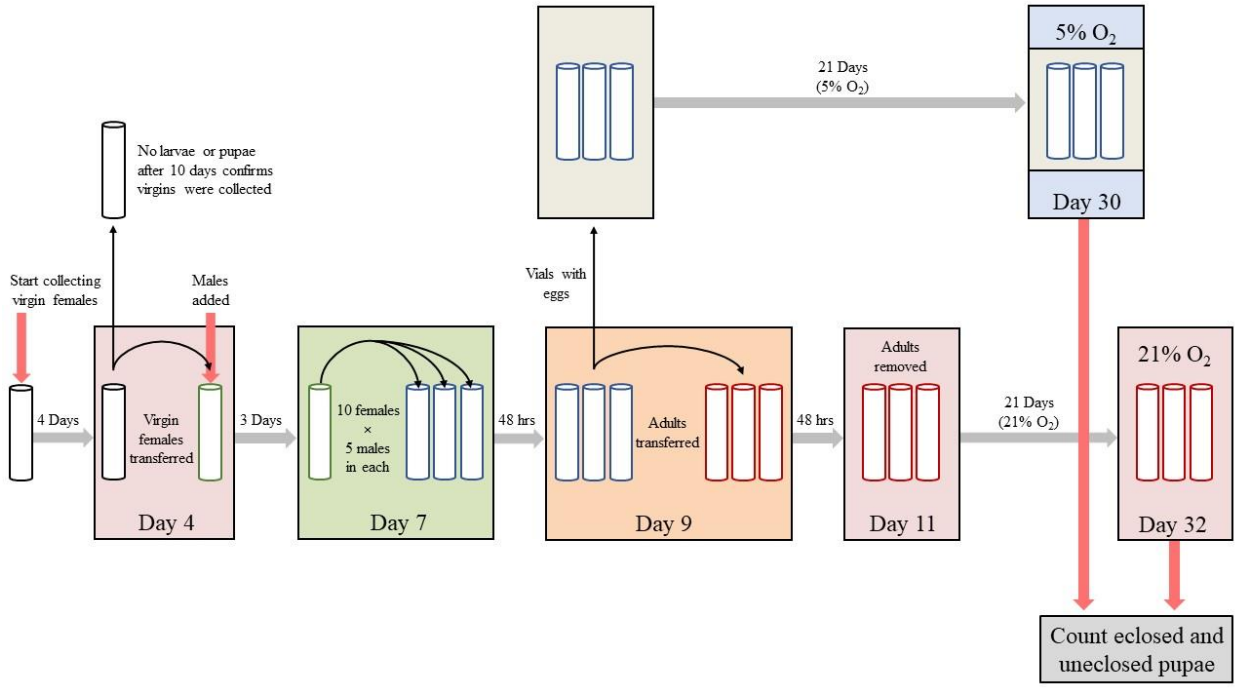

Figure 3 Experimental Paradigm Used to Study Hypoxia Tolerance in *Drosophila melanogaster*

As described in **Fly Rearing and Collection**, above is a detailed visual representation of the experimental procedure performed to calculate the eclosion rate and determine hypoxia tolerance. Briefly, UAS-*RNAi(Hsl)* flies were crossed with tissue-specific GAL4 flies. They were allowed 48 hours for egg-laying in normoxia (21% O_2) followed by 21 days in hypoxia (5% O_2). In the *Drosophila* life cycle there are two critical time points in development which hypoxia impedes: hatching of the embryo, and eclosion to a reproductive adult. To be able to quantify if knockdown of a specific gene confers tolerance to chronic hypoxia, the first stage, hatching of the embryo, was allowed in 21% O_2 followed by development in 5% O_2.

Eclosed	Uneclosed	
		

Figure 4 Eclosed verses Uneclosed Pupal Cases

The images on the top row show eclosed versus uneclosed pupal cases. To determine an accurate eclosion rate, only the pupal cases that completely eclosed to a reproductive adult were counted. The uneclosed cases were dissected to show unsuccessful development of *D. melanogaster*. Necrotic tissue is indicated with a red arrow.

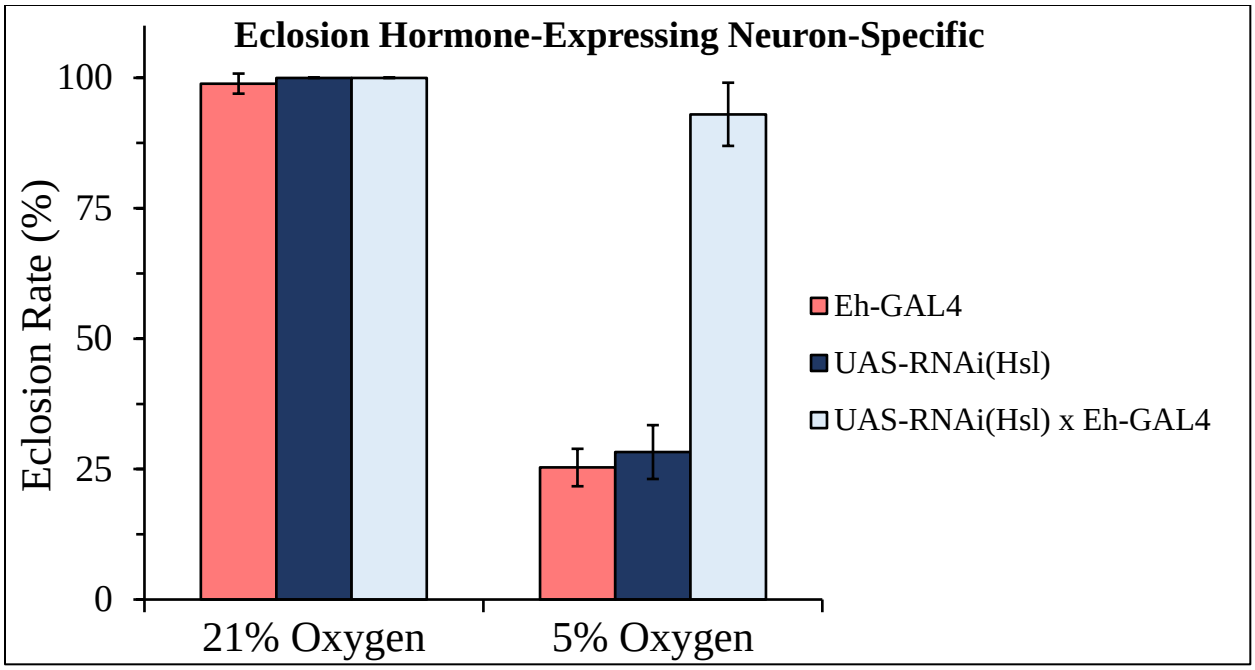

Figure 5 Eclosion Rates of Eclosion Hormone-Expressing Neuron-Specific

The UAS-*RNAi* line for *Hsl* (ortholog of human LIPE) was crossed with *Eh*-GAL4, a driver strain that expresses GAL4 in eclosion hormone-expressing neurons. The level of hypoxia tolerance was determined by measuring eclosion rate in an atmosphere chamber containing 5% O_2. The UAS-*RNAi(Hsl)* and *Eh*-GAL4 self-crosses were used as negative controls. Each bar represents the mean ± standard error of the mean value of three separate tests ($P<0.005$).

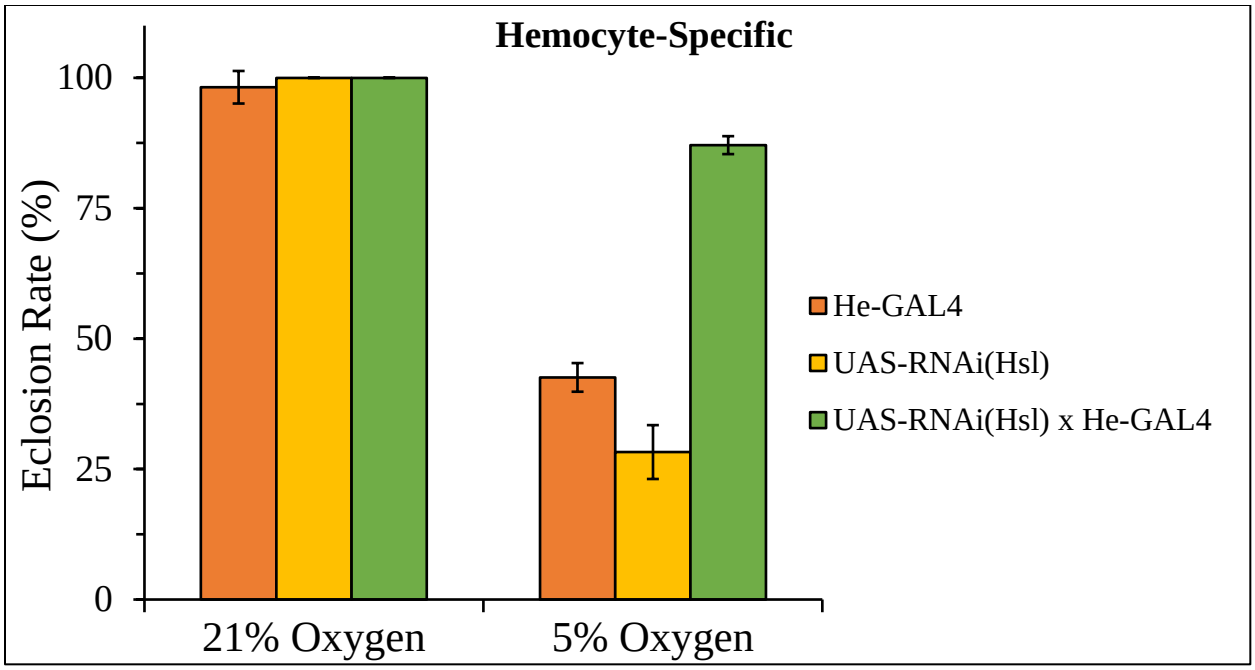

Figure 6 Eclosion Rates of Hemocyte-Specific

The UAS-*RNAi* line for *Hsl* (ortholog of human LIPE) was crossed with *He*-GAL4, a driver strain that expresses GAL4 in hemocytes. The level of hypoxia tolerance was determined by measuring eclosion rate in an atmosphere chamber containing 5% O_2. The UAS-*RNAi(Hsl)* and *He*-GAL4 self-crosses were used as negative controls. Each bar represents the mean ± standard error of the mean value of three separate tests ($P<0.005$).

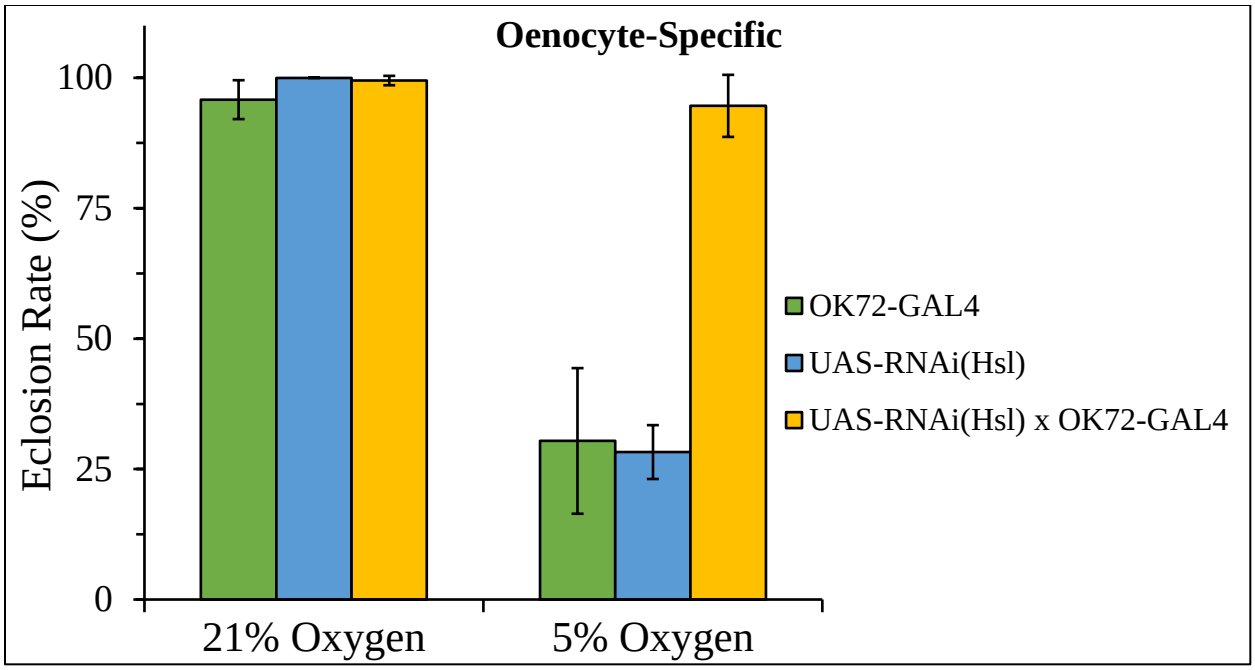

Figure 7 Eclosion Rates of Oenocyte-Specific

The UAS-*RNAi* line for *Hsl* (ortholog of human LIPE) was crossed with *OK72*-GAL4, a driver strain that expresses GAL4 in oenocytes. The level of hypoxia tolerance was determined by measuring eclosion rate in an atmosphere chamber containing 5% O_2. The UAS-*RNAi(Hsl)* and *OK72*-GAL4 self-crosses were used as negative controls. Each bar represents the mean ± standard error of the mean value of three separate tests ($P<0.005$).

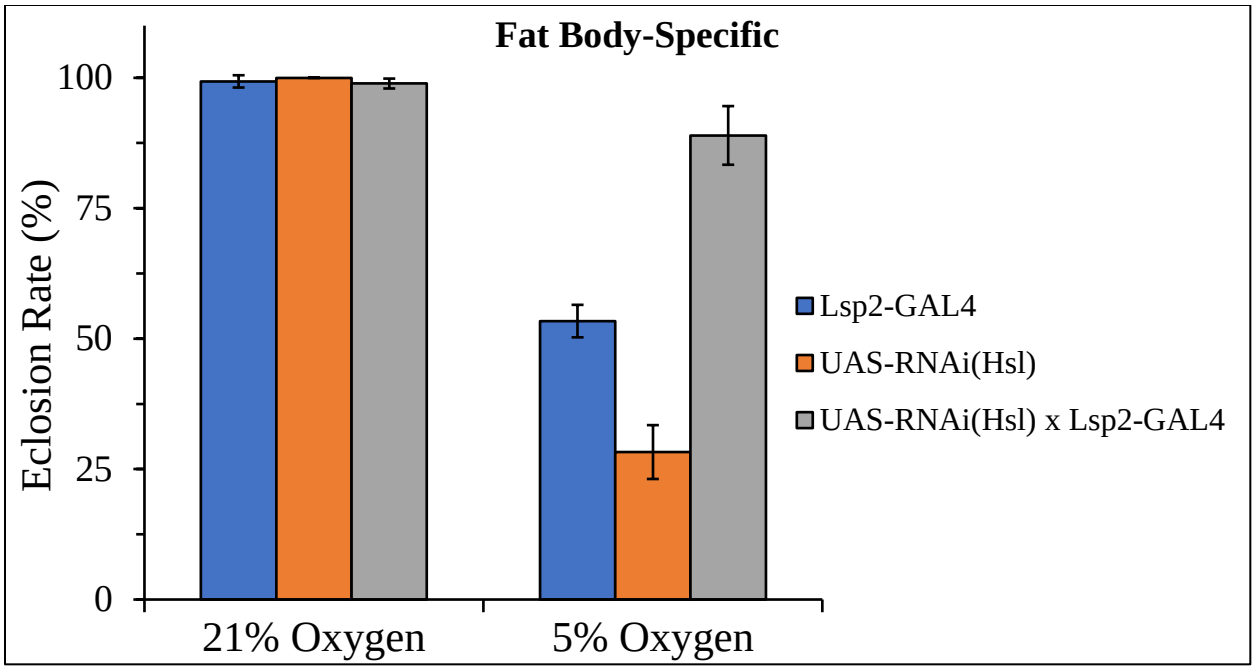

Figure 8 Eclosion Rates of Fat Body-Specific

The UAS-*RNAi* line for *Hsl* (ortholog of human LIPE) was crossed with *Lsp2*-GAL4, a driver strain that expresses GAL4 in third instar larval fat body. The level of hypoxia tolerance was determined by measuring eclosion rate in an atmosphere chamber containing 5% O_2. The UAS-*RNAi(Hsl)* and *Lsp2*-GAL4 self-crosses were used as negative controls. Each bar represents the mean ± standard error of the mean value of three separate tests ($P<0.005$).

Recapitulating Hypoxia Tolerance in Hsl Knockdown Drosophila melanogaster

Ubiquitous knockdown of *Hsl* confers hypoxia tolerance in *D. melanogaster* (Udpa *et al.*, 2014). In order to investigate the tissue specificity of the hypoxia tolerance phenotype, we proposed to systematically knockdown this gene in a specific cell-type(s) or tissue(s) without altering its expression in the rest of the organism. We used 16 different GAL4 drivers that included important cells or tissues known to be adversely affected by hypoxia or participate in lipolysis (**Table 1**). These GAL4 drivers were crossed with the UAS-*RNAi(Hsl)* line, and the eggs laid were kept in a 5% oxygen (O_2) chamber. The eggs of respective self-crossed GAL4 lines and the UAS-*RNAi(Hsl)* line were also included in parallel as negative controls. A detailed schematic of the crosses performed and the methods used can be found in the **MATERIALS AND METHODS** section and in **Figure 3**. The percentage of pupae eclosed, that is, the adult flies that emerge from the pupal case, were measured (refer to **Figure 4**). This eclosion rate was presented both at room air (21% O_2) and at 5% O_2 (refer to **Table 2** in **Appendix E**). A relatively higher eclosion rate at 5% O_2 when compared with the controls indicates hypoxia tolerance.

As a background control, the eclosion rate for W^{1118} in 21% O_2 was measured as 100% while at 5% O_2 the eclosion rate was <50%. Our previous findings on ubiquitous knockdown of *Hsl* leading to a significant hypoxia tolerance was recapitulated by crossing *da*-GAL4 with UAS-*RNAi(Hsl)*. The results were consistent with the previous reports displaying a significantly higher eclosion rate at 5% O_2 in the UAS-*RNAi(Hsl) x da*-GAL4 (~89% compared to the controls, i.e., *da*-GAL4 and UAS-*RNAi(Hsl)* self-crosses ($P<0.005$).

Next, we measured the eclosion rate in the tissue-specific *Hsl* knockdown flies at 21% O_2 and 5% O_2. The eclosion rates at 21% O_2 were 95%-100% for all the crosses using the various GAL4 drivers and their respective self-crossed controls. Since the assay is very sensitive to any

variability that may occur throughout the experimental period, we detected higher eclosion rates at 5% O_2 in many of the GAL4 controls. In order to avoid any false positive results, we first measured the eclosion rates of the control GAL4 lines, that is, the self-crossed GAL4 driver lines both at 21% and 5% O_2. We focused only on those GAL4 drivers for which the eclosion rates were <40%. From the GAL4 driver screen we identified four GAL4 drivers that passed this criterion. These drivers express GAL4 protein in 1) eclosion hormone-expressing neurons; 2) hemocytes; 3) oenocytes; and 4) third instar larval fat body.

Hypoxia Tolerance of Tissue-Specific Hsl Knockdown Drosophila melanogaster

We then measured the eclosion rates of the UAS-*RNAi(Hsl)* crossed with each of the shortlisted GAL4 driver lines. At 21% O_2 the eclosion rates were >95% for all the experimental crosses. Under hypoxic (5% O_2) environments, the eclosion rate of the UAS-*RNAi(Hsl)* line was <30% (**Figure 5**-**Figure 8**).

dHsl Knockdown in Eclosion Hormone-Expressing Neurons and Hypoxia Tolerance

Downregulation of *dHsl* in eclosion hormone-expressing neurons demonstrates a robust response to hypoxia tolerance in *D. melanogaster*. While the eclosion rates for all the lines at 21% O_2 was >98%, the eclosion rate for UAS-*RNAi(Hsl) x Eh*-GAL4 at 5% O_2 was close to its normoxic condition with a 93.01% eclosion rate. This starkly contrasted the eclosion rates for *Eh*-GAL4 and UAS-*RNAi(Hsl)* self-cross controls, with 25.30% and 28.27, respectively ($P<0.005$, **Figure 5**).

dHsl Knockdown in Hemocytes and Hypoxia Tolerance

The other tissue that was validated for *dHsl*-related hypoxia tolerance was hemocytes. The eclosion rate for UAS-*RNAi(Hsl) x He*-GAL4 in 5% O_2 displayed <45% in controls compared to ~87.1% in knockdown progeny (P<0.005, **Figure 6**).

dHsl Knockdown in Oenocytes and Hypoxia Tolerance

A recent study in *Drosophila* reveals a novel role in regulating lipid metabolism for specialized cells called oenocytes that present striking functional similarities to mammalian hepatocytes (Gutierrez *et al.*, 2007). In the experimental cross with *Hsl* knocked down in oenocytes, the eclosion rate for UAS-*RNAi(Hsl) x OK72*-GAL4 was 94.63% at 5% O_2. These results were significantly higher than the corresponding controls with *OK72*-GAL4 at 25.3% and UAS-*RNAi(Hsl)* at 28.27% (P<0.005, **Figure 7**).

dHsl Knockdown in Third Instar Larval Fat Body and Hypoxia Tolerance

Finally, *Lsp2*-GAL4 which expresses GAL4 in third instar larval fat body was another tissue from our screen that passed the hypoxia tolerance assay with 88.96% eclosion rate UAS-*RNAi(Hsl) x Lsp2*-GAL4 at 5% O_2 (P<0.005, **Figure 8**). There was a modestly higher eclosion rate for the *Lsp2*-GAL4 self-cross control 53.37%. However, the difference in eclosion rates were still not as prominent as the UAS-*RNAi(Hsl) x Lsp2*-GAL4 crosses. Upon repeating all of the above experiments, including the *Lsp2*-GAL4 self-cross, the eclosion rate was always <40% in hypoxia

(**Appendix A**-**Appendix D**). In addition, the *Lsp2*-GAL4 self-cross control was as low as 23.16% after re-calibrating the computer-controlled hypoxia chamber (refer to **Figure 18**).

Quantifying Triglyceride Levels in Third Instar Larvae

Earlier we observed that *Hsl* knockdown flies are tolerant to a hypoxic environment. We know that *Hsl* is the rate-limiting enzyme in the degradation of triacylglycerol or triglyceride (TG) to diacylglycerol and free fatty acid. In order to understand the mechanism involving the hypoxia tolerance phenotype of *Hsl* knockdown flies, an obvious step would be to measure if there is an increase in TG levels when lowering the expression of *Hsl*. To study this, first we decided to quantify TG levels in the *Hsl* knockdown flies. We only analyzed the cell- or tissue-specific knockdown of *Hsl* that conferred the most profound response to low oxygen and of which the third instar larvae were alive in 5% oxygen. Second, we detected if there were any quantitative changes to the TG levels in the control and experimental flies that were due to exposure in chronic hypoxic conditions. The objective of measuring triglyceride levels is to provide evidence of any changes in lipid accumulation during hypoxia, as an effect of *Hsl* inhibition.

Additionally, the selection of the specific stage from the fly's life cycle at which the TG levels should be measured is significantly important (**Figure 1**). To begin with, we decided to measure TG levels in the third instar larval stage because this stage provides the energy reservoir necessary for pupation and proper organ development until ecdysis (Mathur *et al.*, 2010). Secondly, this stage precedes the hypoxia tolerance phenotype before eclosion of pharate adult, which we quantify to determine eclosion rate. In addition, in most of the fly lines that we tested, a live third instar larvae could be collected for the TG assay. Lastly, there are numerous studies

reported on fat storage or accumulation at this stage (Géminard *et al.*, 2009; Arrese and Soulages, 2010; Diaconeasa *et al.*, 2013; Xi and Zhang, 2015). We conducted a pilot run to measure the sensitivity of the assay using different number of larvae. On average, two larvae using the W^{1118} line were enough to produce signals that we could use for the TG assay (**MATERIALS AND METHODS**). In addition, a dose response signal was observed with an increase in the number of larvae (**Figure 9**). The concertation levels were not normalized to protein levels. Note, although this assay is called triglyceride content determination, it is based on measuring the glycerol released after fatty acid cleavage and does not distinguish between mono-, di-, and triglycerides.

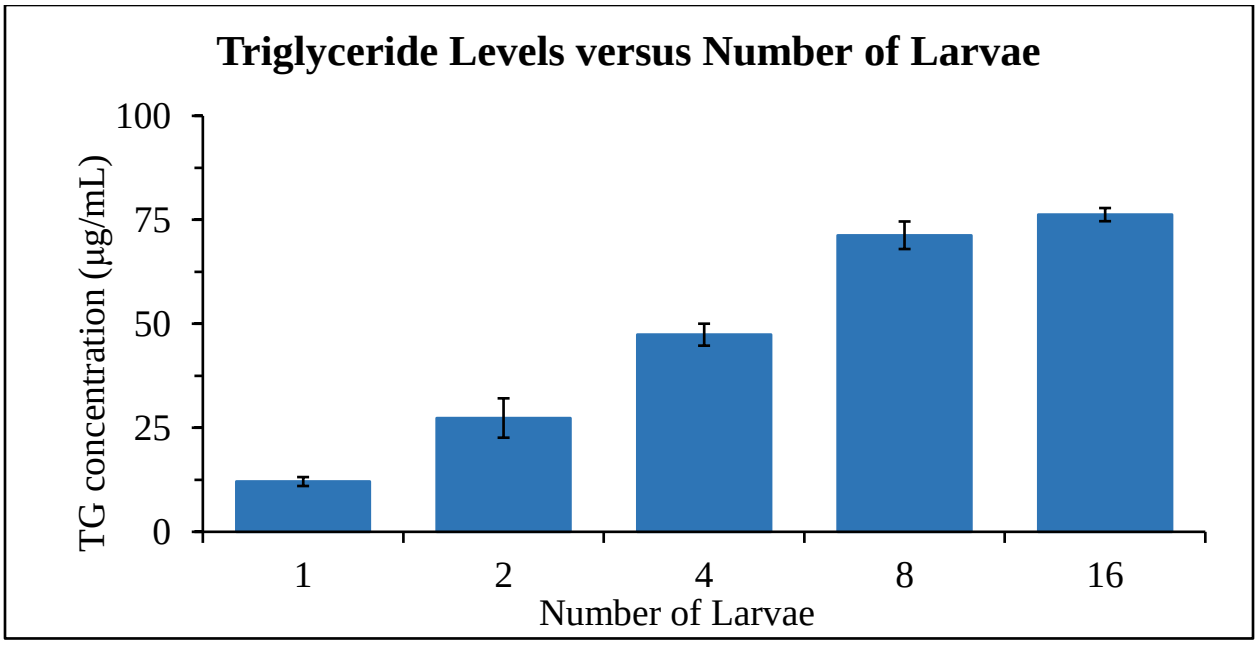

Figure 9 Triglyceride Concentration and the Number of Larvae

The triglyceride concentrations are directly proportional to the number of larvae in the tube. The larvae were collected from W^{1118} vials kept in normoxia. The concentrations here is the direct measurement i.e., without normalizing to the protein levels.

Triglyceride Concentration of Hsl Knockdown Third Instar Larvae in Hypoxic versus Normoxic Conditions

Following the hypoxia tolerance and vulnerability tests, we quantified the triglyceride concentrations of the control and experimental crosses in both normoxia and hypoxia. For all the self-cross controls and W^{1118}, the triglyceride concentrations were always lower in hypoxia than in normoxia (**Figure 10A**). However, for the *Hsl* knockdown crosses UAS-*RNAi(Hsl) x da*-GAL4 and UAS-*RNAi(Hsl) x Eh*-GAL4 the triglyceride concentrations were higher in 5% O_2 than in normoxic conditions, i.e., 158.47 and 136.31 μg/mL in 5% O_2 compared to 99.35 and 106.96 μg/mL in 21% O_2, respectively (**Figure 10A-B**). Knockdown of *Hsl* in UAS-*RNAi(Hsl) x da*-GAL4 and UAS-*RNAi(Hsl) x Eh*-GAL4 augmented triglyceride levels in hypoxia, with a 1.6-fold and 1.27-fold increase, respectively (**Figure 11A-B**). Whereas the triglyceride levels of UAS-*RNAi(Hsl) x Lsp2*-GAL4 were relatively the same in both hypoxia and normoxia with only a 4% increase in hypoxia, i.e., 109.02 μg/mL compared to 113.21 μg/mL (**Figure 10C, Figure 11C**).

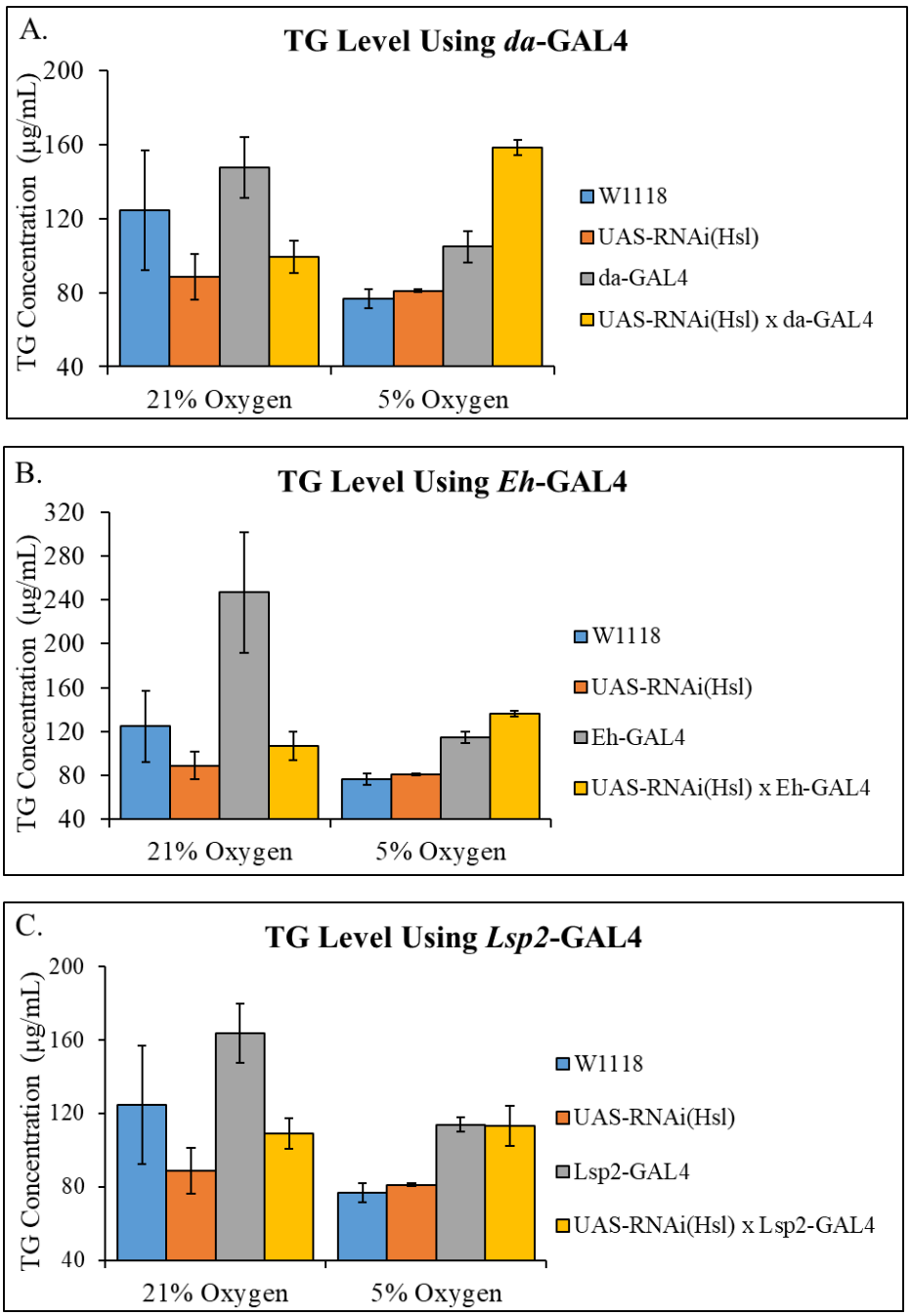

Figure 10 Triglyceride Concentration in Normoxia and Hypoxia

Triglyceride concentrations of UAS-*RNAi(Hsl) x da*-GAL4 (A), UAS-*RNAi(Hsl) x Eh*-GAL4 (B) and UAS-*RNAi(Hsl) x Lsp2*-GAL4 (C) when compared to its respective GAL4 controls. The background controls W^{1118} and UAS-*RNAi(Hsl)* are similar in value. Triglyceride concentrations were measured in µg/mL. Triglyceride concentrations decreases in hypoxia except for UAS-*RNAi(Hsl) x da*-GAL4 (A) and UAS-*RNAi(Hsl) x Eh*-GAL4 (B) where the TG levels increase significantly ($P<0.005$ and $P=0.055$, respectively).

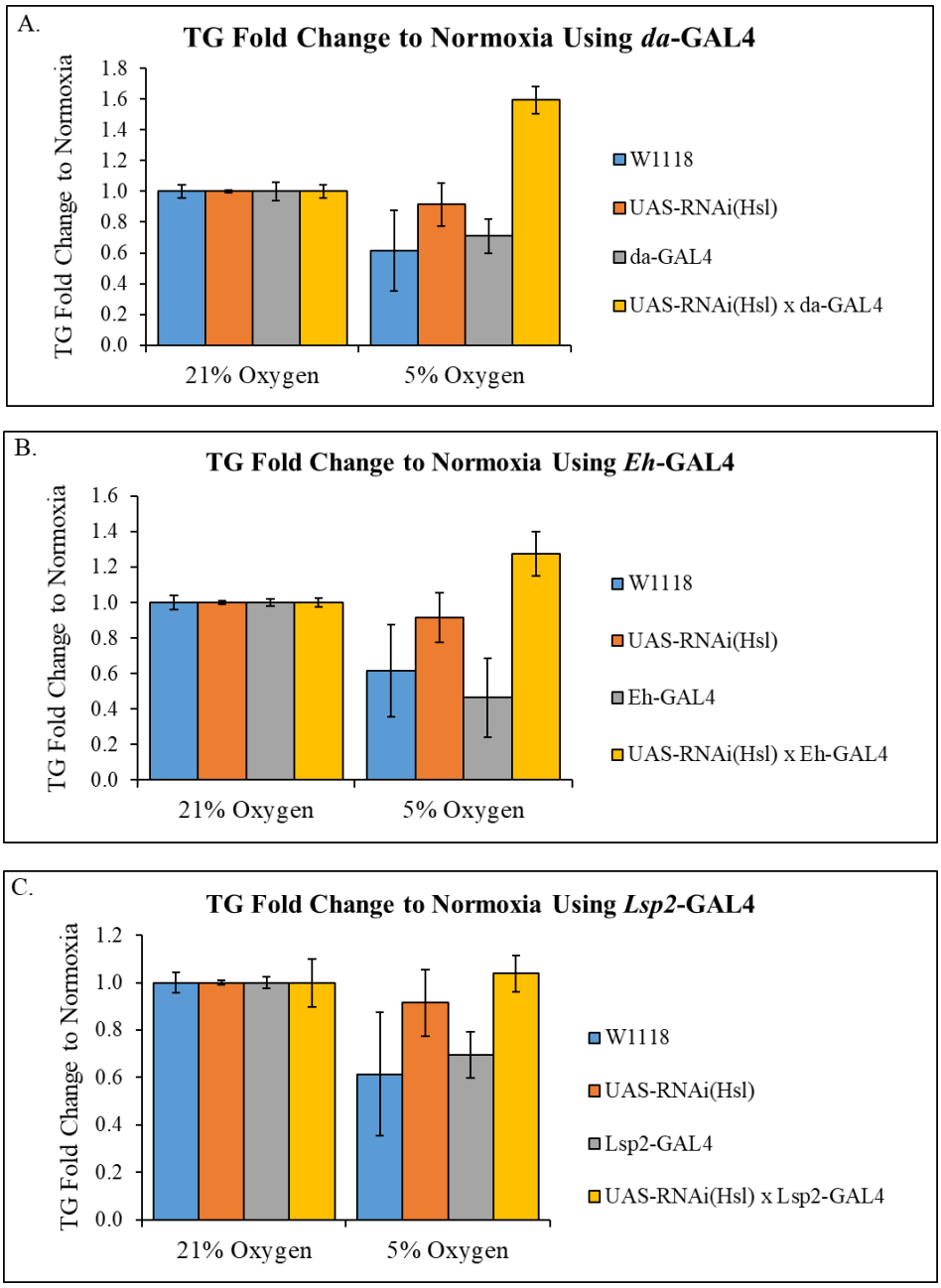

Figure 11 Fold Change of Triglyceride in Hypoxia to Normoxia

The data above represents the fold change ratio between hypoxia to normoxia triglyceride concentrations for UAS-*RNAi(Hsl) x da*-GAL4 (A), UAS-*RNAi(Hsl) x Eh*-GAL4 (B) and UAS-*RNAi(Hsl) x Lsp2*-GAL4 (C) when compared to its respective GAL4 controls. Each bar represents the fold change values to the average TG concentration under normoxia for each fly line respectively. The TG levels increase 1.6-fold for UAS-*RNAi(Hsl) x da*-GAL4 (A) and 1.27-fold for UAS-*RNAi(Hsl) x Eh*-GAL4 (B) in hypoxia.

Triglyceride Concentration of Hsl Knockdown Third Instar Larvae versus Control UAS-RNAi(Hsl) Self-Crosses in Hypoxia

In hypoxia, the UAS-*RNAi(Hsl)* had the lowest eclosion rate whereas the knockdown crosses showed a significantly higher tolerance to low oxygen deprivation, suggesting that inhibiting lipolysis is beneficial and mitigates the damaging effects of hypoxia. When comparing the UAS-*RNAi(Hsl) x da*-GAL4 with the control UAS-*RNAi(Hsl)* line, the triglyceride levels in 21% O_2 are relatively the same, i.e., 99.35 μg/mL and 88.67 μg/mL, respectively (**Figure 10A**). However, in hypoxia, the difference in triglyceride levels in the ubiquitous knockdown show a marked ~2-fold increase compared to UAS-*RNAi(Hsl)* self-cross (81.05 μg/mL compared to 158.47 μg/mL) (**Figure 12A**). When knocking down *Hsl* ubiquitously, it is *only* in hypoxia that this drastic increase is observed (158.47 μg/mL in 5% O_2).

An increase is also observed in the UAS-*RNAi(Hsl) x Eh*-GAL4 cross with a 1.68-fold higher in triglyceride concentration in hypoxia compared to the UAS-*RNAi(Hsl)* self-cross. In hypoxia, the UAS-*RNAi(Hsl) x Eh*-GAL4 was 136.31 μg/mL compared to 81.05 μg/mL for the UAS-*RNAi(Hsl)* control (**Figure 10B, Figure 12B**). This demonstrates that knocking down *Hsl* in eclosion hormone-expressing neurons increases the triglyceride levels by 68% compared to that of the self-cross control.

Interestingly, the UAS-*RNAi(Hsl) x Lsp2*-GAL4 which also showed a strong tolerance to hypoxia displayed a 40% increase relative to the triglyceride levels of the UAS-*RNAi(Hsl)* controls in hypoxia, i.e., 113.21 μg/mL versus 81.05 μg/mL (**Figure 10C, Figure 12C**).

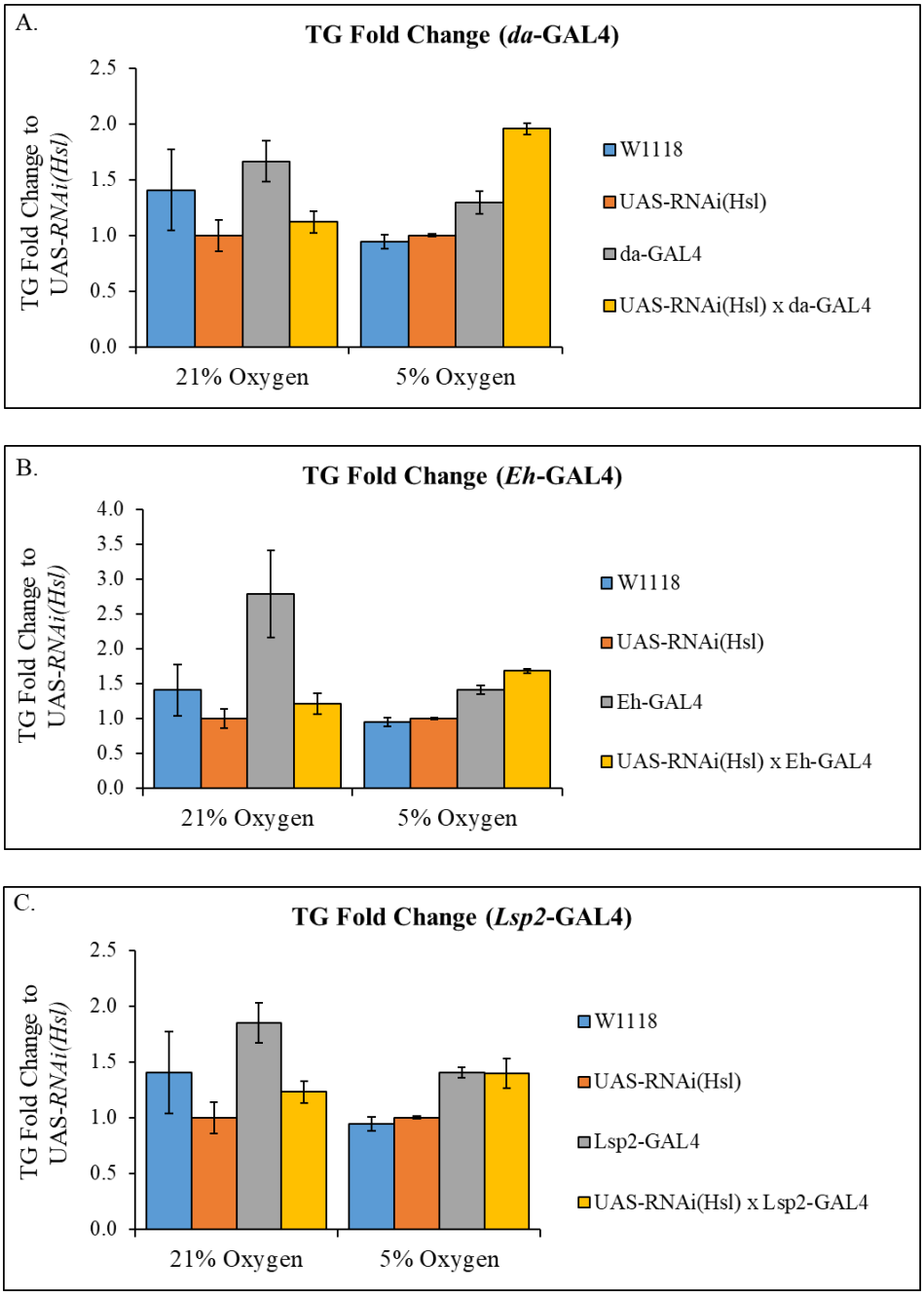

Figure 12 Fold Change of Triglyceride in Different Fly Lines Compared to UAS-*RNAi(Hsl)* Controls in Hypoxia to Normoxia

The bar plot represents the fold change ratio between the different fly line and the UAS-*RNAi(Hsl)* kept under respective environmental conditions (hypoxia or normoxia). The levels were higher in all UAS-*RNAi(Hsl)* crossed with respective GAL4 lines i.e., 2-fold change in *da*-GAL4 (A) 1.8-fold in *Eh*-GAL4 (B) and 1.5-fold in *Lsp2*-GAL4 (C).

Triglyceride Concentration of Hsl Knockdown Third Instar Larvae versus Control GAL4 Self-Crosses in Hypoxia

In addition, it was imperative to compare these knockdown crosses to the other negative control, each respective tissue-specific GAL4 self-cross, which were important in determining hypoxia tolerance and vulnerability tests (**Figure 13**). In hypoxia, the triglyceride levels for UAS-*RNAi(Hsl) x da*-GAL4 were 1.5-times higher than the *da*-GAL4 alone. This is also observed in the UAS-*RNAi(Hsl) x Eh*-GAL4 with only a slight increase by 1.2-fold compared to the *Eh*-GAL4 self-cross. In normoxia, the knockdown crosses were only half the measured triglyceride concentrations of their respective GAL4 self-crosses. Unlike the other knockdown crosses, the UAS-*RNAi(Hsl) x Lsp2*-GAL4 remained unchanged when compared to *Lsp2*-GAL4 control with 113.21 μg/mL and 113.77 μg/mL, respectively.

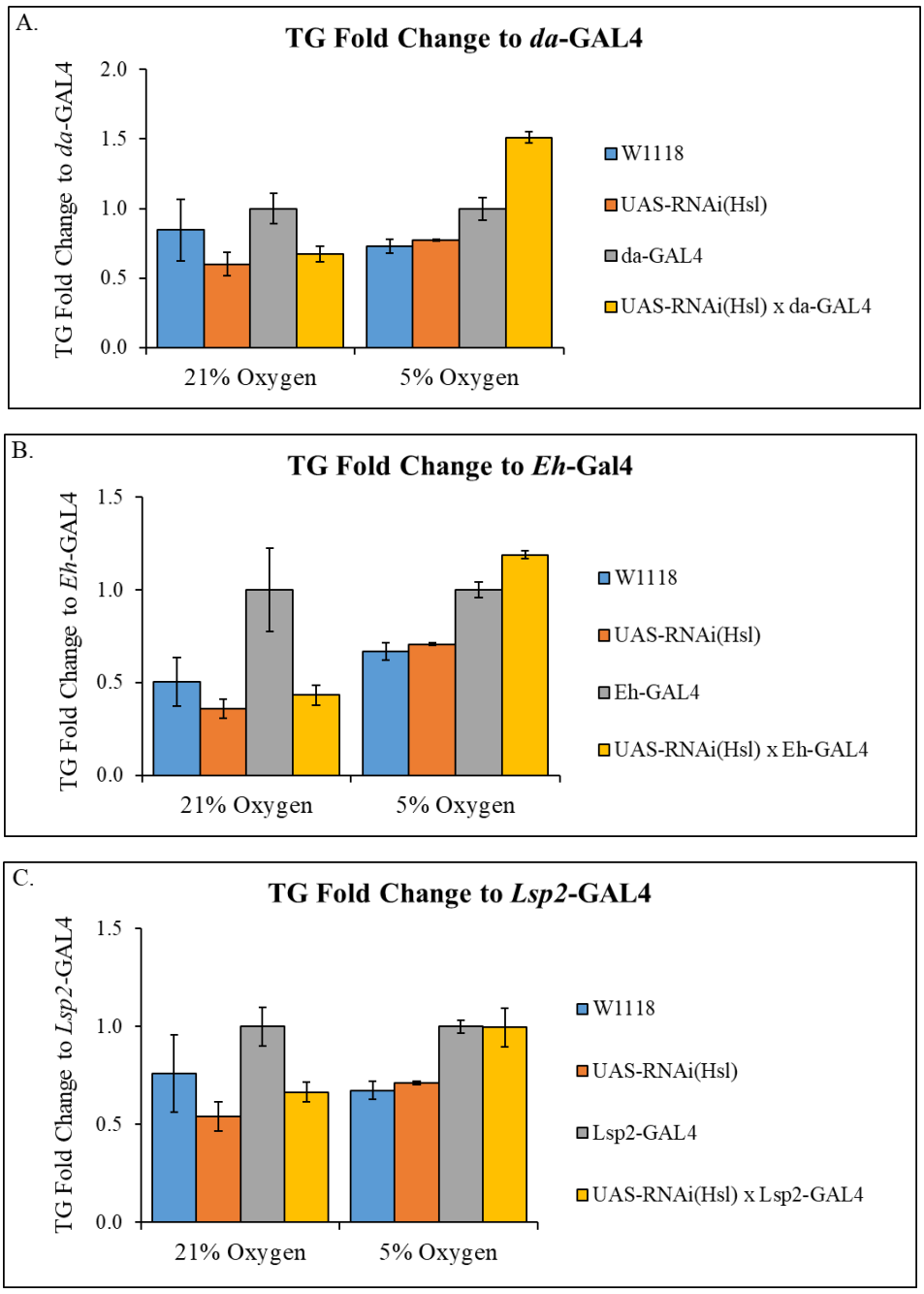

Figure 13 Triglyceride Comparison to Respective GAL4 lines

The data above represents the fold change ratio of triglyceride concentrations of the respective GAL4 self-crosses with *Hsl* knockdown lines. In normoxia, the knockdown crosses have significantly lower triglyceride levels when compared to their respective GAL4 self-crosses i.e., UAS-*RNAi(Hsl) x da*-GAL4 (A), UAS-*RNAi(Hsl) x Eh*-GAL4 (B) and UAS-*RNAi(Hsl) x Lsp2*-GAL4 (C). The reverse is observed in hypoxia; the knockdown crosses have a significantly higher concentration in UAS-*RNAi(Hsl) x da*-GAL4 : *da*-GAL4 = 1.51 fold increase (A) and UAS-*RNAi(Hsl) x Eh*-GAL4 : *Eh*-GAL4 = 1.19 fold increase (B), and no change in UAS-*RNAi(Hsl) x Lsp2*-GAL4 (C).

DISCUSSION

Overview of Hormone-Sensitive Lipase

Hormone-sensitive lipase (*Hsl*) is the rate-limiting enzyme essential for completing hormone-stimulated lipolysis (Sztalryd *et al.*, 1995). In response to catecholamines such as epinephrine and norepinephrine, post-translational phosphorylation of *Hsl* regulates the hydrolysis of cholesteryl esters and diacylglycerol (DAG). *In vitro* studies have shown that this intracellular lipase can also hydrolyze triacylglycerols, monoacylglycerols, and other lipid-soluble and water-soluble substrates (Kraemer and Shen, 2002). Accumulating evidence has defined that *Hsl* regulates numerous aspects of biology, particularly in maintaining homeostasis and energy metabolism (Vaughan *et al.*, 1964; Kramer and Shen, 2006; Zechner *et al.*, 2011; Zechner and Langin, 2014). For instance, when *Hsl* is overexpressed *in vitro*, differentiated adipocytes are unable to accumulate triglyceride (Sztalryd *et al.*, 1995). Conversely, *Hsl* null mice, which are devoid of this neutral cholesteryl ester hydrolase, demonstrate a marked defect in catecholamine-stimulated glycerol release and a decrease in catecholamine-stimulated free fatty acid (FFA) release (Osuga *et al.*, 2000; Wang *et al.*, 2001; Haemmerle *et al.*, 2002). The knowledge gleaned from *Hsl* down-regulation or up-regulation has been applied to circumvent adverse effects of pathophysiological conditions, such as diabetes, tumor cachexia, neutral lipid storage disease with myopathy (NLSDM), and non-alcoholic fatty liver disease (NAFLD) (Mulder *et al.*, 2003; Nakamuta *et al.*, 2005; Agustsson *et al.*, 2007; Albert *et al.*, 2014; Xu *et al.*, 2018). However, high altitude studies have magnified the biological significance of this enzyme by providing compelling new evidence that *Hsl* is imperative for combatting hypoxic damage (Udpa *et al.*, 2014).

Our focus was to apply cell- and tissue-specific RNA interference (*RNAi*) to identify which cell(s) and tissue(s) would benefit from *Hsl* down-regulation in chronic low oxygen conditions. Using *Drosophila melanogaster*, we determined that knockdown in eclosion hormone-expressing neurons, fat body, oenocytes, and hemocytes elicit the adaptive phenotype. Our findings identified key markers that distinguished tolerant flies from the non-tolerant. Specifically, that knockdown crosses displayed a marked increase in eclosion rates and triglyceride levels compared to control flies.

Hsl in the Context of the Hypoxic Response

In the context of the hypoxic response involving *Hsl*, both *in vitro* and *in vivo* studies show that chronic hypoxia leads to an enhanced gene expression of catecholamine-synthesizing enzymes mediated by HIF-1 (Sorriento *et al.*, 2012; Ton and Hammes, 2014). As such, in hypoxia, there is a reduced ability to stimulate beta-adrenergic receptors upstream of *Hsl* activation (Baum and Porte, 1976). The latter increase in catecholamines will stimulate beta-adrenergic receptors, and *Hsl* will be enzymatically-active by phosphorylating an essential serine residue in its catalytic domain by protein kinase A (PKA). Phosphorylated *Hsl* will then translocate from the cytosol to the lipid droplet, which is decorated with perilipins (Kraemer and Shen, 2002). Perilipins are lipid-droplet associated proteins that hinder access of the lipid from enzymes such as *Hsl*. Upon lipolytic stimulation, the perilipins also become phosphorylated and dissociate from the lipid droplet. This in turn will allow the enzymatically-active phosphorylated *Hsl* access and trigger lipolytic activity (**Figure 14**). Thus, *Hsl*, the rate limiting enzyme of lipid metabolism, promotes complete lipolysis during hypoxia, in response to catecholamines induced by HIF-1 accumulation. This suggests that *Hsl* plays a pivotal role in the relationship between metabolic pathways and oxygen levels.

The eclosion rates at 5% O_2 demonstrate the extent to which *Hsl* inactivation is beneficial to experimental flies, while the lack of *Hsl* inactivation is detrimental to control flies. For instance, the eclosion rates for all knockdown crosses were greater than 85% when compared with control self-crosses. Control flies showed eclosion rates less than 40% (refer to **Figure 5-Figure 8**). Although various cells or tissues were used in *Hsl* knockdown, the focus was targeted to those that displayed a robust and differential sensitivity to *Hsl* inactivation during hypoxia. Triglyceride assays were performed to further characterize the metabolic profile of knockdown flies. The triglyceride levels of tolerant flies were compared with control flies in both hypoxia and normoxia. A more detailed analysis connecting other studies to our results revealed potential correlation, that knocking down this lipid lipase in specific cells promotes the intracellular signaling necessary to circumvent the negative effects of chronic hypoxia.

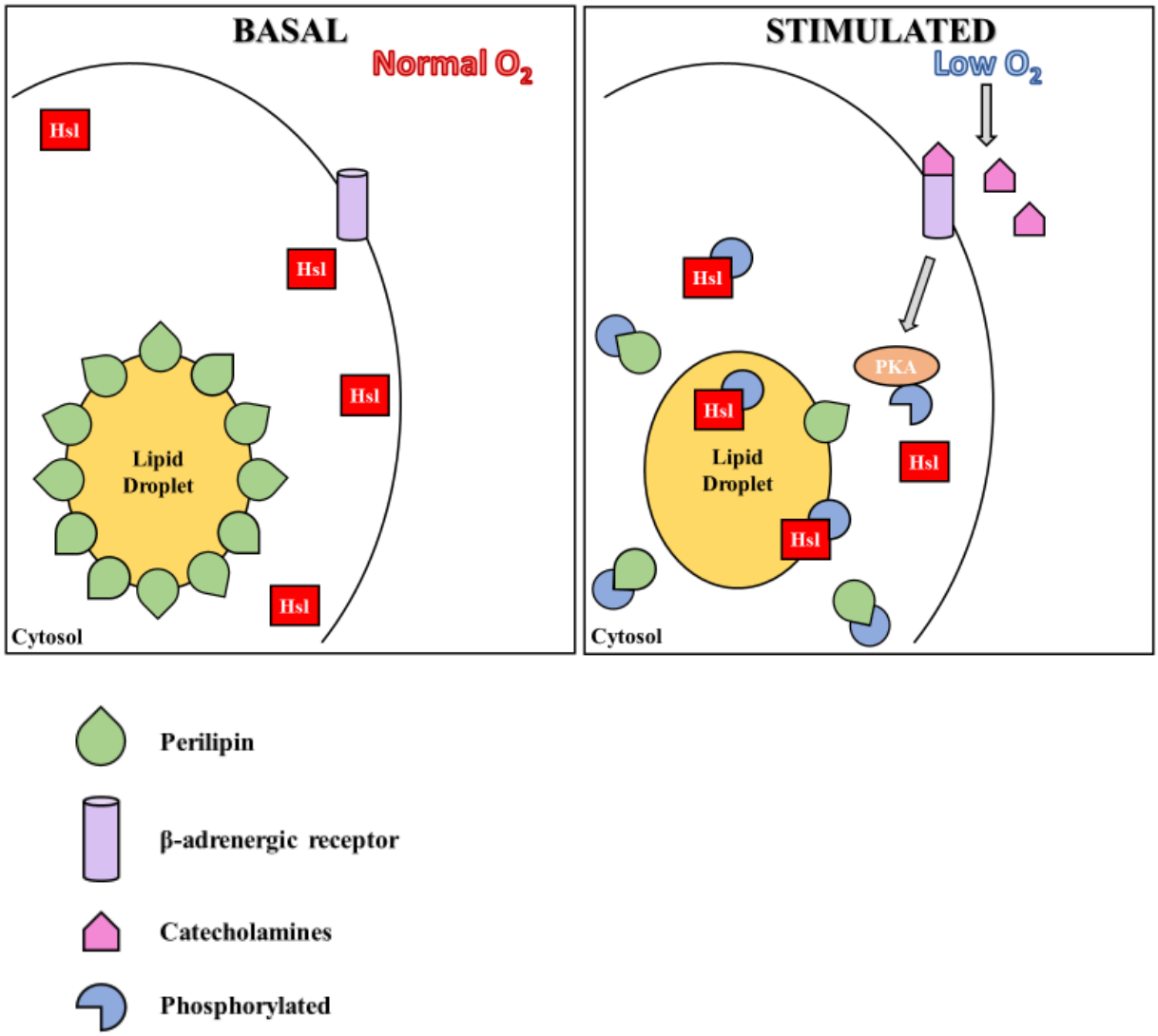

Figure 14 A Simplified Model of Lipid Metabolism Induced by *Hsl*

A simplified mechanism of hormone-stimulated lipolysis is depicted in the above illustrations. Under basal conditions, *Hsl* is not associated with the lipid droplet and is enzymatically inactive. The lipid droplet is decorated with perilipins, or lipid droplet-associated proteins, that prevent *Hsl* access to the lipid droplet. In low oxygen conditions, catecholamine synthesis is enhanced to stimulate β-adrenergic receptors. Catecholamines, such as epinephrine and norepinephrine will bind to β-adrenergic receptors and promote phosphorylation of *Hsl* and perilipins. Phosphorylated perilipins dissociate from the lipid droplet and allow the enzymatically-active phosphorylated *Hsl* access. Phosphorylated *Hsl* will hydrolyze the lipid droplet and release fatty acids.

Hsl Knockdown is Necessary for Development in Chronic Hypoxia

Down-regulation or up-regulation of a specific gene provides information on how interfering with its expression is beneficial or harmful. In our case, knockdown studies were chosen because up-regulation of *Hsl* may be problematic. Specifically, it may be detrimental because of its enzymatic role in an organism. *Hsl* when overexpressed may lead to lipotoxicity and have harmful effects on the organism; thus, fails to answer the question. Experimental evidence supports a detrimental effect of *Hsl* overexpression on metabolism. According to the literature, in a previous study using transgenic mice overexpressing *Hsl*, specifically in beta-cells, resulted in glucose intolerance and severely blunted glucose-stimulated insulin secretion (Winzell, 2003). Interestingly, hypoxia also causes glucose intolerance to humans living at high altitude (Oltmanns *et al.*, 2004; Okumiya *et al.*, 2016). To understand if *Hsl* expression is beneficial or harmful in hypoxia, it would be logical to avoid up-regulation of this gene. *Hsl* overexpression would not combat the harmful effects that are induced by hypoxia, such as, glucose intolerance, but rather compound it. However, using the UAS/GAL4-system, we can knockdown *Hsl* in order to understand the response to hypoxic stress and reaffirm the mechanism of tolerance. In addition, the UAS-*RNAi(Hsl)* line 109336 (from Vienna, Austria) that was used does not have any off-target effects. Off-target effects compromise the specificity of *RNAi*; however, this line allows for undisturbed silencing of *Hsl*.

The cellular and molecular responses that occur when exposed to environmental and systemic signals, such as, hypoxia or ischemia, can be either beneficial or harmful. The aim of *Hsl* knockdown is to react to perturbations in oxygen levels and modulate cellular energy expenditure in order to maintain cell integrity and promote cell proliferation. According to the literature, it has been demonstrated that rats exposed to hypoxia for 1-10 days have significantly greater

phosphorylated-*Hsl* and phosphorylated-perilipin levels compared to the control normoxic group (Xiong *et al.*, 2014). In addition, another study has shown that intermittent hypoxia induces adipose tissue lipolysis and remodeling of which phosphorylation of *Hsl* could be one mechanism mediating this effect (Briançon-Marjollet *et al.*, 2016). We can agree that hypoxia accelerates lipolysis by phosphorylating both *Hsl* and perilipin to induce complete lipolytic activity (refer to **Figure 14**). To understand if interfering with this response is beneficial or harmful in hypoxia became an open question for us to investigate using *Hsl* knockdown studies. For instance, in hypoxia, *Hsl* becomes enzymatically-active, promoting lipolysis and generating ill effects observable through lower eclosion rates and the decline of triglyceride levels in control flies. From our results, we observed a decrease in triglyceride levels of control flies in hypoxia compared to normoxia. Conversely, knockdown crosses had an increase in triglyceride levels in hypoxia, simulating a positive effect. Higher triglyceride levels in the knockdown crosses abrogate a lethal phenotype during an ischemic event, apparent in the concomitant increase in eclosion rates. Altogether, we conclude that *Hsl* knockdown results in an increase in triglyceride levels which avoid pupal lethality and ensure proper cell growth and differentiation.

The aforementioned crosses target knockdown of *Hsl* in eclosion hormone-expressing neurons. Eclosion hormone-expressing neurons play a pivotal role in development. These neurons secrete a peptide factor called eclosion hormone (EH) which endows proliferating and differentiating cells the ability to respond to each developmental stage in the *Drosophila* life cycle (Kingan *et al.*, 2001). Development from egg to reproductive adult requires an appropriate gene program punctuating each stage by shedding the old cuticle (Krüger *et al.*, 2015). This fundamental process is known as ecdysis and is facilitated by the induction or suppression of genes vital to the next stage in *D. melanogaster*. As previously mentioned, we determined there are two critical time

points in development which hypoxia impedes: hatching of the embryo, and eclosion to a reproductive adult. Because eclosion to the reproductive adult is a critical event, we targeted *Hsl* in the neurons that are largely responsible for completing ecdysis.

Hsl knockdown in eclosion hormone-expressing neurons reveals the remarkable ability of a single gene to rescue the entire organism. From our findings, we established that *Hsl* knockdown in eclosion hormone-expressing neurons has an obligatory role in the development of the reproductive adult in chronic low oxygen (**Figure 5**). We hypothesize when the oxygen supply is limited, the use of lipids as an energy source in eclosion hormone-expressing neurons prevents carrying out the ecdysis sequence successfully. Instead, increased fatty acid biosynthesis, followed by esterification into lipids, promotes neuronal-specific adaptation to hypoxia by reducing lactoacidosis in neuronal cells and maintaining reduction potential (Brose *et al.*, 2016). Dysfunction of eclosion hormone-expressing neurons during early metamorphosis of control crosses may be caused by *Hsl* being enzymatically activated rather than inhibited in chronic hypoxia (explained in **Hsl in the Context of the Hypoxic Response**). Consequently, an increase in pupal lethality was observed in hypoxia. These findings demonstrate *Drosophila* invariably survive and develop in chronic oxygen deprivation when *Hsl* is inhibited in eclosion hormone-expressing neurons. Therefore, *Hsl* knockdown is an invaluable gene to mitigate the damaging effects of chronic hypoxia.

Ubiquitous Hsl Knockdown Results in the Highest Triglyceride Accumulation

Ubiquitous knockdown of *Hsl in vivo* shows the strongest change in lipid profiles when comparing knockdown flies with control flies. The result in knockdown flies was a higher triacylglycerol content in hypoxia for third instar larvae, which were collected before the mounting process of pupation (**Figure 10**). Studies have shown that lipid accumulation is a protective mechanism that occurs in both mammals and invertebrates when exposed to critically low oxygen levels (Listenberger *et al.*, 2003; Mylonis *et al.*, 2012; Bensaad *et al.*, 2014; Bailey *et al.*, 2015). The consequence of not inhibiting *Hsl* expression is severe, resulting in the impediment of a fly's development (**Figure 4**). UAS-*RNAi(Hsl) x da*-GAL4 displayed eclosion rates (i.e. ~80% in hypoxia) that were two- to four-fold higher compared to *da*-GAL4 and UAS-*RNAi(Hsl)* control self-crosses respectively (Udpa *et al.*, 2014). Comparison of the control crosses' lipid profiles demonstrated that UAS-*RNAi(Hsl) x da*-GAL4 was 51% greater than *da*-GAL4 self-cross and a staggering 96% greater than UAS-*RNAi(Hsl)* self-cross (**Figure 12A, Figure 13A**). The accumulation of triglycerides in ubiquitous *Hsl* knockdown serve a protective function in hypoxia that can be associated with the marked increase in eclosion rates.

Ubiquitous knockdown using the UAS/GAL4 system is an integral model to study the effects of completely suppressing *Hsl* expression. This experiment addresses an imperative question:

> *Is triglyceride accumulation a direct result of Hsl knockdown, or does hypoxia promote this response?*

The control crosses, *da*-GAL4 and UAS-*RNAi(Hsl),* exhibit 1.5- to 2-times lower triglyceride levels than the ubiquitous knockdown cross respectively. If it were hypoxia that induced triglyceride accumulation, the control and experimental groups would both have increased levels of triglycerides in 5% O_2. In comparison to UAS-*RNAi(Hsl)* self-cross that measured 81.05 μg/mL

and 104.90 μg/mL for *da*-GAL4 self-cross, only the knockdown cross had a marked difference in triglyceride concentration, measuring at 158.47 μg/mL. Our findings strongly correlate with the research done by Haemmerle *et al.,* 2002. They observed that *Hsl* knockout mice accumulate diglycerides in adipose tissue. In our experiment, an increase in triglycerides stimulates a positive effect of tolerance in that it fosters successful development in chronic hypoxia.

Based on the literature, we know that the initial response in hypoxia is triggering lipolytic stimulation due to enhanced catecholamine expression (Rostrup, 1998; Yates *et al.,* 2012) When triglyceride accumulation is impaired, harmful effects such as cell arrest take place (**Figure 4**). Lipid stores seem to circumvent deleterious growth in hypoxia, thus becoming an essential structural unit for healthy tissue growth (Mackenzie *et al.*, 1967; Ackerman *et al.*, 2018). Our results demonstrate that the control crosses' lipid stores were deficient. As such, there were significantly lower eclosion rates in 5% O_2 indicative of unsuccessful development in the fly. We hypothesize that an increase in enzymatic activity of *Hsl* elevated free fatty acid (FFA) levels. Hypoxic or ischemic damage has previously been linked to enhanced lipolysis and increased plasma FFA (Nixon and Brock-Utne 1978; Weinberger *et al.*, 2001; Shin *et al.*, 2011). In turn, a high concentration of circulating FFA may contribute to cell dysfunction and/or cell death, as demonstrated in other studies (Listenberger *et al.*, 2003). According to the literature, abundant levels of circulating FFA promote harmful increases in the rate of reactive oxygen species (ROS) generation (Schonfield and Wojtczak, 2008; Seo and Shen, 2017). The biologically-active and toxic concentrations of ROS can then damage common molecular targets and pathways. In particular, they adversely affect the mitochondria by acting on complexes I, II, and II of the ETC (Wang *et al.*, 2001; Guzy and Schumacker, 2006; Solaini *et al.*, 2010; Kim *et al.*, 2018). Simultaneously, enhanced lipid metabolism will release glycerol, which has been associated with

phospholipid membrane degradation (Clausen *et al.*, 2005). Detecting abundant levels of glycerol allows us to evaluate the severity of ischemic damage (Hillered *et al.*, 1998; Frykholm *et al.*, 2001). Conversely, *Hsl* null mice demonstrate a decrease in catecholamine-stimulated free fatty acid release and blunted catecholamine-stimulated glycerol release (Osuga *et al.*, 2000; Wang *et al.*, 2001; Haemmerle *et al.*, 2002). *Hsl* knockdown *Drosophila* show an increase in triglyceride content. Consequently, we can agree that lipid metabolism (or breakdown into FFA and glycerol) is diminished. Instead, lipid synthesis is being promoted, demonstrating that failure to inhibit *Hsl* in chronic hypoxia results in cellular damage.

This data demonstrates a classic pathobiological response to combat cellular degeneration. The ubiquitous knockdown cross can overcome hypoxic stress because lipid droplets may serve as a structural unit to promote cell growth and proliferation in limited oxygen levels. Consistent with another study, triglyceride accumulation serves a protection function, specifically from fatty acid-induced lipotoxicity (Listenberger *et al.*, 2003). From our experiment, we can conclude that lipid accumulation as a result of *Hsl* knockdown mitigates consequences of hypoxia such as necrosis and accumulation of endogenous toxic products, including FFA, ROS, and glycerol. Impaired cellular function is a direct result of impaired energy balance. Therefore, not inhibiting *Hsl* is lethal and results in cell necrosis (shown in **Figure 4**).

Inhibiting Hsl in the Drosophila Fat Body and Oenocytes

Hsl was first discovered to facilitate the catabolism of fat in adipose tissue. While this epinephrine-sensitive lipase is expressed in different tissue types, such as muscle, adrenal, and testis, it was measured to have the highest expression in adipose tissue (Kramer and Shen, 2002). Lipolysis in adipocytes is complex and can involve different signaling pathways, including catecholamine- and natriuretic peptide-stimulated lipid metabolism (Holm *et al.,* 2003). Hypoxia also utilizes these important signaling pathways to regulate energy homeostasis (Arjamaa and Nikinmaa, 2011; Mahat *et al.*, 2016; Verboven *et al.*, 2017). Therefore, our research efforts were directed toward regulating *Hsl* in the fat body of *Drosophila*. We also performed knockdown studies in cells called oenocytes. Previous research has shown that oenocytes are tightly linked with the fat body and lipid metabolism (Sniderman and Cianflone, 1995; Natarajan *et al.*, 2017). Mammals require specialized cells that coordinate fat metabolism during normal growth and development (Gutierrez *et al.*, 2007). Hepatocytes and adipocytes are the principal cell types involved in lipid-metabolic coupling to regulate lipid use. For example, in a healthy liver, lipolysis is regulated by orchestrating the re-distribution of triacylglycerols from fat droplets (Natarajan *et al.*, 2017). Similarly, during stressful conditions, (such as starvation) fat droplets redistribute and accumulate in hepatocytes (Gibbons *et al.*, 2000). The crosstalk between adipose tissue and hepatic suggests a bi-directional relationship, wherein both tissues serve as major endocrine organs to coordinate energy metabolism.

D. melanogaster also contain specialized hepatocyte-like cells called oenocytes, which process lipids in a similar manner to the mammalian liver during stressful conditions, such as starvation or hypoxia (Gutierrez *et al.*, 2007). From our experiments, we saw that knockdown of *Hsl* in oenocytes avoids pupal lethality and undergoes a successful eclosion in chronic hypoxia

(94.63% for UAS-*RNAi(Hsl) x OK72*-GAL4), whereas control flies arrested in the process of eclosion (30.40% for *OK72*-GAL4). Since oenocytes and fat body cells have a bi-directional relationship to determine lipid uptake, storage, or degradation, we investigated knockdown in the fat body.

To determine whether fat body-specific knockdown was beneficial, we measured the eclosion rate for UAS-*RNAi(Hsl) x Lsp2*-GAL4. This cross demonstrated tolerance to chronic hypoxia, similar to oenocyte-specific knockdown (95.24% for UAS-*RNAi(Hsl)xLsp2*-GAL4) (**Figure 7**, **Figure 8**). Interestingly, *Lsp2*-GAL4, is a temporally restricted driver that is activated only in the third instar larval stage. Knockdown of *Hsl* in the fat body at this specific point in development resulted in a stark difference in eclosion rates in comparison to control self-crosses, suggesting that knockdown at the third instar larval stage is sufficient to yield the adaptive phenotype. *Hsl* inhibition was further examined by measuring triglyceride concentration to determine whether it promotes the accumulation of lipids (the energy source for *Drosophila* development) in chronic hypoxia.

Remarkably, there were no changes in triglyceride levels between *Lsp2*-GAL4 and UAS-*RNAi(Hsl) x Lsp2*-GAL4 in hypoxia. *Lsp2*-GAL4 measured 163.77 μg/mL in normoxia and 113.77 μg/mL in hypoxia (**Figure 10**). In the knockdown cross, the triglyceride concentration was modestly higher in hypoxia than normoxia (i.e. 113.21 μg/mL versus 109.02 μg/mL). Although the experimental and respective GAL4 cross had no differences in triglyceride concentrations, there were still differences in eclosion rates. This suggests that lipid accumulation in the fat body may not be as prominent in hypoxia, or that the lipids may have been redistributed for storage in oenocytes because of their bi-directional relationship. Lipid accumulation of fat body-specific and oenocyte-specific knockdown requires further investigation to reach a definitive conclusion.

However, the inhibition of *Hsl* endorses adequate metabolism and cellular regulatory events required to mediate cell proliferation, differentiation, and survival.

Hemocyte-specific Knockdown Promotes Tolerance to Hypoxia

Hemocytes in *Drosophila melanogaster* are essential intermediaries during injury and immunity in ways similar to their mammalian counterparts. The protective role of *Hsl* knockdown in hemocytes' exposure to chronic hypoxia is remarkable. UAS-*RNAi(Hsl) x He*-GAL4 show 2- to 3-fold increase in eclosion rates compared to *He*-GAL4 and UAS-*RNAi(Hsl)*self-crosses, respectively (**Figure 6**). Although the lipid profiles were not measured for these crosses, we can hypothesize beneficial inductive signals occur as a result of knockdown to combat oxidative stress. *Drosophila* blood cells engage in phagocytosis of apoptotic cells and immune responsive cells by generating signaling molecules and reactive oxygen intermediates (Fogarty *et al.*, 2016). Like the mammalian system, circulating hemocytes that are activated will function as phagocytes and stimulate the fat body to work with oenocytes to remove endogenous toxins (Agaisse *et al.*, 2003; Brennan *et al.*, 2007; Gutierrez *et al.*, 2007). For example, after septic injury, hemocytes communicate with the fat body through a JAK/STAT-dependent pathway (Agaisse *et al.*, 2003). Hemocytes are not only capable of phagocytosis, but contribute to an innate immune response in *Drosophila* larvae by producing signaling molecules that inform distant tissues of injury or infection. Larval hemocytes have proven to be responsible for the nitric oxide signal sent from epithelia to the fat body to induce a specific gene programme (Foley and O'Farrel, 2003). It is possible that there is a causal link between inhibiting *Hsl* in hemocytes and preventing apoptosis by reducing reactive oxygen species (ROS) and hypoxia-induced hydrogen peroxide (H_2O_2) levels (Fogarty *et al.*, 2016). Hemocytes have proven to be a major source of ROS generation, which can

be detrimental to survival in flies (Azad *et al.*, 2011; Azad *et al.*, 2012). The increase in eclosion rates suggests that there may be cytoprotective effects when *Hsl* expression is decreased in hemocytes. Hemocyte-specific *Hsl* knockdown displays the tolerance phenotype in hypoxia.

CONCLUSION

The hypoxic response is a multi-faceted process that requires altering gene expression necessary to provide the precise physiological system for an organism to maintain oxygen and energy homeostasis. Deciphering the genes that participate in hypoxia tolerance allows us to pioneer novel approaches to treat a variety of pathological conditions that contribute to long-term morbidity and mortality. High altitude studies have been insightful in revealing putative hypoxia-tolerant genes. The wide heterogeneity in the hypoxic response between organisms allows us to elect a genetic model, *Drosophila melanogaster*, to establish and define the functional importance of a specific gene, such as LIPE in Ethiopian highlanders.

Ubiquitous knockdown of *Hsl* shows a remarkable tolerance to chronic hypoxia compared to control flies (Udpa *et al.*, 2014). To elucidate the tissue specificity of the adaptive phenotype, systemic knockdown of *Hsl* using cell- or tissue-specific GAL4 drivers and UAS-*RNAi(Hsl)* were crossed without altering its expression in the rest of the organism. There was a marked difference in eclosion rates when *Hsl* was knocked down in eclosion-hormone expressing neurons, third instar larval fat body, oenocytes, and hemocytes. All knockdown crosses constituted a four-fold increase over controls (>87%, $P<0.005$).

Since *Hsl* has a prominent role in lipid metabolism, its inhibition in the cell(s) or tissue(s) that produced the most profound responses to hypoxia tolerance were quantified to provide evidence of any changes in triglyceride levels. An augmented triglyceride concentration was observed in *Hsl* knockdown crosses, specifically in UAS-*RNAi(Hsl) x da*-GAL4 and UAS-*RNAi(Hsl) x Eh*-GAL4 with a 1.5- to 2-fold increase in hypoxia when compared with controls. This data suggests that lipid accumulation as a result of *Hsl* knockdown may be biochemically significant in promoting hypoxia tolerance. An increase in triglycerides as a result of *Hsl*

knockdown demonstrates a mechanism to combat the compromised oxygen availability. The defense response to hypoxia is contingent upon strategies that result in decreased oxygen consumption and adoption of approaches that redistribute essential circulation for proper development.

Through analysis of the differences in lipid accumulation in each cross, our findings suggest that *Hsl* may be acting through a cell-type specific mechanism in response to hypoxia. For instance, UAS-*RNAi(Hsl) x Eh*-GAL4 shows a distinct increase in triglyceride content compared to control crosses, whereas UAS-*RNAi(Hsl) x Lsp2* did not. Further experimentation is required to clarify if *Hsl* acts through a cell-type specific mechanism in response to hypoxia.

Since *Hsl* null mice exist and transgenic mice overexpressing *Hsl* can be created, there is a unique opportunity to spearhead efforts to further study the enzyme and its possible applications to future research topics. Targeting *Hsl* can be advantageous in promoting hypoxia tolerant mechanisms with drugs known to interfere with its enzymatic activity, such as Bosentan. Remarkably, this drug (which is used to treat hypertension and high-altitude sickness) has been reported to target the regulatory module of *Hsl* and de-phosphorylate an essential serine residue in its catalytic domain, thus inhibiting the enzymatic activity of *Hsl (*Briançon-Marjollet *et al.*, 2016*).* With the knowledge we have gleaned from these experiments, it may be possible to use this drug to treat other conditions. Furthermore, it would be compelling to use approaches aimed at overexpressing *Hsl* in cancer cells to promote a reciprocal effect and lead to lipotoxicity. Targeting *Hsl* may be a promising approach to disturb cancerous cell growth and metastatic activity by destroying important reservoirs in O_2 limited cells, such as in glioblastoma cells or renal cell carcinoma (Guo *et al.*, 2013; Agnihotri and Zadeh, 2015). Our research has produced compelling evidence about the role of *Hsl* in broadening our understanding between metabolism and oxygen

deprivation, implying that there is a greater biological significance to be discovered for this unique and promising enzyme.

APPENDIX

Appendix A

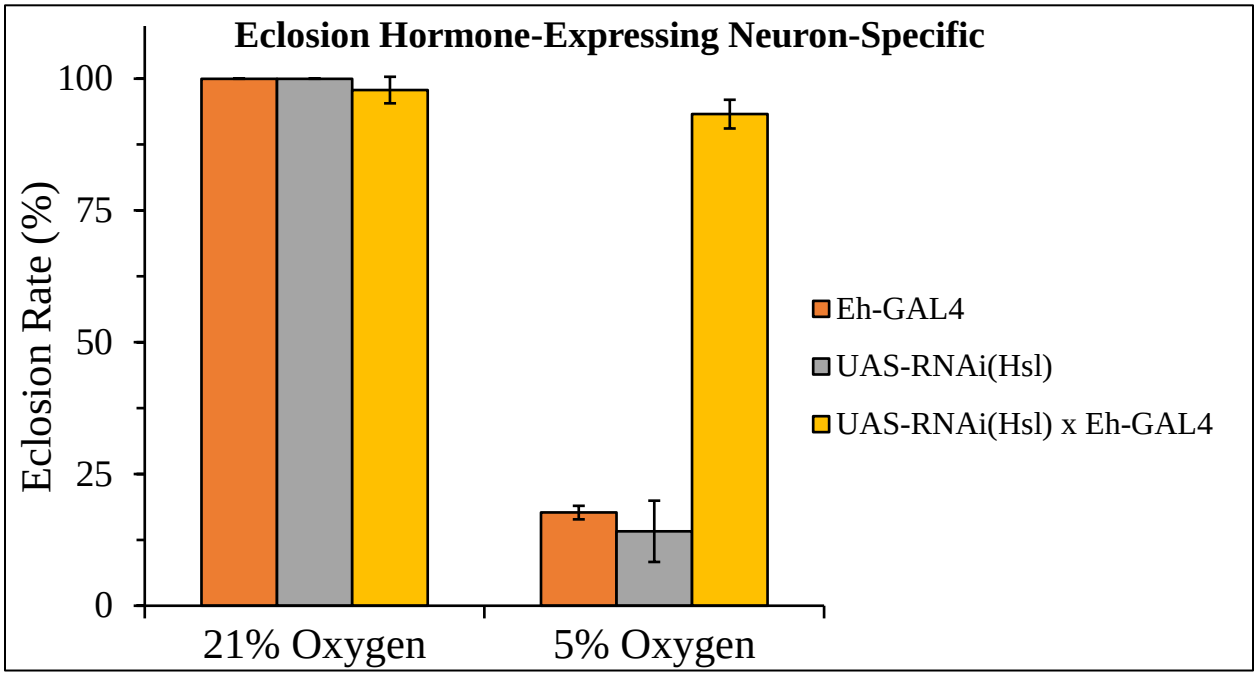

Figure 15 Eclosion Rates of Eclosion Hormone-Expressing Neuron-Specific

The UAS-*RNAi* line for *Hsl* (ortholog of human LIPE) was crossed with *Eh*-GAL4, a driver strain that expresses GAL4 in eclosion hormone-expressing neurons. The level of hypoxia tolerance was determined by measuring eclosion rate in an atmosphere chamber containing 5% O_2. The UAS-*RNAi(Hsl)* and *Eh*-GAL4 self-crosses were used as negative controls. Each bar represents the mean ± standard error of the mean value of three separate tests ($P<0.005$).

Appendix B

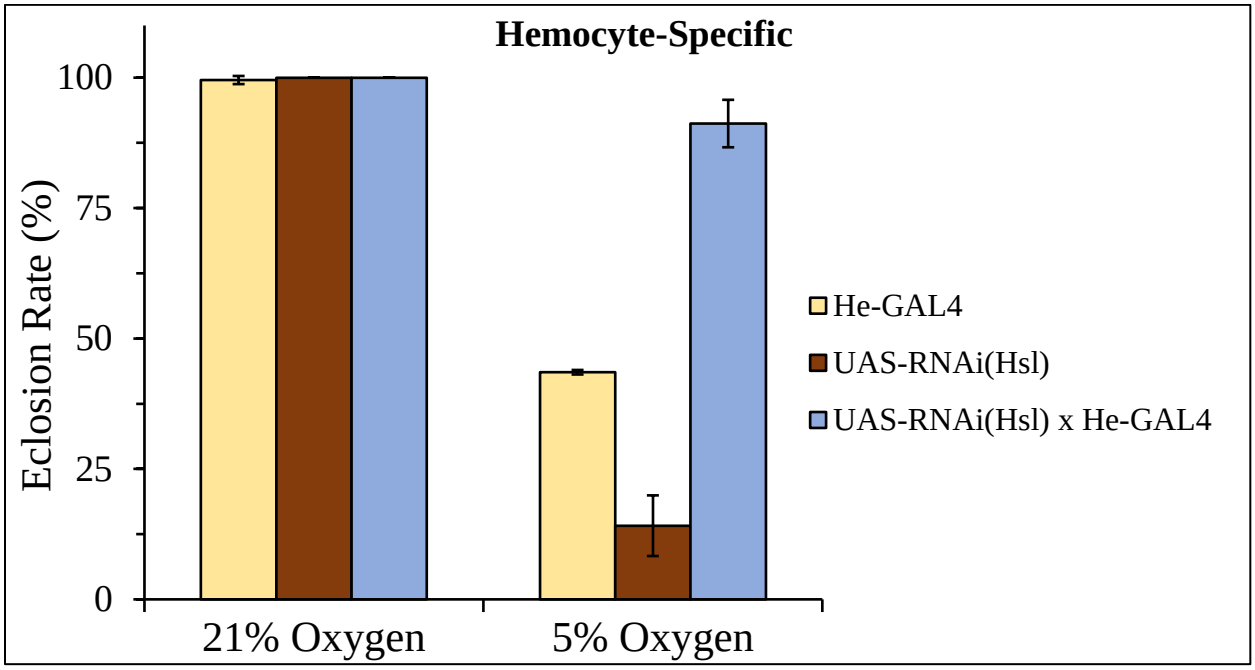

Figure 16 Eclosion Rates of Hemocyte-Specific

The UAS-*RNAi* line for *Hsl* (ortholog of human LIPE) was crossed with *He*-GAL4, a driver strain that expresses GAL4 in hemocytes. The level of hypoxia tolerance was determined by measuring eclosion rate in an atmosphere chamber containing 5% O_2. The UAS-*RNAi(Hsl)* and *He*-GAL4 self-crosses were used as negative controls. Each bar represents the mean ± standard error of the mean value of three separate tests ($P<0.005$).

Appendix C

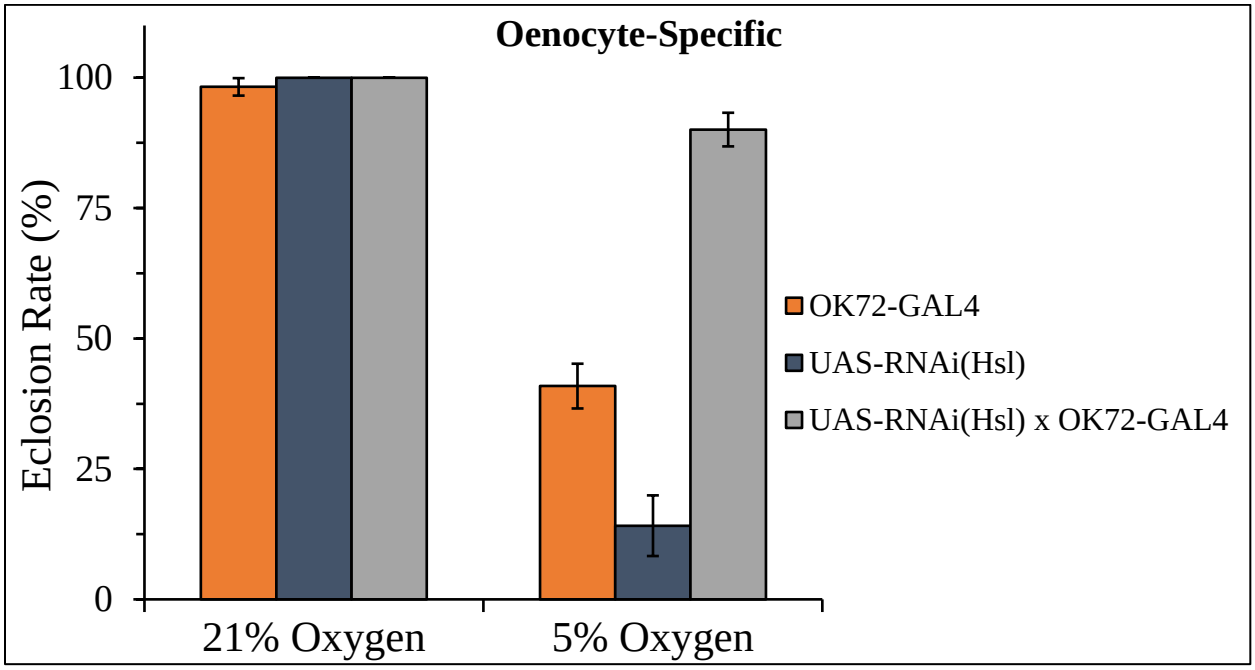

Figure 17 Eclosion Rates of Oenocyte-Specific

The UAS-*RNAi* line for *Hsl* (ortholog of human LIPE) was crossed with *OK72*-GAL4, a driver strain that expresses GAL4 in oenocytes. The level of hypoxia tolerance was determined by measuring eclosion rate in an atmosphere chamber containing 5% O_2. The UAS-*RNAi(Hsl)* and *OK72*-GAL4 self-crosses were used as negative controls. Each bar represents the mean ± standard error of the mean value of three separate tests ($P<0.005$).

Appendix D

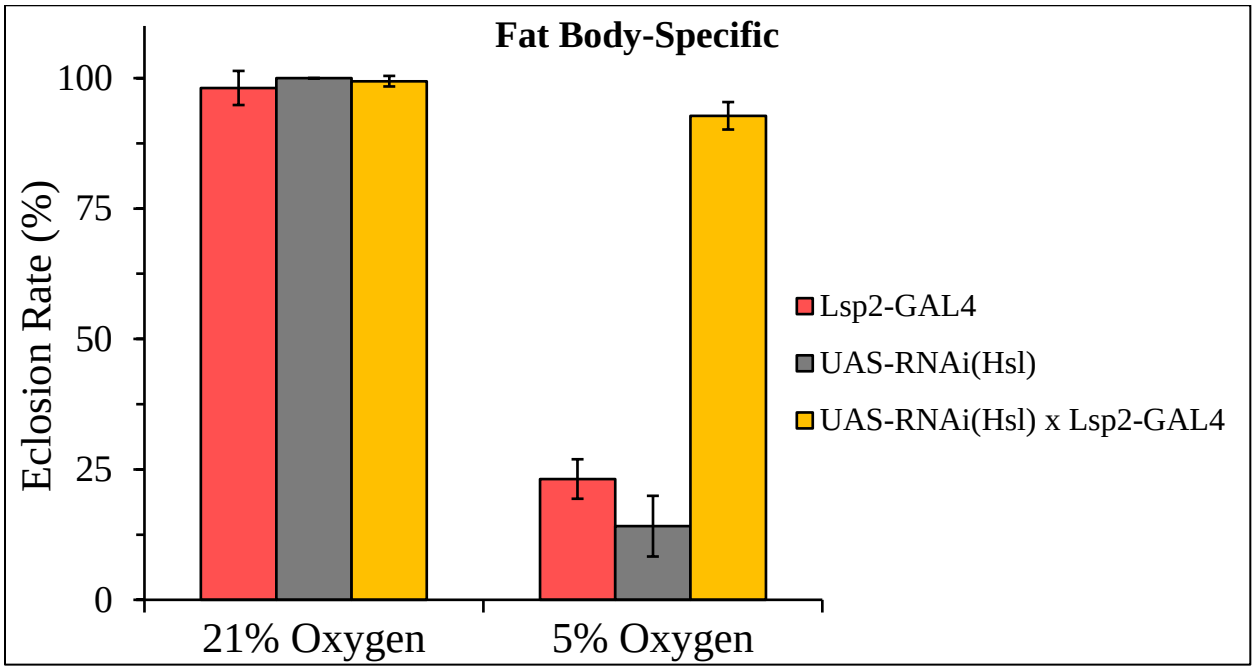

Figure 18 Eclosion Rates of Fat Body-Specific

The UAS-*RNAi* line for *Hsl* (ortholog of human LIPE) was crossed with *Lsp2*-GAL4, a driver strain that expresses GAL4 in third instar larval fat body. The level of hypoxia tolerance was determined by measuring eclosion rate in an atmosphere chamber containing 5% O_2. The UAS-*RNAi(Hsl)* and *Lsp2*-GAL4 self-crosses were used as negative controls. Each bar represents the mean ± standard error of the mean value of three separate tests ($P<0.005$).

Appendix E

Table 2 Eclosion Rates at 21% O_2 and 5% O_2 of Two Independent Trials

	Eclosion (%) at 21 O_2	**Eclosion (%) at 5 O_2**
W^{1118}	100	53.61
UAS-*RNAi(Hsl)*	100	28.27
da -GAL4	99.39	59.37
UAS-*RNAi(Hsl) x da* -GAL4	100	88.15
Eh -GAL4	98.89	25.30
UAS-*RNAi(Hsl) x Eh* -GAL4	100	93.01
He -GAL4	98.20	42.58
UAS-*RNAi(Hsl) x He* -GAL4	100	87.10
OK72 -GAL4	95.82	30.40
UAS-*RNAi(Hsl) x OK72* -GAL4	99.48	94.63
Lsp2 -GAL4	99.32	53.37
UAS-*RNAi(Hsl) x Lsp2* -GAL4	98.91	88.96

	Eclosion (%) at 21 O_2	**Eclosion (%) at 5 O_2**
W^{1118}	100	63.73
UAS-*RNAi(Hsl)*	100	14.12
da -GAL4	100	57.72
UAS-*RNAi(Hsl) x da* -GAL4	100	89.65
Eh -GAL4	100	17.68
UAS-*RNAi(Hsl) x Eh* -GAL4	97.85	93.28
He -GAL4	99.55	43.54
UAS-*RNAi(Hsl) x He* -GAL4	100	91.21
OK72 -GAL4	98.24	40.88
UAS-*RNAi(Hsl) x OK72* -GAL4	100	90.05
Lsp2 -GAL4	98.11	23.16
UAS-*RNAi(Hsl) x Lsp2* -GAL4	99.42	92.78

This Thesis, in full is co-authored by Madlina Babakhanlou, Tsering Stobdan, and Gabriel G. Haddad. The Thesis author was the primary investigator and author of this material.

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