UNIVERSITY OF CALIFORNIA, SAN DIEGO

High Altitude Hearts: Genetic Basis of Cardiac Responses to Long-term Hypoxia Exposures in *Drosophila*

A dissertation submitted in partial satisfaction of the
requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

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The dissertation of Rachel Zarndt is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

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ABSTRACT OF THE DISSERTATION

High Altitude Hearts: Genetic Basis of Cardiac Responses to Long-term Hypoxia Exposures in *Drosophila*

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Cardiomyopathy is a feature of many hypoxia-induced diseases, affecting millions of people worldwide suffering conditions of pulmonary disease, inammation, or high altitude. Interestingly, highlanders with beneficial genetic adaptations to low oxygen have remarkably low incidence of cardiomyopathies. In contrast, cardiac hypertrophy is the hallmark feature in other, poorly adapted highland populations. Detailed mechanisms of these cardiac responses remain largely unknown, yet examination of populations selected for survival in hypoxic environments provides a means to unravel genetic contributions

to hypoxia-related disease.

Drosophila, with its short lifespan and extensive genetic toolbox, allows investigation of conserved and novel pathways underlying cardiac hypoxia responses, particularly through use of a unique population with multi-generational adaptation to hypoxia ('hypoxia-selected'). We advance *Drosophila* models of cardiac hypoxia and show the y heart responds differentially to acute, sustained, chronic and multi-generational hypoxia, responses partly mediated by the *HIFalpha* homolog, *sima*.

We find effects of an acute hypoxia stress on heart function are particularly pronounced in hypoxia-selected flies, even after removal of immediate environmental selection pressure. Most notably, we find persistent reduction in cardiac size in hypoxia-selected flies, but not in control flies exposed to chronic hypoxia, suggesting underlying genetic changes. Using transcriptome analyses of hypoxia-selected and chronically hypoxic fly hearts, we explore contribution of the gene candidate calcineurin to the persistent changes observed in hypoxia-selected fly hearts. Using a heart-specific GAL4 system to modulate expression, we find knockdown of the primary calcineurin A homologues, CanA14F or Pp2B, cause a persistent cardiac restriction which phenocopies effects found in hypoxia-selected flies. We propose a calcineurin pathway as uniquely altered in the hypoxia-selected fly heart, and provide insight on mechanisms underlying cardiac adaptation to high altitude and development of cardiac hypertrophy.

In summary, genes identified from hypoxia-selected populations, human or fly, can alter responses to normal cardioprotective mechanisms; as in HIF*alpha* mutants (first identified in well-adapted humans) and after calcineurin-deficiency (identified in hypoxia-selected flies). We provide new insight into the mechanisms of cardiac remodeling during long-term hypoxia exposures, and evidence of a genetic basis of cardiac adaptations, which may serve as markers of cardiac disease states.

Chapter 1

Introduction

Mountains have invoked awe and wonder in humankind for millennia.

The study of human adaptation to mountainous environments began nearly two thousand years ago with a description of the discomfort of Chinese travelers while traversing ”Big Headache Mountain” (6). These travelers reported the cause of their maladies to be due to the poisonous vapors exhaled from dragons living in the lofty peaks. Many centuries later, a 16th century traveler was the first to attribute ”thin air” to the bodily difficulties of crossing the Peruvian Andes (7).

Despite general physical upsets, the lofty peaks of most of the European Alps were summited by the end of the 19th century. Some of these intrepid early mountaineers mentioned experiencing the symptoms now described as acute mountain sickness; headache, nausea, fatigue, dizziness and general malaise (11). By the 20th century, the relative reduction in oxygen at high altitudes was accepted as the main cause of these altitude related discomforts. However, even today, many questions regarding the fundamental mechanisms of altitude-based diseases remain unanswered.

1.1 Overview of the Dissertation

How humans adapted to lowered oxygen is a puzzle involving a broad array of components including environmental selection pressure, human behavior, genomics, gene expression, diet, physiology and disease progression. This dissertation provides a few of the missing pieces to strengthen the evidence that evolution of genetically encoded traits over a relatively short time span can lead to significant alterations in physiologic performance at altitude.

This dissertation uses the genetic model system*Drosophila* to investigate cardiac responses to varying levels of hypoxia with reoxygenation, and features use of flies selected to survive in extremely low oxygen environments. **The broad ambition of this dissertation is to tease apart effects of acute, chronic and multi-generational hypoxia in a laboratory model of cardiac hypoxia in order to establish core genetic mechanisms underlying cardiac adaptations to hypoxia.**

Insights from flies have greatly informed our understanding of the vertebrate heart, and recent evidence suggests that many aspects of heart function are also conserved and the genes involved in hypoxia response or heart development also play roles in adult heart function. Our findings suggest altered homeostatic responses and alterations to the underlying mechanism of cardiac remodeling in hypoxia-selected populations of *Drosophila*. Further, several of these changes could be explained by alterations in specific genes found in our work to be altered in the fly heart, and have promising ties to current findings of traits found altered in high altitude human populations.

This dissertation unravels some of the complexities of high altitude adaptation and the progression of hypoxia-related cardiac diseases by establishing and using *Drosophila* models of long-term hypoxia exposures, evaluating transcriptional changes underlying these physiological responses, and probing the role of specific genes in the progression

of cardiac remodeling.

1.2 Background

1.2.1 Human expansion into extreme climates and adaptation

A hallmark feature of human evolution includes the expansion from Africa to other continents and environments. Human migration came in waves across the world, and within the last hundred thousand years, humans have come to occupy a vast array of tremendously variable ecological niches. Among these, some sixty million people worldwide live in environments characterized by high altitude, from the highlands of Ethiopia, to the base of the worlds highest mountains in Tibet and Nepal, to the Andean altiplano. Having spent thousands of years in these oxygen poor environments, certain populations have undergone selection for tolerance to life at high altitude.

Genetic selection in human populations living for many generations at high altitude is now well established. Thanks to the enhancement of the sequencing technologies in recent years, different genes were identified which have influenced adaptations of hypoxia tolerance. Those genes found to be fixed in populations, may convey a selective advantage which reflects physiologic adaptations to altitude. Out of these populations, Tibetans are the most extensively studied, although Amhara Ethiopians and Andeans show evidence for certain genetic adaptations as well (30, 35).

Tibetans may have natural protective factors to buffer against the effects of hypoxia due to high altitude. For example erythropoietin (EPO) suppressing genes, may lead to decreased risk of early heart failure and oxidative stress. Multiple studies confirm strong selection in Tibetans and Sherpas for key members of the hypoxia-inducible factor pathway, EPAS1 (HIF2-$alpha$), EGLN1 (PHD2) and PPARA, and correlate these traits

to reduced excessive erythrocyte production (polycythemia), in well adapted populations (8, 13, 25, 31, 32, 34, 35). These genetic changes in key members of hypoxia response pathways may perhaps be considered cardioprotective by preventing right heart pathologic hypertrophy which otherwise arises from chronic polycythemia (2, 9, 22).

Intriguingly, the gene with most extreme signature of positive selection in Tibetans, EPAS1, was found to be acquired in Tibetan populations by introgression of DNA from prehistoric Denisovan populations into the humans who eventually populated the Tibetan plateau (10). Thus, while Denisovans may no longer walk the earth, their beneficial adaptations allow modern Tibetan populations to climb the high Tibetan peaks with relative ease.

In contrast, Andean populations, who likely have been in high altitude environment for a shorter duration, lack Tibetans beneficial genetic adaptations, and are most likely to develop heart failure induced by excessive polycythemia (21, 25). Several genes are now associated with chronic mountain sickness (CMS) in susceptible populations (5, 32, 36). Interestingly, in both populations, the erythrocyte level appears to be controlled genetically, although by differing mechanisms (5, 21, 24, 25, 36). It is not surprising that adaptation and maladaptation may be share and underlying pathway between populations separated by great distance. Mild polycythemia in visitors at high altitude is considered the hallmark sign of acclimatization to high-altitude hypoxia, in order to increase oxygen carrying capacity upon ascent to altitude. Amhara Ethiopian and Tibetan populations have much lower increases than the mild polycythemia exhibited by lowlanders (1, 3, 26). Andeans exhibit erythrocyte levels much higher than Tibetans or sea-level sojourners to altitude on average (33). Andean populations have high incidence of polycythemia, Tibetans have lower incidence than visiting, acclimatized lowlanders.

Humans exhibit extraordinary phenotypic plasticity within individuals, a trait which allows large populations of the same species to exist in climates separated both

by great distance and differing environment conditions (12). Divergence over time as populations adapt to their new environments will draw out the differences in these underlying, core shared mechanisms. While the molecular basis for many altitude-related pathologies are unknown, some sea-level individuals appear predisposed to developing altitude-related illnesses, and reliable predictors are not well established (14, 24, 36). This indicates varying genetic contributions to hypoxia susceptibility even in sea-level populations. **Overall, studying human populations can suggest correlative associations indicative of genetic selection, but experimental evidence from laboratory controlled organisms can solidify the causal effects of genes underlying physiologic adaptations.**

1.2.2 Hypoxia and cardiac disease

In human populations and mammalian models living with chronic hypoxia, the development of cardiopulmonary symptoms, including right heart failure and pulmonary hypertension, are considered maladaptive responses to the prolonged physiological attempts to alleviate hypoxia (15, 35). Even among healthy individuals, the majority of sojourners to elevations over 5,000' encounter cardiopulmonary symptoms related to hypoxia, with an unfortunate few experiencing life-threatening conditions (4, 11). In fact, insufficient tissue oxygenation occurs under a variety of conditions, including high altitude, embryonic and fetal development, inflammation, and thrombotic diseases, often affecting multiple organ systems. Responses and adaptations of the heart to hypoxia are of particular relevance in human cardiovascular and pulmonary diseases, where the effects of hypoxic exposure can range in severity from transient to long-lasting (22).

In mammals, a normal response of the pulmonary circulation to local hypoxia is regional hypertension, a response which directs blood flow toward better oxygenated re-

gions of the lungs (17, 23). However, conditions of chronic hypoxia can cause pulmonary remodeling through thickening of pulmonary vascular smooth muscle after chronic pulmonary hypertension, and thus limit oxygen diffusion and worsen systemic hypoxic illnesses. Further, these individuals perform poorly to increased stress or acute hypoxic illnesses, such as exercise, lung infections or acute cardiac ischemia (9, 21). Chronic pulmonary hypertension or polycythemia can lead to pathological hypertrophy as the heart pumps against increased volume or vascular resistance. Chronic, untreated cardiac hypertrophy can lead to dilated cardiomyopathy, heart failure, and sudden death (9, 16). In contrast, cardiac physiological hypertrophy, as in exercise or pregnancy, is reversible and not typically associated with reduced cardiac function (15).

Hypoxia plays a critical role in the development and pathology of heart disease, cancer, stroke and chronic pulmonary diseases, together accounting for over 60% of deaths in developed countries (9, 15, 22, 27). Diabetes, obesity and cardiomyopathy are also increasing in prevalence, and hypoxia, as a side effect of those illnesses, is an increasing popular research focus. Importantly, these cardiopulmonary diseases represent some of the leading causes of mortality worldwide (1820). The cardiac responses to hypoxia are varied and depend on the duration, location and severity of the stimulus. Mild hypoxia exposures can elicit either reversible physiological acclimations at the systemic level, as observed in sea-level residents traveling to high altitude regions, or tissue remodeling and disease state, as seen at the onset of coronary heart disease (22).

Upon acute, systemic hypoxic exposure, there is an immediate increase in heart rate and myocardial contraction, concomitant increases in intermediary metabolism, and preservation of mitochondrial function at the cellular level. These changes temporarily increase cardiac output and maintain systemic oxygen saturation, while preserving cardiomyocyte integrity as the heart maintains function under lower oxygen conditions. Sustained hypoxia exposure can occur systemically as a result of an underlying disease,

as in pulmonary hypertension, or can arise locally within a tissue as a symptom, such as ischemic heart disease. Sustained hypoxic stimulus, as in the border zone surrounding a myocardial infarction, can lead to changes in transcription required for cellular protection such as a switch from aerobic to glycolytic metabolism, and compromised mitochondrial function (29). Lifetime, chronic exposure to hypoxia can cause the heart to remodel in response to sustained acclimations. Depending on genetic background and hypoxia exposure, this remodeling can lead to heart failure (24).

While both acute and chronic hypoxia exposures are stressors, the return of oxygen, or relative hyperoxia, can be equally damaging. Oxygen reperfusion, referring to reinstated blood flow and the return of oxygen (reoxygenation) to tissues, specifically after an ischemic event, further exacerbates the hypoxia-induced cardiac dysfunction. This damage is thought to occur in response to an increase in reactive oxygen species (ROS) leading to oxidative damage (22, 28). Ischemic heart disease, which involves hypoxia and reoxygenation, is the leading cause of death worldwide. An adequate supply of oxygen is important for the survival of all tissues, but is especially critical for tissues with high energy demands such as the heart.

How do otherwise healthy populations in which hypoxia occurs naturally adjust to the varied responses to conditions of chronic hypoxia? Understanding the mechanisms of hypoxia responses through varied lenses whether multi-generational adaptation, lifetime acclimatization or maladaptive response, or acute injury - allows valuable reflection into hypoxic diseases common in sea-level populations.

1.2.3 Drosophila as a model of cardiac hypoxia

Drosophila melanogaster is an excellent model for exploring hypoxia tolerance (11). These hardy insects tolerate conditions of low oxygen extremely well, even fully

recovering from several hours of complete anoxia (53). However, adaptations to long term hypoxia are accompanied by complex shifts in the transcriptome, a process regulated primarily by the HIF complex (12). Hypoxia-mediated induction of HIF pathways and downstream regulation appears to be well conserved across species, with the oxygen dependent hypoxia-inducible factor-1 *alpha* (HIF-1 *alpha*) present in *Drosophila* as the HIF *alpha* homolog, *sima*. Under conditions of normoxia, HIF-1 *alpha* is degraded after prolyl modification of the oxygen-dependent degradation domain by prolyl hydroxlases and association with the von Hippel-Lindau tumor-suppressor (14, 15). In hypoxic conditions, HIF-1 *alpha* accumulates and allows activation of HIF-dependent transcription pathways. The HIF complex is closely involved with the progression of the hypoxic aspects of many cardiopulmonary diseases (16). Key roles in conserved cellular responses to hypoxia have been confirmed with *sima* (13, 17). Extraordinarily, unlike mammals, *Drosophila* homozygous null mutants of this vital hypoxia response gene can survive development into adulthood, affording the opportunity to design hypoxia experiments in the absence of basal HIF activity.

Cells require a constant supply of oxygen to function normally, and thus are sensitive to hypoxia. Most cells adapt to decreased oxygen by slowing gene transcription and translation, whereas oxygen-responsive genes are up-regulated during hypoxia (12). Cardiomyocytes are remarkably sensitive to hypoxia due to their high energy demand, and require efficient mechanisms to adapt to hypoxia without pathologic consequences for the heart or organism (16).

The relationship between hypoxia and cardiac development and disease progression are largely unknown; Drosophila provides the simplest genetic model for studies of heart development, function, hypoxic injury, and reperfusion. The conservation between mammals and Drosophila in cardiac development is well established, from heart development by the transcription factor tinman to cardiac signaling pathways later in

life, including HIF (21-23). It is likely that the basic mechanisms controlling heart function and adaptations to hypoxia are also highly conserved. For example, previous work established the importance of *dSUR*, a conserved KATP channel component, in protecting the Drosophila heart during hypoxia, and heart-specific over-expression of Hsp70 increases survival during chronic hypoxia in flies (24).

Similarly, previous studies in Drosophila show evolutionarily conserved roles for regulating growth and metabolism during changes in energy state under hypoxic conditions. The insulin/TOR signaling pathways alter functional hypoxia responses and heart function considerably (25,26). Previous deep sequencing and genetic screens from the hypoxia-selected Drosophila population used in the present study identified a number of DNA regions under selection, including genes encoding targets of the Notch and HIF pathways (24,27-29). Most promisingly, past studies have successfully used Drosophila as a screen for novel genes to provide insight into the interplay between hypoxia and cardiac disease (30). Drosophila is a valuable model to study interrelated pathways in the complex conditions of cardiac hypoxia.

1.3 Summaries of dissertation chapters

1.3.1 Chapter 2: Cardiac responses to hypoxia and reoxygenation in *Drosophila*

Since insufficient tissue oxygenation occurs under a variety of systemic conditions and can result in varying durations and severities of cardiovascular disease, we set out to model various hypoxia exposures on the fly heart in a range and severity from transient to long-lasting. Chapter 2 advances hypoxia assays in the *Drosophila* heart, and

establishes cardiac responses to acute (30 minute), sustained (18 hours), chronic (three week lifespan), and multi-generational exposures. We rigorously compare assays in the isolated *Drosophila* heart as a novel genetic model system and determined the fly heart responds differentially to hypoxia insults; recovering fully from acute exposures, and showing signs of cardiac dysfunction and remodeling after longer exposures. Further, determined these responses are partly mediated by signaling via the *HIF1*α homolog *sima*, a key transcriptional regulator of the hypoxia response.

1.3.2 Chapter 3: Altered cardiac responses to hypoxia in hypoxia-selected *Drosophila* populations

In Chapter 3, we explore in detail the hypoxia-selected *Drosophila* response to long-term, multi-generational hypoxia. We exposed hypoxia-selected flies to the acute hypoxia challenge as established in Chapter 2 and found altered recovery upon reoxygenation. In order to tease apart the contribution of lifespan, adult exposure to hypoxia from multi-generational effects, we raised these hypoxia-selected flies under normoxia for two generations. After removing the immediate effects of hypoxic preconditioning, we found persistent altered responses to acute hypoxia exposures, indicating changes in underlying homeostatic mechanisms. In particular, we were intrigued by persistent cardiac restriction observed in hypoxia-selected genotypes, and wished to explore its genetic basis.

1.3.3 Chapter 4: Calcineurin mediates loss of cardiac function after long-term hypoxia exposures in *Drosophila*

Chapter 4 evaluated differentially expressed genes between conditions of hypoxia adaptation and chronic hypoxia to identify shared and unique mechanisms of cardiac remodeling during hypoxia. We compared cardiac-specific microarrays from hypoxia-selected flies (whose cardiac responses we examined in detail in Chapter 3) to cardiac-specific RNA-seq data from *w1118* flies raised under a matched duration of three weeks chronic 4% hypoxia (whose cardiac responses we examined in detail in Chapter 2). Our hypoxia-selected arrays uniquely included strongest down-regulation of the gene candidate CanA14F, as well as down-regulation of its genetic neighbor Pp2B, homologs of human calcineurin A. We thought it was particularly interesting that calcineurin A over-expression is a known marker of cardiac hypertrophy, and our hypoxia-selected flies show persistent cardiac restriction. CanA14F and PP2B were not identified as highly down-regulated in the hypoxic wild type fly array, and these wild type flies also do not exhibit significant cardiac restriction. Thus, we performed heart-specific knockdown of these calcineurin A gene homologs and found evidence that reductions in either gene cause significant cardiac restriction under normoxia or hypoxia, accumulation of whole body triglycerides, and may interact other EGF/sprouty and *HIF/sima* pathways to regulate cardiac growth during hypoxia.

Chapter 2

Cardiac responses to hypoxia and reoxygenation in *Drosophila*

2.1 Abstract

An adequate supply of oxygen is important for the survival of all tissues, but is especially critical for tissues with high energy demands such as the heart. Insufficient tissue oxygenation occurs under a variety of conditions, including high altitude, embryonic and fetal development, inflammation, and thrombotic diseases, often affecting multiple organ systems. Responses and adaptations of the heart to hypoxia are of particular relevance in human cardiovascular and pulmonary diseases, where the effects of hypoxic exposure can range in severity from transient to long-lasting. This study uses the genetic model system *Drosophila* to investigate cardiac responses to acute (30 minutes), sustained (18 hours), and chronic (3 weeks) hypoxia with reoxygenation. Whereas hearts from wild type flies recovered quickly after acute hypoxia, exposure to sustained or chronic hypoxia significantly compromised heart function upon reoxygenation. Hearts from flies with mutations in *sima*, *Drosophila* homolog of the Hypoxia Inducible Factor alpha subunit

(HIFα), exhibited exaggerated reductions in cardiac output in response to hypoxia. Heart function in hypoxia-selected flies, selected over many generations for survival in a low oxygen environment, revealed reduced cardiac output in terms of decreased heart rate and fractional shortening compared to their normoxia controls. Hypoxia-selected flies also had smaller hearts, myofibrillar disorganization and increased extracellular collagen deposition, consistent with the observed reductions in contractility. This study indicates that longer duration hypoxic insults exert deleterious effects on heart function that are mediated in part by *sima* and advances *Drosophila* models for the genetic analysis of cardiac-specific responses to hypoxia and reoxygenation.

2.2 Introduction

Hypoxia is associated with a myriad of physiological and disease states that range from effects at high altitude and during embryonic development to inflammation and cardiopulmonary diseases. In particular, hypoxia is a pivotal factor in cardiopulmonary disorders such as ischemic heart disease, chronic obstructive pulmonary disease and pulmonary hypertension-induced right ventricular dysfunction, among others (38). Importantly, these cardiopulmonary diseases represent some of the leading causes of mortality worldwide (31, 32, 35).

The cardiac responses to hypoxia are varied and depend on the duration, location and severity of this stress. Mild hypoxia exposure can elicit either reversible physiological acclimation at the systemic level, as observed in sea-level residents temporarily traveling to high altitude regions, or tissue remodeling and disease state, as seen at the onset of coronary heart disease (38). Upon acute, systemic hypoxic exposure in healthy humans, there is an immediate increase in heart rate and lung function that temporarily increases

cardiac output and maintains systemic oxygen delivery, while preserving cardiomyocyte integrity. Sustained hypoxia exposure can occur systemically as a result of an underlying disease, as in pulmonary hypertension, or can arise locally within a tissue, such as in ischemic heart disease and can lead to changes in transcription required for cellular protection (44). Further, the return of oxygen (reoxygenation) after acute or sustained exposure causes oxidative damage, further exacerbating the hypoxia-induced cardiac dysfunction (38).

Lifetime, chronic exposure to hypoxia can cause the heart to remodel. Depending on genetic background, this remodeling can lead to improved cardiac performance under hypoxia or to disease leading to heart failure (38, 40). For example, cardiac disease has been documented in Andean human populations living under chronic low oxygen conditions at high altitude, which suffer from cardiovascular symptoms known as chronic mountain sickness. In contrast, other populations, notably the Tibetan Sherpas, are renowned for their remarkable tolerance to high altitude relative to visiting lowlanders (15, 36, 40). Many of the species introduced to high altitude also show signs of genetic and physiologic cardiac adaptation or disease, and are potential models of hypoxia-induced cardiac remodeling (9, 25, 30, 39, 45). However, determining the underlying mechanisms of adaptation and the relationship with human hypoxic disease requires use of an amenable genetic model.

Several pathways are already well known to be activated during hypoxia, and the best studied pathway is mediated by Hypoxia Inducible transcription Factors (HIF). HIF signaling is induced during sustained hypoxia exposures, and affects genes that underlie protection against ischemia and reperfusion (21, 41). The HIFα subunit plays a well-established role in the cardiomyocyte hypoxia response (for a thorough review see 43). The hypoxia-mediated induction of HIF pathways and downstream regulation is widely conserved in insects. In Drosophila, there is only a single HIFα homolog,

encoded by *sima*, that mediates cellular responses to hypoxia (4, 24). Multiple studies show the metabolic and genetic responses to hypoxia in the fly share similarities with vertebrate models (1, 3, 12, 34, 54). Unlike mammals, Drosophila tolerate conditions of low oxygen extremely well, even fully recovering from several hours of complete anoxia (18). Further, Drosophila homozygous null mutants of *sima* can survive to adulthood, whereas homozygous null mammals do not (6, 23), making it difficult to study the effects of loss of function in these models. This ability to survive hypoxia challenges and complete deletion of to completely delete *sima* makes Drosophila an excellent model in which to identify additional pathways that interact with this core hypoxia response gene as well as novel pathways that may mediate the hypoxia response.

We used a genetically tractable model system, the fruit fly *Drosophila melanogaste* to explore genetic mechanisms underlying the cardiac responses to hypoxia. Here we characterize the *Drosophila* cardiac responses to varying levels of hypoxia and subsequent reoxygenation to advance the fly heart as a hypoxia/reoxygenation model. We show that both acute and longer-term exposures to hypoxia alter heart function in the fly and that these responses are partially dependent on *sima* functions. Acute (30 minute) exposure to 1% O_2 caused reductions in heart rate and contractility, which were reversible upon reoxygenation. Notably, hearts from sima mutants exhibited significantly smaller reductions in fractional shortening after acute hypoxia exposure than did wild type flies but significantly greater reductions in fractional shortening after longer term hypoxia exposures. This suggests that the HIF homolog *sima* plays a role in maintaining cardiac contractility. We also examined heart function in populations of *Drosophila* selected for survival in a low oxygen (4% O_2) environment for over 250 generations. Hearts from these 'hypoxia-selected' flies exhibited altered cardiac function, including a slower heart rate, decreased contractility, and disordered arrangement of myofibrils, suggestive of a chronic cardiac stress response to a low oxygen environment (22). Data from our model

systems can be used to probe for novel genes and pathway interactions that contribute to hypoxia-related cardiac diseases.

2.3 Methods

2.3.1 Genetic lines

Specific loss-of-function of sima was achieved using simaKG07607; this line has a P-element insertion in the sima locus, fails to express the hypoxia-inducible LDH-LacZ reporter and is considered a null allele (6, 24). w^{1118}, the laboratory 'wild type control' line, and *sima* mutant heart function was assessed in 1- to 3-week old adult flies. In preliminary studies we did not observe differences in baseline cardiac function between male and female flies, with the exception of cardiac diameters that show sex-dependent differences in size (13, 33). Thus, we chose to use only female flies for cardiac diameter measurements and for structural assessments in response to acute hypoxia/reoxygenation (H/R), sustained H/R and chronic H/R hypoxia exposures. To study long-term adaptive mechanisms we used three *Drosophila* populations selected for more than 250 generations ('hypoxia-selected') for survival at 4% O_2, a level that is normally developmentally lethal. Three distinct and genetically isolated, normoxia-raised populations maintained in parallel with the selected populations were used as outbred control populations ('normoxia controls') (53). To maintain the long-term genetic integrity of the selected populations, only 3 week old, male hypoxia-selected and normoxia control flies were used for cardiac function and structure studies.

2.3.2 Acute hypoxia and reoxygenation assay (acute H/R)

Semi-intact hearts were dissected and equilibrated under normoxic conditions as previously described (13). Dissected preparations were filmed first under 21% O_2, then transferred to a humidified, temperature-controlled glove box and filmed again after 30 min exposure to oxygen levels adjusted to 4% or 1% O_2 using pre-calibrated mixtures (balanced with nitrogen, see Figure 1A). Preparations were then returned to 21% O_2 and filmed after 30 and 60 minutes to assay the effects of reoxygenation on cardiac function. Exposed hearts were perfused with artificial hemolymph bubbled with room air or with the pre-mixed, calibrated O_2 mixtures when making hypoxia measurements. Dissolved O_2 content was monitored and recorded using a Qubit Systems OX1LP polarographic oxygen probe, calibrated and corrected for mean barometric pressure (758 mm Hg), salinity (8.22 °/° °) and perfusate temperatures ($21-22$°C C). The O_2 content was monitored at several time points during experimental sessions. The mean dissolved O_2 content at 1% O_2 was 0.64 mg/L and at 4% O_2 was 1.7 mg/L and these levels remained stable over the 150 minute recording sessions (best fit linear regression, $p<0.0001$; see dissolved oxygen content in Supporting Information). To ensure that individuals in all genotypes received equivalent treatments, each experiment dish contained small numbers of both control and *sima* mutant heart preparations. To simplify interpretation, data quantifying cardiac responses are expressed as a percent of the pre-hypoxia (normoxia) baseline measure for each fly (see Supporting Information for absolute measures).

2.3.3 Sustained hypoxia and reoxygenation assay (sustained H/R)

Previous studies and preliminary dose-response tests in whole *Drosophila* identified critical hypoxia adaptation thresholds at approximately 4% O_2 and 1% O_2 below

which reproduction and lifespan, respectively, were critically attenuated in both wild type and normoxia control fly lines (18, 53). The sustained hypoxia exposure challenge in this study used a humidified chamber (Modular Incubator Chamber, MIC 101, Billups-Rothenberg) kept at room temperature. In preliminary studies, we determined 18 hours at 1% O_2 to be a sustained hypoxia level sufficient to elicit strong phenotypes while still maintaining viability of wild type flies. After the 18 hour sustained hypoxia exposure, the chamber was opened, flies were dissected to generate the semi-intact heart preparation, and exposed hearts were equilibrated for 30 min in artificial hemolymph at 21% O_2 prior to filming (see Figure 3A).

2.3.4 Chronic hypoxia and reoxygenation assay (chronic H/R)

We chose three weeks as the exposure period for the chronic hypoxia challenge because this is the median age for wild type adult flies and, in our experience, heart function is not yet significantly affected by aging-related cardiac effects (34). We chose 4% O_2 because flies can live a normal lifespan at that level of hypoxia, although they cannot reproduce (3, 17, 53). Two- to three-day old adult flies were placed in sealed, humidified chambers at room temperature for three weeks, under 4% O_2. Food (equilibrated for 24 hours at 4% O_2) was changed two times per week in the glove box under 4% O_2 hypoxic conditions. These chambers are stable and reliable for 24-48 hours at 4% O_2 and were flushed daily to maintain stable O2 levels and eliminate the minimal build-up of carbon dioxide. After three weeks, flies were removed from the chamber, dissected under normoxic conditions and equilibrated for 30 min in artificial hemolymph at 21% O_2 prior to filming (see Figure 4A).

2.3.5 Hypoxia-selected fly population

Heart function in hypoxia-selected and normoxia control flies was analyzed using 3-week old males. Hypoxia-selected flies were removed from the 4% O_2 hypoxia chamber, briefly dissected under reduced oxygen conditions (hemolymph with a stream of 4% O2 duration <15 minutes), then returned to their native 4% O_2 for 30 minutes prior to filming under 4% O_2 conditions. To control for possible prolonged effects of exposure to relative hyperoxia during dissection of hypoxia-selected flies, we monitored heart function in both populations under their relative normoxia (4% or 21% O_2) at two time points (60 and 90 minutes) after dissection, and used the average of the two measures as the stable values reported in this study.

2.3.6 Optical Imaging and Heart Function Analysis

Semi-intact hearts were prepared as described previously (13, 47). Direct immersion optics were used in conjunction with a digital high-speed camera (120-150 frames/sec, Hamamatsu EM-CCD) mounted on a Leica DMLFSA microscope (McBain Instruments, Chatsworth, CA) to record 30 second movies of beating hearts; images were captured using HC Image (Hamamatsu Corp.). Cardiac function was analyzed from the high speed movies using Semi-automatic Optical Heartbeat Analysis software (SOHA, free download for research purposes at www.sohasoftware.com) which quantifies diastolic/systolic intervals, cardiac arrhythmia, diastolic/systolic diameters, fractional shortening, and produces M-mode records from the videos (13, 47). Flies displaying no contractions for greater than 25 seconds during the 30 second sampling period were labeled 'asystolic.' For the acute hypoxia assay, asystolic flies were excluded from the dataset if the heart did not resume beating upon reoxygenation (<2% of total dataset,

controls and experimental groups), or if a measurement was > +/- 2 standard deviations from the mean on any parameter (see heart beat histograms in Supporting Information).

2.3.7 Statistical analysis

All statistical analyses were performed using Prism Statistical Software (Graph Pad, Inc, version 6). Data sets were first tested for normal (Gaussian) distributions using the D'Agostino and Pearson omnibus normality test. For data sets that passed this test, we used a regular t-test, 1-way or 2-way ANOVA as appropriate. Most of the studies used two or more genotypes and two experimental conditions (normoxia v. hypoxia) so significance was determined using a 2-way ANOVA followed by multiple comparisons post hoc tests, as appropriate (specific tests indicated in figure legends). Data sets that did not show a normal distribution (heart period and diastolic interval) were analyzed for significance using a nonparametric two-tailed t-test, or Kruskal–Wallis test followed by Dunn multiple comparisons post hoc tests, as appropriate (specific tests indicated in figure legends). We chose to be conservative in our statistical analyses and excluded heart period, systolic/diastolic interval data for hearts that did not beat during exposure to hypoxia. To ensure the non-beating hearts did not represent an aberrant subset we analyzed the beating and non-beating heart data separately and found no significant difference in any of the normoxia-measured parameters between hearts that did or did not beat under hypoxia, thus the normoxia data sets were pooled. In all cases, $P<0.05$ was taken as significant.

2.3.8 Immunofluorescence

Fluorescent labeling of adult cardiac structures was done as previously described (47). Briefly, dissected fly hearts were relaxed with 10 mM EGTA in artificial hemolymph and fixed with 10% methanol-free Formalin for 20 minutes at room temperature followed by 3 rinses in PBST (1X PBS, 0.1% TritonX). Hearts were incubated with 50-100 μL of primary antibody (αPericardin 1:1000, Developmental Studies Hybridoma Bank) overnight at 4°C. Excess antibody was removed by 3 PBSTx washes prior to incubation with secondary antibodies and Alexa594- or Alexa488-Phalloidin (1:500, Molecular Probes) at 4°C overnight. Heart preparations were mounted in Vectashield (Vector Laboratories) and imaged using a Zeiss Imager Z1 equipped with an Apotome (Zeiss), Axiocam MRm camera and Axiovision 4.8.2 software. Exposure settings during image acquisition and processing were kept constant within experiments.

Quantification of the Pericardin network was performed with ImageJ (http://imagej.nih.gov/ij/). Z-stack images (e.g. Figure. 6 D-F) were ”Adjusted” using the ”Threshold” function and all pixels that had intensities above a set threshold (30) were quantified and expressed as a percentage of the total number of pixels in the image. All hearts were stained at the same time and with the same antibody dilution, all images were taken at the same magnification (25X) and with identical exposure settings.

2.4 Results

2.4.1 Wild type cardiac responses to acute hypoxia/ reoxygenation (acute H/R)

We exposed semi-intact fly heart preparations to the acute hypoxia/reoxygenation protocol (acute H/R; 1% or 4% O2 for 30 minutes), illustrated in Figure 1A. Qualitative responses are illustrated in M-modes (Figure 1B), which provide a representative ”snapshot” of heart wall movement over time. A representative response of a heart from a wild type fly to 4% O_2 acute H/R is shown on the left; the response of a different heart to 1% O_2 acute H/R is shown on the right. Both treatments caused the heart rate to slow significantly (Figure 1C,D), but hearts exposed to 1% O_2 tended to exhibit extremely long pauses (>25 seconds, ”asystole”). Histograms displaying every individual heart period, diastolic and systolic interval for each experimental group of hearts show that these asystolic periods were temporary and reversible events. Importantly, post-hypoxia function of these asystolic hearts did not differ significantly compared to hearts that were able to beat during hypoxia. Nevertheless, we used highly conservative statistics and only analyzed flies which beat at each time point to eliminate any statistical bias by these asystolic measures (see Supporting Information).

Cardiac contractility, measured as fractional shortening of the heart tube (34) was only moderately affected under 4% O_2, but was dramatically reduced upon exposure to 1% O_2 (Figure 1E). This suggests that the wild type *Drosophila* heart can respond differentially to short term changes in oxygen content (30 minutes 4% O_2 vs. 1% O_2). Based on these observations we used a 1% O_2 exposure for our comprehensive assessment of heart function in response to acute hypoxia (acute H/R) and sustained with reoxygenation (sustained H/R) treatments.

Exposing hearts to acute hypoxia (Figure 1A) resulted in a robust cardiac response

that included temporary cessation of beating (for 25 seconds or longer) in 40-60% of the heart for both wild type and sima heterozygous (sima+/-) and homozygous (sima-/-) mutants ('asystolic' in Figure 2A). For both wild type and sima mutant hearts that continued to beat during the acute hypoxia exposure, heart period increased as a result of increases in the diastolic interval (Figure 2B-D). Upon reoxygenation, all hearts resumed pre-exposure beating patterns (Figure 2A) and heart period and diastolic intervals returned to their pre-hypoxia exposure levels (Figure 2B,C, T=30 compared to T=60 & 90). Systolic intervals did not change significantly from the baseline during hypoxia or subsequent reoxygenation for both control and sima mutant genotypes (Figure 2D).

Cardiac contractility, measured as fractional shortening, decreased dramatically in all genotypes during acute hypoxia (Figure 2E), in part due to increases in systolic diameters (Figure 2G), suggesting systolic dysfunction in response to hypoxia. The diastolic diameters were not changed during acute hypoxia exposure in wild type flies. However, they were increased in both sima heterozygote and homozygote mutants (Figure 2F). In hearts from wild type flies, fractional shortening and systolic diameters were partially, but not completely, restored following reoxygenation. Interestingly, function in sima mutants appeared to recover almost completely to pre-exposure values. We conclude that chronotropic and ionotropic cardiac function is mostly restored at one hour of reoxygenation post-exposure to extreme hypoxia, consistent with the previously observed overall tolerance and recovery of fruit flies to acute hypoxic/anoxic conditions (2, 18).

2.4.2 Cardiac response to sustained hypoxia/ reoxygenation (sustained H/R)

We examined cardiac performance after more 'sustained' periods of hypoxia lasting hours to days. We determined that a sustained hypoxic exposure for 18 hours at 1% O_2 produced significant changes in cardiac function upon reoxygenation (sustained H/R). Using this protocol (illustrated in Figure 3A), we found that hearts from wild type flies beat significantly faster (reduced heart period) following sustained H/R, primarily due to a decrease in diastolic intervals (Figure 3C-E, white bars). In addition, hearts from wild type flies exposed to sustained H/R became significantly restricted in both diastolic and systolic diameters, resulting in significant decreases in fractional shortening (Figure 3F-H, white bars).

To test whether *sima* is needed to respond to more prolonged periods of hypoxia, where the transcriptional activity of stabilized Sima might be required, we analyzed cardiac function in *sima* heterozygous and homozygous mutants. Significantly, over 70% of *sima*-/- hearts did not beat following our sustained H/R protocol (Figure 3B), indicating that *sima* function is required to appropriately respond to this more sustained hypoxia exposure. Consequently, we focused our heart function analysis only on sima heterozygous mutants (*sima*+/-), where HIFα/*sima* function was only partially compromised. Indeed, we found that *sima*+/- hearts were more susceptible to sustained H/R exposure than wild type controls on several measures (Figure 3C-H, gray bars). In particular, the decreased heart period (increased rate) in response to sustained H/R was more pronounced in *sima*+/- hearts than in wild type controls (Figure 3C). Although both wild type controls and *sima*+/- showed decreases in diastolic intervals (Figure 3D), in *sima*+/- the decrease in heart period was also due to a significant decrease in systolic intervals (Figure 3E).

For both wild type and *sima*+/- flies, hearts exhibited significant reductions in fractional shortening in part because hearts become somewhat constricted following sustained H/R. In hearts from wild type flies, the decreases in diastolic diameter were accompanied by compensatory reductions in systolic diameters that minimize the observed reduction in fractional shortening. In *sima*+/- hearts, although diastolic diameters were also reduced, the systolic diameters do not change in response to sustained H/R (Figure 3F,G). Consequently contractility was significantly more impaired in *sima*+/- because hearts do not shorten as much as hearts in wild type flies. These data suggest that *sima* plays a role in the longer term (sustained H/R) effects of hypoxia exposure on cardiac contractility in *Drosophila*.

2.4.3 Cardiac response to chronic hypoxia/ reoxygenation (chronic H/R)

We monitored cardiac function in wild type flies after three weeks exposure to moderate, chronic H/R (4% O_2), followed by reoxygenation and assessment of cardiac function (illustrated in Figure 4A). In contrast to the shortened heart period observed after sustained H/R (Figure 3C), the heart period and diastolic intervals of wild type flies exposed to chronic H/R lengthened significantly when compared to hearts from normoxia-raised controls (Figure 4B-C, white bars). This response was not observed in sima+/- hearts where heart period and diastolic intervals were unaffected by chronic H/R (Figure 4B-C, gray bars). Chronic H/R had no effect on systolic intervals in wild type controls but caused significant decreases in *sima*+/- (Figure 4D). Contractility in hearts from wild type flies was not significantly affected in chronic H/R whereas hearts from *sima*+/- flies exhibited significant reductions in contractility that were due to decreases in the diastolic diameters (Figure 4E-G, gray bars). This suggests that one cardiac response

to chronic hypoxia exposure is through a sima-mediated reduction in heart rate and maintained cardiac contractility.

2.4.4 Multi-generational response to chronic hypoxia in hypoxia-selected populations

Given the effects of sustained and chronic hypoxia exposure on the *Drosophila* heart, we wondered how heart function is altered in flies that were selected for survival at reduced oxygen levels over many generations ('hypoxia-selected') (53). Heart function was monitored at the 'native' oxygen levels for the hypoxia-selected flies (4% O_2) and their normoxia controls (21% O_2; Figure 5A). The hearts from hypoxia-selected flies beat more slowly (at 4% O_2) compared to the normoxia controls (at 21% O_2), due to increased diastolic intervals, even while the average systolic interval shortened (Figure 5 B-D). Moreover, fractional shortening in hypoxia-selected flies was significantly reduced compared to normoxia controls, due to reductions in both diastolic and systolic diameters (Figure 5E-G). It should be noted that these hypoxia-selected flies were also smaller in overall size than those living under normoxic conditions, due to oxygen limitation during development (53, 55). To confirm that the smaller heart size was not simply due to a reduction in the overall size of the fly we measured the abdominal segment and tibia lengths in both hypoxia-selected and hypoxia-control flies. The average segment length of selected flies was 67% of the abdominal segment length of controls, and similar for tibial measurements (53). When diameter measurements were normalized to correct for this 67% difference in body size could we found that the average diastolic diameters were still significantly smaller for hypoxia-selected flies (54um normoxia, compared to 36um hypoxia-selected uncorrected and 42um corrected, $p<0.001$ 2-way ANOVA).

2.4.5 Prolonged hypoxia and reoxygenation causes myofibrillar and collagen matrix remodeling

Cardiac remodeling of cytoskeletal elements is a common indicator of damage or acclimation of the myocardium in hypoxia-induced diseases. The observed physiological changes in fly hearts, i.e. reduced cardiac diameters and output/contractility in response to H/R treatments, suggested that heart morphology may also be altered. To further explore whether the *Drosophila* heart undergoes hypoxia-dependent remodeling in response to prolonged hypoxia, we examined the myofibrillar organization, which normally exhibits a tightly packed circumferential arrangement of myofibrils (Figure 6A). This arrangement appears to be maintained in response to the different hypoxia treatments (Figure 6A-C) despite the observed constriction and reduced contractility (Figures 3-4). Even after chronic H/R exposure, the circumferential arrangement was maintained although the heart itself becomes slightly restricted.

Another hallmark of cardiac remodeling in response to pathological hypoxia, such as a myocardial infarction, is an increase in fibrosis (7, 16, 26). We examined changes in the extracellular matrix deposition by immunostaining the heart for Pericardin (Prc), a type IV collagen-like protein. Normally Prc is arranged in a ”fish net stocking” pattern and appears to associate with the Z bands of myofibrils (Figure 6D). Exposure to sustained H/R resulted in modest increases in the Prc-positive extracellular collagen network along the heart tube and this increased deposition was also seen following chronic H/R, although the network became significantly more disorganized with this longer hypoxia exposure (Figure 6E-G). Nevertheless, both hypoxia treatments appeared to increase overall Prc collagen fiber density (Figure 6G).

We also investigated whether there was any structural remodeling in fly hearts after multi-generational selection for hypoxia survival. We found that the network of

myofibrils and the collagen fiber network in hearts from hypoxia-selected flies exhibited a rather dramatic rearrangement and disorganization compared to their normoxia-raised controls (Figure 6H-K). In particular, the Prc-positive collagen covered an area that was much wider than the heart itself, despite constriction of the heart tube (Figure 6I) in these flies that can survive and reproduce in 4% O_2.

2.5 Discussion

Hypoxia-induced cardiac dysfunction is a hallmark of many disease states including pulmonary hypertension, ischemic heart attack and chronic mountain sickness. Tolerance or sensitivity to hypoxia depends on complex genetic and molecular mechanisms that have not been clearly established and effects have rarely been compared across different hypoxia severities and durations in one study. Using genetic tools combined with assays of cardiac function, we show that acute, sustained, chronic and multi-generational hypoxia (acute H/R, sustained H/R, chronic H/R, hypoxia-selected, respectively) induce a range of cardiac responses (summarized in Table A.1).

In humans, systemic exposure to hypoxia stimulates compensatory physiological responses that depend on the duration or severity of the hypoxia exposure. These include immediate increases in heart rate in response to moderate hypoxia exposure, or decreases in heart rate during very severe levels of acute hypoxia, which can lead to death (38, 42). Severe, local hypoxia and reoxygenation can lead to irreversible ischemic heart disease and reductions in contractility. In the fly heart, hypoxia and subsequent reoxygenation produces similarly variable responses. Acute hypoxia causes a dramatic slowing of the heart and equally dramatic reductions in contractility in wild type flies (Figure 2). These are likely compensatory responses as they would be expected to reduce ATP demand

in a low oxygen environment (Figures 1,2). In *sima*+/- and *sima*-/- mutants these responses also occurred, but to a lesser degree than for wild type flies and the response was significantly blunted with respect to contractility in *sima*-/- homozygotes. These results suggest that, in the fly, the hearts' sensitivity to changes in oxygen levels is at least partially dependent on components of the HIF hypoxia-sensing pathway, as is the case for other model organisms (24, 28, 43) and may be protective for longer term function. Importantly, most of the responses to acute H/R were reversible within 30-60 minutes.

Sustained and chronic H/R caused variable effects on normal heart function with the heart rate increasing significantly after sustained H/R but slowing significantly after the longer chronic H/R (Figure 3A, 4B). We also observed increased collagen deposition in hearts from wild type flies as a consequence of sustained and chronic hypoxia exposure (Figure 6), which is consistent with known remodeling of the human myocardium upon prolonged exposure to hypoxia that is partially dependent on HIF1α (7, 16, 26). Importantly, these more prolonged hypoxia exposures were not tolerated by sima-/- mutants. Although sima+/- flies survived both types of hypoxia exposure, both treatments produced additional effects that persisted following reoxygenation. Specifically, they caused significant reductions in both cardiac contractility (Figure 3F, 4E) and duration of cardiac contractions (Figure 3E, 4D) compared to hearts from wild type flies. In the *sima*+/- flies, the reductions in fractional shortening, in combination with the shorter contraction times would be expected to result in an overall reduction in cardiac output per beat compared to wild type flies following hypoxia stress. However, even though contractility was compromised in these flies the heart period did not slow as for wild type (Figure 4B). Therefore, even though the per beat cardiac output is reduced, the overall faster heart rate, compared to wild type, might be compensatory in the sima heterozygotes compared to wild type.

In humans, HIF signaling affects genes involved in angiogenesis, erythropoiesis,

glucose metabolism and vasomotor control. HIF-1α may also be regulated by redox signals, thereby playing a role in protecting against ischemia and reperfusion (41). Over-expression of HIF-1α protects the murine heart from myocardial infarction-induced damage by increasing vascularization as well as a likely direct protective effect on cardiomyocytes (21, 41). Conversely, cardiomyocyte-specific deletion of HIF-1α results in reduced heart contractility (20). Although HIF1α has cardioprotective effects following myocardial infarction in murine models, several other studies suggest the HIF pathway is associated with maladaptive responses to chronic hypoxia (5, 51, 54). For example, loss of one copy of HIF1α or HIF2α in mice protects them from chronic hypoxia-induced pulmonary hypertension and right ventricular dysfunction. These observations suggest that activation of the HIF pathway leads to some of the pathologies observed in chronic hypoxia-induced cardiac diseases. Specifically, HIFα stabilization may confer tolerance to acute/sustained hypoxia, but may be detrimental for more chronic hypoxia exposure and our data provide support for this mixed notion. Interestingly, HIF pathway components, including HIF-2α, are under selection in populations that exhibit cardioprotective adaptations to multi-generational residence at high altitude (27, 49), indicating this pathway is also integral to long-term hypoxia tolerance.

In humans, long-term adaptation to chronic hypoxia leads to either enhanced cardiac function or dysfunction in a population- and genotype-specific manner (14, 36). Human populations that are well adapted to chronic hypoxia over many generations, such as Tibetan Sherpas, show beneficial cardiac adaptations to their native high altitudes, including; reduced right ventricular hypertrophy, increased ability to raise maximal heart rate and cardiac output at altitude, and increased myocardial glucose uptake (15). Other high altitude populations exhibit signs of cardiac disease due to multi-generational and continued chronic exposure to hypoxia (8, 50, 54). Interestingly, our hypoxia-selected flies exhibited significant changes to all cardiac parameters, suggesting an overall reduc-

tion in cardiac function under hypoxia (Figure 5). Since the *Drosophila* open circulatory system's primary role is not circulation of oxygen as it is mammalian systems (19), the reductions in heart rate and size would be expected to conserve ATP levels without compromising oxygen delivery (as would occur in mammals).

Metabolomic studies on hypoxia-selected *Drosophila* (whole flies) suggest shifts away from oxygen-expensive pathways and towards increased glycolysis (10, 11, 52, 55). Interestingly, in human populations adapted to high altitude, their 'hypoxic' hearts have increased myocardial glucose uptake and lower cardiac phosphocreatine-to-ATP ratios, even when these high altitude natives live for many years at low altitude (15). However, genetic tools and the long lifespan in vertebrates limit their utility for large-scale genetic screens, and laboratory-controlled, hypoxia-adapted populations do not yet exist. Thus, a hypoxia model in the fly provides an opportunity to probe for novel genetic pathways mediating the cardiac response to hypoxia and disease remodeling. Indeed, previous studies of the hypoxia response in the intact fly heart show the potential of using bioinformatics to determine pathways underlying hypoxia adaptation (12). Compared to previous studies, our system novelly employs rigorously compares the cardiac effects of acute, sustained and chronic hypoxia exposures in varying genetic backgrounds, and reports fractional shortening and diameters in addition to heart rates and rhythmicity. In addition, any examination of heart function in vivo is confounded by hypoxic effects on the nervous system. The model presented in this study permits a more a detailed analysis of heart function and makes use of a denervated heart preparation, allowing us to look at the direct effects of hypoxia on myocardial cells.

2.6 Summary

We have characterized in detail the cardiac response to hypoxia in the *Drosophila* model. Cardiac functional and structural data indicate that the fly heart responds differentially to acute, sustained, chronic and multi-generational hypoxia and that these responses are partly mediated by signaling via the HIF1α homolog *sima*. Cardiac function appears to fully recover following very short term hypoxia exposures (minutes), but displays lasting dysfunction after more prolonged exposure times (hours and days). Our data suggest a role for *sima* in modulating heart rate and maintaining contractility in the fly heart. This model system can be used to probe for additional genes and pathways that contribute to hypoxia-related cardiac diseases.

2.7 Acknowledgments

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Zarndt R, Piloto S, Powell FL, Haddad GG, Bodmer R, Ocorr K. Cardiac responses to hypoxia and reoxygenation in Drosophila. Am J Physiol - Regul Integr Comp Physiol 309: R1347R1357, 2015.

2.8 Figures

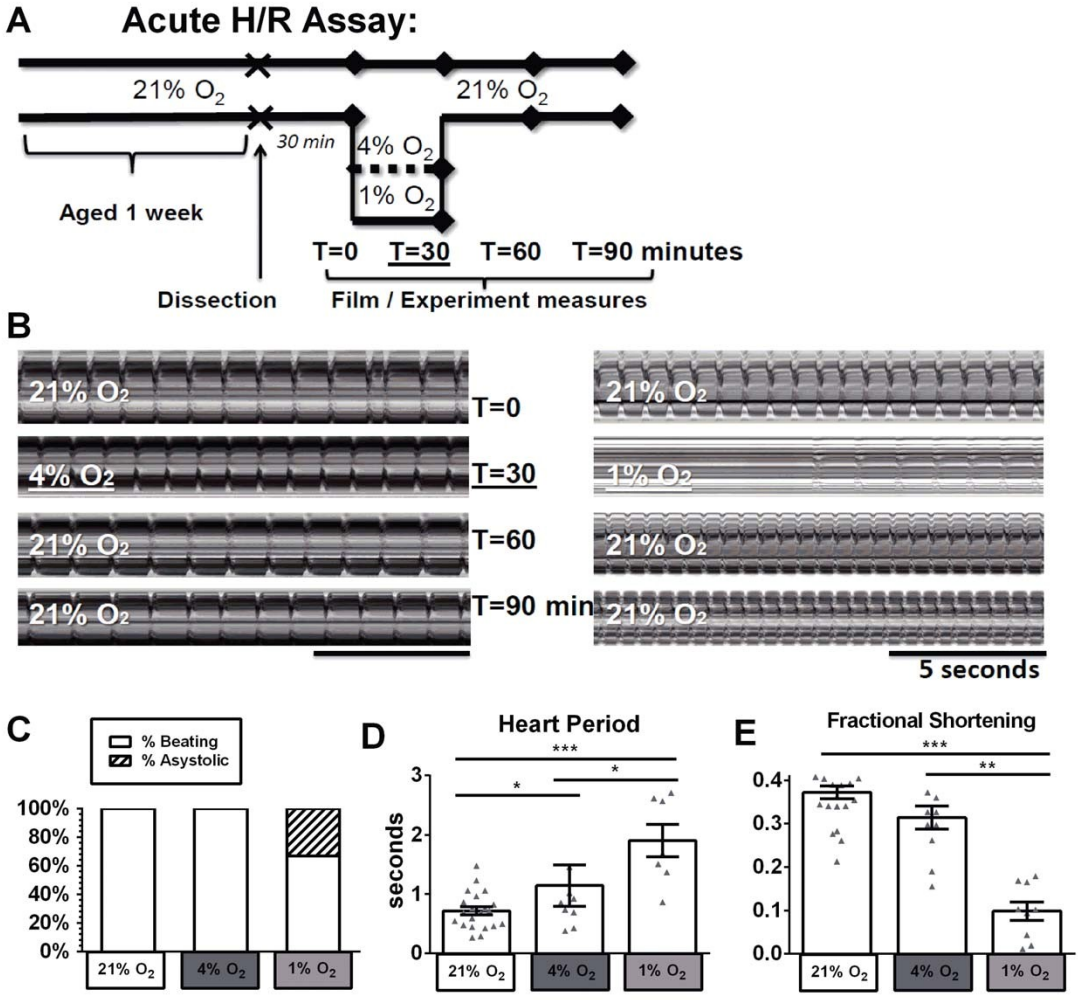
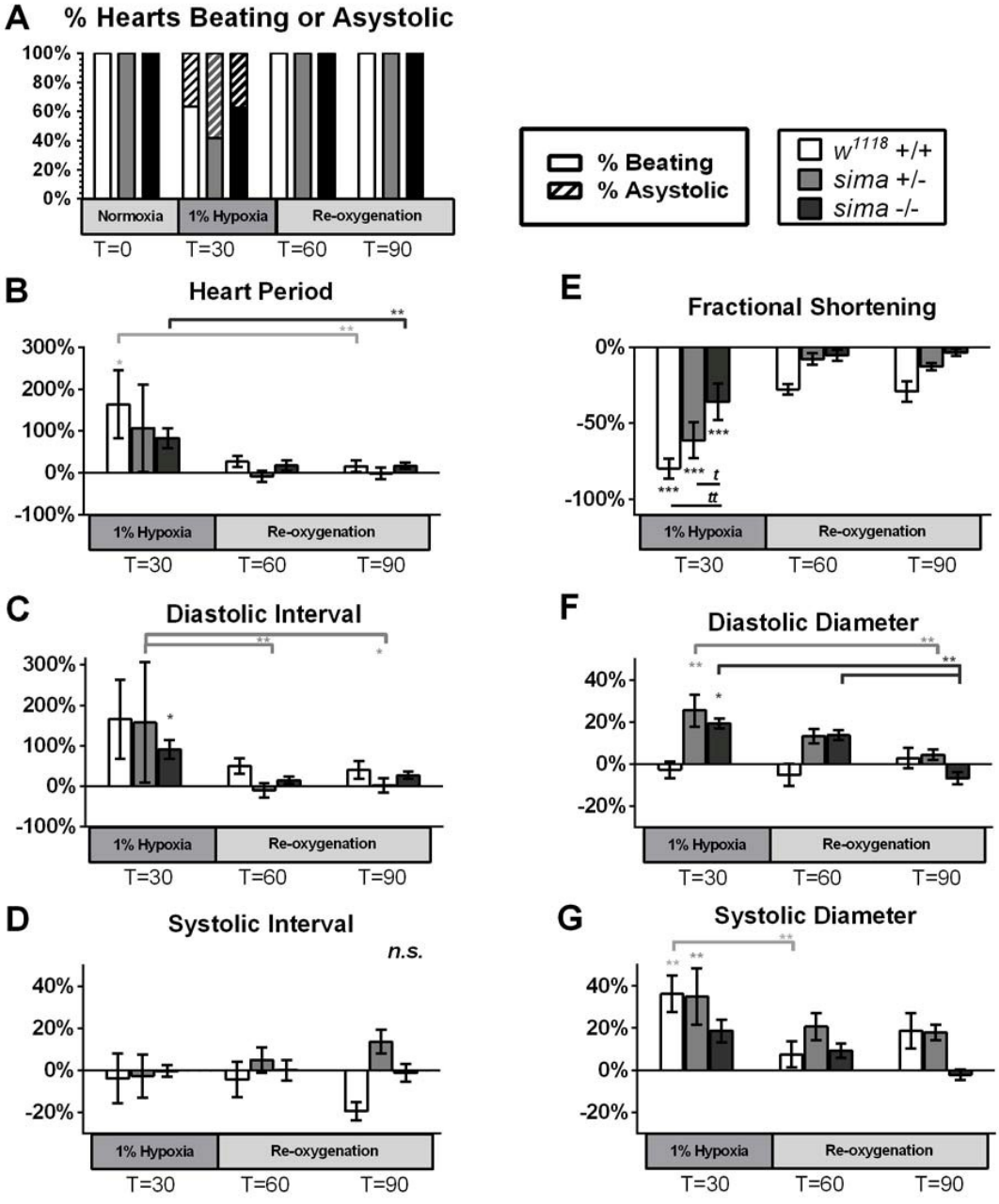


Figure 2.1: **Response of wild type (w1118) controls to acute hypoxia exposure.** A) Schematic showing the acute hypoxia/reoxygenation protocol (acute H/R). B) M-mode records (15 seconds) from movies of beating hearts taken before (T=0), at 30 minutes during (T=30), and at two time points (T=60, 90) following hypoxia treatments. (Left) Representative M-mode showing changes in heart beat in response to 4% O_2. (Right) Representative M-modes showing changes in heart beat in response to 1% O_2. C) Incidence of non-beating or "asystolic" hearts in response to hypoxia exposure, expressed as a percent of total hearts examined. Quantification of changes in D) Heart Period and E) Fractional Shortening under 4% or 1% hypoxia (T=30) relative to pre-hypoxia exposure baseline.

*All values are mean percent change +/- SEM (w1118: N=19 21% O_2, N=10 4% O_2, N=9 1% O_2). Data was analyzed by 1-way ANOVA and Tukey's multiple comparisons post-hoc test; *p¡0.05, **p¡0.01, ***p¡0.001. See Supporting Information for raw data.*

Figure 2.2 (facing page): Acute cardiac response to 30 minutes 1% O_2 hypoxia / reoxygenation (acute H/R) in wild type and sima mutant *Drosophila*
A) Incidence of non-beating or 'asystolic' hearts (> 25 seconds without contractions) in response to hypoxia exposure, expressed as a percent of total hearts examined. Nearly half of wild type and sima mutant hearts entered prolonged periods of asystole at 30 minutes in 1% O2, and all resumed beating on reoxygenation. B)-G) Data is presented as the percent change from the pre-hypoxia baseline during and post hypoxia treatment for wild type, sima+/-, and sima-/-. B) In hearts that beat, heart period (HP) is increased 100% or greater under 1% O_2 hypoxia exposure (T=30) and this reached significance in hearts from wild type flies. HP returned to baseline levels exhibited by normoxia controls in all genotypes post hypoxia exposure (T=90). C) Diastolic intervals (DI) increased in wild type and sima-/- under 1% O2 (T=30) and then returned to baseline levels upon reoxygenation (T=60, 90). D) Systolic intervals did not change significantly across treatments and genotypes. E) For all hearts, fractional shortening was significantly reduced under 1% O_2, but reductions were greatest in wild type and greater in sima+/- than in sima-/- (T=30). F) Diastolic diameters increased significantly in both sima+/- and sima-/- under 1% O_2 and remained larger at 30 minutes post hypoxia exposure compared to controls. G) Systolic diameters were significantly increased during acute hypoxia in both wild type and sima+/-.

*All values are mean percent change +/- SEM (w1118 N=10, N=12 sima+/-, N=16 sima-/-). For heart period, systolic and diastolic interval, asystolic hearts were excluded, while all hearts are included in fractional shortening, diastolic and systolic diameter. Data was analyzed by (B,C) KruskalWallis test and Dunn multiple comparisons post-hoc test and (D-F) 2-way ANOVA and Tukey's multiple comparisons post-hoc test; n.s. = no statistical significance, * <0.05, ** p<0.01, *** p<0.001. Changes at T=30 between genotypes were analyzed by Sidak-Bonferroni t-test; t p<0.05. tt p<0.01. See Appendix for raw numbers.*


A
% Hearts Beating or Asystolic
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300%
200%
100%
0%
-100%
1% Hypoxia
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0%
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40%
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-20%
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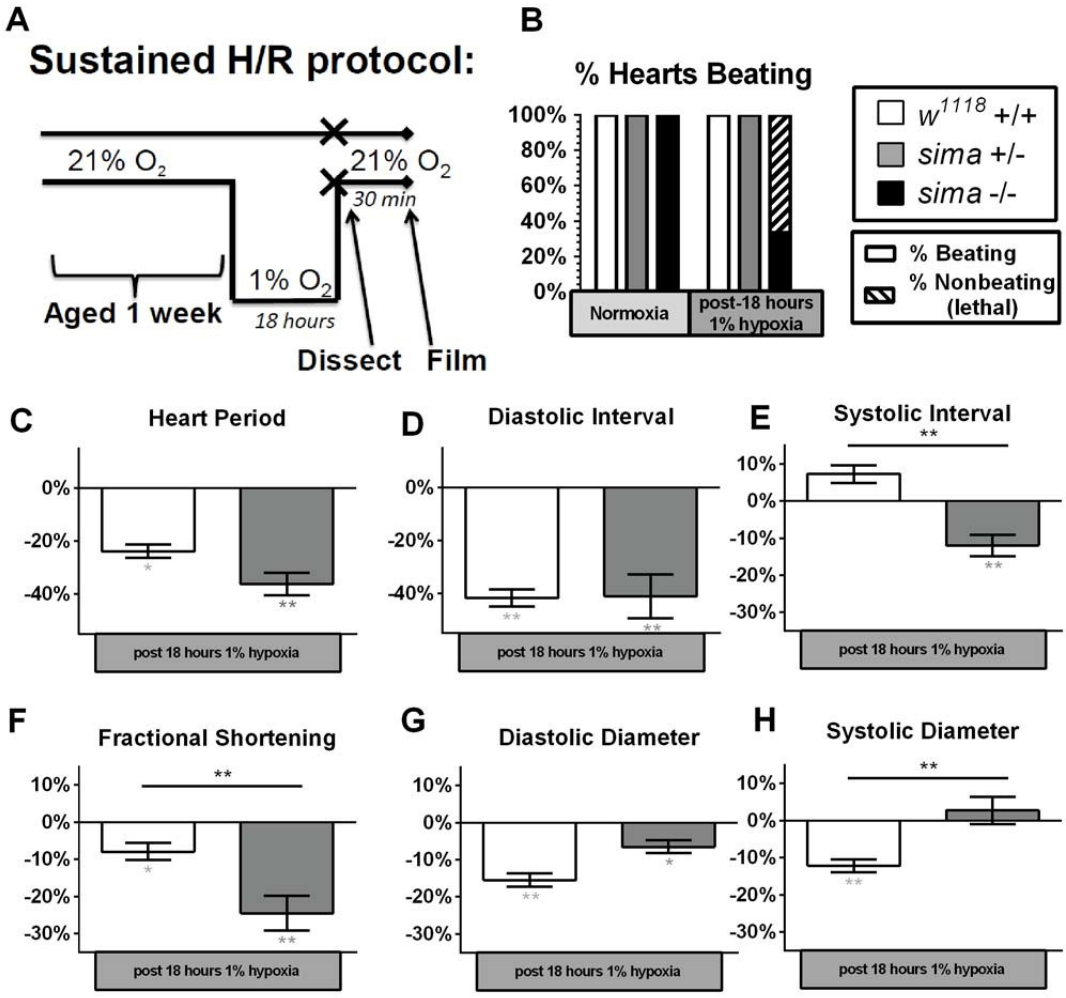
Systolic Diameter

Figure 2.3 (facing page): Cardiac response to sustained hypoxia / reoxygenation (sustained H/R) in wild type and sima mutants.
A) Protocol for sustained hypoxia treatment (sustained H/R). B) Heart period significantly shortened (rate increased) upon exposure to sustained H/R in both wild type and sima+/- hearts. C) Exposure to sustained H/R leads to significant reductions in diastolic interval in both wild type and sima+/- hearts. D) Sustained H/R caused a significant increase in systolic intervals in hearts from wild type controls whereas sima+/- hearts exhibited significant decreases in systolic interval compared to normoxic conditions. E) Fractional shortening decreased in wild type in response to sustained H/R; in sima+/- hearts this decrease was significantly exacerbated compared to controls. F) These changes in fractional shortening were due in part to significant decreases in diastolic diameters in both lines of flies. H) There is no change in systolic diameter in sima+/-, whereas the systolic diameter is significantly reduced in wild type controls in response to sustained H/R. All values are mean percent change +/- SEM for hearts that beat on reperfusion after a sustained hypoxia exposure.

*All values are mean percent change +/- SEM (w1118: N=59 normoxia, N=24 hypoxia; sima+/-: N=17 normoxia, N=20 hypoxia). Data was analyzed by (B,C) Kruskal–Wallis test and Dunn multiple comparisons post-hoc test and (D-H) 2-way ANOVA with Tukey's multiple comparisons post-hoc test; n.s. = no statistical significance, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ Changes at T=30 between genotypes were analyzed by Sidak-Bonferroni t-test; t $p<0.05$. tt $p<0.01$. 2-way ANOVA with Tukey's multiple comparisons post hoc test. n.s. = no statistical significance, *$p<0.05$, **$p<0.01$, ***$p<0.001$. See Supporting Information file for raw data.*


A
Sustained H/R protocol:
21% O2
21% O2
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Aged 1 week
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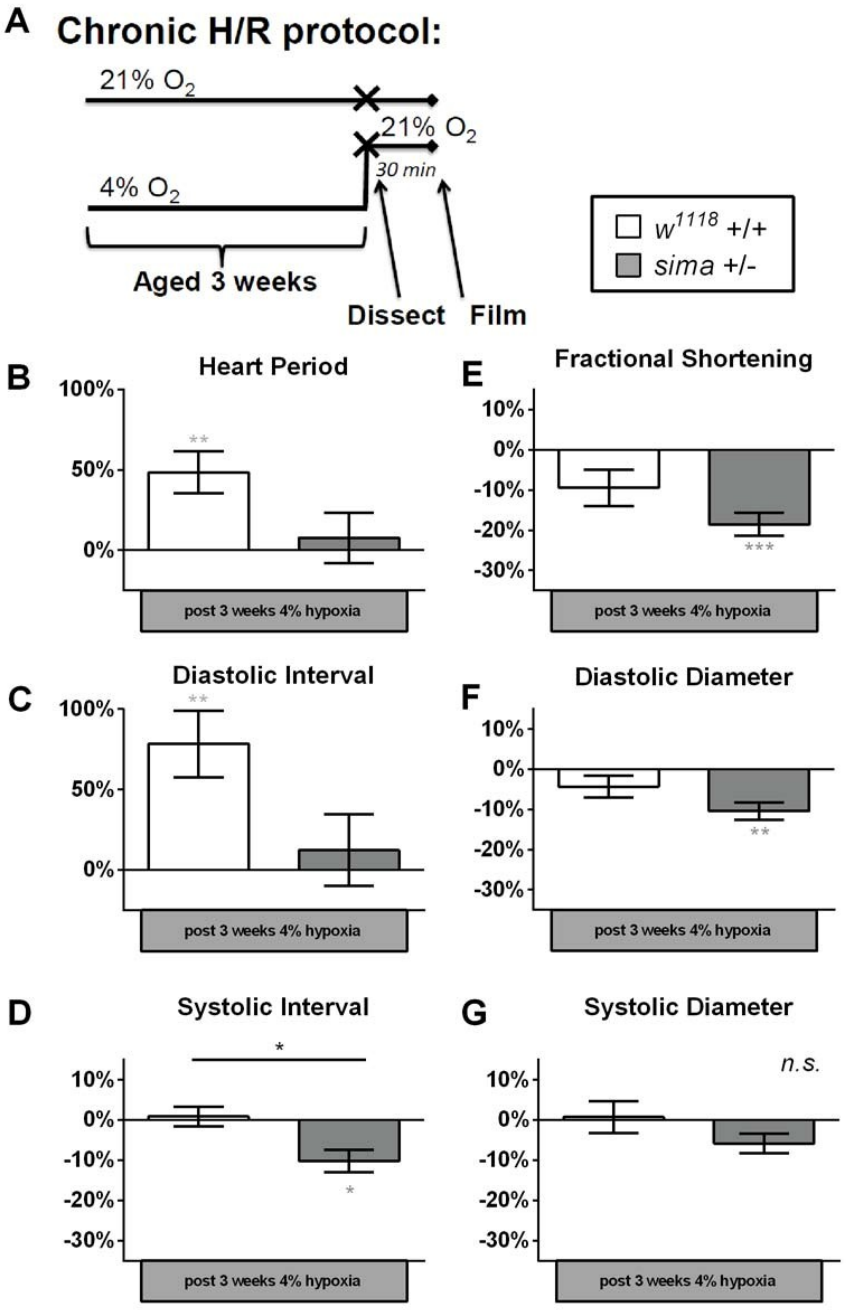
20%
0%
Normoxia
post-18 hours 1% hypoxia
w1118 +/+
sima +/-
sima -/-
% Beating
% Nonbeating (lethal)
C
Heart Period
D
Diastolic Interval
E
Systolic Interval
F
Fractional Shortening
G
Diastolic Diameter
H
Systolic Diameter
post 18 hours 1% hypoxia

Figure 2.4 (facing page): Response to chronic hypoxia / reoxygenation (chronic H/R) in wild type and sima mutants.
A) Protocol for chronic hypoxia treatment (chronic H/R). B) Chronic H/R exposure significantly increased heart period in hearts from w1118 but not in hearts from sima+/- flies when compared to normoxia controls. C) Diastolic intervals were also significantly increased by chronic H/R in w1118 but not sima+/- compared to normoxia controls. D) Systolic intervals were unchanged following chronic H/R in w1118 but were significantly decreased in sima+/- compered to normoxia controls. E) Fractional shortening was also unchanged following chronic H/R in w1118 but decreased significantly in sima+/- compared to normoxia controls. F) Diastolic diameters were unchanged in following chronic H/R in w1118 but were significantly reduced in sima+/- compared to normoxia controls. G) Systolic diameters were unaffected by chronic H/R in both genotypes compared to their normoxia controls.

*All values are mean percent change +/- SEM (w1118: N=36 normoxia, N=41 hypoxia; sima+/-: N=17 normoxia, N=20 hypoxia). Data was analyzed by (B,C) Kruskal–Wallis test and Dunn multiple comparisons post-hoc test and (D-G) 2-way ANOVA with Tukey's multiple comparisons post-hoc test; n.s. = no statistical significance, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ Changes at T=30 between genotypes were analyzed by Sidak-Bonferroni t-test; t $p<0.05$. tt $p<0.01$. 2-way ANOVA with Tukey's multiple comparisons post hoc test. n.s. = no statistical significance, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. See Supporting Information file for raw data.*


A
Chronic H/R protocol:
21% O2
21% O2
30 min
4% O2
Aged 3 weeks
Dissect
Film
w1118 +/+
sima +/-
B
Heart Period
100%
50%
0%
**
post 3 weeks 4% hypoxia
E
Fractional Shortening
10%
0%
-10%
-20%
-30%
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post 3 weeks 4% hypoxia
C
Diastolic Interval
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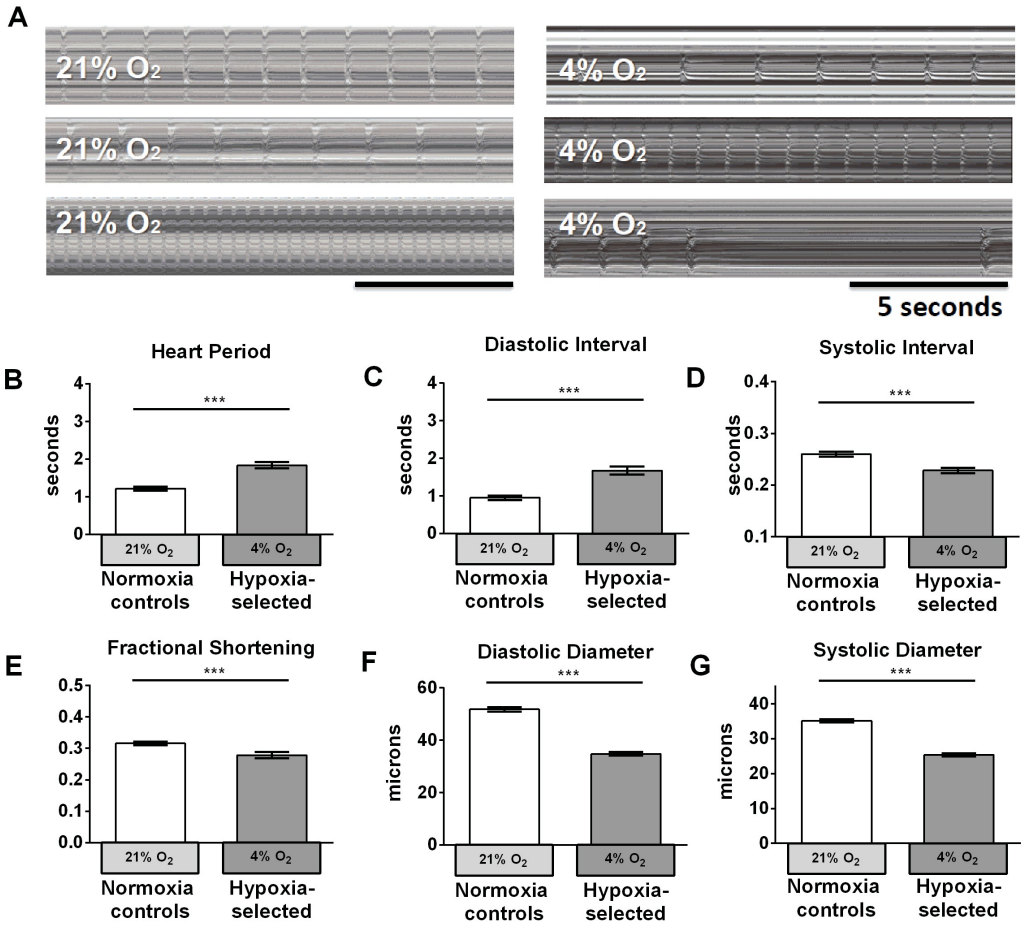

Figure 2.5: **Cardiac parameters of hypoxia-selected and normoxia control populations.** A) Representative 15 sec M-modes showing heart wall movements in three normoxia controls and three hypoxia-selected fly hearts at their relative normoxia; (Left) 21% O_2 for normoxia controls, and (Right) 4% O_2 for hypoxia-selected. B) Under their relative normoxic conditions heart period is significantly longer in hypoxia-selected flies compared to normoxia controls. C) Diastolic intervals are significantly longer hypoxia-selected flies compared to normoxia controls. D) Systolic intervals are significantly decreased in hypoxia-selected flies compared to normoxia controls. E) Fractional shortening is significantly reduced in hypoxia-selected flies, due to reductions in both F) diastolic diameter and G) systolic diameter compared to control populations.
*All values are mean +/- SEM for hearts ***$p<0.001$ Mann-Whitney unpaired t-test; normoxia controls N=87, hypoxia-selected N=72.*

Figure 2.6 (facing page): Hypoxia-induced alterations in cardiac structure and extracellular matrix.
Exposed hearts in semi-intact preparations were stained for F-actin with Phalloidin (A-C,H,I) and for collagen IV with anti-Pericardin antibodies (D-F,J,K). In all images the ventral view of the heart is shown, anterior to the left. A) Heart from a 2-week old w1118 fly exhibits the tightly packed, circumferential arrangement of F-actin stained myofibrils within the myocardial cells. This structure is not affect by B) sustained H/R or C) chronic H/R. D) Pericardin (collagen IV homolog) staining reveals the "fish net" pattern of fibers that associate with sarcomeric structures. E) The collagen network appears denser and is moderately disorganized following sustained H/R and is more disorganized following F) chronic H/R. G) Pericardin staining was quantified from Z stacks of immunostained hearts and shows an increase in intensity following both sustained and chronic H/R. Data is expressed as the percent of total pixels with an intensity above threshold (see methods; number of hearts is indicated in each bar).

*Data was analyzed by 1-way ANOVA and Tukey's multiple comparisons post-hoc test; *$p<0.05$, **$p<0.01$. H) Phalloidin staining of a normoxia control heart showing tightly spaced circumferential myofibrils of the heart tube with overlying longitudinal fibers (non-myocardial). I) Phalloidin staining of a heart from a hypoxia-selected fly showing smaller diameter and disorganized myofibrils. J) Immunostaining of pericardin (collagen IV) in lower magnification images of the terminal region of a normoxia control heart reveals a compact distribution around the region of the outflow tract. K) Pericardin immunostaining of the same terminal region from a hypoxia-selected fly showing an expanded and disorganized deposition of collagen IV. Scale bars are 50 microns.*

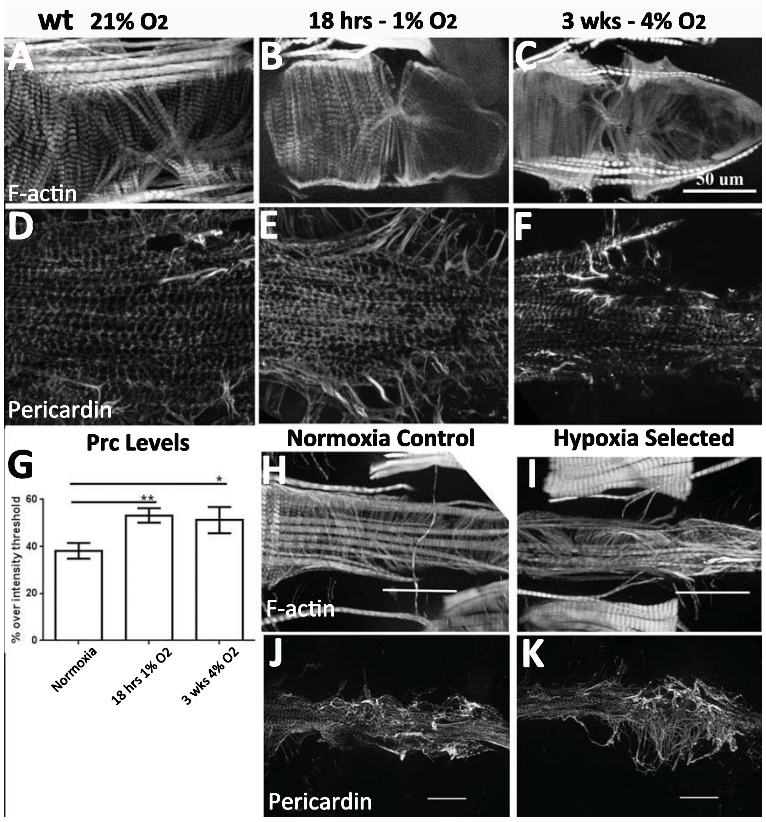
wt 21% O2
18 hrs - 1% O2
3 wks - 4% O2
A
B
C
F-actin
50 um
D
E
F
Pericardin
Prc Levels
Normoxia Control
Hypoxia Selected
G
H
I
% over intensity threshold
60
40
20
0
**
*
Normoxia
18 hrs 1% O2
3 wks 4% O2
F-actin
J
K
Pericardin

Table 2.1: Summary of relative changes in cardiac function observed between genetic backgrounds relative to their normoxia baseline or control population for varying durations of hypoxia or reoxygenation.

Table 1. Summary of relative changes in cardiac function observed between genetic backgrounds relative to their *normoxia baseline* or *control population* for varying durations of hypoxia or reoxygenation

Cardiac measure	Assay	Hypoxia condition	*w*^{1118} +/+	*sima*+/-	*sima*-/-	hypoxia-selected
Heart Period	acute H/R	*30 min at 1% O_2*	↑	↑	↑	
		30 min post 1% O_2	-	-	-	
		60 min post 1% O_2	-	-	-	
	sustained H/R	18 hours 1% O_2	↓	↓		
	chronic H/R	3 weeks 4% O_2	↑	-		↑
Diastolic Interval	acute H/R	*30 min at 1% O_2*	↑	↑	↑	
		30 min post 1% O_2	-	-	-	
		60 min post 1% O_2	-	-	-	
	sustained H/R	18 hours 1% O_2	↓	↓		
	chronic H/R	3 weeks 4% O_2	↑	-		↑
Systolic Interval	acute H/R	*30 min at 1% O_2*	-	-	-	
		30 min post 1% O_2	-	-	-	
		60 min post 1% O_2	-	-	-	
	sustained H/R	18 hours 1% O_2	-	↓↓		
	chronic H/R	3 weeks 4% O_2	-	↓↓		↓
Fractional Shortening	acute H/R	*30 min at 1% O_2*	↓↓↓	↓↓	↓	
		30 min post 1% O_2	-	-	-	
		60 min post 1% O_2	-	-	-	
	sustained H/R	18 hours 1% O_2	↓	↓↓		
	chronic H/R	3 weeks 4% O_2	↓^{t}	↓		↓
Diastolic Diameter	acute H/R	*30 min at 1% O_2*	-	↑	↑	
		30 min post 1% O_2	-	↑^{t}	↑^{t}	
		60 min post 1% O_2	-	-	-	
	sustained H/R	18 hours 1% O_2	↓	↓		
	chronic H/R	3 weeks 4% O_2	-	↓		↓
Systolic Diameter	acute H/R	*30 min at 1% O_2*	↑	↑	↑^{t}	
		30 min post 1% O_2	-	-	-	
		60 min post 1% O_2	-	-	-	
	sustained H/R	18 hours 1% O_2	↓↓	-		
	chronic H/R	3 weeks 4% O_2	-	-		↓

↑ increased; ↓ decreased; - no change or discernible difference; ^{t} trend, significant by 1-way ANOVA within genotype only.
↑/↓ ($p \leq 0.05$); ↓↓ ($p \leq 0.01$); ↓↓↓($p < 0.001$) indicating degree of change from others within same hypoxia condition
30 min at 1% O_2 = measures taken during hypoxia exposure; all other measures taken after reoxygenation = not measured in this study

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Chapter 3

Altered cardiac responses to hypoxia in hypoxia-selected *Drosophila* populations

3.1 Abstract

High altitude provides a constant selection pressure for survival under low oxygen (hypoxia), and long-term occupants in these environments have varying cardiac responses to manage hypoxic stress. These responses are dependent upon the inherited underlying adaptations allowing their multi-generational survival to chronic hypoxia. Cardiac failure is a feature of many hypoxic disease states (e.g., pulmonary hypertension, myocardial infarction) and is the hallmark feature of chronic mountain sickness in presumably poorly adapted populations (e.g., Andeans highlanders). However, cardiac disease is remarkably absent in other high altitude populations, such as Tibetan highlanders. The genetic components underlying development of cardiac responses to hypoxia in these populations is relatively unstudied.

Drosophila, with its short lifespan and extensive genetic toolbox, is a proven, useful model to study the mechanisms of hypoxia. Our understanding of hypoxia adaptations have particularly advanced through use of unique 'hypoxia-selected' populations selected for survival under hypoxia (250 generations at 4% O_2; lethal for wild type). Previously, we found heart function reduced in hypoxia-selected flies, and the present study expands previously established hypoxia-selected *Drosophila* cardiac phenotypes. To probe the hypoxia-selected response, we examined cardiac function after an acute, severe stress (1% O_2), chronic exposure to moderate (4% O2) hypoxia, and in offspring of populations brought back to control level oxygen (21% O_2).

While control hearts exhibit moderate reductions in heart rate to an acute 1% O_2 stress and reductions in heart rate and fractional shortening to chronic 4% O_2 exposure, these changes are recoverable upon re-oxygenation. Hypoxia-selected fly hearts (at native 4% O_2) beat more slowly than controls (at 21% O_2) and further slow when exposed acutely to 1% O_2. However, unlike controls flies after an acute 1% O_2 stress, heart rate does not recover upon re-oxygenation. We also observe persistent altered recovery in F2 generation of hypoxia-selected flies raised at 21% O_2, suggesting the response to acute hypoxia exposure is independent of hypoxic preconditioning by rearing condition. Further, hypoxia-selected populations, whether raised under 21% or 4% O_2, exhibit reductions in cardiac size and systolic interval duration, with preserved fractional shortening relative to controls. We conclude hypoxia-selected *Drosophila* populations exhibit heritable, persistent cardiac responses to hypoxia, suggestive of altered homeostatic adaptations in the hypoxia-selected fly heart.

3.2 Introduction

Over 140,000 people around the world live permanently in the low oxygen conditions of high altitude (>2,500m elevation). Long-term adaptation to chronic hypoxia leads to either enhanced cardiac function or dysfunction in a population- and genotype-specific manner (11, 13, 17, 25). While cardiac disease is found at high incidence in some Andean populations, other populations, most notably the Tibetan Sherpas, are renowned for their remarkable tolerance to altitude relative to visiting lowlanders (13, 17, 23).

In Tibetan populations, who have had thousands of years to adapt to high altitude hypoxia, heart size is larger in relation to body size (physiologic hypertrophy) and overall cardiac performance is more resilient than for European visitors at similar altitudes (13, 23). Maximal heart rate, cardiac output, and myocardial glucose uptake enhance Tibetans' ability to respond to acute hypoxia relative to lowlanders. Further, Tibetan populations have decreased signs of cardiac disease, such as pathological hypertrophy and incidence of congenital heart diseases (13, 23). Relatively little is known about the cardiac response to hypoxia in Ethiopians, who are presumed to have adapted well to high altitude (16, 32). These phenomena are particularly notable when compared to Andean highlanders, where cardiomyopathies lead to congestive heart failure in incidence as high as 20% for some populations (6, 13, 20, 23, 26).

Populations living on the South American Altiplano display cardiovascular symptoms known as chronic mountain sickness (CMS), or Monges disease, from chronic exposure to high altitude. CMS is characterized by an array of symptoms, including congenital heart diseases, and susceptible individuals can suffer from strokes or heart attacks in early adulthood. The hallmark feature of CMS is high hematocrit, known as polycythemia, which increases blood viscosity and if left untreated, causes right-ventricular hypertrophy as the heart pumps against both the thickened blood and pulmonary hyper-

tension induced by hypoxia (7, 25). CMS-induced right heart hypertrophy progresses to right heart dilation, causing reduced cardiac output and eventual death. Recent genomic studies suggest the physiologic traits observed at altitude are sculpted by conditions of chronic hypoxia, and emphasizes the importance of underlying genetic changes for resilience to hypoxia (13, 19, 2628, 31, 32). The mechanisms behind these susceptibilities are largely not yet established in high altitude populations.

Hypoxia models in the genetically tractable model system, the fruit fly *Drosophila melanogaster*, provide opportunities to probe novel genetic pathways mediating the cardiac response to hypoxia and disease remodeling. Although the *Drosophila* heart consists of a linear tube, its genetic specification during development as well as its adult sarcomeric structure and ion channel complement are conserved across species (4, 24, 29, 30, 34). Hypoxia-mediated pathways, including induction of Hypoxia-Inducible factor (HIF) pathway components and downstream regulation, appears to be similarly conserved in mediating cellular responses to hypoxia in the fly (3, 18, 33). Unlike mammals however, *Drosophila* tolerate conditions of low oxygen extremely well, even fully recovering from several hours of complete anoxia (5, 15). This ability to survive hypoxic challenges makes *Drosophila* an excellent model to explore the underlying genetic and physiologic responses. In addition, specific findings from *Drosophila* studies have shown similarities and utility to vertebrate studies in their cardiac responses to hypoxia, for example the function of cardioprotective potassium channels (1, 2, 10, 38).

Use of unique populations of 'hypoxia-selected' *Drosophila* ('HS'), selected for over 250 generations for tolerance to low oxygen (4% O2), allow a controlled examination of the genetic traits underlying survival in low oxygen environments. Indeed, recent genetic studies in HS populations revealed genomic changes surrounding the *Notch* gene locus and gene expression changes in *Wnt* signaling pathways, and implicated these genes in developmental survival under hypoxia (5, 27, 29). Further, studies of the hypoxia

response in the intact wild type fly heart show the potential of using bioinformatics to determine pathways specifically underlying cardiac hypoxia adaptation (8, 9). In addition, our previously established *Drosophila* cardiac models allow detailed investigation of the myogenetic cardiac response to varying durations of hypoxia and reoxygenation (33).

The present study expands our previous knowledge with a detailed analysis of hypoxia-selected (HS) cardiac responses to acute, chronic and multi-generational hypoxia. Further, this study teases apart effects of chronic exposure from multi-generational hypoxia tolerance by examining cardiac function in populations of hypoxia-selected flies reared under normoxia, and exposures of hypoxia in their hypoxia-nave controls. Chronic exposure to hypoxia in control flies increased heart rate upon assay under hypoxia, and reduced contractility, effects that were not completely reversible upon re-oxygenation. These effects were found to be due partly to the immediate effects of acute hypoxia on the heart, and partly to heritable shifts in physiology, since assaying all populations under matched oxygen levels revealed largest chronotropic responses to acute hypoxia to be in the HS and normoxia-reared HS populations. Further, hearts from HS flies were smaller than normoxic controls and exhibited dysfunctional recovery after an acute, severe hypoxia challenge. Acute, hypoxia-induced cardiac dysfunction in HS flies included an increased incidence of asystole (non-beating hearts) and a progressive degradation of contractile function following the insult. This response was not observed in normoxia control populations, suggestive of altered homeostatic responses in these unique populations selected for multi-generational hypoxia survival.

This study expands our understanding of the *Drosophila* cardiac response to chronic and multi-generational hypoxia, and examines contributions of prior hypoxia conditioning to heart function at time of assay. Further, our data suggests underlying heritable changes in HS flies cause persistent cardiac phenotypes in these HS populations specifically selected for long-term hypoxia survival. Our evidence indicates an underlying

genetic basis to the cardiac tolerance and susceptibility to long-term hypoxia exposures.

3.3 Methods

3.3.1 Genetic lines

To study long-term adaptive mechanisms we used three Drosophila populations selected for more than 250 generations ('hypoxia-selected', abbreviated HS) for survival at 4% O_2, a level that is normally developmentally lethal. Previous studies in our and other laboratories in whole *Drosophila* identified critical hypoxia adaptation thresholds at approximately 4% O_2 and 1% O_2 below which reproduction and lifespan, respectively, were critically attenuated in both wild type and normoxia control fly lines (2, 33, 38). Three distinct and genetically isolated, normoxia-raised control populations ('normoxia controls', abbreviated NC) maintained in parallel with the selected populations were used as outbred control populations (35, 38). To maintain the long-term genetic integrity of the HS populations, only three week old, male HS and NC flies were used for cardiac function and structure studies. We chose to assess HS and NC flies at three weeks as this is the median age for wild type adult flies and, from our experience in wild type lines, heart function is not yet significantly affected by aging- related cardiac effects but does show signs of cardiac dysfunction after a chronic hypoxic exposure (21, 22, 33).

3.3.2 Optical Imaging and Heart Function Analysis

Semi-intact hearts were prepared as described previously (21, 22, 33). Direct immersion optics were used in conjunction with a digital high-speed camera (120-150

frames/sec, Hamamatsu EM-CCD) mounted on a Leica DMLFSA microscope (McBain Instruments, Chatsworth, CA) to record 30 second movies of beating hearts; images were captured using HC Image (Hamamatsu Corp.). Cardiac function was analyzed from the high speed movies using Semi-automatic Optical Heartbeat Analysis software (SOHA, free download for research purposes at www.sohasoftware.com) which quantifies diastolic/systolic intervals, cardiac arrhythmia, diastolic/systolic diameters, fractional shortening, and produces M-mode records from the videos (13, 47). Flies displaying no contractions for greater than 25 seconds during the 30 second sampling period were labeled 'asystolic.' Asystolic flies were excluded from the dataset if the heart did not resume beating upon reoxygenation ($<$1% of total dataset, controls and experimental groups) or if a measurement was $>$+/- 2 standard deviations from the mean on any parameter.

3.3.3 Hypoxia rearing conditions

For this study we examine chronic hypoxia at 4% O_2 as hypoxia-nave control flies can live a normal lifespan at that level of hypoxia, although they cannot reproduce except after experimental selection (17, 58, 60). HS fly populations were raised for $>$250 generations in hypoxia chambers at progressively lower O_2 levels down to 4% O_2, as previously established (35). Two- to three-day old male flies were removed from each of the parental population chambers and placed in sealed, humidified chambers (Modular Incubator Chamber, MIC 101, Billups-Rothenberg) at room temperature for three weeks, under 4% or 21% O_2. These chambers are stable and reliable for 24-48 hours at 4% O_2 and were flushed daily to maintain stable O_2 levels and eliminate the minimal build-up of carbon dioxide. Food (equilibrated for 24 hours at 4% O_2) was changed two times per week within a glove box under hypoxic conditions. After three weeks, flies were

removed from the chamber, and assayed as described in methods.

3.3.4 Acute hypoxia/reoxygenation assay (acute H/R)

We wanted to characterize the effects of short term, severe hypoxic stress on heart function in the hypoxia-selected populations to probe underlying mechanisms for extreme hypoxia tolerance. Acute hypoxia/reoxygenation (acute H/R) assays were performed combining our previously established protocols for chronic and acute H/R assays, as presented in Figure 4 A (33). Semi-intact hearts were dissected and equilibrated dependent on rearing condition, as described above, and dissected preparations were assayed first under 21% or 4% O_2. See protocol assays in each figure legend for details related to each experiment. For the acute hypoxic stress, flies were exposed to 1% O_2 using pre-calibrated mixtures balanced with nitrogen. Preparations were then returned to 21% or 4% O_2 and filmed after 30 and 60 minutes after acute hypoxia.

Since 4% and 1% O_2 occasionally causes temporary, reversible periods of 'asysole' greater than the 30 second sampling interval, we show cardiac responses only for hearts that beat, as established previously (33). Importantly, post-asystole function of HS and NC asystolic hearts did not differ significantly compared to hearts that were able to beat during matched time-points. However, we used highly conservative statistics and only analyzed flies which beat at each time point to eliminate the statistical bias of these asystolic measures.

3.3.5 Chronic hypoxia assays

We chose three weeks as the exposure period for the chronic hypoxia challenge as this is the median age for wild type adult flies and, in our experience, heart function is not yet significantly affected by aging-related cardiac effects (21). Chronic hypoxia assays were performed as we established previously in a chronic hypoxia/reoxygenation assay, see protocol schema in Figure 2 A (33). Briefly, we assessed heart function at 21% O_2 after three weeks chronic 4% O_2 exposure. Hypoxia-raised NC (and normoxia-raised HS flies) were dissected and equilibrated for 30 min in artificial hemolymph at 21% O_2, as previously established (26). Further, a subset of hypoxia-raised NC and HS flies were dissected, equilibrated and assayed under 4% O_2 to allow comparison within hypoxia-raised NCs to 4% O_2 (the relative normoxia of HS populations); see protocol schema in Figure 3 A (33).

Exposed hearts were perfused with artificial hemolymph bubbled with room air or with the pre-mixed, calibrated 4% or 1% O_2 mixtures when making hypoxia measurements. Dissolved O_2 content was monitored and recorded using a Qubit Systems OX1LP polarographic oxygen probe, calibrated and corrected for mean barometric pressure (758 mm Hg), salinity (8.22) and perfusate temperatures (21-22C). The O_2 content was monitored at several time points during experimental sessions, with the mean dissolved O_2 content stable over the 150 minute recording sessions at 1% O2 = 0.64 mg/L and at 4% O_2 = 1.7 mg/L (best fit linear regression, p¡0.0001). To ensure that individuals in all populations received equivalent treatments, small numbers of individuals from each population were prepared together, and hypoxia conditions were repeated in at least triplicate for each experiment.

3.3.6 Time-point matched controls

To control for possible prolonged effects of exposure to relative hyperoxia during dissection for assaying baseline measures of hypoxia-raised flies, we monitored heart function in both populations under their relative normoxia (4% or 21% O_2) up to three hours after dissection (See Figure 3.4 A for protocol). We used the average measures of 60 and 90 minutes post dissection as the stable, reference baseline values (33). The full data for time-matched controls are available in Supplementary Information. These time-matched flies were assayed at each time-point of the acute hypoxia/reoxygenation assay to ensure effects were due to the acute assay and not potential destabilization of the heart over time. For all other figures, all assays were assayed at matched timepoints after reoxygenation compared to their controls, and we noted whether flies were conditioned for a 'relative normoxia' of ambient 21% O_2 or hypoxia 4% O_2 by labeling rearing O_2 and assay O_2 in figure legends.

3.3.7 Statistical analysis

All statistical analyses were performed using Prism Statistical Software (Graph Pad, Inc, version 6). Data sets were first tested for normal (Gaussian) distributions using the D'Agostino and Pearson omnibus normality test. For data sets that passed this test, we used a regular t-test, 1-way or 2-way ANOVA as appropriate. Most of the studies used two or more genotypes and two experimental conditions (normoxia vs. hypoxia) so significance was determined using a 2-way ANOVA followed by multiple comparisons post hoc tests, as appropriate (specific tests indicated in figure legends). In tests using only one genotype with multiple conditions a 1-way ANOVA was deemed appropriate. Data sets that did not show a normal distribution (heart period and diastolic interval)

were analyzed for significance using a nonparametric two-tailed t-test, or KruskalWallis test followed by Dunn multiple comparisons post hoc tests, as appropriate (specific tests indicated in figure legends).

We chose to be conservative in our statistical analyses and excluded heart period, systolic/diastolic interval data for hearts that did not beat during exposure to hypoxia. As reported previously, to ensure the non-beating hearts did not represent an aberrant subset, we analyzed the beating and non-beating heart data separately and found no significant difference in any of the normoxia-measured parameters between hearts that did or did not beat under hypoxia, thus the normoxia data sets were pooled (33). In all cases, P<¡0.05 was taken as significant.

3.4 Results

In our previous study, we compared the average cardiac function between control and hypoxia-selected flies, combining the three populations' averages to report baseline cardiac function for each (33). In the present study, we present population-by-population cardiac responses for these same parameters to explore population divergence. Noting more similarities among HS populations than to NC populations, we combine the HS responses to study their acute response to hypoxia, as well as NC response to chronic hypoxia and the acute assay conditions of 4% O_2 in detail.

3.4.1 Baseline cardiac function is reduced in three hypoxia-selected *Drosophila* populations compared to normoxia controls

We examined heart function from three distinct HS and NC populations under their relative normoxia (21% O_2 for NC and 4% O_2 for HS). After dissection under 21% O_2 for NC and reduced O_2 for HS, we assayed flies at multiple time-points to account for any effects of relative hyperoxia in the HS populations (See Supplemental Information). We averaged two time-points at T=30 and T=60 as the stable, baseline measure in these populations at their relative O_2 (See assay protocol Figure 3.1 A).

Heart period (HP) is significantly longer for all HS populations compared to all NCs under their relative normoxic conditions (21% O_2 for NC and 4% O_2 for HS) (Figure 3.1 C). This increase in HP is due to significantly longer diastolic intervals (DI) in HS populations compared to NCs (Figure 3.1 B). In contrast, systolic intervals (SI) are decreased between HS and NC populations. Post-hoc testing revealed two NC populations with significantly lower SI from the other NC populations; these NC Sis were more similar to all three of the HS populations than the outlying NC population (Figure 3.1 C).

On average, fractional shortening (FS) is significantly reduced across HS populations compared to NC. However, by post-hoc testing, FS in one NC population was significantly lower than the other two NC populations, due to a shorter mean diastolic diameter (DD) compared to all NC populations. In HS populations, decreased FS was due to reductions in both DD and SD compared to NCs (Figure 3.1 D-F).

3.4.2 Assaying cardiac function at 4% O_2 reduces contractility in hypoxia-raised control flies

To unravel whether the cardiac responses we observed in HS flies were due to multi-generational hypoxia selection (genetic or epigenetic) or rearing and assaying under 4% hypoxia, we first examined NC populations reared for 3 weeks at 4% O_2 and analyzed at either 21% O_2 (chronic H/R) or at 4% O_2 (see Figure 3.2 A for chronic hypoxia assay protocols).

Instance of asystole was 20% in hypoxia-raised NC assayed at 4% O_2; only data from hearts that beat was used for Figure 3.2 C-H. We observed increased incidence of asystole, HP, DI and SI in hypoxia-raised NCs upon assaying at 4% O_2 over assaying at 21% O_2. This is in line with an acute assay of 4% O_2 in NC flies, which immediately increased HP and DI (but not SI) (see Supplementary materials). Thus we determine the effects of assaying under 4% O_2 to be display stronger cardiac effects than assaying upon reoxygenation after chronic hypoxia exposure.

FS was decreased in both hypoxia-raised NC populations, whether assayed at 21% or 4% O_2 (Figure 3.2 F). In normoxia-raised NC flies assayed at 21% O_2, the decrease in FS was due to non-significant decreases in DD (Figure 3.2 G). In hypoxia-raised NC flies assayed at 4% O_2, the decrease in FS was due to non-significant increases in SD (Figure 3.2 H). This is in line with our previous findings of non-significant DD decreases after chronic H/R, and SD increases upon acute 4% O_2 in wild type lines (33).

3.4.3 Cardiac response to chronic hypoxia and normoxia in control and hypoxia-selected populations

In order to determine whether the altered cardiac function observed in selected flies was due to genetic changes or due to the more immediate effects of lifetime chronic hypoxia exposure, we examined function in HS flies raised at 21% O2 ('HS normoxia raised') for two generations (F2). We also wished to determine effects of chronic, lifespan hypoxia exposure on NC genetic backgrounds ('NC hypoxia raised'). See Figure 3.3 A for chronic hypoxia assay protocols. Hypoxia-raised HS flies were assayed at their native 4% O_2, while a subset were reared under normoxia and the F2 generation assayed again at 4% O_2 for comparison. For comparison to populations without selection pressure, NC flies were exposed to an acute 4% O_2 assay and compared to hypoxia-raised NC flies assayed at 4% O_2.

Interestingly, normoxia-raised HS flies had the most reduction in contractility (HP and FS) compared all other populations. These normoxia-raised HS flies had higher HP and DI at 4% O_2 than either hypoxia-reared NC or HS population (Figure 3.3C-D). Hypoxia-raised HS flies had the lowest SI compared to any other rearing condition when assayed at 4% O_2.

Normoxia-raised HS fly hearts had large reductions in FS, due to decreased DD and lesser reductions in SD (Figure 3.3 F-H). Significant reductions in both DD and SD preserved FS in hypoxia-raised HS flies (Figure 3.3 G,H). Notably, SD was significantly reduced in hypoxia-selected HS flies relative to all other flies assayed at 4% O_2 (Figure 3.3 H).

3.4.4 Acute cardiac response to 30 minutes 1% O2 hypoxia / reoxygenation (acute H/R) in control and hypoxia-selected raised under conditions of normoxia or hypoxia

Acute H/R assay and M-modes of in NC and HS flies

To investigate limits of physiologic plasticity underlying long-term hypoxia adaptation in HS flies, we wished to characterize the effects of short term, severe hypoxic stress on heart function in the hypoxia-selected populations. Acute hypoxia/reoxygenation (acute H/R) assays were performed by measuring heart function before and at two time points after an acute 1% O_2 assay in all populations, see Protocol in Figure 3.4 A. Normoxia-raised NC and HS flies were assayed at 21% O_2 for baseline and reoxygenation measures (left), while hypoxia-raised NC and HS flies were assayed at 4% O2 for baseline and reoxygenation measures (right). Time-matched controls were also assayed, and are presented in Supplementary materials.

Qualitatively, heart function can be illustrated in 15 second M-mode records of individual hearts (Figure 3.4 B), which provide a representative ”snapshot” of heart wall movement (y axis) over time (x axis). Representative M-modes from an individual normoxia-raised NC flys 1% O_2 acute H/R assay is shown on the left; while representative M-modes from an individual hypoxia-raised HS flys 1% O_2 acute H/R assay is shown on the left. Note the long period of asystole in a representative HS fly at 1% O_2, which in this fly, was fully recoverable at 4% O_2. Since 4% O_2 occasionally causes temporary, reversible periods of ’asysole’ greater than the 30 second sampling interval, we show cardiac responses only for hearts that beat, as established previously (33).

Cardiac responses to 30 minutes 1% O2 acute H/R in NC and HS *Drosophila* raised under conditions of normoxia or hypoxia

Exposing hearts to acute hypoxia resulted in a robust cardiac response that included temporary cessation of beating (for 25 seconds or longer) in 40-60% of the hearts at 1% O_2 for both hypoxia-raised NC and HS flies, as well as the normoxia-raised NC flies ('asystolic' in Figure 3.5 A).

All hearts from both NC and HS normoxia-raised flies were found to be beating when assayed at 3 weeks of age under normoxic conditions. In contrast, hearts from both hypoxia raised NC and HS exhibited a small percentage of non-beating hearts ('asystolic') at their baseline 4% hypoxia (8% were asystolic in each line, Figure 3.5 A T=0, Supplement 4). In response to an acute hypoxic challenge, hearts from NC were able to continue beating during an acute 1% O2 challenge. In contrast, roughly half of the hearts from hypoxia-raised NC were asystolic during the same acute hypoxic challenge. More than half of hearts from HS flies also stopped beating regardless of rearing conditions, suggesting that this acute asystolic phenotype can occur after previous parental or chronic exposure to hypoxia (Figure 3.5 A T=30). The post challenge response also appears to differ between the two lines, in that a higher percentage of hearts from HS flies, raised under either normoxia or hypoxia, are unable to continue beating following a hypoxic challenge (Figure 3.5 A T=90). This is in contrast to hearts from normoxia- or hypoxia-raised NCs, which return to baseline on reoxygenation.

For both NC and HS hearts that continued to beat during the acute hypoxia exposure, cardiac responses are reported in Figure 3.5 B-G. HP increased upon 1% O_2 exposure in HS populations as a result of increases in DI (Figure 3.5-C). Upon reoxygenation, all hearts resumed pre-exposure beating patterns, except hypoxia-raised HS flies, whose HP and DI remain elevated 60 minutes after acute 1% O_2 (Figure 3.5 B,C T=30 compared to T=60 & 90). SI increased significantly at 1% O_2 only in normoxia-raised

NC and HS flies. NC flies did not return to pre-hypoxia exposure levels upon subsequent reoxygenation, however normoxia-raised HS flies SI did return to pre-hypoxia measures (Figure 2D).

FS decreased significantly in HS flies of either rearing condition during acute hypoxia, and returned to baseline in normoxia-raised HS but not hypoxia-raised HS at 60 minutes re-oxygenation (Figure 3.5 E). Changes in FS in hypoxia-raised HS flies were due to decreased DD, and non-significant increases in SD (Figure 3.5 F,G). FS and DD were largely unaltered in NC populations of either rearing condition, although SD increases in NCs at 1% O2 were significantly lowered upon first reoxygenation assay (Figure 3.5 G, white bars, T=30 to T=60).

We conclude that chronotropic and ionotropic cardiac function is mostly restored at one hour of reoxygenation post-exposure to extreme acute hypoxia in NC lines, whether normoxia- or hypoxia-reared. This is consistent with the previously observed overall tolerance and recovery of fruit flies to acute hypoxic/anoxic conditions (15, 33). Contractility in hypoxia-raised HS hearts was restored upon reoxygenation, including both diastolic and systolic diameter measurements (Figure 3.5 E-G; gray bars). In contrast, hearts from hypoxia-selected flies while initially recovering fractional shortening toward pre-hypoxia levels, were unable to sustain this level of contractility at 60 minutes post reoxygenation (Figure 3.5 E; T=60, 90). This effect is particularly pronounced when compared to time-matched controls which did not receive an acute hypoxia challenge (see Supplementary materials). This suggests a relative inability of hypoxia-raised HS flies to maintain function in the face of acute hypoxic insults, and emphasizes the severity of the combined multi-generational, chronic and acute hypoxia exposure upon the HS genetic background.

3.5 Discussion

Multi-generational exposure to moderate reduced oxygen environments may increase tolerance to conditions of acute or chronic hypoxia through altered regulatory mechanisms; however, a limit exists to the hypoxic conditions to which species can adapt at extreme high altitudes. Human populations that are well adapted to chronic hypoxia over many generations, such as Tibetan Sherpas, show beneficial cardiac adaptations to their native high altitudes, including; reduced right ventricular hypertrophy, increased ability to raise maximal heart rate and cardiac output at altitude, and increased myocardial glucose uptake (14, 23, 39). Other high altitude populations exhibit signs of cardiac disease due to multi-generational and continued chronic exposure to hypoxia (8, 22, 25, 31). Interestingly, our hypoxia-selected (HS) flies exhibited significant changes to all cardiac parameters, suggesting an overall reduction in cardiac function under hypoxia.

We show adaptations of cardiac responses in HS flies such as reduced rate and fractional shortening that are presumably beneficial given the reduced ability to generate ATP in hypoxia. These adaptations must not affect overall vitality since the HS flies survive to reproduce and live normal lifespans (15, 17, 43, 45). Since the *Drosophila* open circulatory system's primary role is not circulation of oxygen as it is mammalian systems, the reductions in heart rate and size would be expected to conserve ATP levels without compromising oxygen delivery (as would occur in mammals) (10, 19). We raised the HS flies under normoxia (21% O2) for two generations to remove the immediate effects of chronic oxygen-limitation. Notably, under these conditions, the overall HS normoxia-raised body size returned to that of the normoxia control (NC) populations, however mean diastolic diameters (DD) and systolic diameters (SD) remained smaller than the NCs. This suggests the smaller cardiac size in HS flies represents a genetically, stable overall constriction of the heart in response to hypoxia selection.

Compensatory myocardial hypertrophy is the hallmark symptom of chronic hypoxia, and causes of contractility and dilation that culminates in further loss of contractility due to diastolic dysfunction (36). Well adapted human populations, who adapted to chronic hypoxia over many generations, show several parameters of beneficial cardiac adaptation at their native high altitudes, including; reduced right ventricular hypertrophy, increased ability to raise maximal heart rate and cardiac output at altitude, and increased myocardial glucose uptake (14). In NC flies, reductions in HP and FS during acute hypoxia may be cardioprotective, given they completely recover on reoxygenation. Given the smaller body size and reduced baseline function of hypoxia-selected flies they likely can maintain sufficient cardiac output in the face of reduced overall energy production.

In humans, acute, systemic exposure to hypoxia stimulates compensatory physiological responses that include an immediate increase in heart rate and ventilation in response to moderate hypoxia exposures, and decreased heart rate and ventilation upon severe levels of acute hypoxia. These responses are often reversible upon reoxygenation, however, severe or local hypoxia and reoxygenation, such as with cardiac arrest, can lead to irreversible ischemic heart disease (33). After acute H/R in NC *Drosophila* we show cardiac function is initially repressed during the exposure but immediately restored upon reoxygenation. Our previous work showed this phenotype in wild type as well as in HIF/*sima* deficient genotypes, with the HIF-deficient background response significantly blunted with respect to contractility (41).

While contractility (fractional shortening, FS) tended to be reduced in NC flies under 1% O2, this in contrast to hearts from HS flies which show a striking and significant reduction in FS in response to hypoxia. This reduction in fractional shortening under hypoxia was due to both decreases in the DD (reduced relaxation, diastolic dysfunction) and increases in the SD (reduced contraction, systolic dysfunction). Importantly, the relative inability of hypoxia-selected flies to restore function on reoxygenation following

aH/R suggests altered homeostasis. Underlying cardiac remodeling, whether from the continuing physiologic stress of multi-generational chronic hypoxic exposure, or altered underlying genetic mechanisms, does not permit normal cardioprotective mechanisms to operate as seen NC flies and previously in wild type lines. These results suggest adaptation to hypoxia play a role in cardiac contractility during acute hypoxia exposure in HS flies, as shown in other species (22, 31). Our *Drosophila* hypoxia model of long-term adaptation is ideally suited to aid in these discoveries, given the availability of a well-studied hypoxia-selected population and a plethora of innovative genetic tools.

Hypoxia-induced cardiac dysfunction is a hallmark of many disease states at both sea-level or high altitude, including pulmonary hypertension, ischemic heart attack and chronic mountain sickness. Tolerance to hypoxia or development of disease depends on complex genetic and molecular mechanisms that have not been clearly established. Here we use unique hypoxia-selected *Drosophila* populations to elucidate the relationship between environment and disease progression after chronic exposure to hypoxia. We assayed cardiac function under ambient oxygen levels for uniquely hypoxia adapted populations, after further acute hypoxic stress and reoxygenation, or after removing developmental chronic exposure to hypoxia. Even after chronic hypoxia, we found cardiac function to be largely recoverable in control fly populations, even after an acute hypoxic stress. However, populations selected for long-term adaptation to chronic hypoxia showed altered recovery, including persistently small hearts, indicative of underlying homeostatic changes in their cardiac response to hypoxia.

3.6 Acknowledgments

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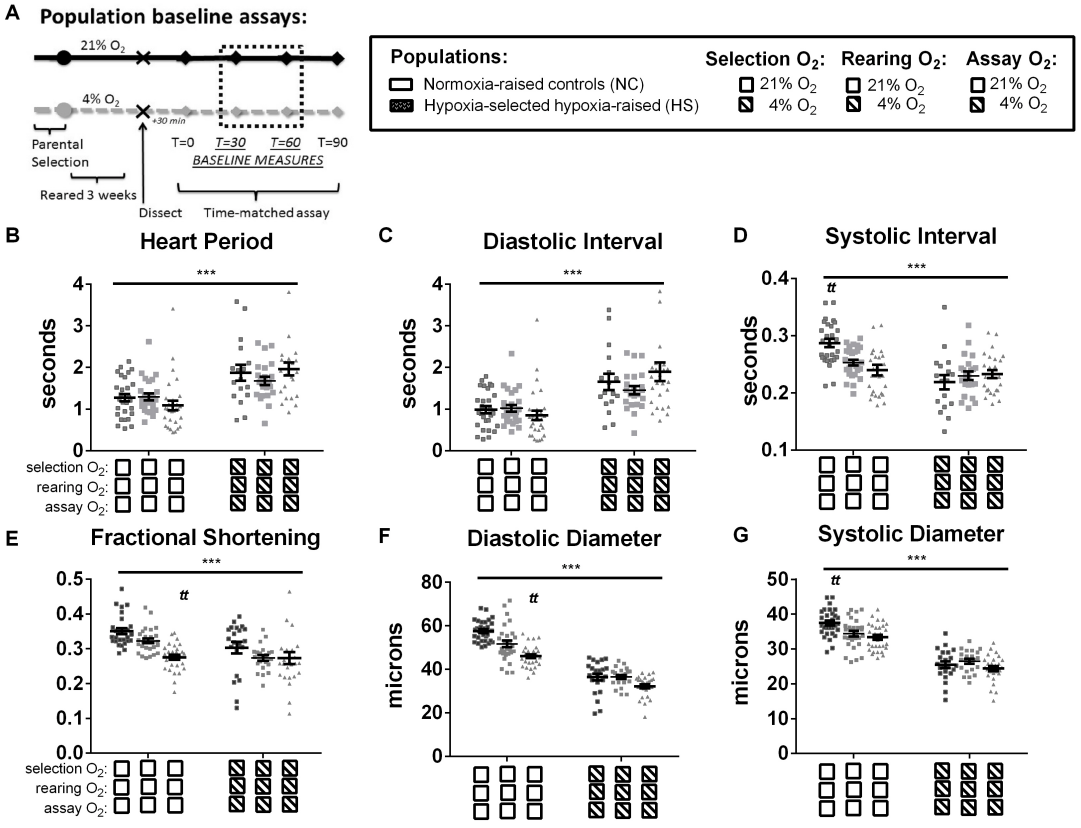
3.7 Figures

Figure 3.1 (facing page): Baseline cardiac function is reduced in three hypoxia-selected *Drosophila* populations compared to normoxia controls.
A) Representative 15 sec M-modes showing heart wall movements in three normoxia controls and three hypoxia-selected fly hearts at their relative normoxia; (Left) 21% O2 for normoxia controls, and (Right) 4% O2 for hypoxia-selected. B) Under their relative normoxic conditions heart period is significantly longer in hypoxia-selected flies compared to normoxia controls. C) Diastolic intervals are significantly longer in hypoxia-selected flies compared to normoxia controls. D) Systolic intervals are significantly decreased in hypoxia-selected flies compared to normoxia controls. One normoxia control population had a significantly longer systolic interval than either of the other two normoxia control populations. E) Fractional shortening is significantly reduced in hypoxia-selected flies, while one normoxia control population has significantly lower fractional shortening than the other two populations, and falls within the mean of the three hypoxia selected populations. Reduction for this normoxia control population as well as the hypoxia-selected population is due to reduction in F) diastolic diameter. G) Further, for hypoxia-selected flies, reduction in systolic diameter still contributes to overall reduction in fractional shortening and systolic diameter compared to control populations. One control population has a slightly higher systolic diameter than the other two populations.

*All values are mean +/- SEM for hearts ***$p<0.001$ 1-way ANOVA. Changes between populations for each condition were analyzed by Sidak-Bonferroni t-test; t $p<0.05$ tt $p<0.01$. Normoxia controls I N=28, II N=26 and III N=29; hypoxia-selected I N=18, II N=22, and III N=24.*


A
Population baseline assays:
21% O2
4% O2
+30 min
Parental Selection
Reared 3 weeks
Dissect
T=0
T=30
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T=90
BASELINE MEASURES
Time-matched assay
Populations:
Normoxia-raised controls (NC)
Hypoxia-selected hypoxia-raised (HS)
Selection O2:
Rearing O2:
Assay O2:
21% O2
4% O2
B
Heart Period
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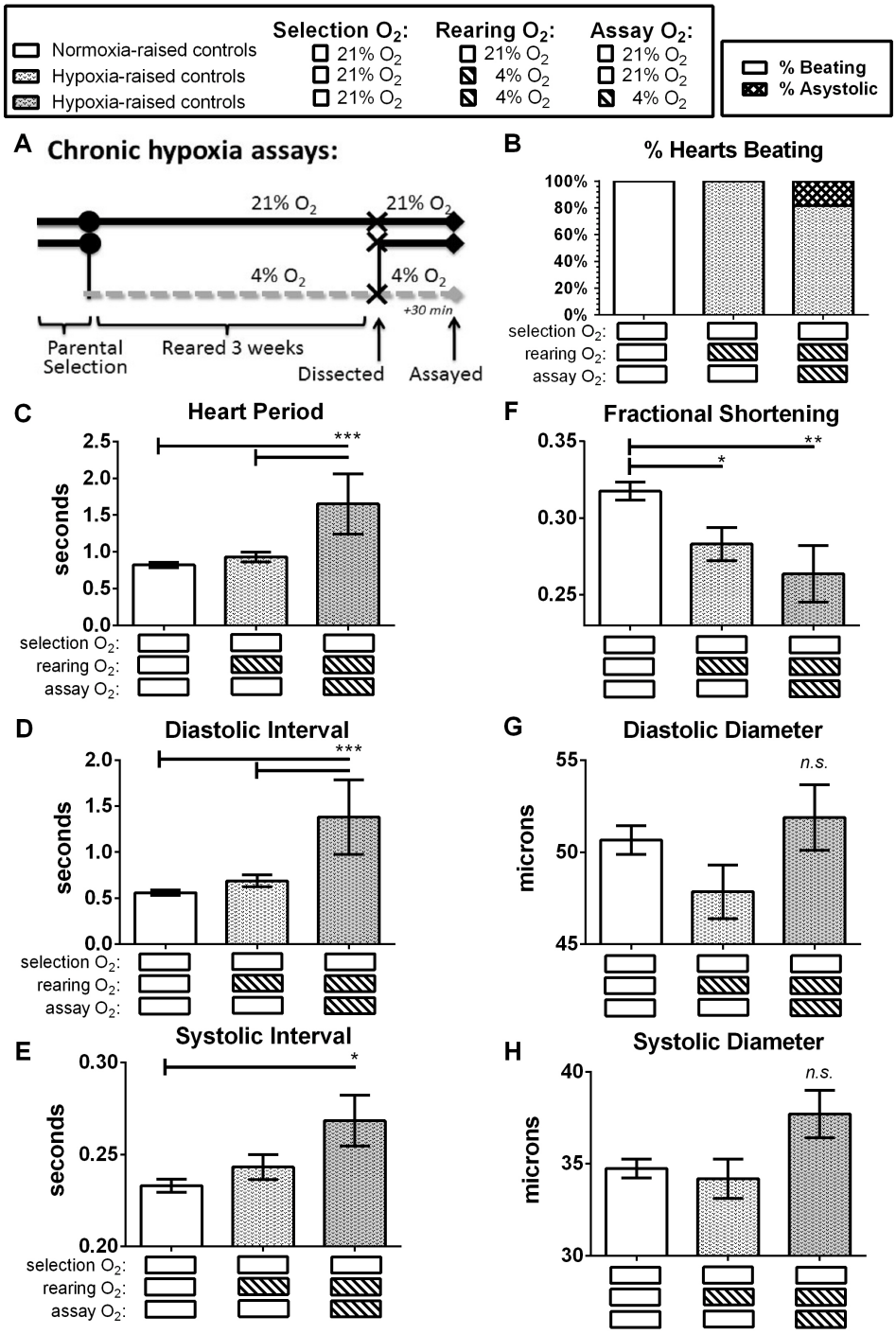
Figure 3.2 (facing page): Cardiac parameters of normoxia control populations raised under hypoxia and assayed at 21% or 4% O2.
A) Schematic showing the chronic hypoxia/reoxygenation protocol to assay relative normoxia at either 4% or 21% O2. B) Instance of asystole was 20% in hypoxia-raised NC assayed at 4% O_2; only data from hearts that beat was used for Figure 3.2 C-H. C) Assay O2 has an impact on the heart period of NC populations; after chronic hypoxia rearing, heart period is significantly longer in NC flies assayed at 4% O2 compared to normoxia- or hypoxia-raised NC assayed at 21% O2. The increase in HP is due to D) significantly longer diastolic intervals and E) systolic intervals in these flies. F) Fractional shortening is significantly reduced in hypoxia-reared NC flies, whether assayed at 21% or 4% O2. No changes were observed based on rearing or assay O2 in NC populations in either G) diastolic diameter or H) systolic interval.

*All values are mean +/- SEM for hearts ***p<0.001 1-way ANOVA. Changes between populations for each condition were analyzed by Sidak-Bonferroni t-test; t p<¡0.05 tt p<¡0.01. NC N=83, HS N=74.*


Selection O_2:
Rearing O_2:
Assay O_2:
Normoxia-raised controls
21% O_2 21% O_2 21% O_2
Hypoxia-raised controls
21% O_2 4% O_2 21% O_2
Hypoxia-raised controls
21% O_2 4% O_2 4% O_2
% Beating
% Asystolic
A Chronic hypoxia assays:
21% O_2
21% O_2
4% O_2
4% O_2
+30 min
Parental Selection
Reared 3 weeks
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Assayed
B % Hearts Beating
100%
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F Fractional Shortening
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G Diastolic Diameter
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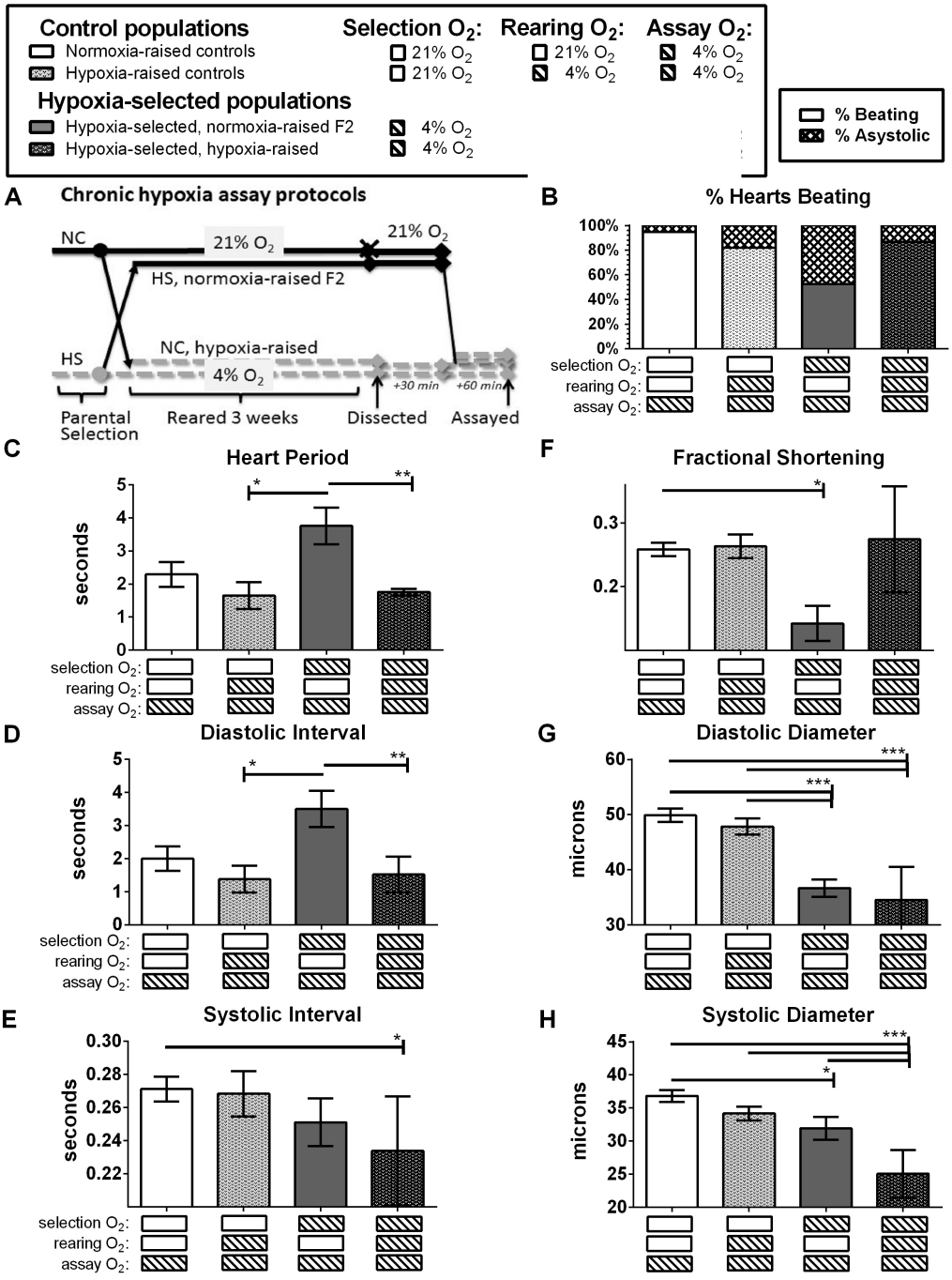
55
50
45
n.s.
H Systolic Diameter
microns
40
35
30
n.s.

Figure 3.3 (facing page): Cardiac response to chronic hypoxia in control and hypoxia-selected populations.
A) Schematic showing the chronic hypoxia or normoxia assay in which NC and HS populations were raised and subsequently assayed at 4% O2. Hypoxia-raised HS flies were assayed at their native 4% O_2, while a subset were reared under normoxia assayed at 4% O_2 for comparison. For comparison to populations without selection pressure, NC flies were exposed to an acute 4% O_2 assay and compared to hypoxia-raised NC flies assayed at 4% O_2. B) Incidence of asystole in HS and NC populations. C) Normoxia-raised HS flies had higher HP than hypoxia-reared NC and HS flies. D) Increases in HP were attributable to increases in DI. E) Hypoxia-raised HS flies had the lowest SI compared to any other rearing condition when assayed at 4% O_2. F) Normoxia-raised HS fly hearts had large reductions in FS. G) Reductions in DD in normoxia- or hypoxia-reared HS flies were significantly lower than either NC population. H). Similarly, reductions in SD in normoxia- or hypoxia-reared HS flies were significantly lower than normoxia-raised NCs, and lower in hypoxia-raised HS flies from hypoxia-raised NC flies.

*All values are mean +/- SEM for hearts ***$p<0.001$ 1-way ANOVA. * $p<0.05$ *** $p<0.001$.*


Control populations
Selection O2:
Rearing O2:
Assay O2:
Normoxia-raised controls
21% O2
21% O2
4% O2
Hypoxia-raised controls
21% O2
4% O2
4% O2
Hypoxia-selected populations
Hypoxia-selected, normoxia-raised F2
4% O2
Hypoxia-selected, hypoxia-raised
4% O2
% Beating
% Asystolic
A
Chronic hypoxia assay protocols
NC
21% O2
21% O2
HS, normoxia-raised F2
HS
NC, hypoxia-raised
4% O2
+30 min
+60 min
Parental Selection
Reared 3 weeks
Dissected
Assayed
B
% Hearts Beating
100%
80%
60%
40%
20%
0%
selection O2:
rearing O2:
assay O2:
C
Heart Period
seconds
D
Diastolic Interval
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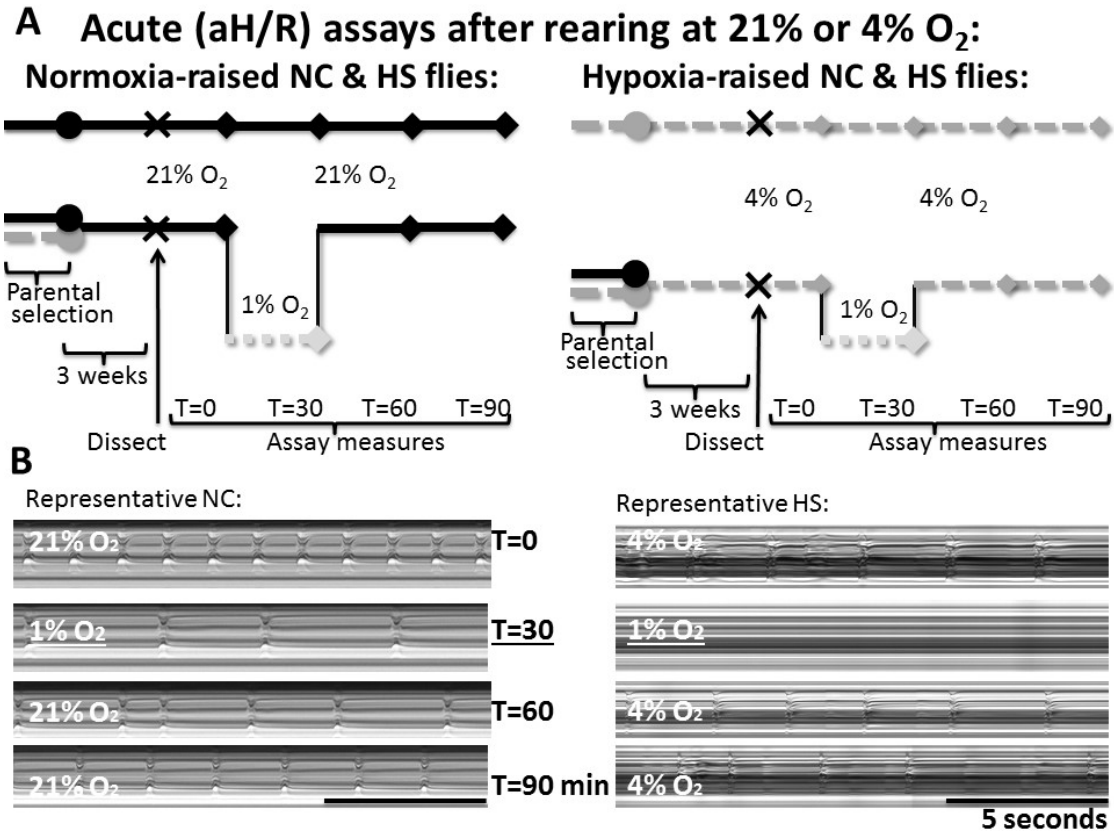

Figure 3.4: **Acute hypoxia/reoxygenation (acute H/R) assay protocol for normoxia control (NC) and hypoxia-selected (HS) flies.** A) Schematic showing the acute H/R protocol in HS and NC control flies raised under either normoxia or hypoxia. B) M-modes records (15 seconds) from movies of beating hearts taken before (T=0), at 30 minutes during (T=30), and at two time points (T=60,90) following hypoxia treatments. (Left) Representative M-modes showing changes in heart beat in response to acute 1% O2 at T=30 from an individual NC fly. (Right) Representative M-modes showing changes in heart beat in response to 1% O2 from an individual HS fly.

Figure 3.5 (facing page): Cardiac responses to 30 minutes 1% O2 acute H/R in NC and HS *Drosophila* raised under conditions of normoxia or hypoxia
A) Incidence of non-beating or 'asystolic' hearts (> 25 seconds without contractions) in response to hypoxia exposure, expressed as a percent of total hearts examined. Nearly half of hearts entered prolonged periods of asystole at 30 minutes in 1% O2, and all resumed beating on reoxygenation. B) In hearts that beat, heart period (HP) is increased under 1% O2 hypoxia exposure (T=30) and this reached significance in hearts from hypoxia-selected flies either normoxia or hypoxia-raised. HP returned to baseline levels exhibited by normoxia controls in all genotypes post hypoxia exposure except hypoxia-selected hypoxia raised flies (T=90). C) Diastolic intervals (DI) increased in hypoxia-selected flies under 1% O2 (T=30) and then returned to baseline levels upon reoxygenation (T=60, 90). D) Systolic intervals increased for normoxia-raised controls and hypoxia-selected genotypes on 1% hypoxia exposure and returned to baseline only in hypoxia-selected, normoxia-raised flies. E) Fractional shortening was significantly reduced under 1% O2 for hypoxia-selected flies either hypoxia- or normoxia-raised (T=30). Fractional shortening returned to baseline for normoxia-raised flies at T=60 and T=90, and at T=60 for hypoxia-raised flies. F) Diastolic diameters is decreased significantly in hypoxia-selected flies, particularly at T=90 from T=30 and T=60 after an acute 1% O2 exposure. G) Systolic diameters were significantly increased during acute hypoxia in hypoxia-selected, normoxia-raised flies (T=30 from T=0); in contrast, systolic diameters reduced in normoxia-raised control and hypoxia-selected hypoxia-raised flies upon reperfusion (T=60,90 from T=30).

*All values are mean percent change +/- SEM (normoxia-raised controls N=23, hypoxia-raised controls N=11, hypoxia-selected normoxia-raised N=23, hypoxia-selected hypoxia-raised N=37). For heart period, systolic and diastolic interval, asystolic hearts were excluded, while all hearts are included in fractional shortening, diastolic and systolic diameter. Data was analyzed by (B,C) KruskalWallis test and Dunn multiple comparisons post-hoc test and (D-F) 2-way ANOVA and Tukey's multiple comparisons post-hoc test; n.s. = no statistical significance, * <0.05, ** p<0.01, *** p < 0.001. Changes between genotypes at each timepoint were analyzed by Sidak-Bonferroni t-test; t p < 0.05. tt p < 0.01.*

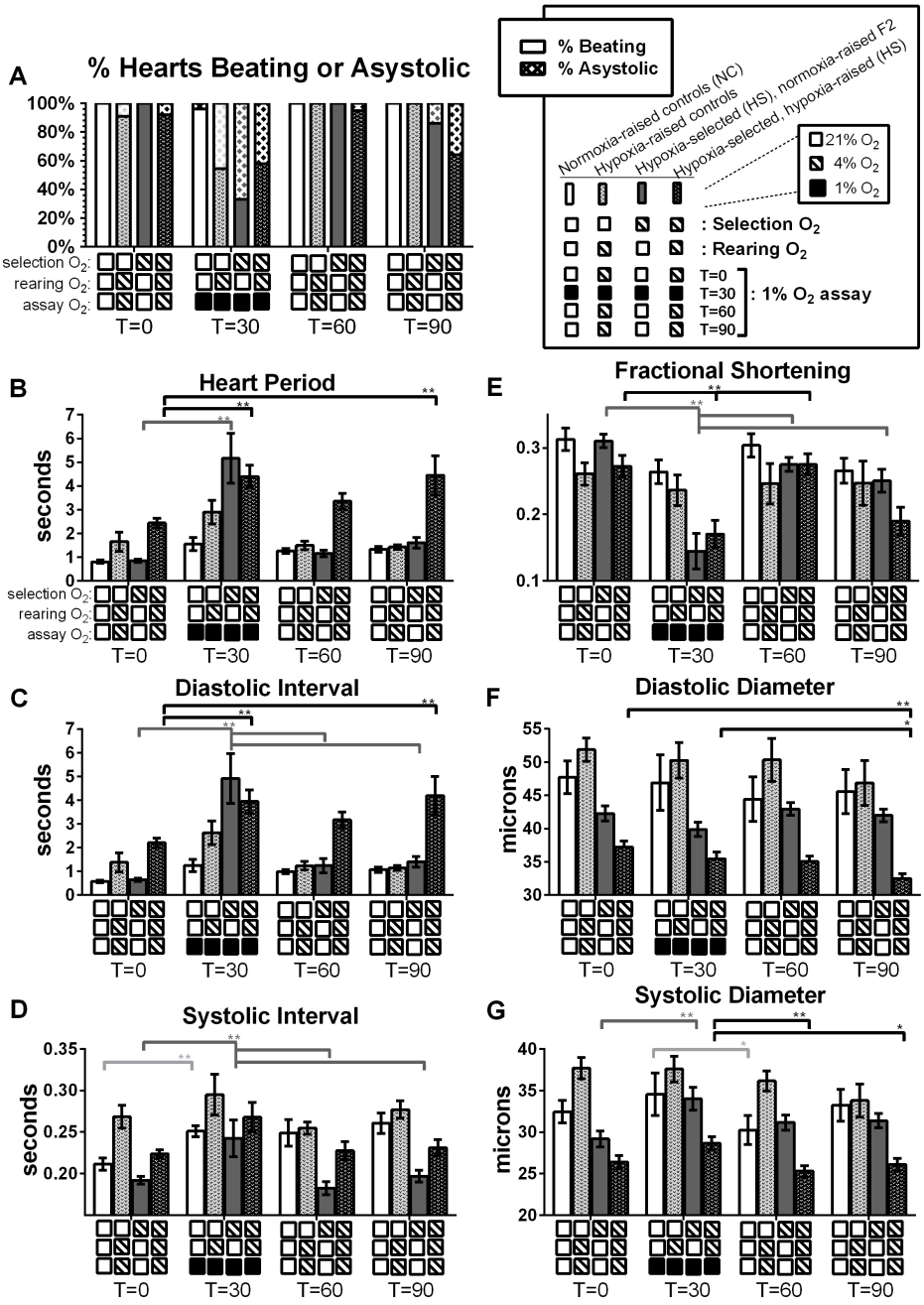
A
% Hearts Beating or Asystolic
100%
80%
60%
40%
20%
0%
selection O2:
rearing O2:
assay O2:
T=0
T=30
T=60
T=90
% Beating
% Asystolic
Normoxia-raised controls (NC)
Hypoxia-raised controls
Hypoxia-selected (HS), normoxia-raised F2
Hypoxia-selected, hypoxia-raised (HS)
21% O2
4% O2
1% O2
: Selection O2
: Rearing O2
T=0
T=30
T=60
T=90
: 1% O2 assay
B
Heart Period
seconds
E
Fractional Shortening
C
Diastolic Interval
seconds
F
Diastolic Diameter
microns
D
Systolic Interval
seconds
G
Systolic Diameter
microns

Table 3.1: Summary of changes in cardiac function observed between genetic backgrounds compared to their relative normoxia baseline (acute H/R) or control population (chronic H/R) for varying durations of hypoxia or reoxygenation.

Summary of changes in cardiac function observed between genetic backgrounds compared to their *relative normoxia baseline* (acute H/R) or *control population* (chronic H/R) for varying durations of hypoxia or reoxygenation						
Cardiac measure	**Assay**	**Hypoxia condition**	**Normoxia-raised controls**	**Hypoxia-raised controls**	**Hypoxia-selected, normoxia-raised**	**Hypoxia-selected, hypoxia-raised**
Heart Period	**acute H/R**	*30 min at 1% O_2*	↑*	↑*	↑	↑
		30 min post 1% O_2	-	-	-	-
		60 min post 1% O_2	-	-	-	↑
	chronic H/R	3 weeks 4% O_2		↑*		↑
Diastolic Interval	**acute H/R**	*30 min at 1% O_2*	↑*	↑*	↑	↑
		30 min post 1% O_2	-	-	-	-
		60 min post 1% O_2	-	-	-	↑
	chronic H/R	3 weeks 4% O_2		↑*		↑
Systolic Interval	**acute H/R**	*30 min at 1% O_2*	↑	-	↑	↑*
		30 min post 1% O_2	↑*	-	-	-
		60 min post 1% O_2	↑*	-	-	-
	chronic H/R	3 weeks 4% O_2		↑*		↓
Fractional Shortening	**acute H/R**	*30 min at 1% O_2*	-	-	↓	↓
		30 min post 1% O_2	-	-	-	-
		60 min post 1% O_2	-	-	-	↓
	chronic H/R	3 weeks 4% O_2		↓*		↓
Diastolic Diameter	**acute H/R**	*30 min at 1% O_2*	-	-	-	-
		30 min post 1% O_2	-	-	-	↓
		60 min post 1% O_2	-	-	-	↓
	chronic H/R	3 weeks 4% O_2		↑*		↓
Systolic Diameter	**acute H/R**	*30 min at 1% O_2*	-	-	↑	↑*
		30 min post 1% O_2	-	-	-	-
		60 min post 1% O_2	-	-	-	-
	chronic H/R	3 weeks 4% O_2		↑*		↓
↑ increased; ↓ decreased; - no change or discernible difference; [†] trend, significant by 1-way ANOVA within genotype only. ↑/↓ ($p \leq 0.05$); ↓↓ ($p \leq 0.01$); ↓↓↓($p < 0.001$) indicating degree of change from others within same hypoxia condition						
30 min at 1% O_2 = measures taken during hypoxia exposure; all other measures taken after reoxygenation						

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Chapter 4

Calcineurin mediates loss of cardiac function after long-term hypoxia exposures in Drosophila

4.1 Abstract

Hypoxia is associated with a myriad of physiological and disease states and is a pivotal factor in cardiopulmonary diseases which represent some of the leading causes of mortality worldwide. Exposure to long-term hypoxia, whether due to cardiopulmonary disease or environmental condition, can cause cardiac hypertrophy and lead to dilated cardiomyopathy, heart failure and sudden cardiac death. Interestingly, cardiac failure is the hallmark feature of chronic mountain sickness yet is largely absent in select, presumably well-adapted, high altitude populations. Recent genomic studies suggest genetic evolution of physiologic traits observed at high altitude are sculpted by conditions of chronic hypoxia, and emphasizes the importance of genetic changes for resilience or susceptibility to hypoxia in both highland and sea-level populations.

Previously, we established models for the effects of long-term hypoxia exposures on the Drosophila heart (62). We found heart function to be altered in chronically hypoxic wild type flies (three weeks at 4% O_2) and in flies selected for multi-generational survival at 4% O_2 ('hypoxia-selected'). In the present study, we explore the function of calcineurin, a gene candidate dramatically down-regulated after hypoxia selection and potentially underlying the ensuing persistent cardiac restriction observed in hypoxia-selected flies. Cardiac dysfunction and calcineurin down-regulation distinguished hypoxia-selected flies from controls, in which chronic hypoxia exposure was not sufficient to significantly reduce calcineurin levels or cardiac size.

We selected the fly homologs of human calcineurin A, CanA14F and Pp2B, due to its known role regulating cardiac hypertrophy in mammals and flies under conditions of normoxia. Calcineurins role in adaptation to multi-generational hypoxia is poorly understood and is completely unstudied in the Drosophila response to hypoxia. We found that knockdown of these homologs of calcineurin caused cardiac restriction under conditions of normoxia and hypoxia, and that effects were both cardiac autonomous and non-autonomous. Our results suggest a key role for calcineurin in cardiac adaptations to long-term hypoxia with implications for high altitude dwellers, and is likely contributes to mechanisms underlying cardiac and hypoxic disease states.

4.2 Introduction

Chronic exposure to hypoxia may lead to compromised cardiopulmonary functions, as seen in patients with obstructive sleep apnea, breathing disorders and pulmonary hypertension (42). In mammals, the normal response of the pulmonary circulation to local hypoxia is regional hypertension, which directs blood flow toward better oxygenated re-

gions of the lungs (38, 43). However, chronic pulmonary hypertension causes remodeling through thickening of vascular smooth muscle and can lead to pathological hypertrophy as the heart pumps against increased volume and vascular resistance (17, 41). Chronic, untreated cardiac hypertrophy can progress to dilated cardiomyopathy, heart failure, and sudden death (17, 34).

Pathological cardiac hypertrophy is also the hallmark feature of chronic mountain sickness, yet is remarkably absent in select, presumably well-adapted, high altitude populations (16, 29, 45, 50, 56, 60, 61). Chronic mountain sickness is characterized by an array of symptoms, including congenital heart diseases, and susceptible individuals can suffer from strokes or heart attacks in early adulthood. Populations living in the Andean highlands of the South American Altiplano display particularly high incidence of chronic mountain sickness, and the disease was recently correlated with increased expression of the genes ANP32D and SENP1 (5). In contrast, EDNRB, a gene identified in well-adapted highland Ethiopians, improved cardiac function during hypoxia in knockout mouse models, emphasizing that certain gene selection at altitude may confer cardioprotection (51). Despite a recent flurry of studies establishing the genetic basis of high altitude adaptations, mechanisms for cardiac remodeling in these populations are largely unstudied (9, 13).

Many pathways are conserved among Drosophila and mammals including the genetic basis of heart development and HIF-1*alpha*-mediated of hypoxia responses (6, 25, 57, 62). With its relatively short lifespan and easily accessible genetic tools, Drosophila is well suited for genetic and cardiac physiologic studies (36, 57). Fly genetics has successfully been used to identify novel genes involved in cardiomyopathies, and detailed cardiac responses may be measured through use of various imaging techniques of the Drosophila heart tube (2, 3, 7, 39, 40, 57). Previously, we established models for the effects of chronic and multi-generational hypoxia exposure in the *Drosophila*

heart (62). We found the effects of chronic hypoxia on heart function are particularly pronounced with HIF deficiency, consistent with the known roles of HIF in mediating these hypoxia responses in human populations (1, 29, 42, 48, 49, 62). Notably we also observed significant cardiac alterations in flies selected for survival to multi-generational hypoxia exposure ('hypoxia-selected', HS), with notable persistent cardiac restriction compared to wild type fly lines that were chronically exposed only as adults to the same reduced oxygen levels (chronic hypoxia, CH). We therefore conducted cardiac studies in Drosophila to further analyze potential underlying homeostatic shifts between HS and CH populations, and identified roles of the mammalian calcineurin A homologs CanA14F and Pp2B during the cardiac response to hypoxia.

Calcineurin promotes a state of cardiac hypertrophy and serves as a well-known marker differentiating pathological heart disease from physiological growth (32, 37, 58). Transgenic mice expressing activated calcineurin show cardiac hypertrophy, while calcineurin inhibition ameliorates induced cardiac hypertrophy in mammalian models (54, 55). Calcineurin is a calcium/calmodulin-dependent protein phosphatase with two subunits, the catalytically active CanA subunit and the regulatory calcium/calmodulin binding CanB subunit. Drosophila homologs include three CanA genes (CanA1, CanA14F, and Pp2B) and two CanB genes (CanB and CanB2; 31). The CanB subunit is essential for Drosophila flight muscle differentiation, and regulates catalytic activity of CanA through stabilizing binding of calcium/calmodulin (14). Reducing CanB expression results in cardiomyopathy in both Drosophila and mice, by impairing cardiomyocyte growth, and in flies causes larval lethality (30, 37, 46). CanA1 is considered the ancestral copy of calcineurin A in Drosophila, and appears to have more recently acquired a novel role in the flys immune response (8, 28). CanA14F and Pp2B share 73-78% and 77%, respective to amino acid sequence identify to the human calcineurin A protein. CanA-14F is highly expressed in early embryonic development, and Pp2B showing expression throughout

development and in adult nervous tissues (4). CanA-14F and Pp2B are neighboring genes on chromosome X, likely arising by gene duplication in Diptera (35). A constitutively active form of the Drosophila Pp2B gene was able to induce cardiac widening in the apical conical chamber under normoxic conditions (26, 37, 59). While inhibiting calcineurin in mammalian models reduced pathological hypertrophy, a role for calcineurin in long-term hypoxia adaptations, where hypertrophy can be a defining feature of chronic disease, has not yet been identified.

Here, we performed cardiac-specific expression profiles from flies exposed to chronic hypoxia (CH, 3 weeks 4% O2) or over many generations (HS, 66). We found that expression of the calcineurin homologs CanA14F and Pp2B to be highly down-regulated in HS flies compared to CH. Heart-specific knockdown of CanA14F or Pp2B in normoxic flies phenocopied the decreased cardiac size observed in HS flies with multi-generational hypoxia exposure. Under hypoxia, this resulted in a further cardiac restriction, although alterations in cardiac function were dependent on whether calcineurin knockdown occurred in the myocardial cells alone or in both myocardial and pericardial cells. Finally, both over-expression of calcineurin A (specifically the Pp2B homolog) and knockdown of the calcineurin repressor, sprouty, preserve cardiac diameters, consistent with a role for calcineurin in mediating hypoxia-induced cardiac remodeling. Our results provide insight into the mechanisms underlying cardiac adaptations to high altitude and the effects of long-term chronic hypoxia exposure on the heart, and define novel roles for calcineurin A as a marker of long-term cardiac disease state.

4.3 Materials and Methods

4.3.1 Genetic lines:

To study long-term adaptive mechanisms we used three *Drosophila* populations selected for more than 250 generations for survival at 4% O_2 ('hypoxia-selected', abbreviated HS), a level that is normally developmentally lethal. Three distinct and genetically isolated, normoxia-raised populations maintained in parallel with the selected populations were used as outbred control populations ('normoxia controls', abbreviated NC) (66). To maintain the long-term genetic integrity of the selected populations, only three week old, male HS and NC flies were used for cardiac function and structure studies.

Specific knockdown of genes was achieved using the Gal4 system, which allows tissue-specific expression of Gal4 and induction or knockout of genes by binding an upstream activation sequence (UAS) of the gene of interest. We used two cardiac-specific drivers, Hand4.2-Gal4 and TinC-Gal4, to modulate gene expression in myocardial cells (TinC), or myocardial and pericardial cells (Hand4.2). As shown in Figure 4.4 A, the myocardial tube is the central dorsal tube in the *Drosophila* abdomen, with pericardial cells surrounding and primarily serving a supportive role to the myocardial cells.

The following UAS-RNAi lines were used to control candidate gene expression; v109858 (CanA14F-RNAi KK), v30105 (CanA14F-RNAi GD), v103144 KK (PP2B-RNAi KK), v46873 GD (PP2B-RNAi GD), v6948 (sty-RNAi/TM3 GD) from the Vienna Drosophila Resource Center (http://stockcenter.vdrc.at/control/main) and B22025 (UAS-PP2B), B31515 and B31516, (sty-TRiP) from the Bloomington stock centers (http://flystocks.bio.indiana.edu/).

Here, female flies were used for cardiac functional and structural assessments, RNA-seq and triglyceride assays in response to chronic hypoxia. Three week old w1118, flies ('wt') were used as the laboratory standard line, as this was the original genetic

line used to previously establish cardiac hypoxia responses (62). For appropriate control comparison to the UAS-RNAi lines, specific background lines, descended from wt, were crossed to drivers and used for the control comparisons within each experiment: 'Luc-TRiP' ('B35789'), 'KK control' (y,w[1118];PattP,y[+],w[3'']), 'GD control' (w[1118]).

4.3.2 Microarray and RNA-seq:

Isolated hearts from the HS flies (at 4% O_2) and NC populations (at 21% O_2) were used for microarray analysis. Hearts were dissected from 50 three week old males for each sample from three each HS and NC populations. Array quality metrics qualified at least one microarray from each population. We also performed RNA-seq, optimized for *Drosophila*, on hearts from 50 three week old female wt flies under conditions of normoxia or chronic hypoxia (three weeks 4% O_2). Transcriptomics data was filtered based on a minimum fold change of +/- 1.2 with a corresponding p-value <0.05 relative to control lines.

Bioinformatic analyses of gene expression changes, including overall fold changes, comparative and KEGG pathway analysis, was performed using available online tools to describe differential patterns between hypoxia-selected and control populations. Gene functional annotation and classification was generated using DAVID bioinformatics module. This module systematically maps large transcriptomics datasets to their associated biological annotation and highlights statistically enriched biological annotation from databases containing linked terms and content. For additional details, please visit david.abcc.ncifcrf.gov/ (19, 20). Additionally, mapping of Drosophila orthologs to known mammalian metabolic pathways was performed using the KEGG array tool. For additional information, please visit genome.jp/kegg/ download/kegtools/ (22). Heat map generation of DAVID and KEGG identified gene subsets was carried out using open

source MeV software provided generously to the public by TIGR (tm4.org/mev) (44).

4.3.3 Heart function analysis experiments:

Semi-intact hearts were dissected and equilibrated under normoxic conditions as previously described (40, 62). Exposed hearts were perfused with artificial hemolymph bubbled with room air and filmed under 21% O_2. Dissolved O_2 content was monitored and recorded using a Qubit Systems OX1LP polarographic oxygen probe, calibrated and corrected for mean barometric pressure (758 mm Hg), salinity (8.22) and perfusate temperatures (21-22C). The O_2 content was monitored at several time points during experimental sessions. To ensure that individuals in all genotypes received equivalent treatments, each experiment dish contained small numbers of both control and experimental heart preparations. Transport for HS or chronic hypoxia exposed NC or wt flies in this study used a humidified chamber (Modular Incubator Chamber, MIC 101, Billups-Rothenberg) filled with calibrated 4% O_2 and kept at room temperature.

Hypoxia-selected fly population:

Heart function in HS and NC flies was analyzed using 3-week old males. HS flies were removed from the 4% O_2 hypoxia chamber, briefly dissected under reduced oxygen conditions (hemolymph with a stream of 4% O_2 duration $<$15 minutes), then returned to their native 4% O_2 for 30 minutes prior to filming under 4% O_2 conditions. To control for possible prolonged effects of exposure to their relative hyperoxia during dissection of HS flies, we monitored heart function in both populations under their relative normoxia (4% or 21% O_2) at two time points (60 and 90 minutes) after dissection, which exhibited similar cardiac parameters, and used the average of the two measures (see Chapter 3 for

detailed studies).

Chronic hypoxia assay:

As previously described, we chose three weeks as the exposure period for the chronic hypoxia challenge as this is the median age for wt adult flies and heart function is not yet significantly affected by aging- related cardiac effects (62). We chose 4% O_2 because flies can live a normal lifespan at that level of hypoxia, although they cannot reproduce, except after experimental selection (i.e. HS flies; 17, 58, 60). Two- to three-day old adult flies were placed in sealed, humidified chambers at room temperature for three weeks, under 4% O_2. These chambers are stable and reliable for 24-48 hours at 4% O_2 and were flushed daily to maintain stable O_2 levels and eliminate the minimal build-up of carbon dioxide. Food (equilibrated for 24 hours at 4% O_2) was changed two times per week in the glove box under hypoxic conditions. After three weeks, flies were removed from the chamber, dissected under normoxic conditions and equilibrated for 30 min in artificial hemolymph at 21% O_2 (NC and all candidate gene knockdown experiments) or 4% O_2 (HS flies) prior to filming.

4.3.4 Optical Imaging and Heart Function Analysis:

Direct immersion optics were used in conjunction with a digital high-speed camera (120-150 frames/sec, Hamamatsu EM-CCD) mounted on a Leica DMLFSA microscope (McBain Instruments, Chatsworth, CA) to record 30 second movies of beating hearts; images were captured using HC Image (Hamamatsu Corp.). Cardiac function was analyzed from the high speed movies using Semi-automatic Optical Heartbeat Analysis software (SOHA, free download for research purposes at www.sohasoftware.com) which

quantifies diastolic/systolic intervals, cardiac arrhythmia, diastolic/systolic diameters, fractional shortening, and produces M-mode records from the videos (39, 40).

4.3.5 Statistical analysis:

All statistical analyses were performed using Prism Statistical Software (Graph Pad, Inc, version 6). Data sets were first tested for normal (Gaussian) distributions using the DAgostino and Pearson omnibus normality test. For data sets that passed this test, we used a regular t-test, 1-way or 2-way ANOVA as appropriate. Most of the studies used two or more genotypes and two experimental conditions (normoxia v. hypoxia) so significance was determined using a 2-way ANOVA followed by multiple comparisons post hoc tests, as appropriate (specific tests indicated in figure legends). Data sets that did not show a normal distribution (heart period and diastolic interval) were analyzed for significance using a nonparametric two-tailed t-test, or KruskalWallis test followed by Dunn multiple comparisons post hoc tests, as appropriate (specific tests indicated in figure legends). We excluded hearts that did not beat after exposure to hypoxia (N $<1\%$ of total dataset, controls and experimental groups) or if cardiac parameters were outlier and fell outside 2 standard deviations of the population. In all cases, $P<0.05$ was taken as significant.

4.4 Results

4.4.1 Multi-generational exposure to chronic hypoxia causes persistent cardiac restriction in hypoxia-selected populations

Previously, we established the effects of chronic hypoxia exposure within a single lifetime in the wild type (wt, *w1118*) heart caused significantly altered cardiac performance. The effects included increased heart period and slight, non-significant decreases in fractional shortening with preserved cardiac diameters. In flies selected for survival at reduced oxygen levels over many generations (hypoxia-selected, HS flies) we observed similar increases in heart period due to increases in diastolic interval, but with significant decreases in systolic interval. We also observed significant decreases in fractional shortening due to significant reductions in both diastolic and systolic diameters (62). We wished to determine which traits were due to the lifetime exposure to chronic hypoxia, and which were due to the effects of selection pressure in order to probe the genetic basis for heritable cardiac responses to hypoxia (explored in further detail in Chapter 3).

We exposed NC flies, which have genetic backgrounds similar to the selected flies, under 4% O_2 for three weeks (chronic hypoxia) using our previously established protocol (Figure 4.1 A). We then quantified cardiac function in these 'NC hypoxia-raised' flies at 21% O_2. For comparison purposes, we also present previously published data for w1118 flies exposed to the same chronic hypoxia protocol ('CH' Figure 4.1). In hearts from the NC hypoxia-raised flies, we observed a tendency toward longer heart periods (HP) although this change was no significant as previously reported for *w1118* flies exposed to CH. Contractility, as measured by fractional shortening (FS), was significantly decreased after chronic hypoxia in NC and CH flies (Figure 4.1 E hatched vs. solid bars). However, hearts from CH and hypoxia-raised NC hearts exhibited reductions in contractility that were due to non-significant decreases in the diastolic diameters (DD) and unchanged

systolic diameters (SD; Figure 4.1 E-G, white and grey bars). These results suggest that hearts from both wt and NC lines response similarly to chronic hypoxia treatment.

To determine which cardiac effects were due to selection pressure, we raised HS flies for two generations flies where environmental selection pressure is removed under 21% O_2 and quantified cardiac function in F2 progeny at 21% O_2 ('HS normoxia-raised'). HS flies raised under hypoxia (4% O_2, their "native" and relative normoxia level), showed the expected longer heart periodicity compared to normoxia-raised controls (Figure 4.1 B, compare dark gray hatched bar with white and solid gray bar). The mean HP in the HS flies raised under 21% O_2 was significantly reduced to levels that were not different from controls. This suggests the chronotropic effects of chronic hypoxia are likely due to the immediate lifetime exposure and are not the result of heritable genetic changes.

The notable differences observed for hearts from HS flies were the significant decreases in mean DD and SD whether HS flies were reared under either 21% O2 or 4% O_2. Strikingly, this did not translate to a significant increase in FS between the HS flies between hearts from HS flies reared under 21% or 4% O_2 or to the NC populations (Figure 4.1E-G, black solid and hatched bars). Overall, this suggests persistent cardiac restriction in both SD and DD in the HS flies, regardless of rearing condition, and which preserves FS relative to controls.

As noted previously, hypoxia-reared HS flies are smaller in overall size than both F2 progeny of HS flies raised under normoxic conditions, as well as NC and *w1118* controls, probably due to oxygen limitation during development (15, 23, 64, 66). To confirm that the smaller heart size was not simply due to a reduction in the overall size of the fly we measured the abdominal segment and tibia lengths in normoxia and hypoxia-reared HS and NC flies. Further, we compared these corrected diameters to the normoxia-raised HS flies, which return to NC body size when raised under normoxia (23). The average segment length of hypoxia raised HS flies was 67% of the abdominal segment length of

either normoxia reared NC or HS, and similar for tibial measurements (62, 66). When diameter measurements were normalized to correct for this 67% difference in body size we found that the average DD was still significantly smaller for HS flies (54.3 um NC, compared to 36.0 um HS uncorrected and 47.9 um HS corrected or 43.1 um HS normoxia-raised, $p<0.001$ 2-way ANOVA; see Supplemental materials for population differences).

4.4.2 Differential expression of genes after chronic or multi- generational hypoxia exposures.

The observed persistent reduction in cardiac size in HS flies but not in CH flies suggested that cardiac restriction is a response to multi-generational hypoxia exposure but not chronic hypoxia exposure in a single generation alone. To identify potential genetic mechanisms underlying the HS cardiac restriction, we performed microarray analysis on isolated hearts from NC and HS lines using transcriptome arrays covering 13,061 total predicted genes from the *Drosophila melanogaster* genome. Significant differential gene expression was defined as genes that were 1.2 fold over- or under-expressed relative to controls with p-values <0.05. We identified a total of 449 genes that were up-regulated and 623 genes that were down-regulated in hearts from HS flies compared to NC.

We also performed RNA-seq analysis on w1118 control and CH exposed flies. We identified a total of 267 genes that were up-regulated and 396 genes that were down-regulated in hearts from CH flies compared to their wt controls. When we compared the up-regulated genes that were up-regulated in both HS and CH hears, we identified 22 genes that were commonly over-expressed and 50 that were commonly under-expressed. A further 22 genes were contra-regulated between HS and CH populations; 7 up- in HS only and down- in CH and 15 up- in CH only and down- in HS. Figure 4.2A features a

Venn diagram summarizing the significant 1.2 fold over- or under-expressed genes from the CH and HS populations. A volcano plot of significant log2 fold changes highlights ”outlier” genes in each category with the highest differential expression changes in each category (Figure 4.2C).

We generated heatmap groupings based on known KEGG metabolic pathways and DAVID gene ontology clustering, which uses statistical approaches to group genes based on multiple ontology databases, and looks for enrichments within our differentially expressed genes. This analysis identified genes in broad categories relating to cardiac remodeling; calcineurins, proteasome, mitochondrial, oxidoreductase, tracheal system, extracellular matrix genes and biological responses, including stress (Figure 5.1C). We identified genes with as yet unknown functional ontologies, labeled as ”unknown” (Figure 4.2 B,C). The ”various” category includes the 22 contra-regulated genes between HS- and CH populations. Heatmaps for HS and CH only differential expression can be found in the supplemental information, as well as a volcano plot including all genes with significant expression change.

We looked for gene candidates which were unique to HS populations and which may underlay the persistence of cardiac restriction in HS flies compared to CH populations. Importantly, we found that while the calcineurin gene family shows similar misregulation in hearts from both CH and HS flies, the CanA14F and Pp2B homologs are significantly more down-regulated in the HS hearts, and CanA14F is the most down-regulated gene in HS arrays (down 25 fold). We chose to focus on the calcineurin family for further cardiac function studies, given calcineurins known role in mammalian and Drosophila cardiac hypertrophy and metabolism when over-expressed, but relatively poorly understood role in cardiac metabolism and restriction when repressed (14, 26, 47, 55, 59).

4.4.3 Cardiac response to chronic hypoxia with knockdown of calcineurins in pericardial and myocardial cells.

We quantified heart function in flies after three weeks of either normoxia or hypoxia exposure in flies with knockdown of either of the two primary *Drosophila* Calcineurin A homologues: CanA14 or Pp2B (see Figure 4 A for assay protocol). Calcineurin gene knockdown was first performed using the Hand4.2-Gal4 driver which expresses selectively in cardiomyocytes and pericardial cells from early development (see Figure Figure 4 A), since microarrays and RNA-sequencing were performed on tissue dissections containing primarily these cell types. Notably, the Pp2B-RNAi flies survived to three weeks in normoxia conditions, few Pp2B-RNAi flies survived to three weeks under chronic hypoxia conditions (empty bar on right; full survivorship data in Supplemental data) suggesting this level of knockdown in the heart and supporting pericardial cells is too severe during hypoxia exposure.

We observed decreases in mean DI in both Pp2B-RNAi and CanA14F-RNAi flies compared to controls at normoxia (Figure 4.3 B,C). In the case of Pp2B-RNAi the decreased DI also resulted in significant decreases in overall HP (Figure 4.3 B,C). Compared to Figure 4.1 A where we saw significant increases in HP after chronic hypoxia exposures in wild type lines, HP and DI were reduced after three weeks chronic hypoxia in the Hand4.2-Gal4 >KK control fly line (Figure 4.3 B,C). We attribute the variations observed in control heart rate changes after chronic hypoxia exposure to the dynamic nature of heart rate and diversity in genetic backgrounds response to stress. SI only changed significantly in CanA14F flies, where it increased after chronic hypoxia compared to hypoxia controls (Figure 4.3 D).

Heart size, measured as DD, was dramatically decreased in response to cardiac knockdown of both CanA14F-RNAi and Pp2B-RNAi under normoxia conditions

compared to controls (Figure 4.3 F). SD in Pp2B-RNAi knockdown hearts were also significantly reduced, whereas in the CanA14F-RNAi hearts showed a non-significant trend toward smaller SD (Figure 4.3 G). Consequently, Fractional shortening (FS, a measure of contractility) was preserved for all genotypes under normoxia (Figure 4.3 E). After three weeks chronic hypoxia, both DD and SD were significantly reduced in CanA14F-RNAi hearts compared to normoxia control hearts and caused significant decreases in FS (Figure 4.3 E-G).

In order to rule out an effect of genetic background, we repeated the pericardial and myocardial specific knockdown using a separate UAS-RNAi lines with different genetic backgrounds ("GD" lines, VDRC), that also target CanA14F-RNAi and PP2B-RNAi and observed largely similar effects (see Supplemental Information).

4.4.4 Cardiac response to chronic hypoxia with knockdown of calcineurins in myocardial cells.

We next wondered if persistent cardiac restriction by inhibiting expression of either Drosophila homolog of Calcineurin A could be found by knocking down expression in myocardial cells alone, without additional knockdown in the associated pericardial cells (Figure 4.3 A for assay protocol; Figure 4.4 A for tissue expression). We used the TinC-Gal4 driver that expresses selectively only in cardiomyocytes from early development, in contrast to the Hand4.2-Gal4 driver which expresses in both cardiomyocytes and pericardial cells. While the Hand4.2>Pp2B-RNAi flies largely did not survive three weeks chronic hypoxia conditions (Figure 4.3), TinC >Pp2B-RNAi knockdown flies did survive and allowed assessment of cardiac function after chronic hypoxia exposure (Figure 4.4).

We observed decreased HP and DI due to myocardial specific Pp2B-RNAi knock-

down flies, similar to what we observed for Hand4.2-Gal4 mediated knockdown in both myocardial and pericardial cells (Figure 4.3 B,C). Interestingly, we observed increases in both parameters in the CanA14F-RNAi knockdown hearts. Following chronic hypoxia exposure, we observed significant decreases in HP and DI in control and CanA14F-RNAi knockdown hearts compared to normoxia, as seen previously for the Hand4.2-Gal4 mediated knockdown in controls. SI was significantly reduced in control lines after chronic hypoxia exposure (Figure 4.4 D). SI was significantly higher in CanA14F-RNAi flies after chronic hypoxia compared to hypoxic control flies, similar to the results using the Hand4.2-Gal4 driver (Figure 4.4 D).

DD was significantly reduced compared to controls in both CanA14F-RNAi and Pp2B-RNAi knockdown hearts under both normoxia or after chronic hypoxia (Figure 4.4 F). These results are consistent with our findings using the Hand4.2-Gal4 driver (compare to Figure 4.3 F). Mean SD under normoxia did not change in response to CanA14F-RNAi knockdown but there was a significant reduction in Pp2B-RNAi hearts. Following chronic hypoxia, the mean SDs in the knockdown hearts tended to be smaller but this did not reach significance as following knockdown with the stronger Hand4.2-Gal4 driver (compare to Figure 4.3 G). Decreases in FS in CanA14F-RNAi under normoxia were due to decreases in DD, not SD, and copy non-significant trends observed using the Hand4.2-Gal4 driver (Figure 4.4 E-G). Interestingly, only the TinC >Pp2B-RNAi flies show significantly reduced FS under chronic hypoxia (Figure 4.4 E). We again repeated the myocardial specific knockdown using a separate genetic background (”GD”) of CanA14F-RNAi and PP2B-RNAi to largely similar effect (see Supplemental Information).

4.4.5 Over-expression of Pp2B and knockdown of the calcineurin suppressor, sprouty, alter cardiac function under normoxia

Since cardiac hypertrophy is orchestrated by over-expression of calcineurin through well-established pathways in mammals, we sought to emulate over-expression of calcineurin in Drosophila under conditions of hypoxia, where we typically see knockdown of calcineurin, and cardiac restriction. Using the TinC-Gal4 driver, we performed knockdown of sprouty, using sty-TRiP, which is suggested to act in negative feedback to repress calcineurin (53). and over-expression of calcineurin A using UAS-Pp2B line, which is known to cause cardiac hypertrophy in the conical regions of the Drosophila heart (26). The available GD lines of sty knockdown did not survive development when crossed to TinC drivers. However a TRiP line sty knockdown did survive to three weeks, but only under normoxia, and thus was used in studies compared to its Luc-TRiP control. Heart function in all flies was measured at 21% O2.

In Figure 4.5 A, we present a schema of negative regulation of sty by calcineurin and downstream activation of hypertrophy. Predicted pathways using Drosophila homologs are on left, with the established mammalian pathway on right. In mammals, calcineurin is a known marker distinguishing progression of pathological hypertrophy from reversible physiologic growth.

Heart period (HP) is reduced in sty-TRiP compared to Luc-TRiP controls (Figure 4.5 B). In contrast, HP is reduced in Pp2B-RNAi, but not CanA14F-RNAi knockdown, and elevated with UAS-Pp2B over-expression in comparison to Pp2B-RNAi. These changes are explained by identical changes in DI, while SI are unchanged in UAS-Pp2B over-expression or knockdown backgrounds, but significantly reduced in sty-TRiP knockdown (Figure 4.5 C,D).

Knockdown of sprouty, using sty-TRiP, and over-expression of calcineurin A

using UAS-Pp2B line, both caused reductions in FS under normoxia due to increases in SD with non-significant alterations in DD (Figure 4.5 E-G). This is particularly in contrast to knockdown observed with Pp2B-RNAi, in which FS is preserved due to reduced DD and SD. Interestingly, the sprouty knockdown mimics CanA14F over-expression in preserving or slightly elevating DD, and increasing SD, causing the observed reduction in FS. This supports our hypothesis that it is reduced calcineurin signaling which causes cardiac restriction observed originally in hypoxia-selected flies, which showed significant reduction in CanA14F that was not observed in chronically hypoxic flies.

4.4.6 Hypoxia-selected flies exhibited a cardiac specific, calcineurin dependent shift toward glycolysis and triglyceride storage

In order to explore the underlying metabolic shifts indicated by differential gene expression, we performed gene ontology based pathway analysis for glycolysis, oxidative phosphorylation and triglyceride synthesis pathways (see materials and methods, Figure 4.6). A network analysis from within our HS mircroarrays indicates a shift in HS flies towards genes involved in glycolysis and away from genes involved in fatty acid oxidation and triglyceride synthesis (Figure 5.6 A, top). On the other hand, hearts from CH exposed flies showed increased expression of lactate dehydrogenase, suggesting a shift toward anaerobic metabolism (Figure 5.6 A bottom).

To test whether knockdown of calcineurin and sprouty were also involved in metabolic shifts during normoxia or hypoxia, we performed whole fly triglyceride assays after knocking down sty-TRiP or CanA14F-RNAi in the heart (Figure 5.6 B). Interestingly, knockdown of sty-TRiP decreased triglyceride accumulation in relation to Luc-TRiP controls under normoxia. We did not observe triglyceride accumulation after knockdown of CanA14F-RNAi under normoxia. Accumulation of triglycerides is

commonly found after exposure to chronic hypoxia, as the body shifts toward glycolysis (32, 59). We observed similar shifts in our KK control lines after chronic hypoxia, but even greater whole fly body triglyceride accumulation after hypoxia with knockdown of CanA14F-RNAi, indicating a potential role shifting HS flies further toward glycolysis and away from oxidative phosphorylation.

4.5 Discussion

In humans, multi-generational adaptation to chronic hypoxia leads to either enhanced cardiac function or dysfunction in a population- and genotype-specific manner (8, 15, 21, 28, 38). Our laboratory model of hypoxia-selected flies (HS, selected for multi-generational survival under chronic hypoxia) exhibited significant changes in all cardiac parameters measured (Figure 5.1; Chapter 3, 40). Our data from HS flies raised for two generations under normoxia indicate that the chronotropic effects are likely due to the hypoxia exposure during an individuals lifetime and are not the result of heritable genetic changes. On the other hand, the cardiac restriction we observed in hearts from HS flies persisted even after removing immediate selection pressure on the HS populations and assaying at 21% O_2 (62). Notably, we do not see significant cardiac restriction when rearing control populations under lifelong chronic hypoxia (CH), suggesting cardiac restriction in HS flies is a heritable trait.

In order to identify the genes which may explain the persistent reduction in HS cardiac size, we analyzed transcriptome data obtained from microarrays and RNAseq performed on isolated hearts from both HS and CH wild type flies, to seek differentially expressed genes in the HS arrays. While we found shared regulation of genes in mitochondrial and proteosomal families (down-regulation), and tracheal, oxidoreduction

and immune response (up-regulation), many of these pathways have been previously reported for organisms facing chronic hypoxia exposure, and do not appear unique to HS fly hearts (13, 27, 31, 32). Further, of all shared and differential genetic changes in up- and down-regulation of genes between both hypoxia-selected and chronically hypoxic fly arrays, the calcineurin A homolog CanA14F was the most significantly reduced gene, leading us to predict calcineurin knockdown would cause cardiac restriction in hypoxia-nave flies. Calcineurin is a well known mediator of metabolism and cardiac hypertrophy during hypoxia exposure in mammals, thus we wished to understand the role calcineurin plays in the heart during chronic hypoxia and to improve our understanding of the progression of cardiac disease.

Indeed, using established assays of cardiac function, we found that knockdown of the primary calcineurin A homologues, CanA14F or Pp2B, caused cardiac restriction similar to HS flies with multi-generational selection, notably, a persistent restriction in wild type flies under normoxia conditions that was reminiscent of what we observed for HS flies with multi-generational hypoxia selection. Further, the restriction caused by knockdown of calcineurin homologs became worse under hypoxia, causing a reduction in fractional shortening. Given that strong knockdown of Pp2B-RNAi in both pericardial/myocardial cells survive under normoxia but not under chronic hypoxia, it is likely that this gene plays an important role in hypoxia stress. Further, myocardial-specific Pp2B-RNAi knockdown was sufficient to induce the changes observed in response to combined pericardial/myocardial tissues and in HS flies, suggesting that this response in heart-autonomous. Further, over-expression of UAS-CanA14F or knockdown of sprouty, a known negative regulator of calcineurin, both preserved cardiac size relative to controls.

In human populations, hearts from well-adapted Tibetans exhibit increased myocardial glucose uptake and lower cardiac phosphocreatine-to-ATP ratios, even when these high altitude natives live for many years at low altitude (13, 16, 24, 33). Calcineurins

have known roles promoting the metabolic shift toward glycolysis during pathological cardiomyopathies. Further, active calcineurin acts to stabilize the hypoxia-inducible factor, HIF, by preventing HIF from being targeted for degradation and thus allowing HIF to further promote metabolic shifts during hypoxia toward glycolysis and away from fatty acid oxidation (42, 58). In support of this, we found knockdown of CanA14F in the fly heart had little effect on whole fly body triglyceride accumulation under normoxia, but appeared to cause triglyceride accumulation during hypoxia. Past studies have shown metabolic alterations in whole HS flies genetically and in flight muscle indicating a shift from oxygen-expensive pathways and towards increased glycolysis (11, 12, 63, 67). Notably, our KEGG bioinformatic analysis of HS cardiac microarrays also noted a shift from genes involved in oxidative phosphorylation and toward genes involved in glycolysis and triglyceride synthesis in the HS heart, a shift that was not found in our chronically hypoxic fly arrays. Further, several of the glycolysis and triglyceride synthesis promoting genes we identified as up-regulated in the HS hearts were found to have predicted hypoxia-responsive elements (HREs), consensus binding sites for HIF/sima, whose role we previously established in the Drosophila cardiac response to hypoxia (62).

Human populations that are well adapted to chronic hypoxia over many generations, such as Tibetan Sherpas, show beneficial cardiac adaptations to their native high altitudes, including; reduced right ventricular hypertrophy, increased ability to raise maximal heart rate and cardiac output at altitude, and increased myocardial glucose uptake (16, 29). Other high altitude populations exhibit signs of cardiac disease due to multi-generational and continued chronic exposure to hypoxia (10, 61, 65). Recent evidence from high altitude and model organisms selected for long-term adaptation indicates shared genetic and physiologic adaptations (21, 38, 52). Intriguingly, one of the genomic regions found to be under positive selection in both HS fly and multiple well-adapted human populations, contains SPRY, the fly homolog sprouty, a proposed

negative regulator of EGF via calcineurin, although no mechanism was suggested for this gene in adaptation (21, 53). We suggest sprouty may serve as an important regulator during development and organism growth in both flies and humans, allowing survival or growth in multi-generational hypoxia with downstream effects on the regulation of calcineurin A causing cardiac effects under chronic hypoxia.

A previous study in the HS flies examined whole body HS fly arrays and similarly altered regulation of EGF signaling (67). As further validation of the results of our own cardiac-specific HS arrays from these same HS populations, we also noted significant contra-regulation of the transcriptional suppressor hairy, which was previously found to coordinate metabolic suppression via Notch signaling in whole body HS fly arrays (67). More recent whole genome sequencing of HS populations and HS populations raised under normoxia for 12 generations were performed on HS flies to examine transcriptome changes critical to development, and thus survival, during hypoxia. Intriguingly, this genomic study highlighted Wnt pathway activation in normoxia-raised HS whole flies, with key polymorphisms noted near Pp2B, CanA14 and the Drosophila homolog of NFAT (15). We propose alterations in Wnt/EGF/sprouty signaling, identified previously as vital to hypoxia survival in HS flies, serves to reduce expression of the calcineurin A homologs, Pp2B and CanA14F, in the HS fly heart and cause persistent cardiac restriction.

Based on these studies as well as the present evidence, we propose a mechanism for calcineurin/EGF signaling during chronic hypoxia in the heart (Figure 5.6). Calcium influx, stabilizes calmodulin binding to calcineurin B, thus promoting activation of calcineurin A (CanA14F and Pp2B in *Drosophila*). Calcineurin serves many functions in the cell, including dephosphorylation of NFAT (in mammals) which allows translocation into the nucleus and activation of transcription factors, including GATA4 and MEF2, to induce hypertrophic genes. A reduction in calcineurin signaling would then reduce induction of hypertrophic genes, potentially via GATA4 or MEF2, leading to the cardiac restriction

phenotype we observe in HS and CanA14F/Pp2B knockdown hearts. Notably, NFAT signaling has not yet been identified in the Drosophila heart, and thus other transcription factors may be regulating cardiac growth directly by direct calcineurin activation (as in MEF2, GATA4), or through separate pathways. In mammals, calcineurin stabilizes HIF activity by preventing dimerization of the RACK complex, which marks HIF for degradation under normoxic conditions. HIF can then activate, by direct transcriptional activation on genes with HREs, such as VEGF, EPO and glycolytic genes. In mammals, VEGF is known to activate Calcineurin A directly, or through association with SPRY (*sprouty* in *Drosophila*). Given prolonged hypoxia, these genes orchestrate the switch from physiologic growth to pathologic hypertrophy as cardiomyocytes switch to hypertrophic gene activation, a switch away from glycolysis and increase in oxidative phosphorylation, and decrease in triglyceride accumulation.

We found knockdowns in calcineurin restrict cardiac size under normoxic and hypoxic conditions, and suggest Drosophila Pp2B and CanA14F have roles of cardiac remodeling during lifetime or chronic hypoxia exposure. In support of known hypertrophy pathways, a reduction in calcineurin would decrease hypertrophic genes and account for the observed cardiac restriction. Calcineurin and *HIF/sima* orchestrate metabolic changes in the heart, with reductions in calcineurin decreasing oxidative phosphorylation and increase glycolysis, which we saw as whole body accumulation of triglycerides during chronic hypoxia, and bioinformatics analysis suggesting switches to triglyceride accumulation. Further, when we altered sprouty function in Drosophila, we potentially released negative regulation of calcineurin, as observed in increased systolic diameter. Thus alterations in cardaiac diameters requires caclineurin knockdown, and may occur through suppression via the sprouty- or calcineurin/sprouty- mediated EGFR pathway.

In summary, we examined heart function and transcriptional changes in Drosophila populations selected through multi-generational exposure to severe hypoxic conditions.

Several adaptive genetic changes were identified in these HS flies that include down-regulation of calcineurin, down-regulation of oxidative phosphorylation genes and up-regulation of glycolysis. While multiple pathways undoubtedly contribute to cardiac remodeling observed in HS flies, we demonstrate that two Drosophila homologs of calcineurin A, CanA14F and Pp2B, are important in causing restriction in the Drosophila heart under normoxia or hypoxia. The cardiac remodeling mechanisms identified in this model may also play a crucial role in mammalian cardiac adaptation or the progression toward disease during prolonged hypoxia.

4.6 Acknowledgments

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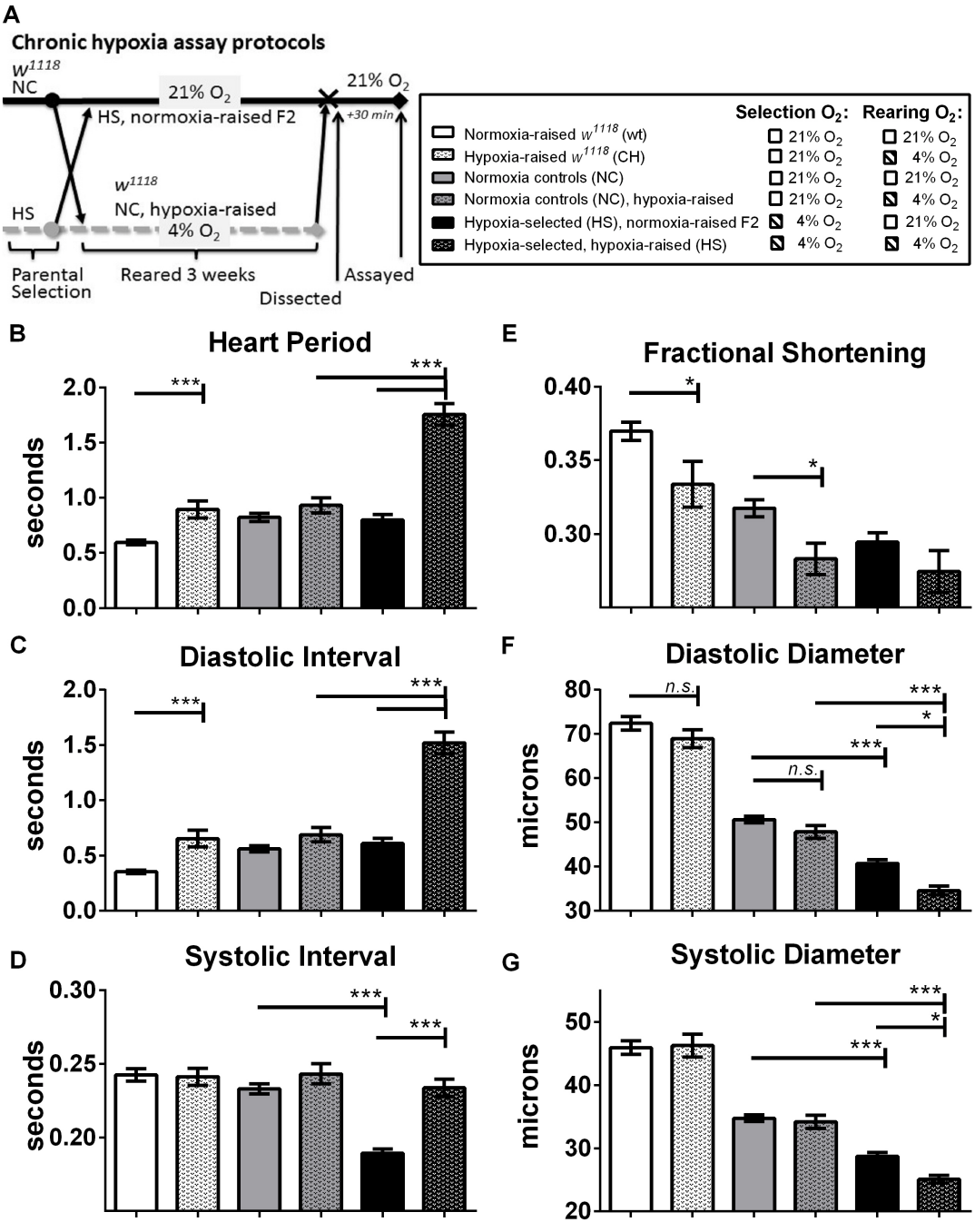
4.7 Figures

Figure 4.1 (facing page): Cardiac response to chronic hypoxia in w1118 flies (CH) compared to wild types and hypoxia-selected flies (HS) compared to their normoxia control (NC) populations, reared under either hypoxia or normoxia.
A) Protocol for chronic hypoxia treatment in w1118 flies, HS and NC populations. B) Exposure of w1118 flies to chronic hypoxia exposure (CH) significantly increased heart period compared to their wild type controls (wt), Normoxia-raised HS flies had heart periods that were indistinguishable from NC hearts and significantly faster than hearts from the same HS flies reared under their chronic hypoxia selection conditions. C) Diastolic intervals were significantly increased in CH hearts compared to wt, and HS flies in normoxia were again indistinguishable from NC whereas HS flies under hypoxia had significantly longer DI compared to normoxia-raised HS flies and hypoxia-reared NC flies. D) Systolic intervals were unchanged following chronic hypoxia in all groups except normoxia-raised HS flies, where systolic interval is significantly decreased compared to NC and HS. E) Fractional shortening is significantly decreased in hearts from both wt flies and NC flies exposed to chronic hypoxia. F) Diastolic diameters were unchanged following chronic hypoxia in w1118 and NC, but were significantly reduced in normoxia-raised HS compared to NC, and hypoxia-raised HS compared to both NC and hypoxia-raised HS flies. G) Similarly, systolic diameters were unaffected by chronic hypoxia in w1118 or NC lines, but are reduced in normoxia-raised HS compared to NC, and hypoxia-raised HS compared to both NC and hypoxia-raised HS flies.

*All values are mean +/- SEM for hearts (normoxia-raised w1118: N=75, hypoxia-raised w1118: N=41, NC: N=57, hypoxia-raised NC: N=41, normoxia-raised HS: N=60 HS: N=36). Data was analyzed by 2-way ANOVA with Tukey's multiple comparisons post-hoc test; n.s. = no statistical significance, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. See previous publications for further data on these populations.*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Chronic hypoxia assay protocols
w1118
NC
21% O2
21% O2
+30 min
HS, normoxia-raised F2
w1118
NC, hypoxia-raised
HS
4% O2
Parental Selection
Reared 3 weeks
Dissected
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Selection O2:
Rearing O2:
Normoxia-raised w1118 (wt) 21% O2 21% O2
Hypoxia-raised w1118 (CH) 21% O2 4% O2
Normoxia controls (NC) 21% O2 21% O2
Normoxia controls (NC), hypoxia-raised 21% O2 4% O2
Hypoxia-selected (HS), normoxia-raised F2 4% O2 21% O2
Hypoxia-selected, hypoxia-raised (HS) 4% O2 4% O2
B
Heart Period
seconds
C
Diastolic Interval
seconds
D
Systolic Interval
seconds
E
Fractional Shortening
F
Diastolic Diameter
microns
n.s.
G
Systolic Diameter
microns

Figure 4.2 (facing page): Cardiac-specific differential gene expression in hypoxia-selected populations and in wild type flies exposed to chronic hypoxia
Transcriptome analysis of RNA from isolated *Drosophila* hearts containing both myocardial and pericardial cells. A) Venn diagram depicting the number of genes differentially expressed in common or uniquely in hypoxia-selected flies (HS) compared to chronic hypoxia-exposed *w1118* flies (CH) under 4% O2. B) Relative log2 fold-change expression from HS vs. CH populations for genes with >1.2 fold up-regulated (red) or >1.2 fold down-regulated (green) and p-values <0.05. Contra-regulated genes between the populations are shown in yellow. Genes that change only in HS- or CH- are indicated with open triangles or open circles, respectively. C) Heat maps for gene families from each of the representative groups up-regulated, down-regulated or contra-regulated between HS and CH populations.

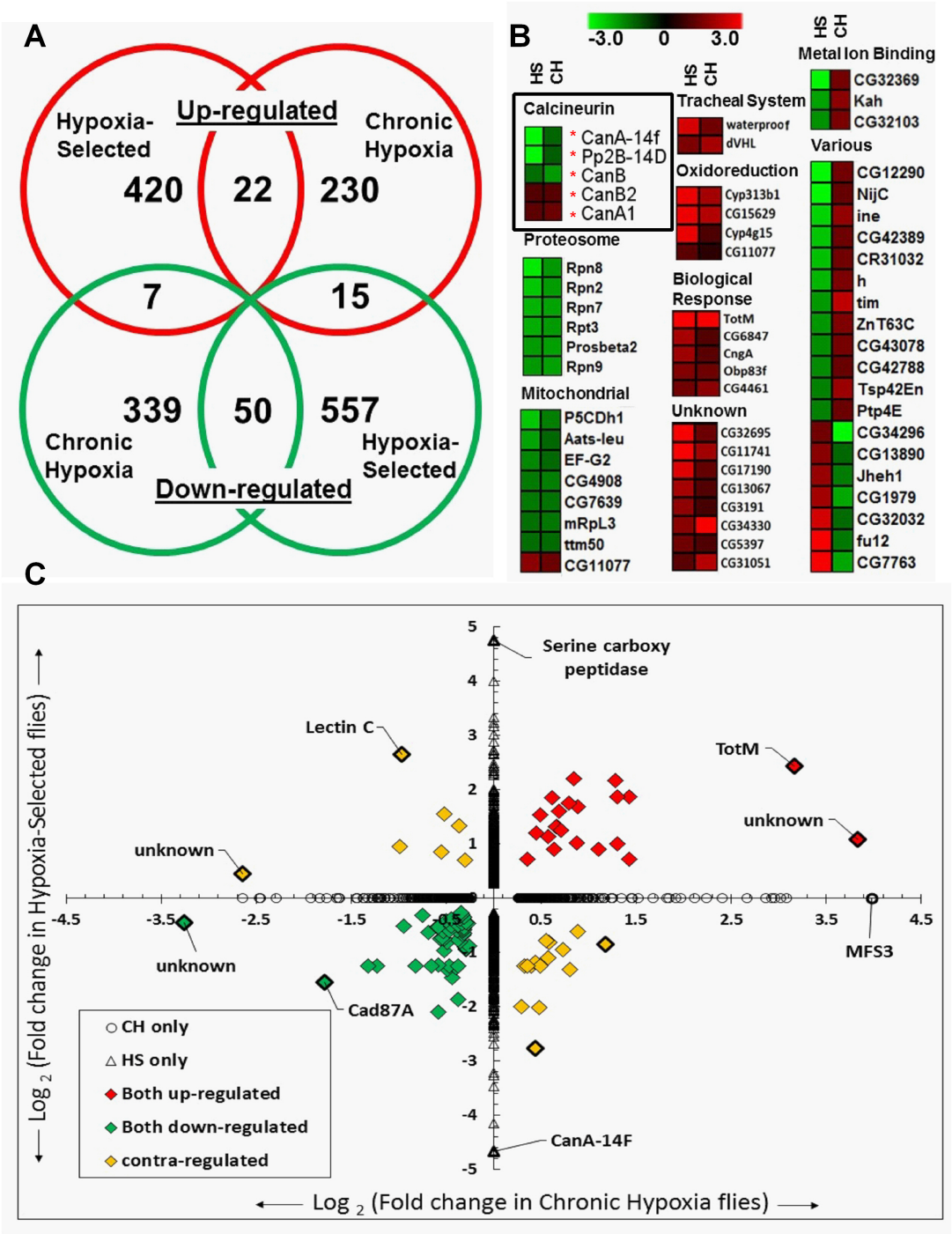

A
Up-regulated
Hypoxia-Selected
Chronic Hypoxia
420
22
230
7
15
339
50
557
Chronic Hypoxia
Hypoxia-Selected
Down-regulated
B
-3.0 0 3.0
HS CH
Calcineurin
* CanA-14f
* Pp2B-14D
* CanB
* CanB2
* CanA1
Proteosome
Rpn8
Rpn2
Rpn7
Rpt3
Prosbeta2
Rpn9
Mitochondrial
P5CDh1
Aats-leu
EF-G2
CG4908
CG7639
mRpL3
ttm50
CG11077
Tracheal System
waterproof
dVHL
Oxidoreduction
Cyp313b1
CG15629
Cyp4g15
CG11077
Biological Response
TotM
CG6847
CngA
Obp83f
CG4461
Unknown
CG32695
CG11741
CG17190
CG13067
CG3191
CG34330
CG5397
CG31051
Metal Ion Binding
CG32369
Kah
CG32103
Various
CG12290
NijC
ine
CG42389
CR31032
h
tim
ZnT63C
CG43078
CG42788
Tsp42En
Ptp4E
CG34296
CG13890
Jheh1
CG1979
CG32032
fu12
CG7763
C
Serine carboxy peptidase
Lectin C
TotM
unknown
unknown
unknown
MFS3
Cad87A
CanA-14F
CH only
HS only
Both up-regulated
Both down-regulated
contra-regulated
Log 2 (Fold change in Hypoxia-Selected flies)
Log 2 (Fold change in Chronic Hypoxia flies)

Figure 4.3 (facing page): Effect of chronic hypoxia on heart function in hearts with myocardial and pericardial knockdown of calcineurins.
A) Protocol for chronic hypoxia assay. B)-G) Heart function data is presented in normoxia controls on the left (white bars) and in flies exposed to three weeks chronic hypoxia on the right (grey bars) for controls, CanA14F-RNAi knockdown, and Pp2B-RNAi knockdown crossed to a Hand4.2-Gal4 driver causing selective expression in pericardial and myocardial cells from early development; hearts were dissected and assayed under 21% O2 conditions after three weeks at 21% or 4% O2 conditions. Note that while the PP2B-RNAi flies survived to three weeks in normoxia conditions, very few survived to three weeks under chronic hypoxia conditions (empty bars; full survivorship data in Supplemental data). B) Compared to normoxia controls, heart period (HP) was reduced in normoxic Pp2B-RNAi flies and after three weeks chronic hypoxia in control flies (condition: F=14.24, P=0.0002; genotype: F=8.089, P=0.0004; Interaction = 2.693, P=0.0704). C) Similarly, compared to normoxic controls, diastolic intervals (DI) were reduced in both CanA14F-RNAi and Pp2B-RNAi flies under normoxia, as well as after chronic hypoxia in control flies (DI condition: F=12.93, P=0.0004; genotype: F=3.787, P=0.0246; Interaction = 2.075, P=0.1286). D) Systolic intervals (SI) were significantly higher only in CanA14F flies after chronic hypoxia compared to hypoxic control flies (SI condition: F=0.6345, P=0.4268; genotype: F=7.793, P=0.0006; Interaction=1.189, P=0.3070). E) Fractional shortening (FS) was similar for all genotypes in normoxic flies, however, compared to hypoxic controls, CanA14F-RNAi shows reduced FS after three weeks chronic hypoxia exposure (condition: F=0.5326, P=0.4665; genotype: F=19.85, P¡0.0001; Interaction=9.043, P=0.0002). F) Diastolic diameters were decreased significantly in both CanA14F-RNAi and PP2B-RNAi backgrounds at three weeks normoxia conditions, as well as in CanA14F-RNAi background after chronic hypoxia exposure (condition: F=7.277, P=0.0009; genotype: F=13.77, P=0.0003; Interaction=7.277, P=0.0009). G) Systolic diameters were similarly significantly decreased in CanA14F and Pp2B backgrounds at three weeks normoxia, and in CanA14F flies after chronic hypoxia (condition: F=11.36, P=0.0009; genotype: F=1.347, P=0.2626; Interaction=F=2.948, P=0.0550).

*All values are mean +/- SEM (normoxia: N=37 KK control, N=33 CanA14F-RNAi, N=32 Pp2B-RNAi; chronic hypoxia: N=43 KK control, N=20 CanA14F-RNAi, N=20 Pp2B-RNAi). Data was analyzed by 2-way ANOVA and Sidak or Dunn multiple comparisons tests; n.s. = no statistical significance, * <0.05, ** p<0.01, *** p<0.001.*

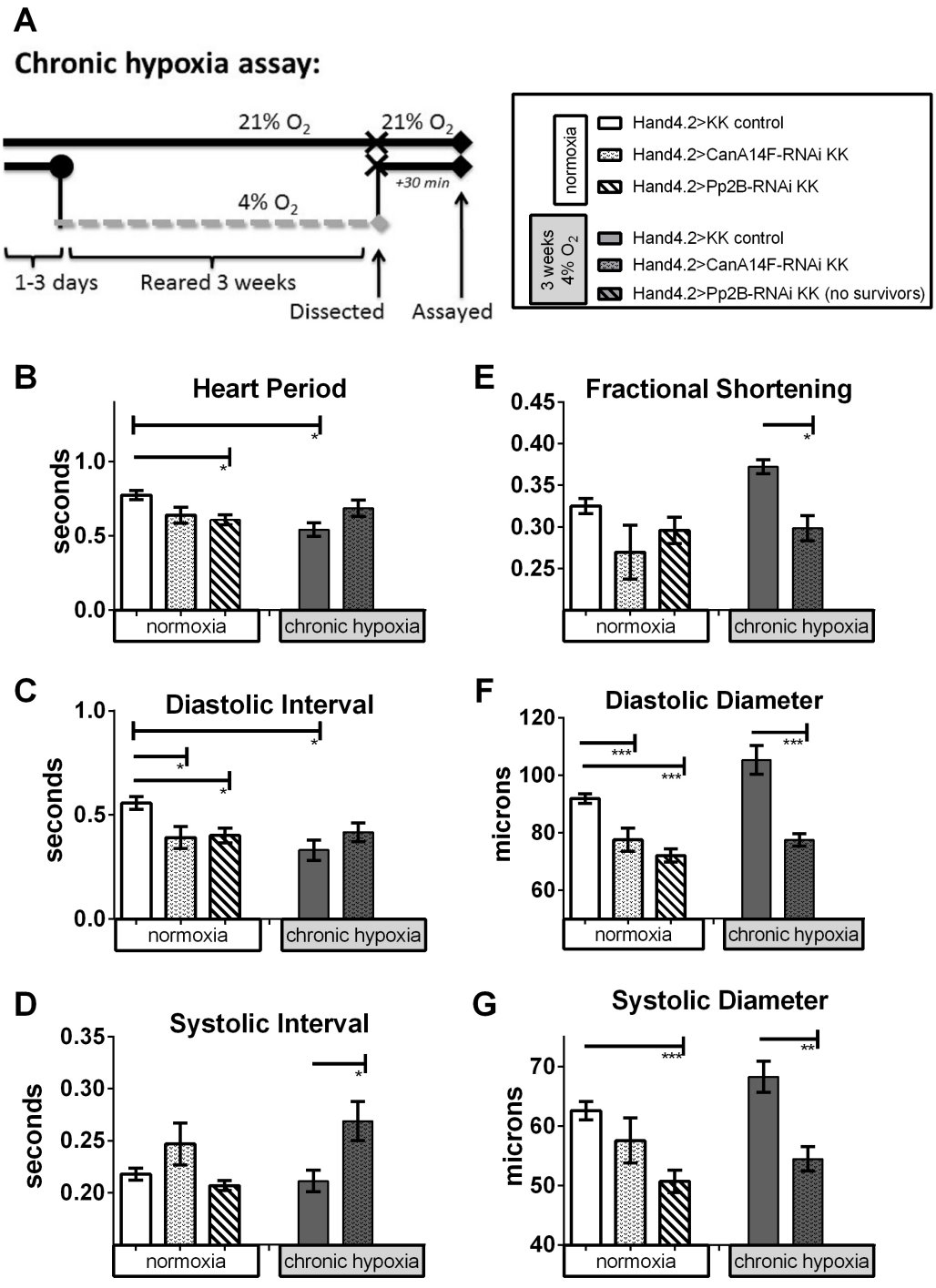

A
Chronic hypoxia assay:
21% O2
21% O2
+30 min
4% O2
1-3 days
Reared 3 weeks
Dissected
Assayed
normoxia
Hand4.2>KK control
Hand4.2>CanA14F-RNAi KK
Hand4.2>Pp2B-RNAi KK
3 weeks 4% O2
Hand4.2>KK control
Hand4.2>CanA14F-RNAi KK
Hand4.2>Pp2B-RNAi KK (no survivors)
B
Heart Period
seconds
1.0
0.5
0.0
normoxia
chronic hypoxia
E
Fractional Shortening
0.45
0.40
0.35
0.30
0.25
normoxia
chronic hypoxia
C
Diastolic Interval
seconds
1.0
0.5
0.0
normoxia
chronic hypoxia
F
Diastolic Diameter
microns
120
100
80
60
normoxia
chronic hypoxia
D
Systolic Interval
seconds
0.35
0.30
0.25
0.20
normoxia
chronic hypoxia
G
Systolic Diameter
microns
70
60
50
40
normoxia
chronic hypoxia

Figure 4.4 (facing page): Effect of chronic hypoxia on heart function in hearts with myocardial-selective knockdown of calcineurins.]
A) Schema of *Drosophila* heart tube lying within middle of abdomen, (adapted from Vogler, 2012 with permission; 25). Schema shows TinC-Gal4 myocardial specific expression (central tube, in red) compared to additional pericardial and cardiac valve expression from Hand4.2-Gal4 driver (cells, in pink). B)-G) Heart function data is presented in normoxia controls on the left (white bars) and in flies exposed to three weeks chronic hypoxia on the right (grey bars) for controls (TinC>KK control), CanA14F-RNAi knockdown, and Pp2B-RNAi knockdown crossed to a TinC-Gal4 driver expressing selectively in myocardial cells from early development; heart function in all flies was measured at 21% O2 after three weeks 21% or 4% O2 conditions. B) Heart period (HP) was elevated in CanA14F-RNai but lowered in Pp2B-RNAi flies under normoxic conditions. After three weeks chronic hypoxia, HP was lowered for control and CanA14F genotypes compared to their normoxia counterparts (condition: F=14.24, P=0.0002; genotype: F=8.089, P=0.0004; Interaction = 2.693, P=0.0704). C) Changes in HP can be fully explained by fluctuations in diastolic intervals (condition: F=12.93, P=0.0004; genotype: F=3.787, P=0.0246; Interaction = 2.075, P=0.1286). D) Systolic intervals (SI) were lowered in control lines after chronic hypoxia, and raised in hypoxic CanA14F compared to hypoxic controls (condition: F=0.6345, P=0.4268; genotype: F=7.793, P=0.0006; Interaction=1.189, P=0.3070). E) Fractional shortening (FS) interacted between genotypes and conditions, FS was significantly reduced in CanA14F-RNAi background at normoxia compared to normoxic controls and hypoxic CanA14F-RNAi flies, and Pp2B-RNAi showed FS reduction after three weeks chronic hypoxia exposure compared to hypoxic controls (condition: F=0.5326, P=0.4665; genotype: F=19.85, P¡0.0001; Interaction=9.043, P=0.0002). F) Diastolic diameters were decreased significantly in both CanA14F-RNAi and Pp2B-RNAi backgrounds under normoxia, and after three weeks hypoxia conditions (condition: F=7.277, P=0.0009; genotype: F=13.77, P=0.0003; Interaction=7.277, P=0.0009). F) Systolic diameters were significantly decreased only in Pp2B-RNAi flies at normoxia. (condition: F=11.36, P=0.0009; genotype: F=1.347, P=0.2626; Interaction=F=2.948, P=0.0550).

*All values are mean +/- SEM (normoxia: N=32 KK control, N=30 CanA14F-RNAi, N=38 Pp2B-RNAi; chronic hypoxia: N=28 KK control, N=22 CanA14F-RNAi, N=20 Pp2B-RNAi). Data was analyzed by (A,B) KruskalWallis test and Dunn multiple comparisons post-hoc test and (C-E) 2-way ANOVA and Tukey's multiple comparisons post-hoc test; n.s. = no statistical significance, * <0.05, ** p<0.01, *** p<0.001.*

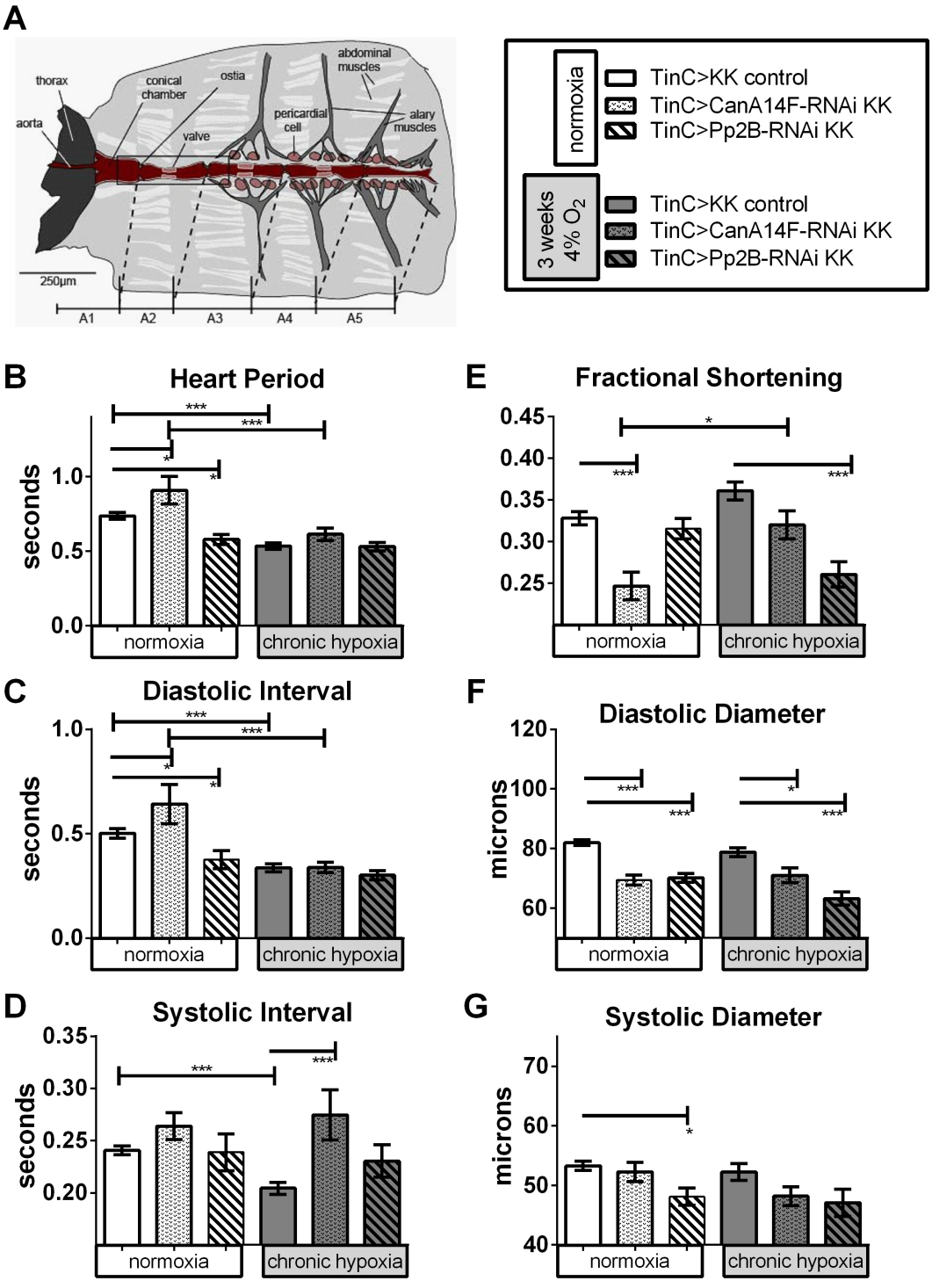

A
thorax
aorta
conical chamber
ostia
valve
pericardial cell
abdominal muscles
alary muscles
250µm
A1
A2
A3
A4
A5
normoxia
TinC>KK control
TinC>CanA14F-RNAi KK
TinC>Pp2B-RNAi KK
3 weeks 4% O2
TinC>KK control
TinC>CanA14F-RNAi KK
TinC>Pp2B-RNAi KK
B
Heart Period
seconds
normoxia
chronic hypoxia
E
Fractional Shortening
normoxia
chronic hypoxia
C
Diastolic Interval
seconds
normoxia
chronic hypoxia
F
Diastolic Diameter
microns
normoxia
chronic hypoxia
D
Systolic Interval
seconds
normoxia
chronic hypoxia
G
Systolic Diameter
microns
normoxia
chronic hypoxia

Figure 4.5 (facing page): Over-expression of Pp2B (UAS-Pp2B) and knockdown of negative regulation of calcineurin, sprouty (sty), cardiac function after 3 weeks normoxia in *Drosophila* myocardial cells.
A) Schema of negative regulation of sty by calcineurin and downstream activation of hypertrophy. Drosophila homologs are on left, with known mammalian pathway on right. In mammals, calcineurin is a known marker distinguishing progression of pathological hypertrophy from reversible physiologic growth. B)-G) Heart function data is presented under normoxic conditions for controls (TinC>KK control and TinC>), CanA-14F-RNAi knockdown, and Pp2B-RNAi knockdown and UAS-Pp2B over-expression. Two sty KK lines were unable to produce viable TinC crosses to three weeks, thus a sty-TRiP line was used instead to knockdown sty expression and relieve regulation on calcineurin; heart function in all flies was measured at 21% O2. B) Heart period (HP) is reduced in Pp2B-RNAi knockdown, and elevated with UAS-Pp2B activation. Similarly, sty-TRiP reduces HP compared to Luc-TRiP controls. These changes are explained by identical changes in C) Diastolic intervals. D) Systolic intervals (SI) are unchanged in TinC backgrounds, but significantly reduced in sty-TRiP background. E) Fractional shortening (FS) is significantly reduced in CanA14F-RNAi, UAS-Pp2B, and sty-TRiP backgrounds at normoxia. F) Diastolic diameters are decreased significantly in both CanA14F-RNAi, Pp2B-RNAi backgrounds under normoxia, as shown previously, but not in UAS-Pp2B or sty-TRiP backgrounds. F) Systolic diameters are significantly decreased in Pp2B-RNAi, and elevated in UAS-Pp2B.

*Data was analyzed by 1-way ANOVA and Tukey's multiple comparisons post-hoc test; *$p<0.05$, **$p<0.01$.*

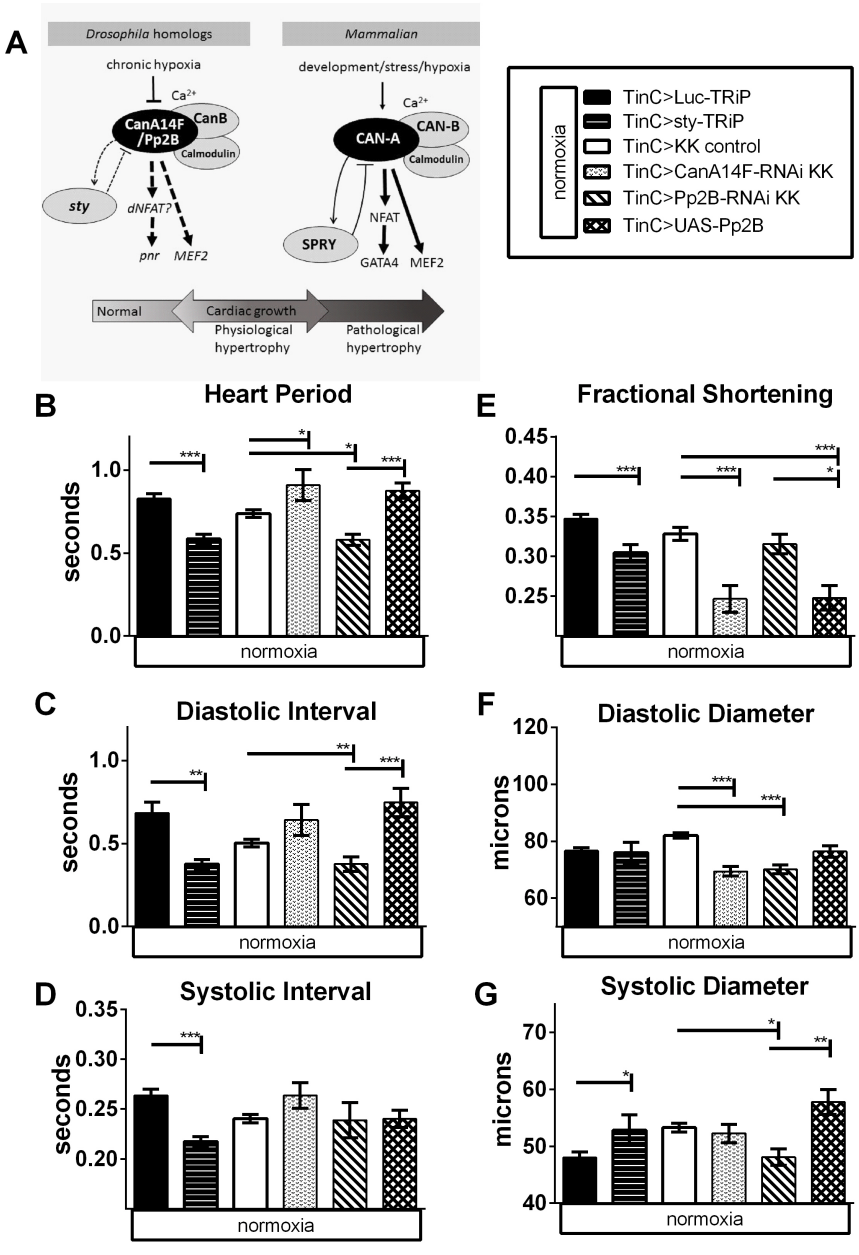
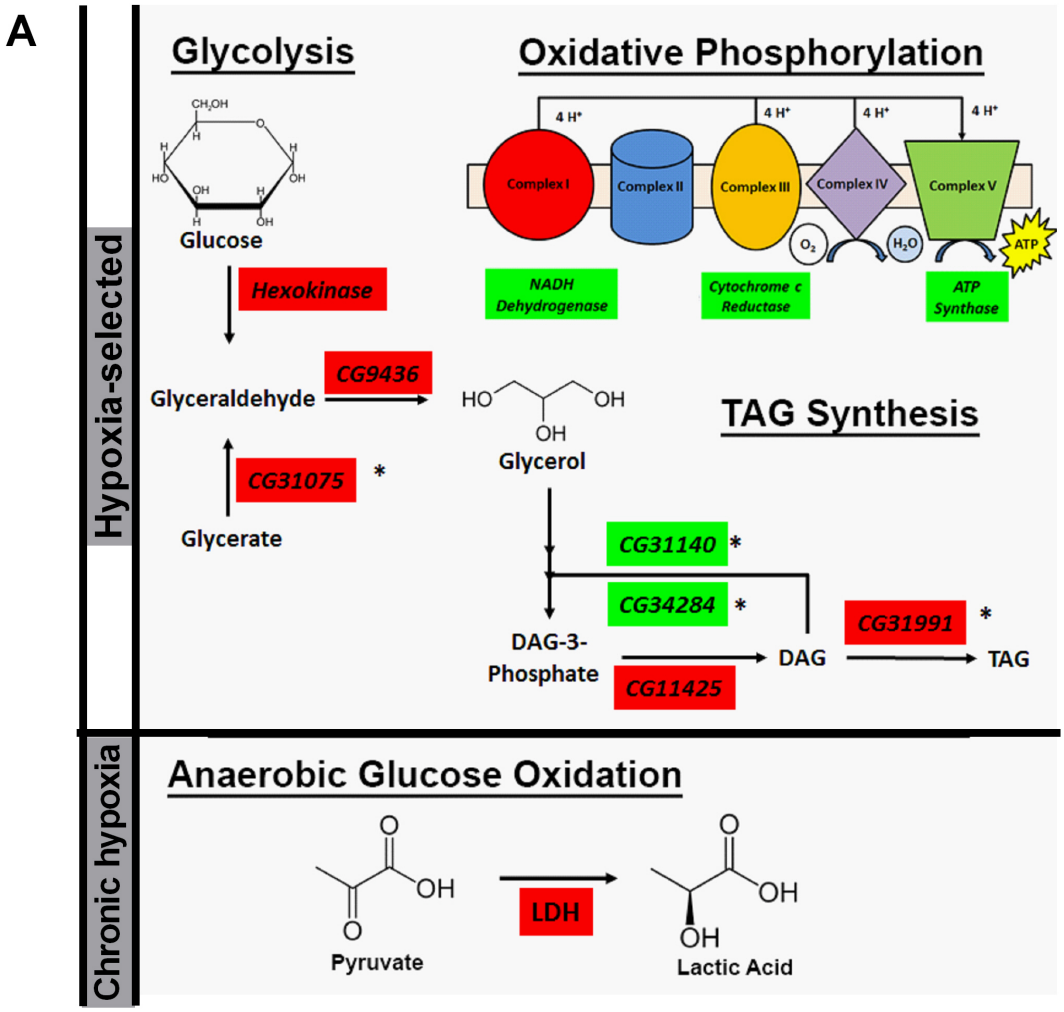
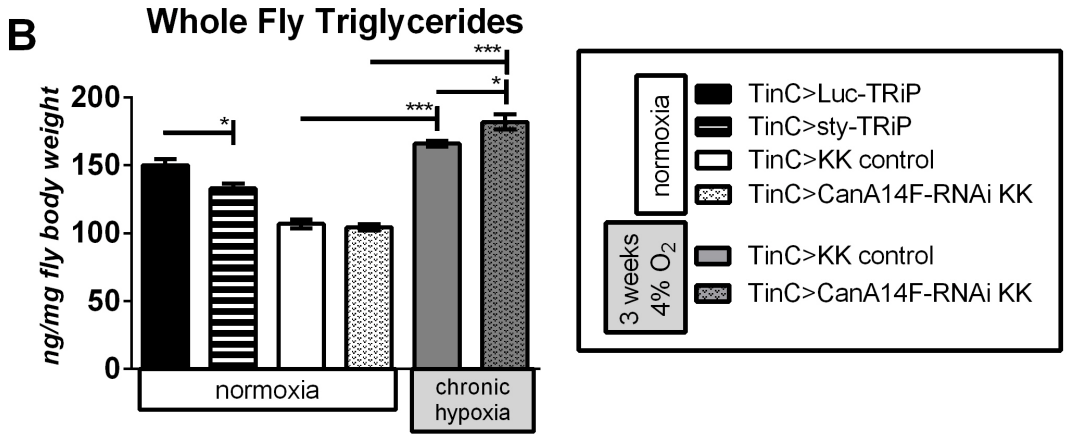

A
Drosophila homologs
Mammalian
chronic hypoxia
development/stress/hypoxia
Ca2+
CanB
CanA14F /Pp2B
Calmodulin
sty
dNFAT?
pnr
MEF2
CAN-A
CAN-B
NFAT
SPRY
GATA4
MEF2
Normal
Cardiac growth
Physiological hypertrophy
Pathological hypertrophy
normoxia
TinC>Luc-TRiP
TinC>sty-TRiP
TinC>KK control
TinC>CanA14F-RNAi KK
TinC>Pp2B-RNAi KK
TinC>UAS-Pp2B
B
Heart Period
seconds
C
Diastolic Interval
D
Systolic Interval
E
Fractional Shortening
F
Diastolic Diameter
microns
G
Systolic Diameter

Figure 4.6 (facing page): Hypoxia-selected flies exhibited a cardiac-specific and calcineurin-dependent shift toward glycolysis and triglyceride storage.
We performed gene ontology based pathway analysis for glycolysis, oxidative phosphorylation and triglyceride synthesis pathways. A) (top) network analysis from within our HS mircroarrays indicates a shift in HS flies towards genes involved in glycolysis and away from genes involved in fatty acid oxidation and triglyceride synthesis. B) (bottom) Hearts from CH exposed flies showed increased expression of lactate dehydrogenase, suggesting a shift toward anaerobic metabolism.
B) Whole fly triglyceride assays after knocking down sty-TRiP or CanA14F-RNAi in the heart. Knockdown of sty-TRiP, but not CanA14F-RNAi, decreased triglyceride accumulation in relation to their appropriate controls under normoxia. Triglyceride levels increases in KK controls after chronic hypoxia, but even greater increases are observed in whole fly body triglyceride accumulation after hypoxia with knockdown of CanA14F-RNAi.

*Data was analyzed by 2-way ANOVA and Tukey's multiple comparisons post-hoc test; *$p<0.05$, **$p<0.01$.*


A
Hypoxia-selected
Glycolysis
Glucose
Hexokinase
Glyceraldehyde
CG9436
CG31075
*
Glycerate
Oxidative Phosphorylation
4 H+
Complex I
Complex II
Complex III
Complex IV
Complex V
NADH Dehydrogenase
Cytochrome c Reductase
ATP Synthase
ATP
TAG Synthesis
Glycerol
CG31140
*
CG34284
*
CG31991
*
DAG-3-Phosphate
DAG
TAG
CG11425
Chronic hypoxia
Anaerobic Glucose Oxidation
Pyruvate
LDH
Lactic Acid


B
Whole Fly Triglycerides
ng/mg fly body weight
200
150
100
50
0
*
***
*
***
normoxia
chronic hypoxia
normoxia
TinC>Luc-TRiP
TinC>sty-TRiP
TinC>KK control
TinC>CanA14F-RNAi KK
3 weeks 4% O2
TinC>KK control
TinC>CanA14F-RNAi KK

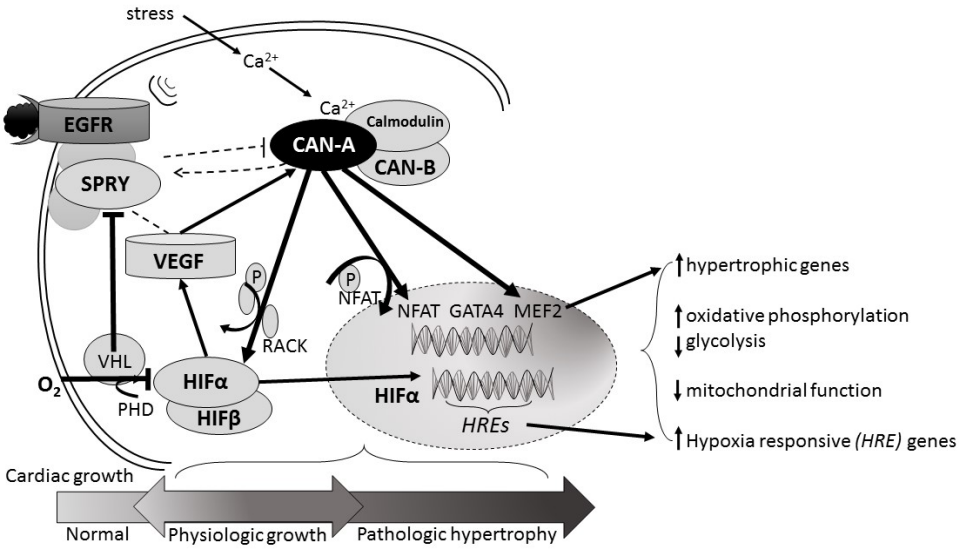


Figure 4.7: **Schema of negative regulation of sty by calcineurin and downstream activation of hypertrophy.** Calcium influx, stabilizes calmodulin binding to calcineurin B, thus promoting activation of calcineurin A (CanA14F and Pp2B in *Drosophila*). Calcineurin serves many functions in the cell, including dephosphorylation of NFAT (in mammals) which allows translocation into the nucleus and activation of transcription factors, including GATA4 and MEF2, to induce hypertrophic genes. A reduction in calcineurin signaling would then reduce induction of hypertrophic genes, potentially via GATA4 or MEF2, leading to the cardiac restriction phenotype we observe in HS and CanA14F/Pp2B knockdown hearts. Notably, NFAT signaling has not yet been identified in the Drosophila heart, and thus other transcription factors may be regulating cardiac growth directly by direct calcineurin activation (as in MEF2, GATA4), or through separate pathways. In mammals, calcineurin stabilizes HIF activity by preventing dimerization of the RACK complex, which marks HIF for degradation under normoxic conditions. HIF can then activate, by direct transcriptional activation on genes with HREs, such as VEGF, EPO and glycolytic genes. In mammals, VEGF is known to activate Calcineurin A directly, or through association with SPRY (*sprouty* in *Drosophila*). Given prolonged hypoxia, these genes orchestrate the switch from physiologic growth to pathologic hypertrophy as cardiomyocytes switch to hypertrophic gene activation, a switch away from glycolysis and increase in oxidative phosphorylation, and decrease in triglyceride accumulation.

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Chapter 5

Concluding Remarks

Humans are unique among species for our remarkable ability to travel between and live within disparate environments. Modern humans are expert explorers in every environment on (and off) earth. However, as a species, we are not adapted for continual habitation in every available ecological niche.

Even within as little as a single generation, we humans can re-establish ourselves. This desire to explore our environment around ourselves is accompanied by a ready physical ability to adapt to new environments. While not all individuals show a penchant for travel, all humans retain a remarkable physical plasticity to survive a range of environments. It is this phenomenon that originally piqued my interest in high altitude adaptations. *Why is it some individuals seem predisposed to the thin air of high peaks, while others suffer?* In the course of my dissertation work, I set out to explore the biological basis of this plasticity; from genomic through phenotypic, from fruit flies to human evolution. *Which genes are conserved through organisms that aid our ability to respond to stressful environments? What responses are encoded by these genes, and can we alter them to adapt an organism to be better suited to its environment?*

Five years ago, I traveled from the wintery mountains of Bozeman Montana to

explore high altitude adaptations at the University of California San Diego (UCSD). In 2010, not much was known about the underlying genetic basis of acclimatization, altitude sickness or adaptation. There were very few reliable predictors of altitude illness, and it was the one topic that would keep me up late at night (even before graduate school!) searching PubMed for answers.

I've been a mountaineer for over a decade, and would reliably develop symptoms of acute mountain sickness even at the relatively low altitude of 9,000, while equally fit friends would pass me, skipping up the mountains with ease. We'd join at the top for a summit antic, and, while feeling better on the descent, I felt burdened by questions. I was determined to find out why some people fared better than others, and was fascinated by mountain super-heros like Reinhold Messner (who has summited all fourteen 8,000 meter peaks without supplemental oxygen). Before joining UCSD, I started my studies by amassed a small mountain-sized pile of books after visiting Powells bookstore in Portland Oregon: I took home every single piece of high altitude literature (from scientific to novellas) that I could carry home on the plane in my mountaineering pack. However, I felt the big questions in the field were unanswered Why are some people susceptible to high altitude while others are less so? Surely there is a genetic basis? Is it shared with other high altitude dwellers?

When humans travel to high altitude, several physiologic responses compensate the body to gradually adjust to the reduced availability of atmospheric oxygen (hypoxia). While this acclimatization allows short sojourns to intolerable environments, no human can live for prolonged periods at extremely high altitude. Even visitors to Everests base camp are in a state of slow deterioration. However, several human populations, including highland Ethiopians, Tibetans and Andeans, have uniquely adapted over thousands of years and live quite well at moderately high altitudes (less than 4,300m). Several physiologic compensations are now well-known to be adaptive, and today, even the underlying

genetic basis is considered well established in these populations.

I set out to find novel genetic pathways underlying physiologic adaptations to hypoxia through an interdisciplinary, collaborative approach. Joining UCSD's Anthropogeny sub-specialization track for PhDs, I learned how to translate my research into its broadest terms and ask the Big Questions. *Who are we? Why are we here? How did we get here? Are we unique as a species?* Through incredibly fascinating symposium and journal clubs, I learned some of what makes humans so special among all living things. An appreciation developed for the subtle signatures of shared common ancestry which connects our incredibly complex genetic and physiologic responses to even the simplest of critters. I was able to travel to Tanzania and Ethiopia with my professors to study sites essential to the study of human evolution. Then to highland Ras Dejen, Ethiopia. There, while traveling solo on a "potential future research mission" I met some of the Amhara people living above 4,200m. People whose response to the question - How do you do so well at high altitude? - was answered with shrugs and grins. Research in these areas is remarkably difficult, thus not much is known about these people. Unfortunately, outside development projects and environmental climate change leading to drought and erosion may forever limit our understanding of these people's unique physiological adaptations as they are relocated into camps nearer sea-level. I wished I could stay and rigorously study the people living in the Ethiopian highlands, and contented myself to return home with a "...someday."

Through Howard Hughes Medical Institutes (HHMI) Med-into-Grad program, I learned translational aspects of complex research questions. Speaking with clinicians and patients about hypoxia-related diseases, I realized the impact basic research questions can make when applied to complex disease processes. HHMI even sent me to highland Bolivia, where I attended the High Altitude Pathology Institutes V Chronic Hypoxia Symposium. As a guest speaker, I gave a speech on models of cardiac disease with

international clinical colleagues, floating in the Bolivian navy's yacht on Lake Titicaca (yes, Bolivia has a navy). The highlight of the trip was being introduced by Prof. Dr Gustavo Zubieta-Calleja Sr to people living in La Paz with chronic mountain sickness. I learned how to integrate disciplines, organ systems, and even some of the clinicians' lingo. Perhaps the most tangible benefit of my studies at UCSD was the acquired ability to see through a question to its heart.

In the lab, I used a unique population of hypoxia-adapted fruit flies (*Drosophila melanogaster*) to investigate key genetic pathways underlying physiologic response of the heart during low oxygen exposure. This research unraveled novel physiologic responses and genetic mechanisms occurring during acute and chronic hypoxia, and features that distinguished these responses from multi-generational adaptation. In the future, I strongly wish to determine the relevance of these findings to human high altitude adaptations, particularly through use of recent genetic screens and samples from Asia, South America and Africa. Highland Ethiopia still fascinates me; the fact it is relatively scientifically unexplored makes it feel like a wild frontier of high altitude research. For my lowland dwelling friends, I hope findings from my research project may determine future medical interventions and alleviate symptoms of cardiac disease. Or perhaps serve as cardiac biomarkers in maladaptive humans who struggle to acclimatize or show signs of altitude illness.

Appendix A

Supporting Information for Chapter 1

A.1 Establishing and refining models of *Drosphila* cardiac hypoxia

Prior to publishing our *Drosophila* models of acute, sustained, chronic and multi-generational cardiac hypoxia exposure, we spent significant time stablizing this novel method of assessing cardiac function during hypoxia. A previous high-throughput assay developed in a collaborating laboratory (Feala, 2009), enabled a higher throughput phenotyping assay for short-term (minutes) hypoxia exposure. Dissertation work here refined these methods for integration with our previously established SOHA cardiac phenotyping applications (Ocorr, 2007). The combination of these two assays combines the best of the high-throughput in the previously published design with the detailed image analysis and cardiac physiology of the SOHA method. We made additional novel improvements to allow maximal application to establishing high-throughput hypoxia assays. The final design was implemented to screen a list of candidate genes identified from comprehensive literature search of high-altitude species' genomes for gene candidates underlying conserved adaptation to chronic hypoxic disease. The background, methods and results

are presented below.

A.2 Comparative candidate gene screen

Our preliminary experiments show flies have increases in heart period to all levels of hypoxia applied; a phenotype which is not restored to normoxia levels even on re-oxygenation in sustained hypoxia flies, or after re-oxygenation from acute hypoxia exposure in hypoxia-selected flies, indicating altered homeostatic mechanisms. As a partial explanation for chronotropic cardiac responses observed, we proposed that down-regulation of candidate genes from the dopamine pathway would be involved in the reduced heart rate phenotype observed during chronic hypoxia.

The primary aim of this comparative, candidate screen was to identify conserved gene homologs which reveal pathways underlying adaptation to chronic hypoxia for further extensive study using the *Drosophila* heart model. Given the significant alterations in expression for the dopamine pathway in a conserved, cardiac-specific pattern in chronic hypoxia, we proposed a cardiac role during chronic hypoxia, investigated in detail here.

We investigated genes selected from a list of candidate genes identified in human genome studies correlated to high altitude adaptation. To investigate mechanisms underlying hypoxia adaptation, we compared cardiac expression profiles of our hypoxia-selected fly populations to those from other high altitude species hearts. We tested candidate genes in contractile, transcriptional and signaling processes in our fly heart model for a potential role in cardiac-specific hypoxia adaptation. A small number of genes were similarly differentially expressed in these chronically hypoxic hearts, and provided a basis to study the underlying conserved mechanisms by which genetic selection might alter the remodeling of hearts of high altitude natives. Specifically, we probe the hypoxia-

dependent and HIF-dependent gene interactions in the fly heart using the promising candidate gene, dopa-decarboxylase (ddc), which is implicated in both the HIF pathway and an altered hypoxia response, but whose cardiac role has not yet been explored in detail.

A.2.1 Background information

In the mammalian heart, the catecholamine pathway has well-known cardioaccelatory effects, as well as regulating arousal, courtship wound-healing and release of glucose stores, all of which may be important to long-term or short-term hypoxia adaptation. Further, studies in Drosophila show the effects of dopamine pathway constituents are conserved on heart rate and behavior, and, of particular interest, down-regulated during stress responses. While many studies investigate roles for myocardial dopamine receptors and/or the effect of catecholamines on heart rate, very few studies examine dopamine pathway expression in the myocardial tissue itself during hypoxi. In humans, prolonged exposure to hypoxia causes a decrease in maximal heart rate during exercise, even with pharmacologic increase of circulating levels of the cardiac stimulant, noradrenaline. Current literature suggests limited heart rate under severe hypoxia and prevention of cardiac failure during hypertrophy may be caused by a down-regulation or blocking of -adrenergic receptors. Further, the catecholamine pathway interacts directly with HIF1 to maintain cardiac function. As little is known about myocardial dopamine expression and its regulation under hypoxia, examined the dopamine pathway in hearts of the tractable model *Drosophila*.

A.2.2 Microarray

We compared the cardiac-specific gene expression profiles from our 'hypoxia-selected' microarrays to cardiac profiles of other hypoxia-tolerant species or laboratory models of hypoxia available on the GEO database, seeking a conserved genetic signature common to the cardiac hypoxia response. The present study presents a meta-analysis of cardiac microarrays from populations exposed to longterm hypoxia (lifetime chronic hypoxia, and/or multi-generational hypoxia) to identify a common, differentially-expressed transcriptional signature of longterm hypoxia. Specifically, this approach employs meta-analyses of GEO Affymetrix microarray datasets, with inter-set comparisons to identify a pooled, conserved differential gene expression set by homology alignment between datasets. To perform this meta-analysis, we pooled genes with significant differential expression of +/- 1.2 fold in hypoxic hearts of Tibeten and Chinese chickens, zebrafish and our 'hypoxia-selected' Drosophila and revealed a small subset of conserved gene homologs.

A.2.3 Bioinformatic analysis

As previously reported in whole fly arrays from the hypoxia-selected fly populations, the Notch target and transcriptional repressor *hairy* regulates a metabolic switch away from aerobic metabolism, a phenotype which persists in normoxia and is indicative of underlying genetic changes. While the present study corroborates changes in hairy expression, we report no significant expression changes in the conserved core members of the hypoxia-response pathway (Table 1). Despite the lifetime, chronic exposure to hypoxia, unchanged expression of *sima* is not entirely unexpected given its known stabilization and induced transcriptional activity under acute hypoxic conditions, and

degradation during prolonged chronic exposure16. A search for transcription factor binding in the up- and down-regulated genes of hypoxia-selected fly hearts revealed an enrichment of *sima*, tango, and hairy binding sites, further evidence of their transcriptional activity in these flies.

KEGG pathway mapping from microarray data allows discovery of gene enrichment in mapped metabolic pathways (Kyoto Encyclopedia of Genes and Genome: www.genome.jp/kegg). KEGG analyses of differential expression in the hypoxia-selected fly can suggest metabolic adaptations to hypoxia from which gene candidates may be selected for further detailed study. Previous studies using whole fly microarrays and simulated flight muscle metabolic adaptations from these hypoxia-selected populations found overall suppression of both the tricarboxylic acid (TCA) cycle and oxidative phosphorylation genes as well as a down-regulation of glycolysis. Our cardiac-specific analysis similarly finds a shift away from oxygen dependent complexes, but, in contrast, a significant shift toward glycolysis.

In particular, out of the 126 mapped genes in the oxidative phosphorylation pathway, we find 9 genes to be significantly down-regulated >1.2 fold (Figure 6). This 7.1% pathway down-regulation represents 2-fold enrichment of the oxidative-phosphorylation pathway over all genes significantly down-regulated >1.2 in the hypoxia-selected Drosophila heart.

Overall, gene expression indicates pathways that increase flux through the oxygen-using TCA cycle are down-regulated; for example, significant down-regulation of valine, leucine and isoleucine degradation pathways may limit generation of acetyl CoA. Combining our results with previous studies indicates the TCA cycle is strongly suppressed in whole fly, flight muscle and hearts of adapted flies, likely from hairy Notch up-regulation as reported previously.

However, in contrast to previous studies with this population, fly hearts appear

to have up-regulation of glycolysis. Pyruvate hexokinase A and the pentose-phosphate pathway are over-expressed, signifying these reactions may be necessary to increase flux through glycolytic pathways. These metabolic findings have much in common to cardiac adaptations regulated by HIF in vertebrates. We propose this shift to glycolysis to be an adaptation in Drosophila hearts, allowing maintenance of contraction during states of prolonged hypoxia.

A.2.4 Selection of conserved gene candidates

Candidate genes are the homologs of dopa-decarboxylase, fibrinogen and GATA3. None of these candidates, or their associated pathways, were particularly distinguished by KEGG, GEO pathway clustering, or other available tools. More remarkably, the genes identified by homology alignment were only part of entire pathways or paralogs of differentially expressed genes, when expression within a family of related genes was examined individually (Table 2).

To test cardiac function, we used Drosophila tinc-GAL4 lines crossed to UAS- or UAS-RNAi constructs; this allowed heart-specific overexpression or knockdown of candidate genes. Thus far, examination of cardiac responses at 3 weeks while over- or under-expressing ddc or scabrous under normoxia has not revealed compelling phenotypes, GATAd lines were unavailable at time of survey.

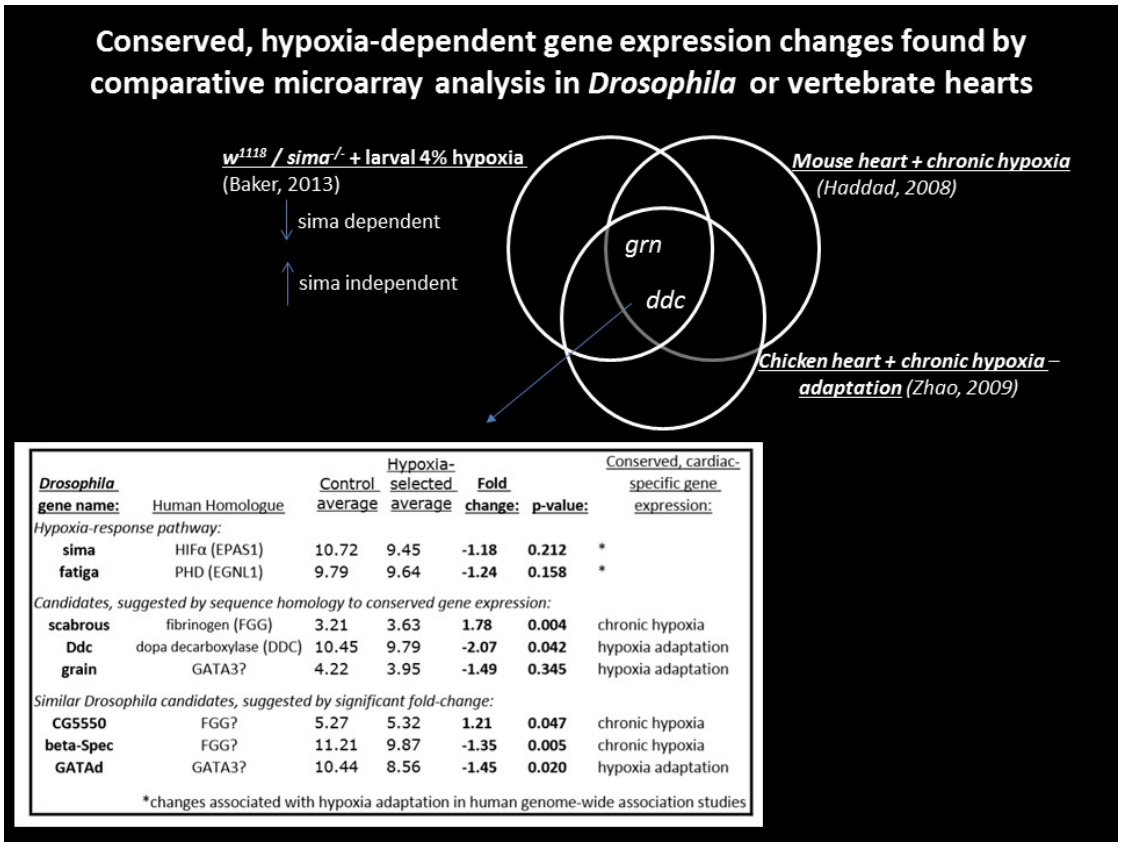
A.2.5 Cardiac-specific candidate gene expression

To determine the interaction of dopamine with the hypoxia-response pathway, we examined candidate gene interaction with *sima* in the fly heart. A search for

hypoxia-responsive elements in the 500 bp promoter region indicates such in *ddc*. These hypoxia-responsive elements imply binding with *sima* for hypoxia-specific regulation, and dopamines interaction with HIF*alpha* is confirmed in vertebrate models.

Using cardiac-specific microarrays from several species, homologous Affy sequences were aligned to find shared gene expression patterns (up- or down- 1.2 fold, p¡0.05). These candidates are commonly over- or under- expressed selectively in hypoxic conditions. We examined cardiac function of genes found by bioinformatics analysis by over- and under-expression of specifically identified candidates (scabrous, ddc, GATAd) as well as candidates found by similar sequence homology (GATA transcriptional regulation: gata-e; dopamine pathway genes: pale, Skeletor, ebony, knickkopf; fibrinogen genes; CG5550, beta-Spec). By using the GAL4-upstream activation sequence (UAS) binary expression system (37), we drove expression in myocardial cells using the tinc4-GAL4 driver, and in myocardial and pericardial cells using the Hand4.2-GAL4 driver. Interactions of candidates with the Hif *alpha* homolog, *sima* were performed through use of a simaK07607G mutant line balanced with TM3 and crossed with the Hand4.2-GAL4 driver for cardiac-specific expression by crossing to RNAi genes of interest. We used trans-heterozygote lines in a systemic sima null background combined with tinc4-GAL4 expression of candidate genes.

Table A.1: Gene candidates identified by comparative, conserved microarray meta-analysis.



Drosophila gene name:	Human Homologue	Control average	Hypoxia-selected average	Fold change:	p-value:	Conserved, cardiac-specific gene expression:
Hypoxia-response pathway:						
sima	HIFα (EPAS1)	10.72	9.45	**-1.18**	**0.212**	*
fatiga	PHD (EGNL1)	9.79	9.64	**-1.24**	**0.158**	*
Candidates, suggested by sequence homology to conserved gene expression:						
scabrous	fibrinogen (FGG)	3.21	3.63	**1.78**	**0.004**	chronic hypoxia
Ddc	dopa decarboxylase (DDC)	10.45	9.79	**-2.07**	**0.042**	hypoxia adaptation
grain	GATA3?	4.22	3.95	**-1.49**	**0.345**	hypoxia adaptation
Similar Drosophila candidates, suggested by significant fold-change:						
CG5550	FGG?	5.27	5.32	**1.21**	**0.047**	chronic hypoxia
beta-Spec	FGG?	11.21	9.87	**-1.35**	**0.005**	chronic hypoxia
GATAd	GATA3?	10.44	8.56	**-1.45**	**0.020**	hypoxia adaptation

*changes associated with hypoxia adaptation in human genome-wide association studies

Appendix B

Supporting Information for Chapter 2

This appendix contains the raw data (”absolute measurements”) and figures representing these data for Figures in Chapter 2 in an .xls file, submitted separately. Further, this appendix establishes the stability of dissolved oxygen content for hypoxia/reoxygenation protocols used in Chapters 1-4.

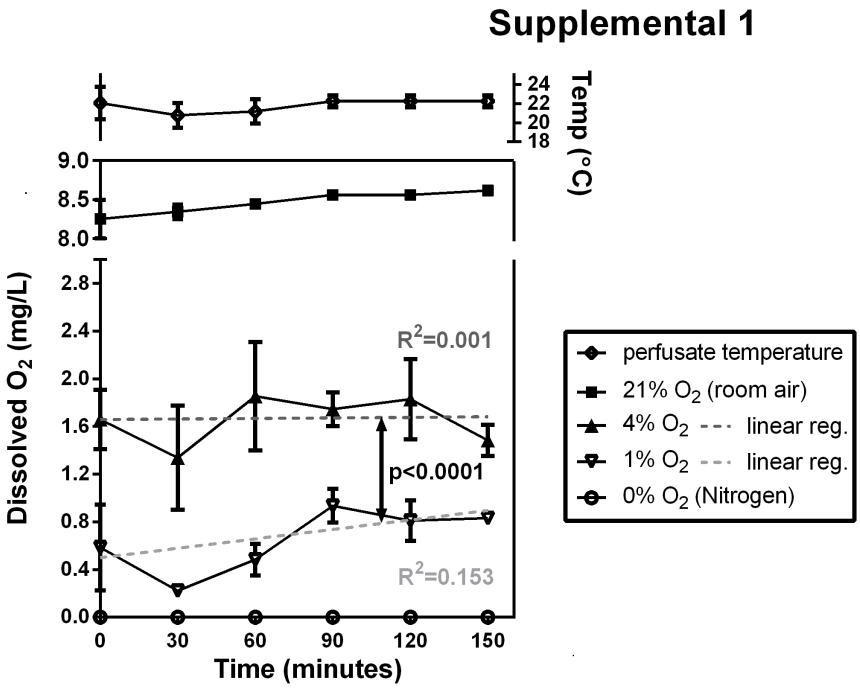


Figure B.1: **Dissolved Oxygen (DO) measures for Drosophila artificial hemolymph.** Measures of dissolved O_2 (DO) content were monitored and recorded over several time points and experimental days to ensure a stable level of reduced oxygen using a Qubit Systems OX1LP polarographic oxygen probe, calibrated and corrected for mean barometric pressure (758 mm Hg), salinity (8.22) and perfusate temperatures (21-22C). We measured mean dissolved oxygen content in artificial Drosophila hemolymph perfusate to be 0.64 mg/L at 1% O_2 and 1.7 mg/L at 4% O_2 to be, and stable over 150 minutes (best fit linear regression, $p<0.0001$.)

Appendix C

Supporting Information for Chapter 3

This appendix contains the time-matched raw data (”absolute measurements”) and figures representing these data for data in Chapter 3.

Figure C.1 (facing page): Acute cardiac response to 30 minutes 4% O2 hypoxia / reoxygenation (acute H/R) in control and hypoxia-selected *Drosophila* raised under conditions of normoxia or hypoxia.
A) Incidence of non-beating or 'asystolic' hearts (> 25 seconds without contractions) in response to hypoxia exposure, expressed as a percent of total hearts examined. Nearly half of normoxia-raised HS hearts entered prolonged periods of asystole at 30 minutes in 4% O2, and all resumed beating on reoxygenation. B) In hearts that beat, HP is increased under 4% O2 hypoxia exposure (T=30) and this reached significance in hearts from hypoxia-selected flies either normoxia or hypoxia-raised. HP returned to baseline levels exhibited by NC in all genotypes post hypoxia exposure except HS hypoxia raised flies (T=90). C) DI increased in HS flies under 4% O2 (T=30) and then returned to baseline levels upon reoxygenation (T=60, 90). D) SI increased for normoxia-raised controls and HS genotypes on 4% hypoxia exposure and returned to baseline only in NC and HS , normoxia-raised flies. E) FS was significantly reduced under 1% O2 for HS flies either hypoxia- or normoxia-raised (T=30). FS returned to baseline for normoxia-raised flies at T=60 and T=90, and at T=60 for hypoxia-raised flies. F) DD was unchanged after an acute 4% O2 exposure. G) SD was significantly increased during acute hypoxia in hypoxia-selected, normoxia-raised flies (T=30 from T=0).

*All values are mean percent change +/- SEM (normoxia-raised controls N=23, hypoxia-raised controls N=11, hypoxia-selected normoxia-raised N=23, hypoxia-selected hypoxia-raised N=37). For heart period, systolic and diastolic interval, asystolic hearts were excluded, while all hearts are included in fractional shortening, diastolic and systolic diameter. Data was analyzed by (B,C) KruskalWallis test and Dunn multiple comparisons post-hoc test and (D-F) 2-way ANOVA and Tukey's multiple comparisons post-hoc test; n.s. = no statistical significance, * <0.05, ** p<0.01, *** p < 0.001. Changes between genotypes at each timepoint were analyzed by Sidak-Bonferroni t-test; t p < 0.05. tt p < 0.01.*

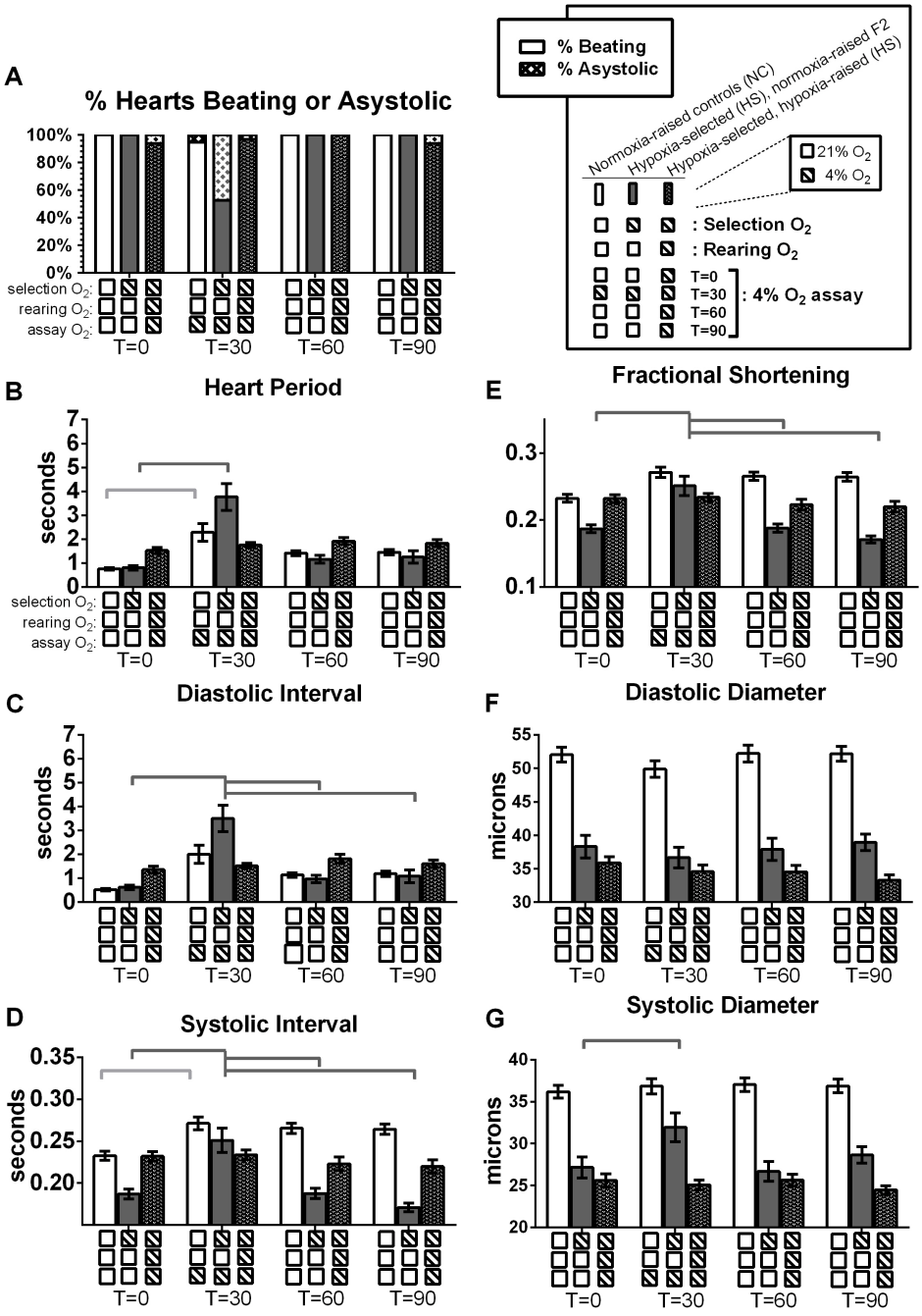
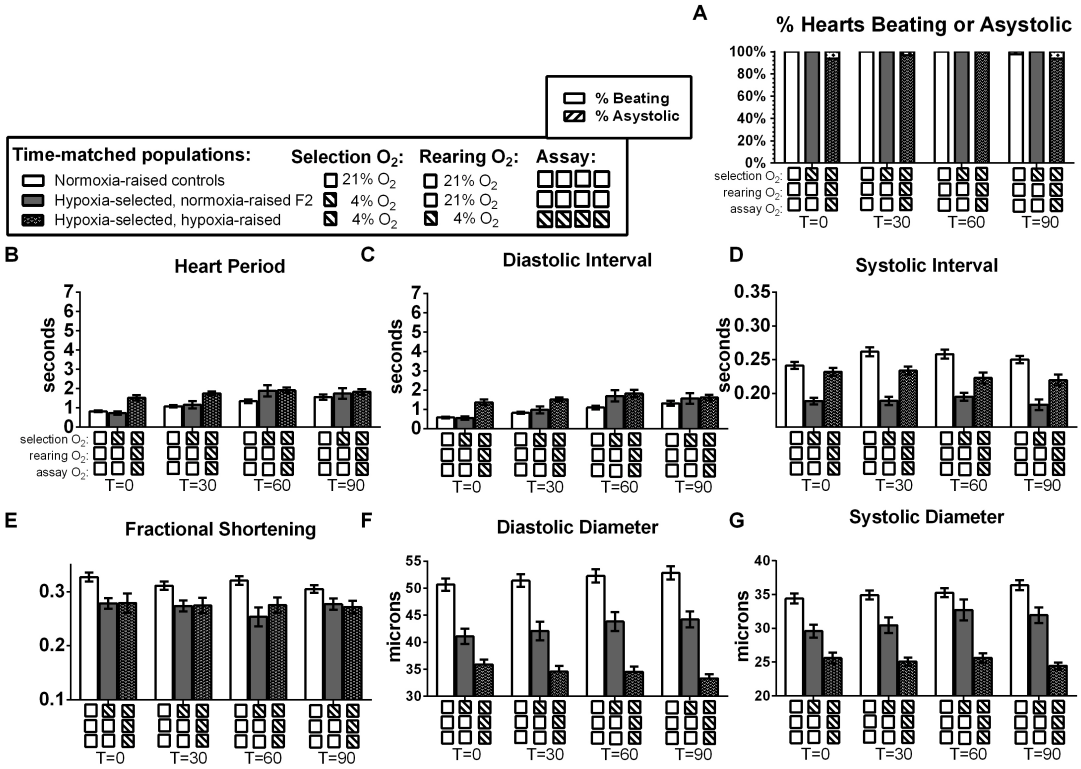

A
% Hearts Beating or Asystolic
100%
80%
60%
40%
20%
0%
selection O2:
rearing O2:
assay O2:
T=0
T=30
T=60
T=90
% Beating
% Asystolic
Normoxia-raised controls (NC)
Hypoxia-selected (HS), normoxia-raised F2
Hypoxia-selected, hypoxia-raised (HS)
21% O2
4% O2
: Selection O2
: Rearing O2
T=0
T=30
T=60
T=90
: 4% O2 assay
B
Heart Period
seconds
C
Diastolic Interval
seconds
D
Systolic Interval
seconds
E
Fractional Shortening
F
Diastolic Diameter
microns
G
Systolic Diameter
microns

Figure C.2 (facing page): Time-matched controls for acute H/R in control and hypoxia-selected *Drosophila* raised under conditions of normoxia or hypoxia
A) Incidence of non-beating or 'asystolic' hearts ($>$ 25 seconds without contractions) in response to hypoxia exposure, expressed as a percent of total hearts examined. B)-G) HP, DI, SI, FS, DD and SD cardiac parameters do not change significantly from T=0 to T=30, T=30 to T=60, or T=60 to T=90.

*All values are mean percent change +/- SEM. For heart period, systolic and diastolic interval, asystolic hearts were excluded, while all hearts are included in fractional shortening, diastolic and systolic diameter. Data was analyzed by (B,C) KruskalWallis test and Dunn multiple comparisons post-hoc test and (D-F) 2-way ANOVA and Tukey's multiple comparisons post-hoc test; n.s. = no statistical significance, * $<$0.05, ** $p<0.01$, *** $p < 0.001$. Changes between genotypes at each timepoint were analyzed by Sidak-Bonferroni t-test; t $p < 0.05$. tt $p < 0.01$.*


A
% Hearts Beating or Asystolic
100%
80%
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40%
20%
0%
% Beating
% Asystolic
Time-matched populations:
Normoxia-raised controls
Hypoxia-selected, normoxia-raised F2
Hypoxia-selected, hypoxia-raised
Selection O2:
21% O2
4% O2
4% O2
Rearing O2:
21% O2
21% O2
4% O2
Assay:
selection O2:
rearing O2:
assay O2:
T=0
T=30
T=60
T=90
B
Heart Period
seconds
C
Diastolic Interval
D
Systolic Interval
E
Fractional Shortening
F
Diastolic Diameter
microns
G
Systolic Diameter

Appendix D

Supporting Information for Chapter 4

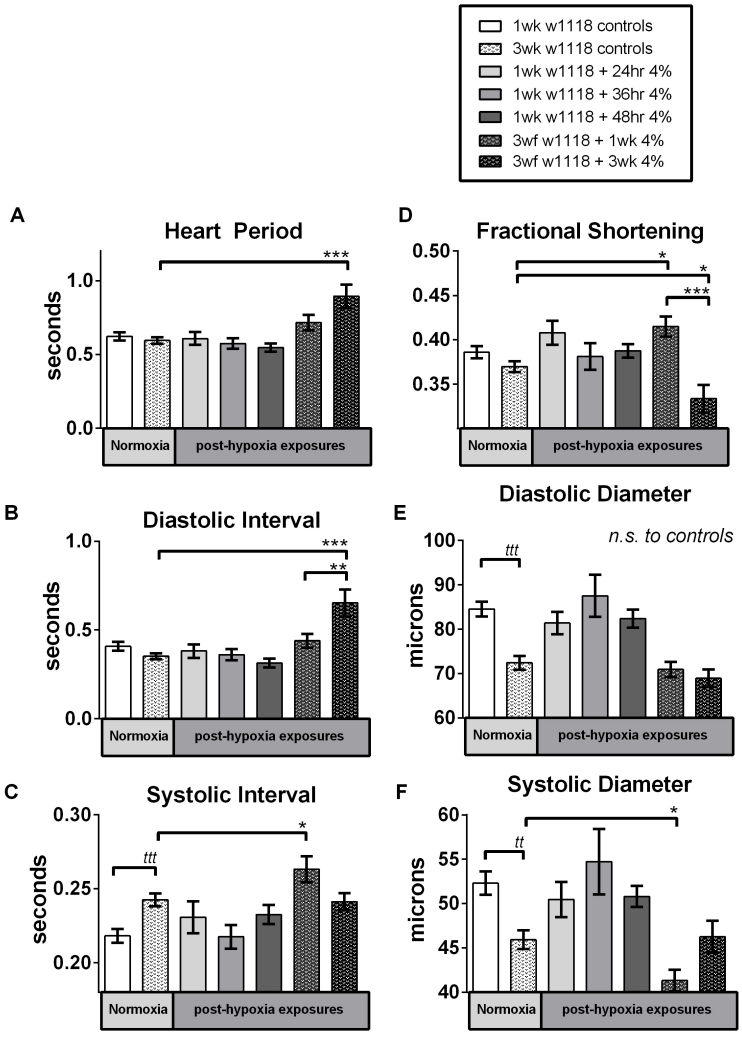

Figure D.1: **Cardiac responses in wild type flies after exposure to varying durations of hypoxia.** Cardiac responses in wild type flies after exposure to varying durations of hypoxia from 24 hours through 3 weeks adult exposure.

*All values are mean percent change +/- SEM. * p<0.05 2-way ANOVA ** p<0.01 2-way ANOVA *** p<0.001 2-way ANOVA*

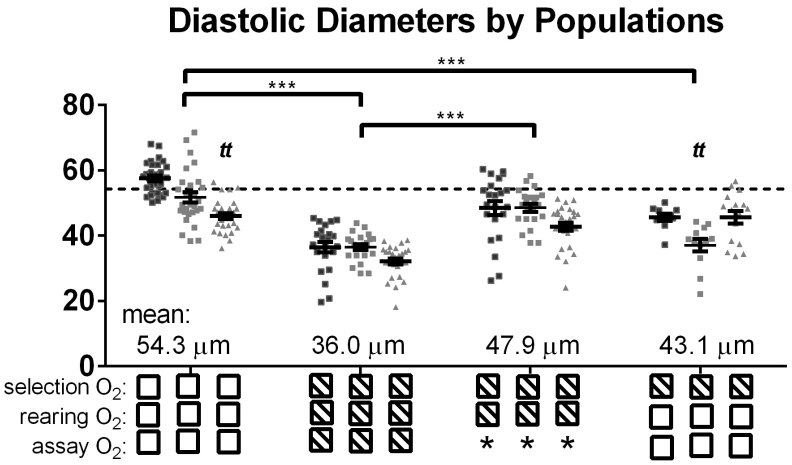

Figure D.2: **Diastolic diameter size correction for hypoxia-selected flies' smaller body size.** As noted previously, hypoxia-reared HS flies are smaller in overall size than both F2 progeny of HS flies raised under normoxic conditions, as well as NC and w1118 controls, probably due to oxygen limitation during development (14, 58, 60). To confirm that the smaller heart size was not simply due to a reduction in the overall size of the fly we measured the abdominal segment and tibia lengths in normoxia and hypoxia-reared HS and NC flies. The average segment length of hypoxia raised HS flies was 67% of the abdominal segment length of either normoxia reared NC or HS, and similar for tibial measurements (56, 60). When diameter measurements were normalized to correct for this 67% difference in body size we found that the average DD was still significantly smaller for HS flies. Combined population means are presented below population scatter plots: 54.3 um NC, compared to 36.0 um HS uncorrected and 47.9 um HS corrected or 43.1 HS normoxia-raised.

Overall averages of diastolic diameter in NC flies is consistent at 54.3 um (at 21% O2), yet only 36.0 um HS hypoxia-raised flies (at 4% O2), and 47.9 um with relative size correction (at 4%). Overall total body size in HS flies returns toward normoxia size after release of 4% selection pressure, but the heart size does not similarly return to normoxia size, staying at an average 43.1 um for normoxia-raised HS flies ($p<0.001$ 2-way ANOVA). Two HS populations in particular experience a persistent, reduction in cardiac size despite being normoxia-raised. These results led us to explore whether this hypoxia-induced reduction in cardiac size is genetically controlled and/or could be related to genetic adaptations in the hypoxia-selected populations. Our previous study showed no significant cardiac size reduction after three weeks chronic 4% hypoxia exposure in a control line of w1118 flies. This exposure matches the hypoxia-selected lifespan exposure, and indicates cardiac restriction in hypoxia-selected flies may be a persistent genetically-selected trait, particularly given the variation between populations.
All values are mean percent change +/- SEM. p<¡0.001 2-way ANOVA

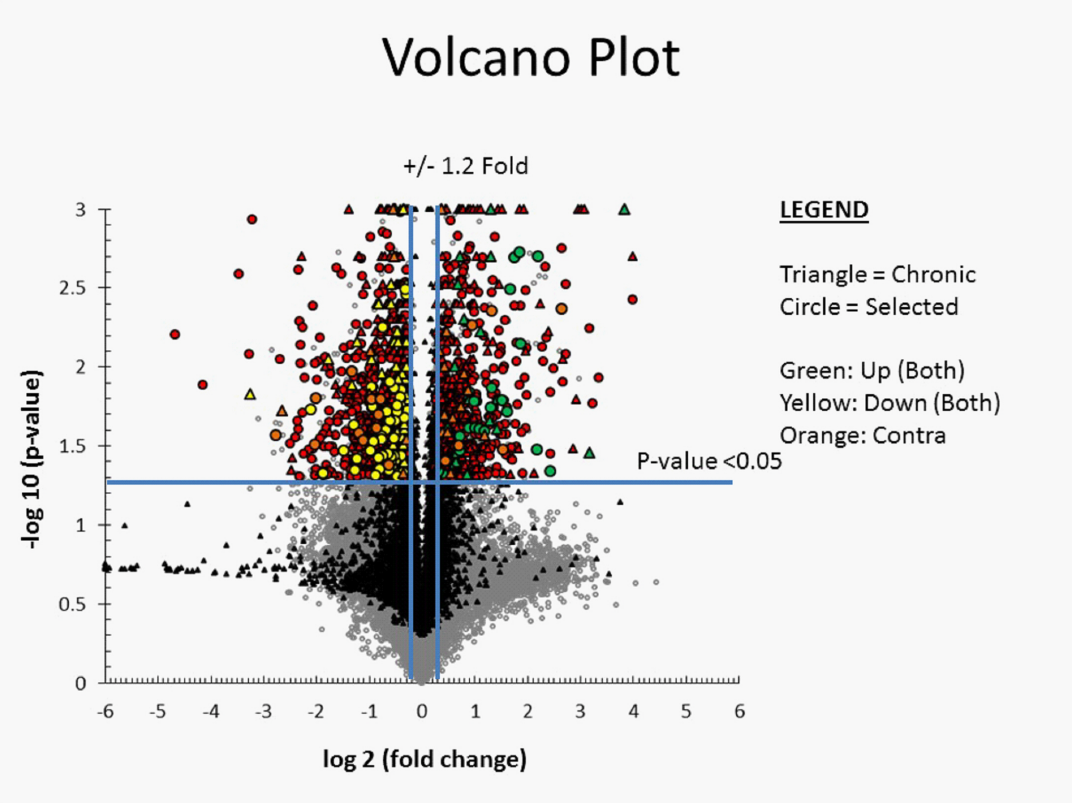

Figure D.3: **All cardiac-specific genes differentially expressed in hypoxia-selected and chronic hypoxia exposed fly populations.** Transcriptome analysis of RNA from *Drosophila* myocardial and pericardial cells for cardiac-specific, differential gene expression in hypoxia-selected flies (HS) compared to chronic hypoxia-exposed *w1118* flies (CH). Relative log2 fold-change expression from HS vs. CH populations between genes found $>$1.2 fold up-regulated (red) or $>$1.2 fold down-regulated (green) in both populations, contra-regulated between the populations (yellow) or found in HS- or CH- only with transcriptome changes (open circle or triangle points).

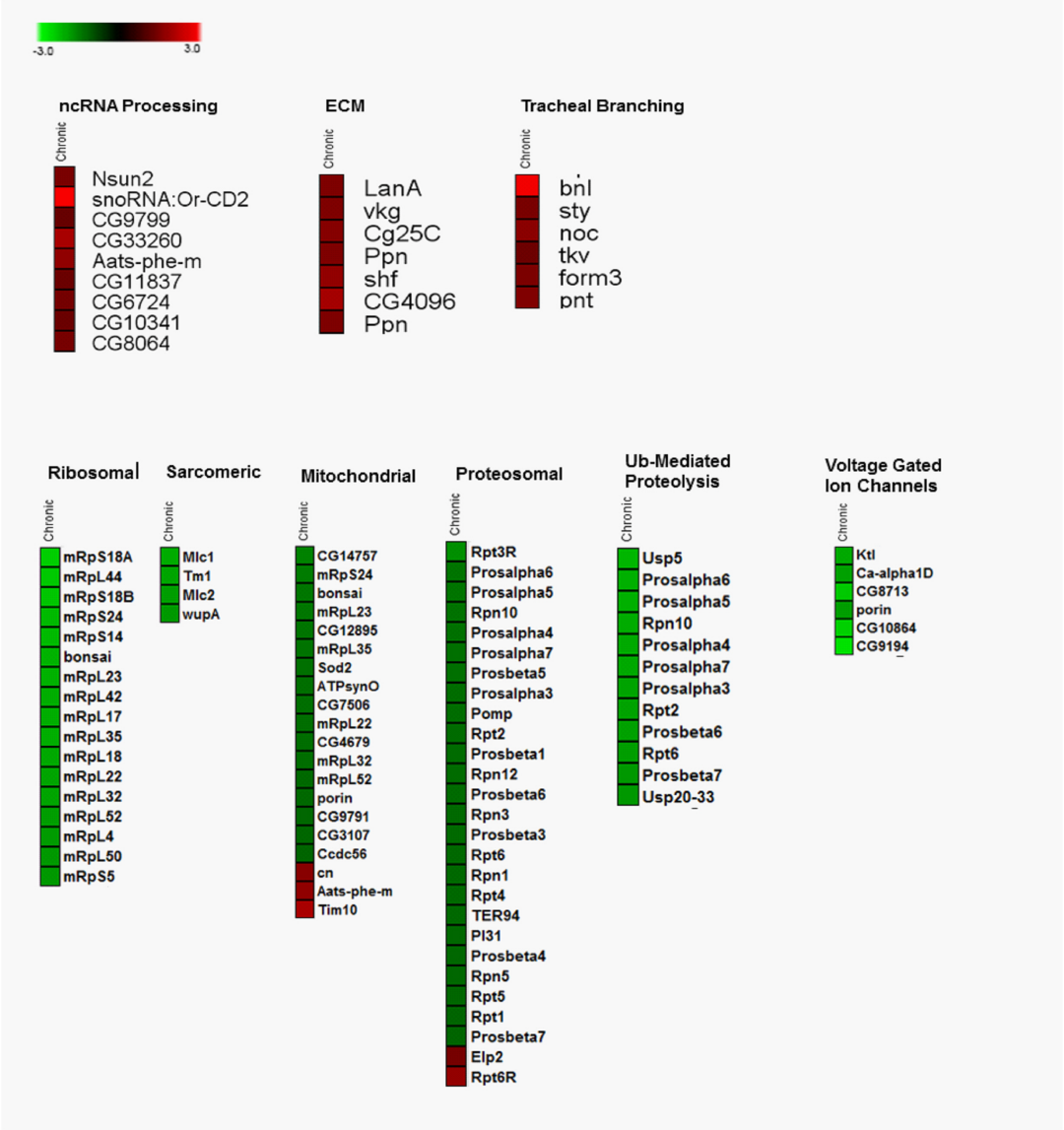

Figure D.4: **Cardiac-specific genes differentially expressed only in chronic hypoxia exposed fly populations.** Transcriptome analysis of RNA from *Drosophila* myocardial and pericardial cells using RNA-seq for cardiac-specific, differential gene expression unique to chronic hypoxia exposed *w1118* flies (CH) and not found in hypoxia-selected flies. Gene familes are grouped by KEGG and DAVID ontologies by biological processes including; non-coding RNA processing, extra-cellular matrix, trachael branching (up-regulated); ribosomal, sarcomeric, mitochondrial, proteosomal, ubiquitin-mediated proteolysis and voltage-gated ion channels (mainly down-regualted).

Figure D.5 (facing page): Cardiac-specific genes differentially expressed only in hypoxia-selected fly populations.
Transcriptome analysis of RNA from *Drosophila* myocardial and pericardial cells using RNA-seq for cardiac-specific, differential gene expression unique to hypoxia-selected flies (HS) and not found in chronic hypoxia exposed flies (HS). Gene familes are grouped by KEGG and DAVID ontologies by biological processes including; non-coding RNA processing, extra-cellular matrix, trachael branching (up-regulated); ribosomal, sarcomeric, mitochondrial, proteosomal, ubiquitin-mediated proteolysis and voltage-gated ion channels (mainly down-regulated).


-3.0
3.0
Cytochrome P450
Adaptive
Cyp9b1
Cyp318a1
Cyp12a4
Cyp4ac2
Cyp6a17
Cyp4d1
sad
Cyp6a2
Cyp12d1-d
Cyp6a8
Cyp6a22
Immune Response
Adaptive
IM23
IM1
psidin
IMPPP
psh
PGRP-SD
Hf
IM2
TotX
sphinx1
Endopeptidase
Adaptive
CG15369
CG16704
Spn42Dd
Reck
Cys
CG16713
IM33
CG10031
Spn43Ad
CG31777
Cell Cycle
Adaptive
Spc25
U2af38
Cdk4
ric8a
sced
Mis12
JNK Cascade
Adaptive
Gadd45
Rac2
kay
puc
Aplip1
Jra
Sarcomeric
Adaptive
sls
Prm
mib2
Mf
Stress Response
Adaptive
Hsp67Bc
Hsp70Bc
Hsp27
Hsp70Bb
Hsp23
Hsp68
CG15100
Hsp26
GstO2
Trxr-1
GstO3
CG32641
CG6852
GstD3
GstD5
GstZ1
TotX
GstD7
GstE11