Cardiac responses to hypoxia and reoxygenation in Drosophila

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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: R1347–R1357, 2015. First published September 16, 2015; doi:10.1152/ajpregu.00164.2015.—An adequate supply of oxygen is important for the survival of all tissues, but it 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, in which 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 min), sustained (18 h), and chronic (3 wk) 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, the Drosophila homolog of the hypoxia-inducible factor alpha subunit (HIF-α), 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 with 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.

Drosophila; heart; hypoxia; hypoxia-inducible factor; sima; genetic selection; reoxygenation

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 physiological 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 HIF-α subunit plays a well-established role in the cardiomyocyte hypoxia response (for a thorough review, see Ref. 43). The hypoxia-mediated induction of HIF pathways and downstream regulation is widely conserved in insects. In Drosophila, there is only a single HIF-α homolog, encoded by sima, which 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 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 melanogaster, 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 min) exposure to 1% O2 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 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% O2) environment for more than 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).

METHODS

**Genetic lines.** Specific loss-of-function of sima was achieved using sima(K07607); 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). w1118, the laboratory wild-type control line, and sima mutant heart function was assessed in 1- to 3-wk 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% O2, 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-wk-old, male hypoxia-selected and normoxia control flies were used for cardiac function and structure studies.

**Acute hypoxia and reoxygenation assay.** Semi-intact hearts were dissected and equilibrated under normoxic conditions, as previously described (13). Dissected preparations were filmed first under 21% O2, and then transferred to a humidified, temperature-controlled glove box and filmed again after 30 min of exposure to oxygen levels adjusted to 4% or 1% O2 using precalibrated mixtures (balanced with nitrogen, see Fig. 1A). Preparations were then returned to 21% O2 and filmed after 30 and 60 min to assay the effects of reoxygenation on cardiac function. Exposed hearts were perfused with artificial hemolymph bubbled with room air or with the premixed, calibrated O2.
mixtures when making hypoxia measurements. Dissolved O₂ content was monitored and recorded using a Qubit Systems OX1LP polargraphic oxygen probe, calibrated, and corrected for mean barometric pressure (758 mmHg), salinity (8.22‰), and perfusate temperatures (21–22°C). The O₂ content was monitored at several time points during experimental sessions. The mean dissolved O₂ content at 1% O₂ was 0.64 mg/l and at 4% O₂ was 1.7 mg/l, and these levels remained stable over the 150-min 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 percentage of the prehypoxia (normoxia) baseline measure for each fly (see Supporting Information for absolute measures).

### Sustained hypoxia and reoxygenation assay

Previous studies and preliminary dose-response tests in whole Drosophila identified critical hypoxia adaptation thresholds at ~4% O₂ and 1% O₂ 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 h at 1% O₂ to be a sustained hypoxia level sufficient to elicit strong phenotypes, while still maintaining viability of wild-type flies. After the 18 h of 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% O₂ prior to filming (see Fig. 3A).

### Chronic hypoxia and reoxygenation assay

We chose 3 wk 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% O₂ because flies can live a normal life span 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 3 wk, under 4% O₂. Food (equilibrated for 24 h at 4% O₂) was changed 2 times per week in the glove box under 4% O₂ hypoxic conditions. These chambers are stable and reliable (24–48 h at 4% O₂ and were flushed daily to maintain stable O₂ levels and eliminate the minimal buildup of carbon dioxide. After 3 wk, flies were removed from the chamber, dissected under normoxic conditions and equilibrated for 30 min in artificial hemolymph at 21% O₂ prior to filming (see Fig. 4A).

### Adapted fly population

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

### 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/s; Hamamatsu EM-CCD) mounted on a Leica DMLFA microscope (McBain Instruments, Chatsworth, CA) to record 30-s movies of beating hearts; images were captured using HC Image (Hamamatsu). 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 s during the 30-s sampling period were labeled “asystolic.” For the acute hypoxia assay, asystolic flies were excluded from the data set if the heart did not resume beating upon reoxygenation (<2% of total dataset, controls, and experimental groups), or if a measurement was more than ± 2 standard deviations from the mean on any parameter (see heart beat histograms in Supporting Information).

### Statistical analysis

All statistical analyses were performed using Prism Statistical Software (version 6, Graph Pad, San Diego, CA). 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, one-way or two-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 two-way ANOVA followed by 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 nonbeating hearts did not represent an aberrant subset, we analyzed the beating and nonbeating 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.

### 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 min at room temperature followed by three rinses in PBST (1× PBS, 0.1% TritonX). Hearts were incubated with 50–100 μl of primary antibody (αPericardin 1:1,000, Developmental Studies Hybridoma Bank) overnight at 4°C. Excess antibody was removed by 3× PBST washes prior to incubation with secondary antibodies and Alexa Fluor 594- or Alexa Fluor 488-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), Axioscam 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

Quantification of the Pericardin network was performed with ImageJ software (http://imagej.nih.gov/ij/). Z-stack images (e.g., Fig. 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 (×25) and with identical exposure settings.

### RESULTS

#### Wild-type cardiac responses to acute hypoxia/reoxygenation

We exposed semi-intact fly heart preparations to the acute hypoxia/reoxygenation protocol (acute H/R; 1% or 4% O₂ for 30 min), illustrated in Fig. 1A. Qualitative responses are illustrated in M-modes (Fig. 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% O₂ acute H/R is shown on the left; the response of a different heart to 1% O₂ acute H/R is shown on the right. Both treatments caused the heart rate to slow significantly (Fig. 1, C and D), but hearts exposed to 1% O₂ tended to exhibit extremely
long pauses (>25 s, “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, posthypoxia function of these asystolic hearts did not differ significantly compared with hearts that were able to beat during hypoxia. Nevertheless, we used highly conservative statistics and only analyzed flies that 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% O2 but was dramatically reduced upon exposure to 1% O2 (Fig. 1E). This suggests that the wild-type Drosophila heart can respond differentially to short-term changes in oxygen content (30 min 4% O2 vs. 1% O2). On the basis of these observations,

Fig. 2. Acute cardiac response to 30 min 1% O2 acute H/R in wild-type and sima mutant Drosophila. A: incidence of nonbeating or “asystolic” hearts (>25 s without contractions) in response to hypoxia exposure, expressed as a percentage of total hearts examined. Nearly half of wild-type and sima mutant hearts entered prolonged periods of asystole at 30 min in 1% O2, and all resumed beating on reoxygenation. B–G: data are presented as the percent change from the prehypoxia baseline during and after hypoxia treatment for wild-type, sima+/+, and sima−/−. B: in hearts that beat, heart period (HP) is increased 100% or greater under 1% O2 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 after 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% O2, 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% O2 and remained larger at 30 min after hypoxia exposure compared with controls. G: systolic diameters were significantly increased during acute hypoxia in both wild-type and sima−/+ . All values are mean percent change ± SE (n = 10 for w1118; n = 12 for sima+/+; n = 16 for sima−/−). For heart period, systolic and diastolic interval, asystolic hearts were excluded, while all hearts are included in fractional shortening and diastolic and systolic diameter. Data were analyzed by Kruskal-Wallis test (B, C) and Dunn multiple-comparison post hoc test (D–F), and two-way ANOVA and Tukey’s multiple-comparisons post hoc test. n.s., no statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant. Changes at T = 30 between genotypes were analyzed by Sidak-Bonferroni t-test. tP < 0.05; ttP < 0.01. See Supporting Information for raw numbers.
we used a 1% O2 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 (Fig. 1A) resulted in a robust cardiac response that included temporary cessation of beating (for 25 s or longer) in 40–60% of the heart for both wild-type and sima heterozygous (sima+/−) and homozygous (sima−/−) mutants (“asystolic” in Fig. 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 (Fig. 2, B–D). Upon reoxygenation, all hearts resumed preexposure beating patterns (Fig. 2A), and heart period and diastolic intervals returned to their prehypoxia exposure levels (Fig. 2, B and C; T = 30 compared with T = 60 and 90). Systolic intervals did not change significantly from the baseline during hypoxia or subsequent reoxygenation for both control and sima mutant genotypes (Fig. 2D).

Cardiac contractility, measured as fractional shortening, decreased dramatically in all genotypes during acute hypoxia (Fig. 2E), in part, due to increases in systolic diameters (Fig. 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 (Fig. 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 preexposure values. We conclude that chronotropic and ionotropic cardiac function is mostly restored at 1 h of reoxygenation postexposure to extreme hypoxia, consistent with the previously observed overall tolerance and recovery of fruit flies to acute hypoxic/anoxic conditions (2, 18).

**Cardiac response to sustained hypoxia/reoxygenation.** We examined cardiac performance after more sustained periods of hypoxia lasting hours to days. We determined that a sustained hypoxic exposure for 18 h at 1% O2 produced significant changes in cardiac function upon reoxygenation (sustained H/R). Using this protocol (illustrated in Fig. 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 (Fig. 3, C–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 (Fig. 3, F–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, more than 70% of sima−/− hearts did not beat following our sustained H/R protocol (Fig. 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+/−), in which 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.

Fig. 3. Cardiac response to sustained H/R in wild-type and sima mutants. A: protocol for sustained hypoxia treatment (sustained H/R). B: percent of hearts beating during normoxia and post-18 h 1% hypoxia. C: heart period significantly shortened (rate increased) upon exposure to sustained H/R in both wild-type and sima+/− hearts. D: exposure to sustained H/R leads to significant reductions in diastolic interval in both wild-type and sima+/− hearts. E: 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 with normoxic conditions. F: fractional shortening decreased in wild type in response to sustained H/R; in sima+/− hearts, this decrease was significantly exacerbated compared with controls. G: these changes in fractional shortening were due, in part, to significant increases 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 ± SE for hearts that beat on reperfusion after a sustained hypoxia exposure (w1118; n = 59 normoxia, n = 24 hypoxia; sima+/−; n = 17 normoxia, n = 20 hypoxia). Data were analyzed by Kruskal-Wallis test and Dunn multiple-comparisons post hoc test (C, D) and two-way ANOVA with Tukey’s multiple-comparison post hoc test (E–H). *P < 0.05, **P < 0.01, ***P < 0.001. See Supporting Information for raw data.
ures (Fig. 3, C–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 (Fig. 3C). Although both wild-type controls and sima\(^{+/--}\) showed decreases in diastolic intervals (Fig. 3D), in sima\(^{+/--}\), the decrease in heart period was also due to a significant decrease in systolic intervals (Fig. 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 diameters 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 (Fig. 3, F and 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.

**Cardiac response to chronic hypoxia/reoxygenation.** We monitored cardiac function in wild-type flies after a 3-wk exposure to moderate, chronic H/R (4% O\(_2\)), followed by reoxygenation and assessment of cardiac function (illustrated in Fig. 4A). In contrast to the shortened heart period observed after sustained H/R (Fig. 3C), the heart period and diastolic intervals of wild-type flies exposed to chronic H/R lengthened significantly compared with hearts from normoxia-raised controls (Fig. 4, B and C, white bars). This response was not observed in sima\(^{+/--}\) hearts in which heart period and diastolic intervals were unaffected by chronic H/R (Fig. 4, B and C, gray bars). Chronic H/R had no effect on systolic intervals in wild-type controls but caused significant decreases in sima\(^{+/--}\) (Fig. 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 (Fig. 4, E–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.

**Multigenerational 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\(_2\); Fig. 5A). The hearts from hypoxia-selected flies beat more slowly (at 4% O\(_2\)) compared with the normoxia controls (at 21% O\(_2\)), as a result of increased diastolic intervals, even while the average systolic interval shortened (Fig. 5, B–D). Moreover, fractional shortening in hypoxia-selected flies was significantly reduced compared with normoxia controls, due to reductions in both diastolic and systolic diameters (Fig. 5, E–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, we found that the average diastolic diameters were still significantly smaller for hypoxia-selected flies (54 \(\mu\)m for normoxia, compared with 36 \(\mu\)m for hypoxia-selected uncorrected and 42...
μm for hypoxia-selected corrected, \( P < 0.001 \) two-way ANOVA).

Prolonged hypoxia and reoxygenation cause 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 (Fig. 6A). This arrangement appears to be maintained in response to the different hypoxia treatments (Fig. 6, A–C), despite the observed constriction and reduced contractility (Figs. 3 and 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 (Fig. 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 (Fig. 6, E–G). Nevertheless, both hypoxia treatments appeared to increase overall Prc collagen fiber density (Fig. 6G).

We also investigated whether there was any structural remodeling in fly hearts after multigenerational 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 with their normoxia-raised controls (Fig. 6, H–K). In particular, the Prc-positive collagen covered an area that was much wider than the heart itself, despite constriction of the heart tube (Fig. 6I) in these flies that can survive and reproduce in 4% \( \text{O}_2 \).

### 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 multigenerational hypoxia (acute H/R, sustained H/R, chronic H/R,
and hypoxia-selected, respectively) induce a range of cardiac responses (summarized in Fig. 7).

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 produce similarly variable responses. Acute hypoxia causes a dramatic slowing of the heart and equally dramatic reductions in contractility in wild-type flies (Fig. 2). These are likely compensatory responses, as they would be expected to reduce ATP demand in a low-oxygen environment (Figs. 1 and 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 min.

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

Fig. 6. 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-wk old w¹¹¹⁸ fly exhibits the tightly packed, circumferential arrangement of F-actin-stained myofibrils within the myocardial cells. This structure is not affected by sustained H/R (B) or chronic H/R (C). D: pericardin (collagen IV homolog) staining reveals the “fish net” pattern of fibers that associate with sarcomeric structures. E: collagen network appears denser and is moderately disorganized following sustained H/R and is more disorganized following chronic H/R (F). G: pericardin staining that was quantified from Z stacks of immunostained hearts shows an increase in intensity following both sustained and chronic H/R. Data are expressed as the percent of total pixels with an intensity above threshold (see Methods; number of hearts (l to r) = 13, 11, 7). Data were analyzed by one-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 (nonmyocardial). 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: 50 μm.
H/R (Figs. 3A and 4B). We also observed increased collagen deposition in hearts from wild-type flies as a consequence of sustained and chronic hypoxia exposure (Fig. 6), which is consistent with known remodeling of the human myocardium upon prolonged exposure to hypoxia that is partially dependent on HIF-1α (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 (Figs. 3F and 4E) and duration of cardiac contractions (Figs. 3E and 4D) compared with 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 with 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 flies (Fig. 4B). Therefore, even though the per beat cardiac output is reduced, the overall faster heart rate might be compensatory in the sima heterozygotes compared with wild-type flies.

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). Overexpression 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 HIF-1α 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 HIF-1α or HIF-2α 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, HIF-α 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 multigenerational 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 multigenerational and continued chronic exposure to hypoxia (8, 50, 54). Interestingly, in human populations adapted to high altitude, their “hypoxic” hearts have increased myocardial glucose uptake compared with wild-type flies.

### Table: 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>
<thead>
<tr>
<th>Cardiac measure</th>
<th>Hypoxia condition</th>
<th>Heart Period</th>
<th>Diastolic Interval</th>
<th>Systolic Interval</th>
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<tbody>
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<td></td>
<td></td>
<td>acute H/R</td>
<td>sustained H/R</td>
<td>acute H/R</td>
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<tr>
<td></td>
<td></td>
<td>30 min 1% O₂</td>
<td>18 hours 1% O₂</td>
<td>30 min 1% O₂</td>
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<td></td>
<td></td>
<td>30 min post 1% O₂</td>
<td>3 weeks 4% O₂</td>
<td>30 min post 1% O₂</td>
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<tr>
<td></td>
<td></td>
<td>60 min post 1% O₂</td>
<td>3 weeks 4% O₂</td>
<td>60 min post 1% O₂</td>
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<td>sustained H/R chronic H/R</td>
<td>acute H/R</td>
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<td>18 hours 1% O₂</td>
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<td>chronic H/R</td>
<td>sustained H/R</td>
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<td>chronic H/R</td>
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<td>60 min post 1% O₂</td>
<td>3 weeks 4% O₂</td>
<td>60 min post 1% O₂</td>
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† increased; ‡ decreased; ‡ no change or discernible difference; † trend, significant by 1-way ANOVA within genotype only.

Fig. 7. A 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 is shown.
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 life span 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 with previous studies, our system 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 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.

Perspectives and Significance

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 multigenerational hypoxia and that these responses are partly mediated by signaling via the HIF-1α 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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15. Gilbert-Kawai ET, Milledge JS, Grocott MPW, Martin DS. Cardiovascular responses to altitude (15). However, genetic tools and the long life span 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 with previous studies, our system 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 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.
CARDIAC RESPONSES TO HYPOXIA


