Moderate and severe hypoxia elicit divergent effects on cardiovascular function and physiological rhythms

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Key Points Summary

- Here we provide evidence for divergent physiological responses to moderate compared to severe hypoxia—addressing an important knowledge gap related to severity, duration and after-effects of hypoxia encountered in cardiopulmonary situations.
- The physiological responses to moderate and severe hypoxia were not proportional, linear or concurrent with the time-of-day.
- Hypoxia elicited severity-dependent physiological responses that either persisted or fluctuated throughout normoxic recovery.
- The physiological basis for these distinct cardiovascular responses implicates a shift in the sympathovagal set point and not likely molecular changes at the artery due to hypoxic stress.

Key points word count: 92

Abstract

Hypoxia is both a consequence and cause of many acute and chronic diseases. Severe hypoxia causes hypertension with cardiovascular sequelae, however, the rare studies using moderate severities of hypoxia support that it can be beneficial, suggesting hypoxia may not always be detrimental. Comparisons between studies are difficult due to varied classifications of hypoxic severities, methods of delivery and use of anesthetics. Thus, to investigate the long-term effects of moderate hypoxia on cardiovascular health, radio-telemetry was used to obtain *in vivo* physiological measurements in unanesthetized mice during 24-hours of either moderate (F_IO_2=0.15) or severe (F_IO_2=0.09) hypoxia, followed by 72-hours of normoxic recovery. Systolic blood pressure was decreased during recovery following moderate hypoxia but increased following severe. Moderate and severe hypoxia increased heme oxygenase-1 expression during recovery, suggesting parity in hypoxic stress at the level of the artery. Severe, but not moderate, hypoxia increased the low/high frequency ratio of heart rate variability 72 hours post-hypoxia, indicating a shift in sympathovagal balance. Moderate hypoxia dampened the amplitude of circadian rhythm while severe disrupted rhythm during the entire insult, with perturbations persisting throughout normoxic recovery. Thus, hypoxic severity differentially regulates circadian blood pressure.

Abbreviations

COPD, chronic obstructive pulmonary disease

EPO, erythropoietin

F_IO_2, fraction of inspired oxygen

HF, high frequency

HIF, hypoxia-inducible factor

HMOX1, heme oxygenase 1

LF, low frequency

NOS, nitric oxide synthase

PaO_2, arterial oxygen pressure

RMSSD, root mean square of successive normal R-R interval differences

SBP, systolic blood pressure

SDNN, standard deviations of normal R-R intervals

Introduction

Impairments in oxygen delivery are both a cause and consequence of many acute and chronic disease states (e.g. obstructive sleep apnea, heart failure, chronic obstructive pulmonary disease [COPD]) and associated with a reduced quality of life and increased mortality. Investigation of the pathophysiological consequences of hypoxia illustrate primarily detrimental outcomes of sustained (Sheedy *et al.*, 1996; Viganò *et al.*, 2011; Simpson & Iscoe, 2014) or intermittent (Fletcher *et al.*, 1992; Campen *et al.*, 2005; Simpson *et al.*, 2008) severe hypoxia. Whether hypoxia is caused by breathing low oxygen (~F_IO_2 <0.10) or the application of a respiratory load, the resultant hypoxic outcome is equivalent to what would be observed physiologically at an elevation of >6500 m above sea level (ranging between the peaks of Mount Kilimanjaro to Mount Everest). The nature of hypoxia is a product of available oxygen, prevailing pressure, duration of exposure, adaptation and metabolic demand (including organ-specific hypoxia). However, hypoxia is also associated with beneficial outcomes in cognitive performance (Leconte *et al.*, 2012). Importantly, there is no standardization of hypoxic thresholds and it is difficult to reconcile the conditions to which each apply due to disagreements in the classification of severities (e.g. mild, moderate and severe), method of delivery, duration, and (in some cases) use of anaesthetic. Further, direct comparisons of moderate and severe hypoxia are seldom (Frappell *et al.*, 1991; Morgan *et al.*, 2014) and studies investigating the pathophysiology of moderate hypoxia are rare (Haider *et al.*, 2009). This is an

important omission given that the clinical gradation of hypoxia in most disease states is typically mild to moderate (approximately equivalent to F_IO_2=0.15; ~2500 m above sea level, e.g. Aspen, CO) (Thomas *et al.*, 1961; Hayashi, 1976; Tuck *et al.*, 1984; Oswald-Mammosser *et al.*, 1995; Mannino *et al.*, 2002).

Systemic reductions in arterial oxygen pressure (PaO_2), either by reducing the fraction of inspired oxygen (F_IO_2) or hemoglobin content, does not necessarily equate to similar hypoxia of various organs. Activation of compensatory neural and vascular mechanisms attempt to maintain sufficient oxygenation of vital organs. Following decreases in PaO_2, expression of hypoxia inducible factor (HIF)-1α, a highly-conserved, oxygen-sensitive transcript factor, is elevated in some organs (e.g. brain) but remains unresponsive until PaO_2 is severely reduced in others (e.g. kidney) (Stroka *et al.*, 2001). The time profile of HIF-1α expression also appears to be organ-specific and differ between moderate and severe hypoxia (Stroka *et al.*, 2001). This organ-specific transcriptional response to hypoxia is also seen in anemia, where in response to mild, moderate and severe anemia, heterogeneous expression of HIF-1α occurs in the brain, kidney and liver (Tsui *et al.*, 2014; Mistry *et al.*, 2018). These patterns are not necessarily reflected in the expression of HIF down-stream targets (e.g. heme-oxygenase I [HMOX-1], erythropoietin [EPO]) (Tsui *et al.*, 2014), suggesting that HIF alone is not sufficient to predict expression. The severity of hypoxia also produces different metabolic responses. Both moderate and severe hypoxia depress aerobic metabolism but only severe hypoxia increases anaerobic metabolism; changes which persist following normoxic recovery (Frappell *et al.*, 1991). These data support the concept that the molecular and biochemical responses to moderate and severe hypoxia are heterogeneous.

Further discrepancies between moderate and severe hypoxia are also present in the cardiovascular response following hypoxia. Exposure to both intermittent and sustained severe hypoxia leads to hypertension in animals (Fletcher *et al.*, 1992; Vaziri & Wang, 1996; Campen *et al.*, 2005; Zoccal *et al.*, 2007) and humans (Olea *et al.*, 2014). In contrast, individuals living in high-altitude, moderately hypoxic environments do not show elevations in blood pressure (Ruiz & Peñaloza, 1977; Bruno *et al.*, 2014); however, the latter findings could be the result of long-term genetic adaptations (Hochachka *et al.*, 1996; Moore, 2001; Lorenzo *et al.*, 2014). Interestingly, exposure to mild, intermittent hypoxia can be cardioprotective (Navarrete-Opazo & Mitchell, 2014; Mateika *et al.*, 2015; El-Chami *et al.*, 2017), but the corresponding effects in health are unknown. To

determine pathophyisological mechanisms, it is important to first establish the effect of variable hypoxic gradations in health.

The objective of the present study was to compare the cardiovascular responses to moderate and severe hypoxia followed by normoxic recovery. We hypothesized that the physiological response to moderate hypoxia is not simply a scaled down response to severe hypoxia. Radio-telemetry provided unanesthetized, unrestrained and continuous *in vivo* physiological measurements (Kim *et al.*, 2013) before, during and after either moderate or severe hypoxia. We found distinct cardiovascular responses between moderate and severe hypoxia that are neither proportional nor linear nor concurrent with the time-of-day. Divergent changes in sympathovagal activity could be the cause for the observed differences. Finally, recovery from moderate and severe hypoxia elicited either persistent or fluctuating cardiovascular changes during normoxic recovery.

Methods and Materials

Ethical Approval

Adult male C57Bl/6J mice were bred in our facility and aged 8-12 weeks (~25 g body weight) prior to surgery. Animal housing was maintained at 24˚C, 45% humidity and kept to a 12-hour light-dark cycle (lights on: 08:00h; lights off: 20:00h). Following telemetry implantation, animals were housed individually with food and water provided *ad libitum*. Housing and experimental procedures were approved by the Animal Care Committee at the University of Guelph in conformity with the guidelines of the Canadian Council on Animal Care.

Telemetry

HDX11 murine telemetry transmitters (Data Science International, St Paul, MN, USA) were used to measure systolic blood pressure (SBP), heart rate, core body temperature and physical activity. Briefly, mice were anesthetized with isoflurane/oxygen (2%:100%), intubated and body temperature was maintained using a water-filled heating pad. A local anesthetic 50:50 mix of lidocaine (3 mg/kg) and bupivicaine (1.5 mg/kg) was administered subcutaneously at the incision sites. The right carotid artery was isolated and the pressure catheter was inserted and secured in

place using 7-0 suture and vet bond (3M, London, ON, Canada). To accurately measure core body temperature, the telemetry units were implanted in the abdomen—a 7 cm pressure catheter is superior to the standard 5 cm length to minimize kinking of the pressure catheter, which can cause signal dropout of the blood pressure tracing. Following insertion, the transmitter was advanced subcutaneously to the abdomen and secured intraperitoneally. Two electrocardiography leads were placed subcutaneously, one above the rib cage and the second above the abdominal wall, and secured to the underlying muscle layer. Animals recovered on a warming bed and carefully monitored for post-surgical complications. Post-operative analgesic buprenorphine (0.1 mg/kg) was given upon awakening and at 8 and 24 hours postoperatively; subsequent analgesic was given as required.

Two-weeks post-operatively, mice were individually housed within an environmental chamber (Figure 1; 830-ABB, Plas Labs, Lansing, MI, USA) where oxygen levels could be titrated accordingly (ProOx 110, BioSpherix, New York, NY, USA). Drierite (W.A. Hammond Drierite Company, Xenia, OH, USA) and calcium carbonate were added to the chamber to maintain constant ambient humidity and prevent elevations in carbon dioxide. Each cage was placed on a telemetry receiver (RPC-1, Data Science International, St. Paul, MN, USA) within custom made Faraday cages. Telemetry signals were collected every 5 minutes for 30 seconds. Ambient temperature (C10T, Data Science International) and pressure (APR-1, Data Science International) were also recorded throughout the duration of the study. All signals were collected using computer acquisition software (Dataquest ART V.3.3, Data Science International) and exported to Microsoft Excel for further analysis (Excel 2011, Microsoft, Redmond, WA, USA).

Hypoxia Study Design

Each animal was exposed to only one hypoxic insult (either moderate or severe; maximum 3 animals at a time). Baseline recordings were obtained over a weekend and hypoxia (moderate [F_IO_2=0.15] or severe [F_IO_2=0.09]) was gradually induced Monday morning at 08:00h over 15 minutes. After 24 hours of hypoxia, ambient oxygen levels were restored and telemetry continued for an additional 72 hours.

qPCR Analysis

At the end of the normoxic recovery, animals were re-anesthetized with isoflurane. Mesenteric artery samples were isolated and excised using a dissection microscope. Following excision, samples were immediately frozen in liquid nitrogen and stored at -80˚C until analysis. RNA extraction was performed on ~50 mg of mesenteric arteries (pooled from 3 animals) with Trizol reagent according to the manufacturer's instructions (Invitrogen, Life Technologies, Burlington, ON, Canada). RNA samples were then treated using a RNase Free DNase (Qiagen), according to manufacturer's instructions. Concentrations of isolated RNA were quantified using a spectrophotometer (NanoDrop, ND1000, Thermo Scientific, Mississauga, ON, Canada). Generation of cDNA was completed using qScript cDNA SuperMix (Quanta Biosciences, Beverly, MD, USA), according to the manufacturer's instructions, using standardized 100 ng of RNA per sample. Quantitative real-time PCR was performed using SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies) with a CFX Connect Real-Time PCR Detection System (BioRad) and primers for HMOX1, EPO and GAPDH, as listed in Table 1. All RNA data are expressed relative to GAPDH, which was stable across all states with no difference in the raw C_T values observed between groups (p>0.05).

Immunoblotting

Samples were homogenized in buffer with a phosphatase and protease inhibitor cocktail and total protein content was measured by BCA assay as previously described (Foster *et al.*, 2017). Briefly, samples were loaded onto a 4-20% Criterion TGX precast gel (BioRad, Mississauga, ON, Canada) alongside 10 µl Precision Plus Protein Standards Kaleidoscope ladder (BioRad, Mississauga, ON, Canada) and were separated by SDS-PAGE followed by immunoblotting. Nitrocellulose membranes were rinsed in ddH_2O and then incubated in Pierce Reversible Memcode Stain (Thermo Fisher, Burlington, ON, Canada) for 5 minutes to confirm equal protein transfer. The blot was imaged using a ChemiDoc MP Imaging System (BioRad, Mississauga, ON, Canada) prior to stain removal (Pierce Stain Eraser, Thermo Fisher, Burlington, ON, Canada). Membranes were blocked (5% non-fat dry milk in 1X TBS) and incubated with a primary anti-HMOX1 antibody [1:1000] (Cat: 82585, Abcam, Toronto, ON, Canada) overnight at 4˚C. Membranes were washed and subsequently incubated with a goat anti-rabbit horseradish peroxidase conjugated secondary antibody [1:2000]

(Cat: 2054, Santa Cruz, Dallas, TX, USA). All antibody dilutions were completed in 1% non-fat dry milk and membrane washes were completed in 1X TBS with 0.5% Tween. Signal was detected by chemiluminescence (Thermo Fisher Scientific), imaged (ChemiDoc, Bio-Rad) and quantified using Image Lab software (Bio-Rad). Values were obtained by measuring the target band relative to the total protein of the lane.

Heart Rate Variability

Frequency-domain heart rate variability analysis was conducted using Kubios Heart Rate Variability Analysis Software 2.2 for Windows (University of Kuopio, Finland). The continuous R-R interval signal was re-sampled to 20 Hz and analysed by fast Fourier transformation. Spectral analysis was completed on one 30-second epoch taken at the beginning of each hour during a segment of the lights on period (10:00 to 18:00h–corresponding to 2 to 10 and 122 to 130 hours Zeitgeber Time for baseline and normoxic recovery, respectively). Results are presented as the mean value of these nine segments. This method was selected to ensure signal stationarity and improve overall reproducibility (Thireau *et al.*, 2008). Each file was visually inspected to verify the absence of ectopic beats or signal artifact, defined as <5% of the total number of beats. If present, abnormal beats were corrected using a piecewise cubic spline interpolation method. As recommended for mice, frequency cut-offs of 0.15-1.5 Hz were selected as the low frequency (LF) range and 1.5-5.0 Hz as the high frequency (HF) range, which has been validated pharmacologically (Thireau *et al.*, 2008). The LF and HF spectral values are presented in relative (%) and normalized power (nu). Normalized power removes the contributions of very low frequency (0.00-0.15 Hz) to total power. Total power consists of the area over the whole frequency spectrum (0.0-5.0 Hz). The LF/HF ratio was calculated as a general marker of sympathovagal balance (Nunn *et al.*, 2013).

Data Analysis

Raw data for SBP, heart rate, body temperature and physical activity were averaged for each hour to obtain hourly means. For baseline measurements, data means for each parameter were organized into 48-hour periods and then averaged between all animals. For hypoxia and normoxic recovery, data means were averaged in its entirety between all animals. Each animal was recorded

at baseline prior to hypoxic exposure allowing for them to serve as their own control. Circadian mesor (mean value around which the wave oscillates), amplitude (difference between peak/trough and mean) and acrophase (time at which peak occurs) values were calculated and analysed using cosinor analysis as previously described (Munakata *et al.*, 1990; Refinetti *et al.*, 2007). For telemetry data, one-way repeated measures ANOVA were performed on ten 1-hour averages from each animal during both light and dark cycles (i.e. excluding the four 1-hour intervals that bordered both cycles to remove the influence of transition periods). If a significant main effect of time was detected, Holm-Sidak post-hoc analysis was performed on data sets that were normally distributed. For non-normally distributed data, Friedman's test was used with Dunn's post-hoc. A 5 x 2 (time x group) mixed model ANOVA was also performed on 10-hour averaged telemetry data to determine whether there were differences between the hypoxic conditions during lights on and lights off. If there was a significant interaction, Holm-Sidak post-hoc analysis was performed to compare differences between severe and moderate hypoxia at the same time point. Statistical analysis of heart rate variability data was completed using a 2 x 2 (time x group) mixed model ANOVA, and Holm-Sidak post-hoc analysis was performed when appropriate. Baseline and 72 hours post-hypoxia were chosen for HRV analysis as the latter showed the most divergent response in SBP. HRV analyses for all time points using a 5 x 2 (time x group) mixed model ANOVA are also presented. A 2 x 2 (time x group) mixed model ANOVA was performed for mRNA expression, except time and group were both between subject comparisons. For protein data, one-tailed Mann-Whitney tests were performed comparing hypoxic conditions to normoxia. Graphical and data analyses were completed using GraphPad (Prism 6, GraphPad Software Inc, LaJolla, CA, USA). Differences were considered significant at $p<0.05$.

Results

Body temperature and activity responses to moderate and severe hypoxia

To confirm mice responded to hypoxia, we measured body temperature for hypoxia-induced anapyrexia. Not surprisingly, and in agreement with previous research (Yuen *et al.*, 2012), severe hypoxia decreased body temperature during lights on and off (Figure 2A, C and D). To be certain whether this was a simple static difference or an effect on circadian rhythm, we performed cosinor analysis. Indeed, rhythm during severe hypoxia was disrupted with a mild disagreement of R^2

(goodness-of-fit), decreased mesor and increased amplitude (Table 2, Figure 3). During moderate hypoxia, body temperature was slightly decreased during lights off but not lights on (Figure 2B, E and F); rhythm was unaffected (Table 2, Figure 4). To investigate whether hypoxia had any residual effects on physiological parameters, we continued our analysis after return to normoxia (Figure 2A-F, Table 2). While severe hypoxia had a rebound change in body temperature that persisted during lights off, moderate hypoxia had only a modest increase in body temperature in the first 12 hours of recovery, with another modest increase compared to baseline at 72 hours post-hypoxia. Normoxic recovery from severe hypoxia also had a rebound effect on mesor and amplitude with a strong disagreement of R^2–where the former persisted and the latter two dissipated after 24 hours. Normoxic recovery from moderate hypoxia did not significantly affect rhythm. A two-way (time x group) ANOVA indicated a divergent response in body temperature to hypoxic severity (Figure 2G and H). Severe hypoxia induced a rebound in body temperature that exceeded baseline levels 24 hours post-hypoxia, while moderate induced a mild decrease in body temperature that did not persist during normoxic recovery.

Similar to body temperature, severe hypoxia decreased activity during lights on and off (Figure 5A, C and D). Rhythm was disrupted, evidenced by a strong disagreement in R^2 and a decreased mesor (Table 2, Figure 3). In contrast, moderate hypoxia had no effect on overall activity (Figure 5B, E and F) or rhythm (Table 2, Figure 4). Severe hypoxia had no effects on activity during normoxic recovery, including rhythm. Interestingly, moderate hypoxia increased activity only at 72 hours post-hypoxia during lights off (i.e. the last 12 hours of recording). Rhythm was also disrupted at 72 hours post-hypoxia as R^2 was in mild disagreement and mesor and amplitude were increased. A two-way (time x group) ANOVA confirmed a divergent response in activity to hypoxic severity (Figure 5G and H) with severe, but not moderate, hypoxia causing a decrease in activity. Thus, activity changes in response to hypoxia did not explain changes in body temperature during normoxic recovery. In addition, there were no differences in activity between groups at baseline or at any time post-hypoxia (data not shown), suggesting that arousal state was similar.

Systolic blood pressure responses to moderate and severe hypoxia

To determine the physiological risk for hypertension due to severity of hypoxia, we assessed ambulatory SBP. Severe hypoxia decreased SBP during lights on but not lights off (Figure 6A, C and

D). Rhythm was disrupted during severe hypoxia with a mild disagreement of R^2, decreased mesor and increased amplitude, with no change in acrophase (Table 2, Figure 3). In contrast, moderate hypoxia decreased SBP during lights off but not lights on (Figure 6B, E and F); rhythm was disrupted with a mild disagreement of R^2, decreased mesor with no change in amplitude or acrophase (Table 2, Figure 4). Surprisingly, while severe hypoxia had a rebound change in SBP, moderate hypoxia had a persistent change over 72 hours. Severe hypoxia also had a rebound effect on mesor and amplitude with a strong disagreement of R^2—where the former persisted and the latter two dissipated after 24 hours. Moderate hypoxia only had a persistent effect on mesor throughout 72 hours. Further, the after-effects of severe and moderate hypoxia were most salient in lights on or lights off, respectively. A two-way (time x group) ANOVA confirmed a divergent response in SBP to hypoxic severity (Figure 6G and H) acutely and in recovery, thus indicating hypoxic severity differentially regulates circadian blood pressure. Body temperature and activity were similar for this subset of animals compared to the full cohort (data not shown).

Heart rate responses to severe and moderate hypoxia

Next, we examined whether changes in blood pressure were associated with corresponding changes in heart rate. Severe hypoxia decreased heart rate during lights on and lights off (Figure 7A, C and D). Heart rate rhythm was also disrupted during severe hypoxia with a mild disagreement of R^2 and decreased mesor (Table 2, Figure 3). In contrast, moderate hypoxia increased heart rate during lights on, but not lights off (Figure 7B, E and F); rhythm was disrupted with a strong disagreement of R^2 with increased mesor and decreased acrophase (Table 2, Figure 4). During normoxic recovery, heart rate rebounded initially following severe hypoxia with fluctuations during the 72-hour period; then returning to baseline (Figure 7C and D). There was a strong disagreement of R^2 at 24 hours following severe hypoxia, but returned to baseline by 72 hours; mesor was decreased throughout. Conversely, moderate hypoxia decreased heart rate throughout the majority of the normoxic recovery period. Rhythm was disrupted following moderate hypoxia, evidenced by a mild decrease in R^2 and a sustained decrease in mesor. A two-way (time x group) ANOVA confirmed a divergent response in heart rate to hypoxic severity (Figure 7G and H). Thus, severe and moderate hypoxia had opposing effects on heart rate during hypoxic stress with a general reduction in heart rate during recovery.

Heart rate variability responses to moderate and severe hypoxia

To further investigate the mechanism underlying changes in SBP and heart rate, we utilized heart rate variability analysis 72 hours post hypoxia as this represented the greatest difference in divergent SBP response (Table 3; HRV data for all time points are presented in Table 4). R-R interval increased only in response to severe hypoxia (Figure 8A, Table 3). Moderate hypoxia increased the standard deviations of normal R-R intervals (SDNN) and root mean square of successive normal R-R interval differences (RMSSD) 72 hours post hypoxia compared to baseline (Table 3). Total spectral power was also generally increased in response to hypoxia. Hypoxic severity induced a divergent response to the LF/HF ratio; there was no change in the LF/HF ratio following moderate hypoxia while severe increased it. The change in the LF/HF ratio in response to severe hypoxia was mediated by an increase in the relative and normalized power of the LF band following severe hypoxia and a corresponding decrease in the HF band. Following moderate hypoxia, the relative and normalized power of the LF and HF bands were decreased and increased, respectively (Figure 8B and C, Table 3). In addition, LF power was higher and HF power was lower 72 hours following severe hypoxia compared to moderate. This was also evidenced by an increase in the LF/HF ratio following severe compared to moderate hypoxia (Figure 8D). Thus, severe hypoxia induced a shift in sympathovagal balance towards sympathetic dominance, while moderate hypoxia increased parasympathetic activity with a potential decrease in sympathetic activation.

Effect of moderate and severe hypoxia on mesenteric resistance arteries

Divergence in SBP recovery from moderate and severe hypoxia was most consistent and robust at the end of the study. Thus, to determine whether localized molecular mechanisms of hypoxic stress in resistance arteries could account, at least in part, for the observed divergent physiological responses, we examined gene expression of canonical targets of HIF (EPO and HMOX1) in mesenteric arteries. Both moderate and severe hypoxia increased HMOX1 mRNA expression while EPO was unchanged (Figure 9A and B). HMOX1 protein levels (Figure 9C-E) were in agreement with mRNA expression. This suggests residual oxidative stress, but not tissue hypoxia, is observed in resistance blood vessels. Physiologically, the consequence of hypoxia on SBP resides in a summation

of inputs—both systemic and localized. Here, we find agreement in localized stress but disagreement in systemic sympathetic dominance.

Discussion

We demonstrate, for the first time, contrasting hemodynamic responses during normoxic recovery following moderate and severe hypoxia. These results highlight the importance of hypoxic severity in mediating the physiological response. Moderate and severe hypoxia both decreased SBP during the hypoxic insult, but induced divergent hypertensive and hypotensive responses, respectively, following normoxic recovery. While both moderate and severe hypoxia increased expression of HMOX1, a potent hypoxia-induced vasodilator, only severe hypoxia induced a shift in sympathovagal balance towards sympathetic dominance. Conversely, moderate hypoxia resulted in an increase in parasympathetic activity with a potential decrease in sympathetic dominance. Thus, the effects of hypoxia on SBP likely represent the net balance between the increased vasodilatory effects of HMOX1 and the opposing sympathetic vasoconstriction, secondary to chemoreflex activation. Further, both moderate and severe hypoxia disrupted circadian rhythm during the hypoxic insult and transiently during normoxic recovery. Such observations have major implications for our understanding of basic physiology and the role of hypoxia in disease progression.

Though rare, severe reductions in PaO_2 do occur pathologically in some end-stage patients (Edell *et al.*, 1989; Dubois *et al.*, 1994; Ferrer *et al.*, 2003). These severe consequences are often the final result of disease progression. For the majority of patients suffering from conditions where hypoxia is a salient feature, the reductions in PaO_2 are more moderate (Thomas *et al.*, 1961; Hayashi, 1976; Oswald-Mammosser *et al.*, 1995; Mannino *et al.*, 2002). Despite moderate hypoxia being typical for many physiological (i.e. exercise, altitude) and pathological (e.g. COPD, heart failure) conditions, severe hypoxia is more commonly used in research. While we are not the first to investigate the physiological effects of moderate hypoxia, previous work focused largely on the metabolic and ventilatory responses (Frappell *et al.*, 1991; Morgan *et al.*, 2014). In those animal models, the relationship between moderate and severe hypoxia is scaled, similar to our findings in body temperature and activity. However, the effects on cardiovascular measures are less clear. While we also report divergent responses in heart rate and SBP during the hypoxic insult, there is

little support from the literature, largely attributed to the uniqueness and novelty of radio-telemetry methodology.

Circadian rhythms are fundamental to our homeostasis, occur in virtually every organ in the body, and when disrupted, exacerbate disease pathogenesis (Martino *et al.*, 2007; Podobed *et al.*, 2014). Recent profiling of the mouse genome reveals that 43% of all protein-coding genes display biological rhythm, most in an organ specific manner (Zhang *et al.*, 2014). Loss or disruption of circadian rhythm, or chronodisruption, is associated with worsened pathology in numerous conditions including cancer (Sephton *et al.*, 2000), obesity (Lamia *et al.*, 2009) and cardiovascular disease (de la Sierra *et al.*, 2009; Martino *et al.*, 2011). Further, despite evidence of hypoxic influence on circadian rhythm through interactions between clock genes Period1 and BMAL1 with HIF-1α, studies directed at understanding the effects of hypoxia on circadian rhythm are rare (Chilov *et al.*, 2001; Peek *et al.*, 2017). Here we report that severe hypoxia suddenly and dramatically decreased SBP, while moderate resulted in a delayed and gradual decrease. This might be explained by differential alternations in the circadian clock (as suggested by differences in altered circadian rhythm between hypoxic severities), resulting in altered expression/activation of HIF-1α via BMAL1 (Peek *et al.*, 2017). We also report that severe hypoxia disrupts circadian rhythm of SBP, temperature, heart rate and activity in mice. Notably, we are the first to demonstrate chronodisruption in response to a more clinically relevant level of moderate hypoxia. Amplitude dampening is associated with worsened disease progression and increased mortality (Hurd & Ralph, 1998; Mormont *et al.*, 2000). Thus, while moderate hypoxia may not result in abolishment of circadian rhythm, the alterations in amplitude may be indicative of pathology and hold significant implications for patients suffering from chronic or nocturnal hypoxia. To fully understand the pathophysiological consequences of hypoxia, it is important to evaluate different severities and explore how they affect circadian rhythm and other factors that would play a crucial role in the etiology of disease.

Heterogeneous activation of the HIF pathway occurs in response to different hypoxic severities following reductions in hemoglobin concentration (anemic hypoxia) (Tsui *et al.*, 2014) and F_IO_2 (hypoxic hypoxia) (Stroka *et al.*, 2001). Further, different severities of anemia also induce differential expression of HIF-dependent genes, suggesting a corresponding functional difference in the physiological response (Tsui *et al.*, 2014; Mistry *et al.*, 2018). Expression of EPO, nitric oxide synthase (NOS), and monocarboxylate transporter 4 are all differentially activated between mild,

moderate and severe anemia in an organ-specific manner (Tsui *et al.*, 2014). Our results demonstrate that both moderate and severe hypoxia is associated with corresponding increases in HMOX1 mRNA and protein. HMOX1 is an inducible enzyme responsible for catabolizing heme into ferrous iron, biliverdin and carbon monoxide (Liu *et al.*, 2007; Brunt *et al.*, 2009; Allwood *et al.*, 2014). HMOX-derived carbon monoxide is a potent vasodilator, similar to NO, and is involved in regulating vascular tone (Thorup *et al.*, 1999). Further, HMOX-derived carbon monoxide also inhibits endothelial NOS expression (Thorup *et al.*, 1999), which is supported by decreased endothelial NOS gene expression following both moderate and severe hypoxia in our model (data not shown).

We believe that the observed hypotension following moderate hypoxia is due to alterations in local vascular tone resulting from increased production of HMOX-derived carbon monoxide, despite potential reductions in endothelial NOS expression. However, following severe hypoxia, SBP is increased due to concomitant sympathetic activation, as demonstrated by the increased LF/HF ratio, likely as a result of chemoreflex activation. Severe hypoxia has been demonstrated previously to increase sympathetic drive (Greenberg *et al.*, 1999; Zoccal *et al.*, 2007), further supporting our findings. Differences in the cardiovascular response during the hypoxic insult between severe and moderate hypoxia may be due, at least in part, to a physiological response via hypoxia-induced anapyrexia. This is a well-characterized response to the proportion of hypoxia, where the thermoregulatory set-point is decreased to reduce metabolic demands and protect tissues from cellular damage (Steiner & Branco, 2002). This response occurs both in rodents (Robinson & Milberg, 1970; Steiner *et al.*, 2000) and humans (Kottke & Phalen, 1948; Robinson & Haymes, 1990), however, as body temperature is linearly associated with heart rate in mice, this reduction in body temperature during severe hypoxia was accompanied by depressions in heart rate and blood pressure in our model. During severe hypoxia, there is also an acute systemic vasodilatory effect (Fredricks *et al.*, 1994; Marshall, 2000; Weisbrod *et al.*, 2001) which is proposed to cause a decrease in mean arterial pressure in rodents (Campen *et al.*, 2005; Gonzalez *et al.*, 2007; Marcus *et al.*, 2009). In agreement with this, we observed a sudden and drastic decrease in SBP during severe hypoxia which we did not observe during moderate. In contrast, chronic exposure to severe hypoxia results in elevated mean arterial pressure in humans (Calbet, 2003; Parati *et al.*, 2014) and rodents (Campen *et al.*, 2005; Marcus *et al.*, 2009). Thus, the differential cardiovascular responses observed following moderate and severe hypoxia represent the net balance between local vasodilatory factors and central neural sympathoexcitatory regulation of vasculature tone.

Although we used activity as a surrogate marker of arousal, a limitation of our study is the absence of ventilation and arousal state (i.e. EEG) recordings for each animal, which may influence heart rate variability. In addition, telemetry units were set to record only 30 seconds of data every 5 minutes. While we acknowledge the limitation that our segment length is below the 1-3 minutes used in other studies, we found it easier to find stationarity of the signal using shorter time lengths. To accommodate the shorter time length, we used nine 30-second segments. Indeed, the averaging of multiple 1-minute segments produces comparable means as 3-minute data segments (Thireau *et al.*, 2008). Finally, though we observed disruptions to circadian rhythm during and following hypoxia, longer durations of hypoxic stress and recovery should also be investigated. This could provide valuable insight to whether moderate hypoxia disrupts circadian rhythm and contributes to diseases like hypertension and mild COPD.

Impaired tissue oxygenation is present in numerous chronic diseases and is associated with worse quality of life and clinical outcomes. Decades of research have almost exclusively focused on investigating the effects of severe hypoxia in pathophysiological states, while the same effects of moderate hypoxia remain uninvestigated. Further, there is no standardization for the classification of hypoxic severities with the same reduction in F_IO_2 being classified as mild, moderate and severe, depending on the study design. In contrast to hypoxic hypoxia, anemic hypoxia has defined haemoglobin concentrations recommended by the World Health Organization for the classification of mild, moderate and severe anemia. This lack of standardization represents a significant barrier in the interpretation and comparison of results from different studies using reduced F_IO_2 as the primary insult.

In summary, we demonstrate, for the first time, differential pressor responses during normoxic recovery following moderate and severe hypoxia. These effects appear to be mediated, at least in part, by different autonomic nervous system responses. These results should stimulate additional studies investigating the therapeutic potential of moderate hypoxic exposure to improve overall cardiovascular health. The findings of this study illustrate a critical need to revisit the basic pathophysiology of hypoxia to promote standardization, reconcile our understanding of the literature, and improve clinical translation.

Additional Information

Competing Interests

None.

Author Contributions

Conception and design of the experiments: M.A.A., J.A.S.; collection, analysis and interpretation of data: M.A.A., B.A.E., J.S.H., N.R., A.E., P.J.M., K.R.B., J.A.S.; drafting the article or revising it critically for important intellectual content: M.A.A., B.A.E., J.S.H., N.R., A.E., P.J.M., K.R.B., J.A.S. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Table 1. Sequence Information

Gene	Sequence	GenBank Accession #	Tm (ºC)
HMOX1	5’-GGTGATGGCTTCCTTGTACC-3’ 5’-AGTGAGGCCCATACCAGAAG-3’	NM_010442.2	58
EPO	5’-CATCTGCGACAGTCGAGTTCTG-3’ 5’-CACACCCATCGTGACATTTTC-3’	NM_007942.2	61
GAPDH	5’-GCACAGTCAAGGCCGAGAAT-3’ 5’-GCCTTCTCCATGGTGGTGAA-3’	NM_001289726.1 NM_008084.3	60

Table 2. Cosinor analysis of physiological parameters

	Mesor	Amplitude	Acrophase, hours	R^2
		9% O_2		
SBP (mmHg)				
Baseline	117.6 ± 0.6	4.3 ± 0.9	14.5 ± 0.2	0.54
Hypoxia	110.8 ± 1.2*	10.3 ± 1.7*	20.8 ± 0.2	0.66
24h Post Hypoxia	121.1 ± 0.8*	1.6 ± 1.1*	20.4 ± 0.7	0.09
48h Post Hypoxia	124.2 ± 0.6*	4.3 ± 0.8	14.6 ± 0.2	0.58
72h Post Hypoxia	123.5 ± 0.6*	2.8 ± 0.8	20.7 ± 0.3	0.35
HR (bpm)				
Baseline	481 ± 5	29 ± 7	15.0 ± 0.2	0.44
Hypoxia	376 ± 5*	42 ± 8	15.4 ± 0.2	0.62
24h Post Hypoxia	459 ± 6*	23 ± 9	14.3 ± 0.4	0.25
48h Post Hypoxia	454 ± 6*	37 ± 8	14.6 ± 0.3	0.46
72h Post Hypoxia	456 ± 7*	41 ± 10	14.6 ± 0.2	0.44
Temperature (°C)				
Baseline	36.4 ± 0.1	0.5 ± 0.1	14.3 ± 0.2	0.51
Hypoxia	33.9 ± 0.1*	1.4 ± 0.2*	20.7 ± 0.2	0.71
24h Post Hypoxia	36.9 ± 0.1*	0.1 ± 0.1*	14.7 ± 1.6	0.02
48h Post Hypoxia	36.7 ± 0.1*	0.7 ± 0.1	14.3 ± 0.2	0.62
72h Post Hypoxia	36.7 ± 0.1*	0.7 ± 0.1	20.5 ± 0.2	0.65
Activity (AU)				
Baseline	4.0 ± 0.5	1.7 ± 0.7	14.3 ± 0.4	0.21

Hypoxia	1.2 ± 0.2*	0.4 ± 0.2	14.1 ± 0.6	0.15
24h Post Hypoxia	4.6 ± 0.6	1.8 ± 0.9	13.5 ± 0.5	0.16
48h Post Hypoxia	4.6 ± 0.7	3.0 ± 0.9	20.7 ± 0.3	0.33
72h Post Hypoxia	4.5 ± 0.7	2.5 ± 1.0	20.5 ± 0.4	0.25

15% O_2

SBP (mmHg)

Baseline	117.1 ± 0.9	6.0 ± 1.2	14.3 ± 0.2	0.54
Hypoxia	114.2 ± 0.9*	4.9 ± 1.3	20.5 ± 0.3	0.45
24h Post Hypoxia	114.6 ± 1.0	4.2 ± 1.4	14.0 ± 0.3	0.30
48h Post Hypoxia	110.6 ± 0.7*	4.6 ± 1.1	20.0 ± 0.2	0.47
72h Post Hypoxia	109.0 ± 0.9*	6.2 ± 1.3	20.3 ± 0.2	0.54

HR (bpm)

Baseline	516 ± 4	33 ± 6	14.5 ± 0.2	0.59
Hypoxia	537 ± 6*	16 ± 8*	1.0 ± 0.5*	0.16
24h Post Hypoxia	491 ± 6*	34 ± 8	14.3 ± 0.2	0.48
48h Post Hypoxia	490 ± 6*	29 ± 8	14.4 ± 0.3	0.40
72h Post Hypoxia	491 ± 7*	44 ± 10	14.4 ± 0.2	0.50

Temperature (°C)

Baseline	36.3 ± 0.1	0.5 ± 0.1	20.4 ± 0.1	0.73
Hypoxia	36.2 ± 0.1	0.6 ± 0.1	14.0 ± 0.2	0.71
24h Post Hypoxia	36.4 ± 0.1	0.6 ± 0.1	20.4 ± 0.2	0.69
48h Post Hypoxia	36.4 ± 0.1	0.6 ± 0.1	14.1 ± 0.2	0.68
72h Post Hypoxia	36.4 ± 0.1	0.7 ± 0.1	20.4 ± 0.1	0.76

Activity (AU)				
Baseline	4.0 ± 0.3	1.6 ± 0.4	20.3 ± 0.3	0.39
Hypoxia	4.5 ± 0.4	2.3 ± 0.6	20.3 ± 0.3	0.42
24h Post Hypoxia	4.9 ± 0.4	2.2 ± 0.6	20.3 ± 0.3	0.39
48h Post Hypoxia	4.4 ± 0.5	2.4 ± 0.6	20.2 ± 0.3	0.40
72h Post Hypoxia	5.5 ± 0.5*	3.6 ± 0.7*	20.1 ± 0.2	0.53

Data are mean ± SEM. SBP, systolic blood pressure; HR, heart rate; mesor, midline estimating statistic of rhythm; amplitude, half the extent of predictable variation within a cycle; acrophase, the time of overall high values recurring in each cycle; R^2, degree of curve fit. *p<0.05 compared to baseline.

Table 3. Frequency and time domain heart rate variability analysis

	15% O_2		*9% O_2*	
	Baseline	72h Post Hypoxia	Baseline	72h Post Hypoxia
Mean R-R interval (ms) [b]	123.49 ± 13.38	141.21 ± 17.14	129.43 ± 7.28	150.43 ± 18.87*
SDNN (ms) [b]	5.52 ± 2.70	7.89 ± 2.61*	5.39 ± 1.45	7.24 ± 1.83
RMSSD (ms) [b]	6.57 ± 3.30	10.59 ± 3.92*	6.36 ± 2.16	8.47 ± 2.76
LF (nu) [a]	62.18 ± 8.72	57.08 ± 6.17*	62.12 ± 6.85	73.59 ± 4.91*[†]
HF (nu) [a,c]	37.82 ± 8.72	42.91 ± 6.17*	37.88 ± 6.85	26.41 ± 4.91*[†]
Total power (ms^2) [b]	43.35 ± 37.76	73.19 ± 36.72	31.84 ± 15.66	60.55 ± 33.49
LF/HF [a,c]	2.32 ± 0.87	1.79 ± 0.45	2.56 ± 1.00	3.53 ± 0.79*[†]

Data are mean ± SD. SDNN, standard deviations of normal R-R intervals; RMSSD, root mean square of successive normal R-R interval differences; LF, low frequency; HF, high frequency; a, significant interaction; b, significant main effect of time; c, significant main effect of group. *p<0.05 as compared to baseline; [†]p<0.05 compared to moderate hypoxia at the same time point (n=7 per group).

Table 4. Heart rate variability analysis at baseline, hypoxia, and normoxic recovery

Group	*9% O_2*	*15% O_2*
Mean R-R interval (ms) [a,b,c]		
Baseline	129.43 ± 7.28	123.49 ± 13.38
Hypoxia	175.16 ± 22.53*[†]	121.32 ± 13.55
24h Post Hypoxia	150.25 ± 16.82*	140.84 ± 15.05*
48h Post Hypoxia	155.77 ± 5.15*	141.72 ± 19.68*
72h Post Hypoxia	150.43 ± 18.87*	141.21 ± 17.14*
SDNN (ms) [a,b,c]		
Baseline	5.39 ± 1.45	5.52 ± 2.70
Hypoxia	25.20 ± 9.85*[†]	4.52 ± 2.20
24h Post Hypoxia	11.69 ± 3.29*	6.92 ± 3.24
48h Post Hypoxia	8.36 ± 2.25	6.32 ± 2.78
72h Post Hypoxia	7.24 ± 1.83	7.89 ± 2.61
RMSSD (ms) [a,b,c]		
Baseline	6.36 ± 2.16	6.57 ± 3.30
Hypoxia	33.60 ± 15.08*[†]	4.94 ± 2.49
24h Post Hypoxia	14.60 ± 5.14*	8.71 ± 4.31
48h Post Hypoxia	9.50 ± 3.39	7.77 ± 3.82
72h Post Hypoxia	8.47 ± 2.76	10.59 ± 3.92
LF (nu) [a,b]		
Baseline	62.12 ± 6.85	62.18 ± 8.72
Hypoxia	72.17 ± 10.00*	68.37 ± 6.45
24h Post Hypoxia	65.49 ± 13.86	60.75 ± 7.84
48h Post Hypoxia	72.91 ± 10.97*	60.93 ± 9.52
72h Post Hypoxia	73.59 ± 4.91*[†]	57.08 ± 6.17

HF (nu) [a,b]		
Baseline	37.88 ± 6.85	37.82 ± 8.72
Hypoxia	27.82 ± 10.00*	31.62 ± 6.45
24h Post Hypoxia	34.50 ± 13.85	39.25 ± 7.84
48h Post Hypoxia	27.09 ± 10.97*	39.07 ± 9.52
72h Post Hypoxia	26.41 ± 4.91*†	42.91 ± 6.17
Total power (ms^2) [a,b,c]		
Baseline	31.84 ± 15.66	43.35 ± 37.76
Hypoxia	971.04 ± 686.84*†	25.84 ± 22.81
24h Post Hypoxia	154.51 ± 86.83	50.97 ± 46.43
48h Post Hypoxia	70.33 ± 32.30	42.43 ± 29.83
72h Post Hypoxia	60.55 ± 33.49	73.19 ± 36.72
LF/HF [b,c]		
Baseline	2.56 ± 1.00	2.32 ± 0.87
Hypoxia	4.30 ± 1.35*	3.14 ± 0.67
24h Post Hypoxia	3.43 ± 3.00	2.11 ± 0.71
48h Post Hypoxia	4.10 ± 2.27*†	2.02 ± 1.01
72h Post Hypoxia	3.53 ± 0.79	1.79 ± 0.45

Data are mean ± SD. SDNN, standard deviations of normal R-R intervals; RMSSD, root mean square of successive normal R-R interval differences; LF, low frequency; HF, high frequency; a, significant interaction; b, significant main effect of time; c, significant main effect of group. *p<0.05 as compared to baseline; †p<0.05 compared to moderate hypoxia at the same time point (n=7).

Figure Legends

Figure 1. Hypoxia chamber and telemetry unit setup.

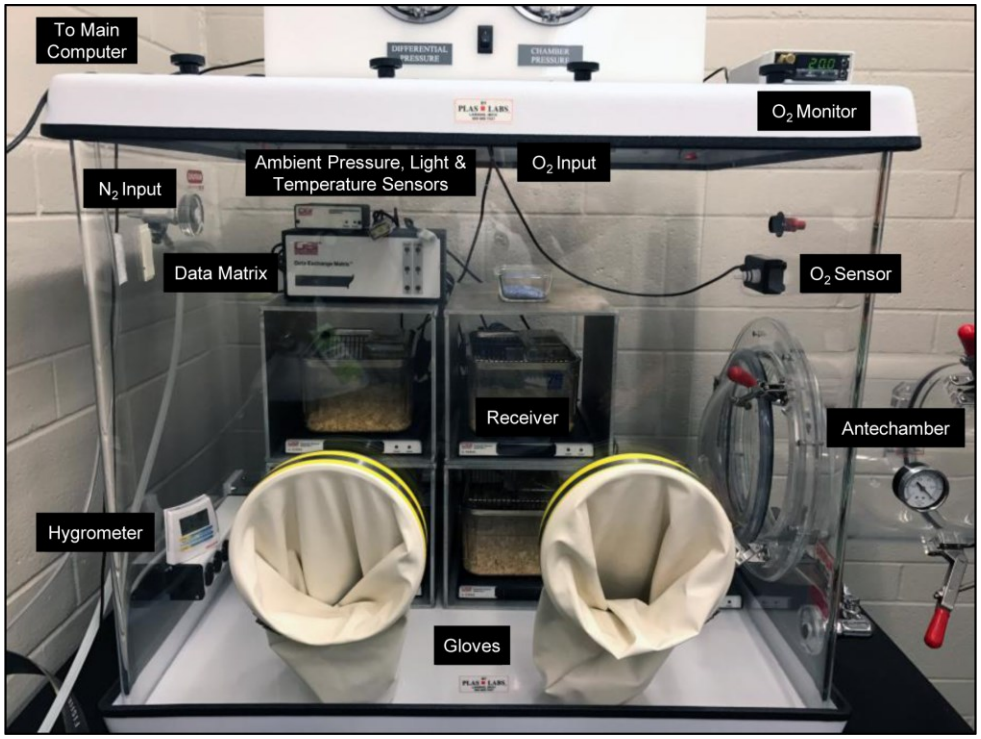

Figure 2. Physiological responses of body temperature during baseline, severe (A) or moderate (B) hypoxia, and 72 hours of normoxic recovery. Average body temperatures recorded following normoxia, severe hypoxia, 24 hours post-hypoxia, 48 hours post-hypoxia, and 72 hours post-hypoxia during lights on (C; $\chi^2(4)=235.3$, $p<0.0001$) and lights off (D; $F(4,316)=269.8$, $p<0.0001$). Average body temperatures recorded following baseline, moderate hypoxia, 24 hours post-hypoxia, 48 hours post-hypoxia, and 72 hours post-hypoxia during lights on (E; $\chi^2(4)=23.4$, $p=0.0001$) and lights off (F; $F(4,316)=7.4$, $p<0.0001$). Two-way ANOVA of body temperature during lights on (G; interaction $F(4,56)=94.0$, $p<0.0001$; main effect of time $F(4,56)=113.1$, $p<0.0001$; main effect of group $F(1,14)=1.1$, $p=0.3158$) and lights off (H; interaction $F(4,56)=20.8$, $p<0.0001$; main effect of time $F(4,56)=29.8$, $p<0.0001$; main effect of group $F(1,14)=0.1$, $p=0.7579$). a, significant interaction; b,

significant main effect of time; c, significant main effect of group. For all panels, *p<0.05 compared to baseline, $^{\dagger}p$<0.05 compared to moderate hypoxia at the same time point. Values expressed are mean ± SEM (n=8 per group). Note: y-axes for panels A and B are broader than panels C to H for visual clarity.

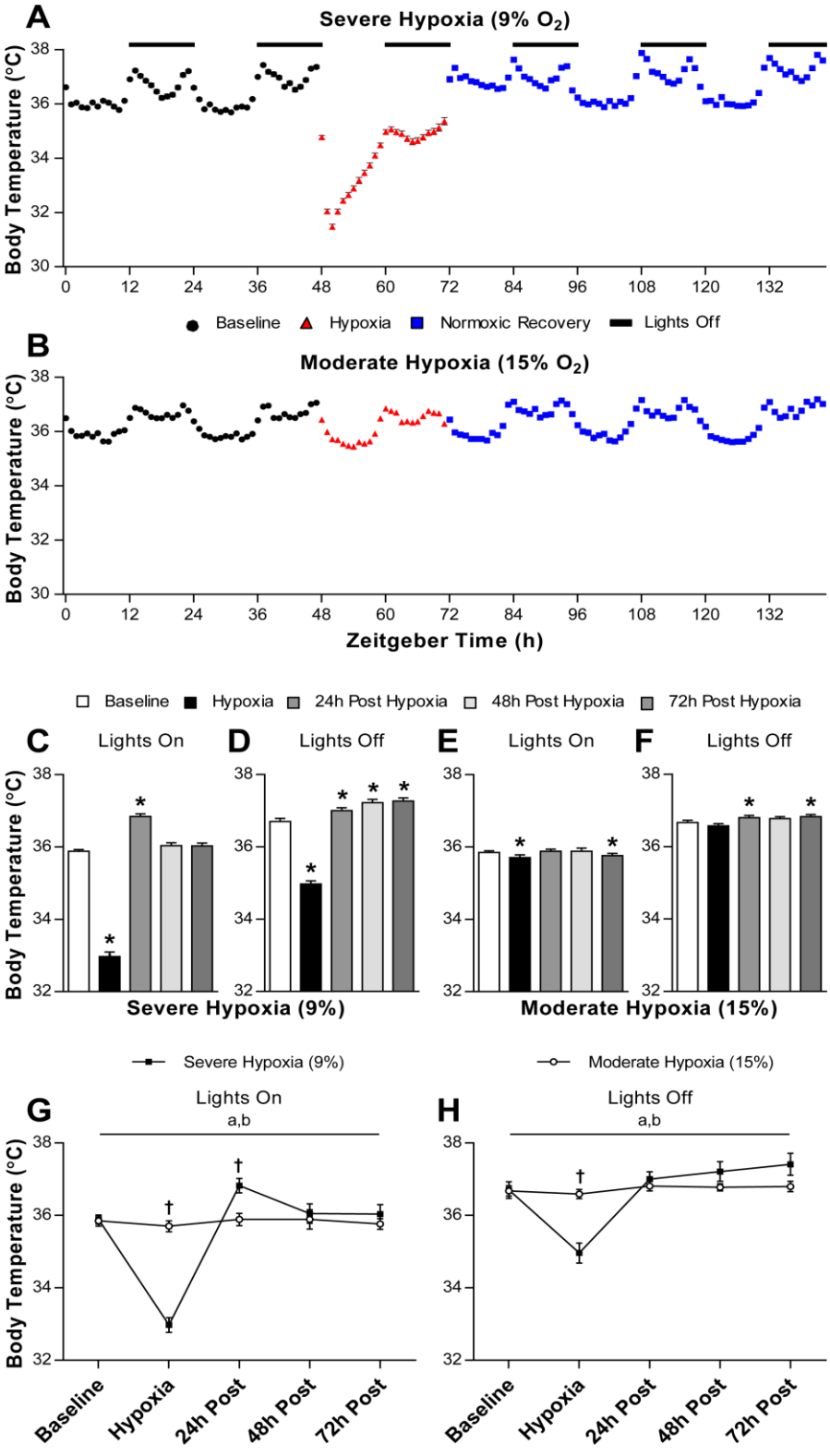

Figure 3. Graphical representation of cosinor analysis of severe hypoxia (9% O_2).

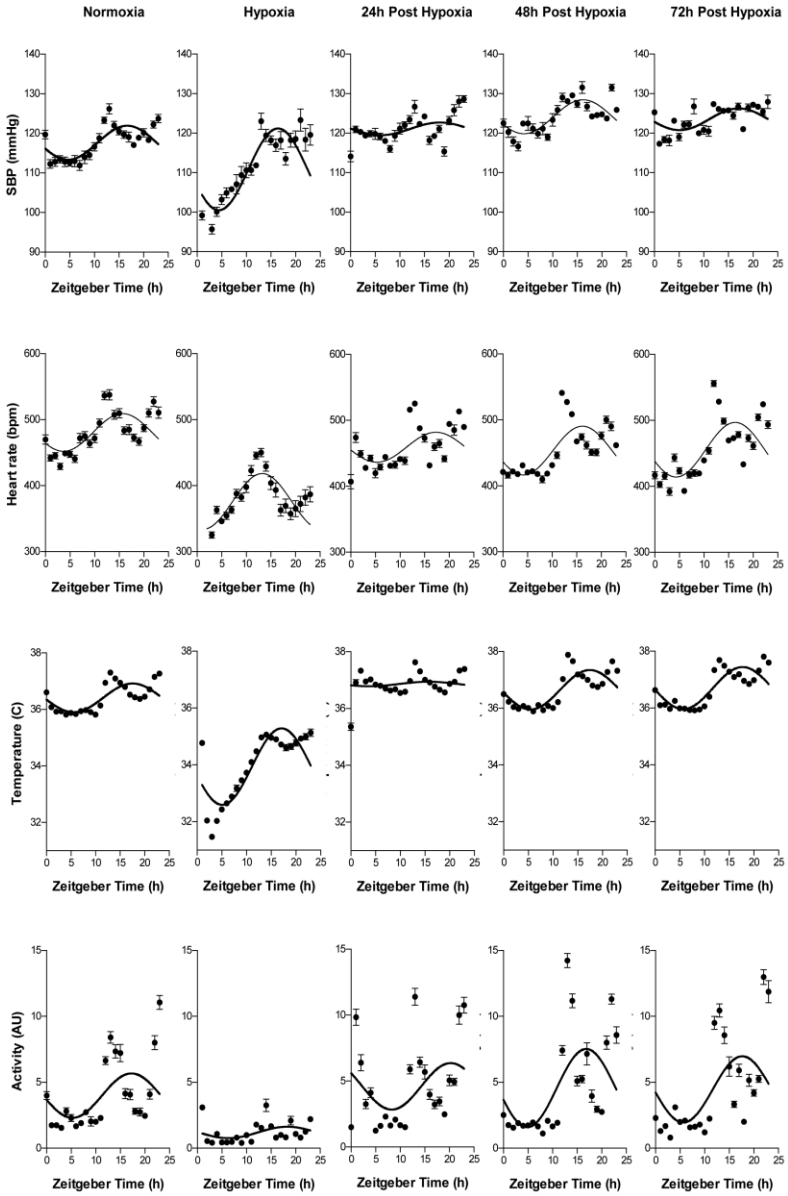

Figure 4. Graphical representation of cosinor analysis of moderate hypoxia (15% O_2).

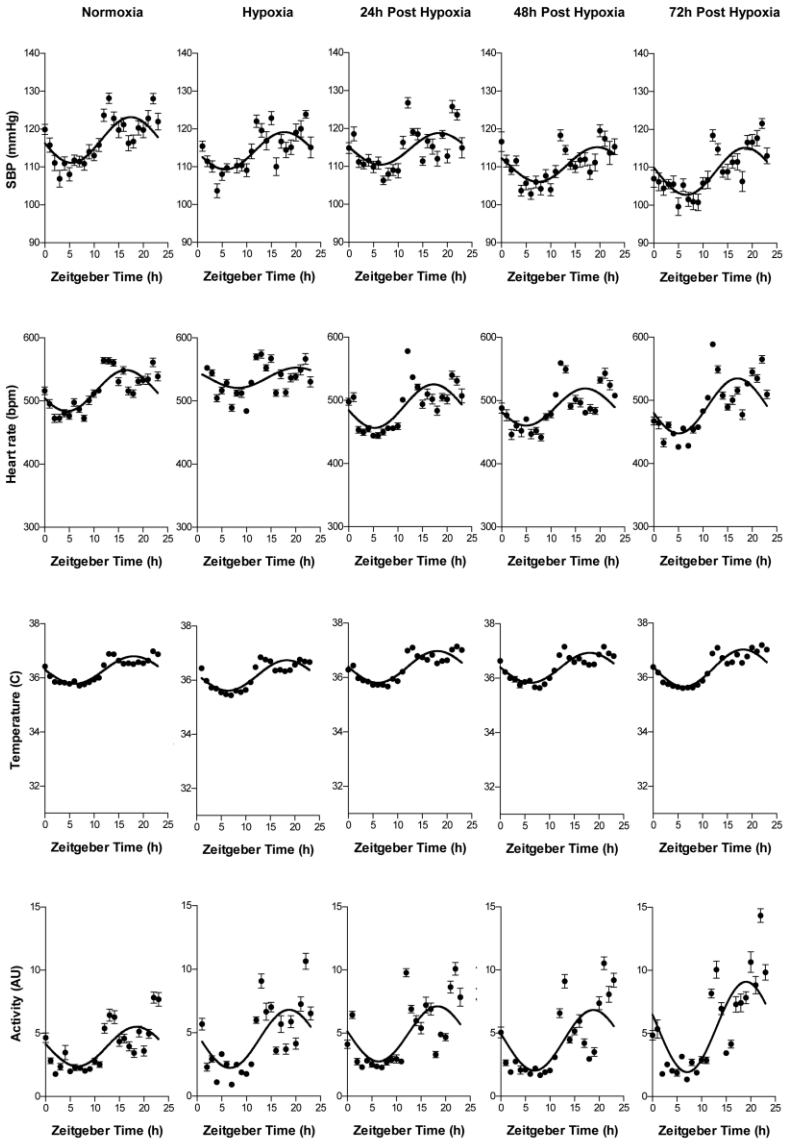

Figure 5. Physiological responses of activity during baseline, severe (A) or moderate (B) hypoxia, and 72 hours of normoxic recovery. Average activity recorded following baseline, severe hypoxia, 24 hours post-hypoxia, 48 hours post-hypoxia, and 72 hours post-hypoxia during lights on (C; $\chi^2(4)=80.7$, $p<0.0001$) and lights off (D; $\chi^2(4)=112.8$, $p<0.0001$). Average body temperatures

recorded following baseline, moderate hypoxia, 24 hours post-hypoxia, 48 hours post-hypoxia, and 72 hours post-hypoxia during lights on (E; $\chi^2(4)=14.7$, $p=0.0055$) and lights off (F; $\chi^2(4)=10.1$, $p=0.0396$). Two-way ANOVA of activity during lights on (G; interaction $F(4,56)=4.0$, $p=0.0062$; main effect of time $F(4,56)=10.0$, $p<0.0001$; main effect of group $F(1,14)=1.3$, $p=0.2791$) and lights off (H; interaction $F(4,56)=11.8$, $p<0.0001$; main effect of time $F(4,56)=13.5$, $p<0.0001$; main effect of group $F(1,14)=3.2$, $p=0.0945$). a, significant interaction; b, significant main effect of time; c, significant main effect of group. For all panels, *$p<0.05$ compared to baseline, $^{\dagger}p<0.05$ compared to moderate hypoxia at the same time point. Values expressed are mean ± SEM ($n=8$ per group). Note: y-axes for panels A and B are broader than panels C to H for visual clarity.

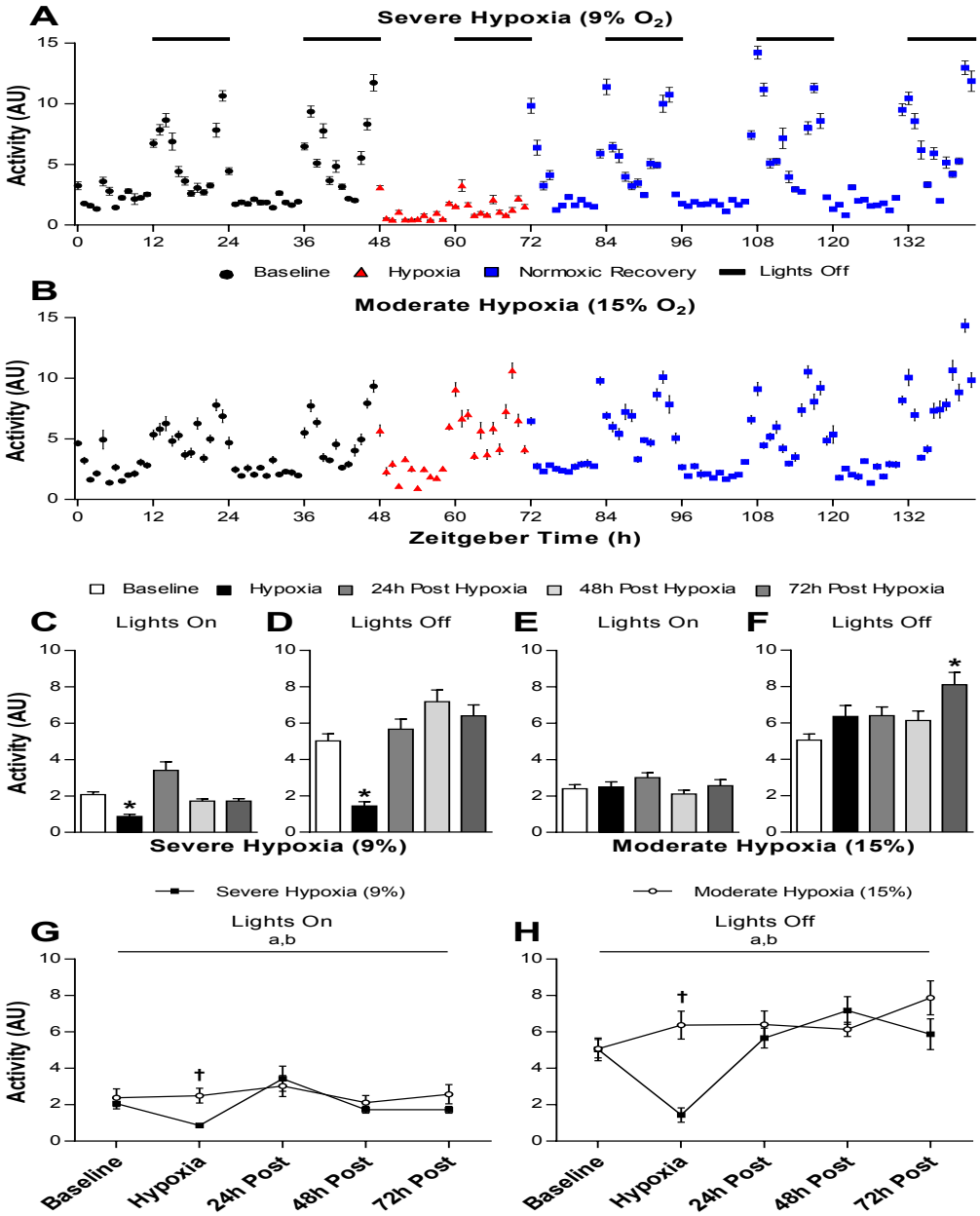

Figure 6. Physiological responses of systolic blood pressure (SBP) during baseline, severe (A) or moderate (B) hypoxia, and 72 hours of normoxic recovery. Average SBP recorded following baseline, severe hypoxia, 24 hours post-hypoxia, 48 hours post-hypoxia, and 72 hours post-hypoxia during lights on (C; $\chi^2(4)=85.4$, $p<0.0001$) and lights off (D; $\chi^2(4)=41.2$, $p<0.0001$). Average SBP recorded following baseline, moderate hypoxia, 24 hours post-hypoxia, 48 hours post-hypoxia, and 72 hours post-hypoxia during lights on (E; $\chi^2(4)=50.9$, $p<0.0001$) and lights off (F; $\chi^2(4)=52.5$, $p<0.0001$). Two-way ANOVA of SBP during lights on (G; interaction $F(4,24)=11.5$, $p<0.0001$; main effect of time $F(4,24)=4.6$, $p=0.0067$; main effect of group $F(1,6)=1.4$, $p=0.2779$) and lights off (H; interaction $F(4,24)=3.3$, $p=0.0265$; main effect of time $F(4,24)=0.1$, $p=0.9830$; main effect of group $F(1,6)=0.7$, $p=0.4329$). a, significant interaction; b, significant main effect of time; c, significant main effect of group. For all panels, $^*p<0.05$ compared to baseline, $^{\dagger}p<0.05$ compared to moderate hypoxia at the same time point. Values expressed are mean ± SEM (severe, $n=4$; moderate, $n=5$).

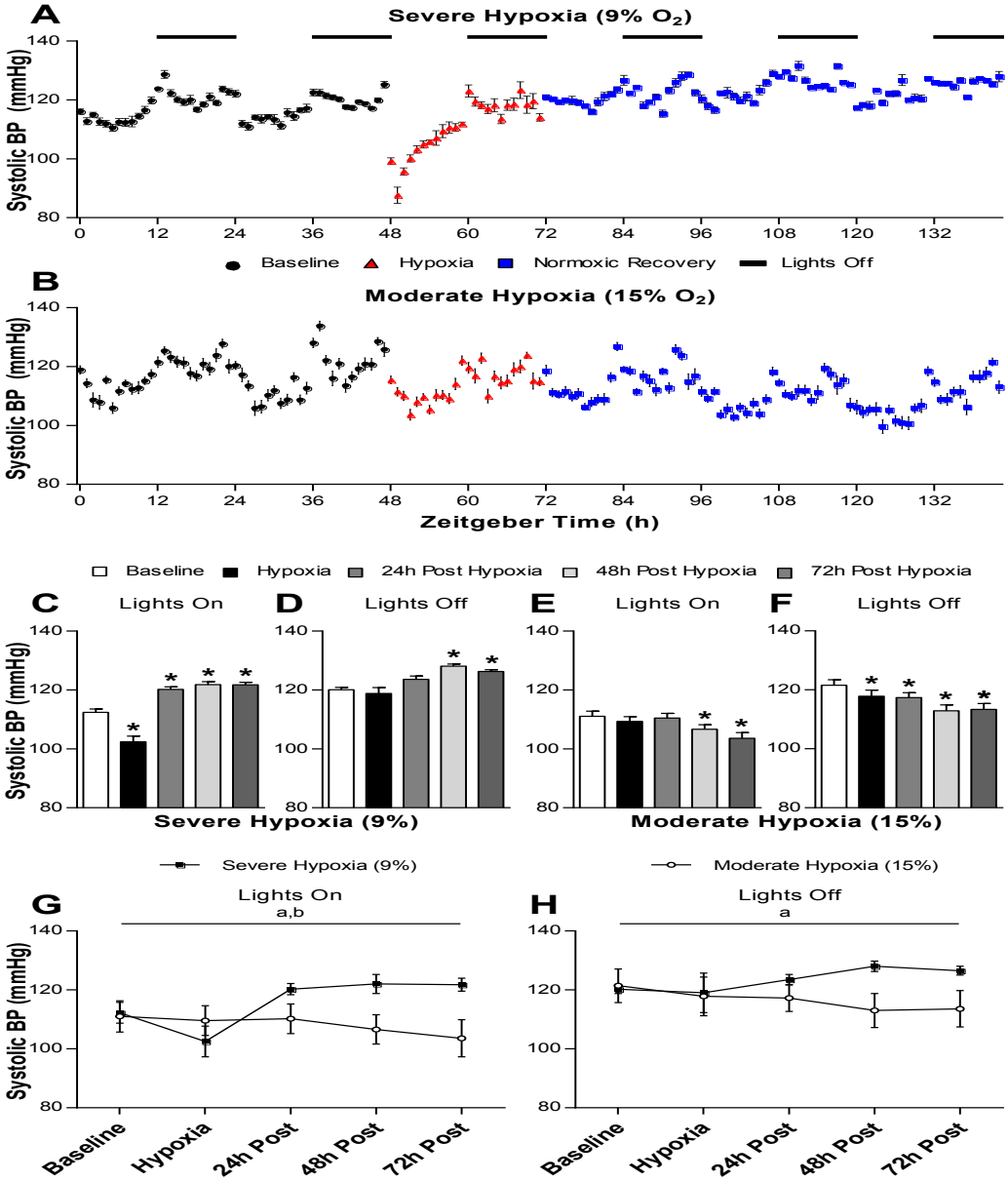

Figure 7. Physiological responses of heart rate during baseline, severe (A) or moderate (B) moderate hypoxia, and 72 hours of normoxic recovery. Average heart rate recorded following baseline, severe hypoxia, 24 hours post-hypoxia, 48 hours post-hypoxia, and 72 hours post-hypoxia during lights on (C; $\chi^2(4)=91.8$, $p<0.0001$) and lights off (D; $F(4,276)=78.2$, $p<0.0001$). Average heart rate recorded following normoxia, moderate hypoxia, 24 hours post-hypoxia, 48 hours post-hypoxia, and 72 hours post-hypoxia during lights on (E; $\chi^2(4)=98.4$, $p<0.0001$) and lights off (F; $\chi^2(4)=24.7$, $p<0.0001$). Two-way ANOVA of heart rate during lights on (G; interaction $F(4,52)=19.7$, $p<0.0001$; main effect of time $F(4,52)=5.0$, $p=0.0018$; main effect of group $F(1,13)=10.9$, $p=0.0057$) and lights off (H; interaction $F(4,52)=11.8$, $p<0.0001$; main effect of time $F(4,52)=5.9$, $p=0.0005$; main effect of group $F(1,13)=13.7$, $p=0.0026$). a, significant interaction; b, significant main effect of time; c, significant main effect of group. For all panels, $^*p<0.05$ compared to baseline, $^{\dagger}p<0.05$ compared to moderate hypoxia at the same time point. Values expressed are mean ± SEM (severe, $n=7$; moderate, $n=8$). Note: y-axes for panels A and B are broader than panels C to H for visual clarity.

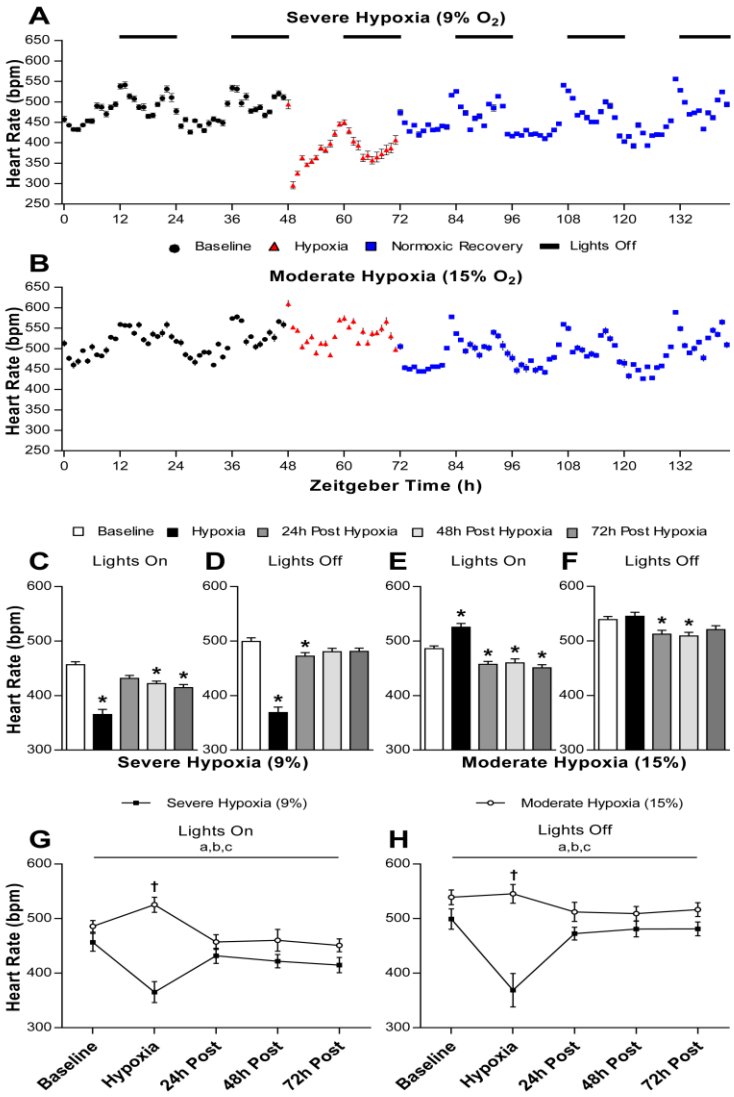

Figure 8. Mean R-R interval ± standard deviation of normal R-R intervals (SDNN) at baseline and 72 hours post severe and moderate hypoxia (A; interaction $F(1,12)=0.1$, $p=0.7543$; main effect of time $F(1,12)=14.3$, $p=0.0026$; main effect of group $F(1,12)=1.6$, $p=0.2352$). Low frequency (LF) spectral power (B; interaction $F(1,12)=18.5$, $p=0.0010$; main effect of time $F(1,12)=1.5$, $p=0.2500$; main effect of group $F(1,12)=4.7$, $p=0.0515$), high frequency (HF) spectral power (C; interaction $F(1,12)=26.2$, $p=0.0003$; main effect of time $F(1,12)=3.9$, $p=0.0723$; main effect of group $F(1,12)=6.4$, $p=0.0266$) and the ratio of LF/HF (D; $t(6)=3.0$, $p=0.0110$) at baseline and 72 hours post severe or moderate hypoxia. *$p<0.05$ compared to baseline; $^{\dagger}p<0.05$ compared to moderate hypoxia; a, significant interaction; b, significant main effect of time; c, significant main effect of group; values expressed are mean ± SD (n=7 per group).

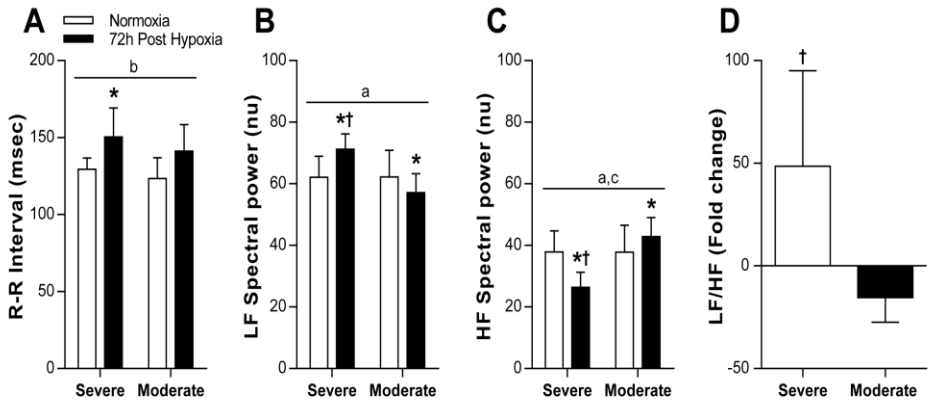

Figure 9. Mesenteric artery gene expression of erythropoietin (EPO; A; interaction $F(1,15)=0.3$, $p=0.3227$; main effect of time $F(1,15)=1.1$, $p=0.3085$; main effect of group $F(1,15)=0.2$, $p=0.6631$) and heme oxygenase 1 (HMOX1; B; interaction $F(1,20)=0.6$, $p=0.4327$; main effect of time $F(1,15)=20.2$, $p=0.0002$; main effect of group $F(1,15)=0.4$, $p=0.5102$) during baseline and following 24 hours of severe or moderate hypoxia (n=4-8 per group). b, significant main effect of time. HMOX1 protein levels 72 hours post severe and moderate hypoxia (C; severe $U=0.0$, $p=0.0119$; moderate

U=6.0, *p*=0.0325; *n*=6 for normoxia and moderate, *n*=3 for severe; where 2 animals were required per sample). For all panels, **p*<0.05 compared to normoxia. Values expressed are mean ± SD.