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

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Key points

- In the present study, 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 probably not molecular changes at the artery resulting from hypoxic stress.

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 indicate that it can be beneficial, suggesting that hypoxia may not always be detrimental. Comparisons between studies are difficult because of the varied classifications of hypoxic severities, methods of delivery and use of anaesthetics. Thus, to investigate the long-term effects of moderate hypoxia on cardiovascular health, radiotelemetry was used to obtain *in vivo* physiological measurements in unanaesthetized mice during 24 h of either moderate ($F_IO_2 = 0.15$) or severe ($F_IO_2 = 0.09$) hypoxia, followed by 72 h of normoxic recovery. Systolic blood pressure was decreased during recovery following moderate hypoxia but increased following severe hypoxia. Moderate and severe hypoxia increased haeme 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 h post-hypoxia, indicating a shift in sympathovagal balance. Moderate hypoxia dampened the

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amplitude of circadian rhythm, whereas severe disrupted rhythm during the entire insult, with perturbations persisting throughout normoxic recovery. Thus, hypoxic severity differentially regulates circadian blood pressure.

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Introduction

Impairments in oxygen delivery are both a cause and consequence of many acute and chronic disease states, such as obstructive sleep apnoea, heart failure and chronic obstructive pulmonary disease (COPD), and are associated with a reduced quality of life and increased mortality. Investigations of the pathophysiological consequences of hypoxia primarily illustrate the 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 ($\sim F_IO_2 < 0.10$) 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 applies as a result of disagreements in the classification of severities (e.g. mild, moderate and severe), method of delivery, duration and, in some cases, use of anaesthetic. Furthermore, direct comparisons of moderate and severe hypoxia are scarce (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 (P_aO_2), either by reducing the fraction of inspired oxygen (F_IO_2) or haemoglobin content, do not necessarily equate to similar hypoxia of various organs. Activation of compensatory neural and vascular mechanisms attempts to maintain sufficient oxygenation of vital organs. Following decreases in P_aO_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 P_aO_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 differs between moderate and severe hypoxia (Stroka *et al.* 2001). This organ-specific transcriptional response to hypoxia is also seen in anaemia, where, in response to mild, moderate and severe anaemia, 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 downstream targets [e.g. haeme-oxygenase I (HMOX1), 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, whereas only severe hypoxia increases anaerobic metabolism, with changes that 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). By 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), although the corresponding effects in health are unknown. To determine pathophysological mechanisms, it is important to first establish the effect of variable hypoxic gradations in health.

The present study aimed 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. Radiotelemetry

provided unanaesthetized, 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 were not proportional, linear or 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

Ethical approval

Adult male C57Bl/6J mice were bred in our facility and were aged 8–12 weeks (~25 g body weight) prior to surgery. Animals were housed under a 12:12 h light/dark cycle (lights on 08.00 h) at 24°C and 45% relative humidity. Following telemetry implantation, animals were housed individually with food and water being provided *ad libitum*. Housing and experimental procedures were approved by the Animal Care Committee at the University of Guelph and conformed 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 anaesthetized with isoflurane/oxygen (2%:100%), intubated and body temperature was maintained using a water-filled heating pad. A local anaesthetic 50:50 mix of lidocaine (3 mg kg^{-1}) and bupivicaine (1.5 mg kg^{-1}) was administered S.C. 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 for minimizing kinking of the pressure catheter, which can cause signal dropout of the blood pressure tracing. Following insertion, the transmitter was advanced S.C. to the abdomen and secured intraperitoneally. Two electrocardiography leads were placed S.C., 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. Postoperative analgesic buprenorphine (0.1 mg kg^{-1}) was given upon awakening and at 8 and 24 h postoperatively; subsequent analgesic was given as required.

Two-weeks postoperatively, mice were individually housed within an environmental chamber (830-ABB; Plas Labs, Lansing, MI, USA) (Fig. 1) where oxygen levels could be titrated accordingly (ProOx 110; BioSpherix, New York, NY, USA). Drierite (WA Hammond Drierite Company, Xenia, OH, USA) and calcium carbonate were added to the chamber to maintain constant ambient humidity and prevent an elevation in carbon dioxide. Each cage was placed on a telemetry receiver (RPC-1; Data Science International) within custom made Faraday cages. Telemetry signals were collected every 5 min for 30 s. 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 Excel 2011 (Microsoft Corp., Redmond, WA, USA) for further analysis.

Hypoxia study design

Each animal was exposed to only one hypoxic insult (either moderate or severe; a maximum of three 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 on the monday morning at 08.00 h over 15 min. After 24 h of hypoxia, ambient oxygen levels were restored and telemetry continued for an additional 72 h.

Quantitative PCR analysis

At the end of the normoxic recovery, animals were re-anaesthetized 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 three animals) with Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, Life Technologies, Burlington, ON, Canada). RNA samples were then treated using a RNase Free DNase (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. Concentrations of isolated RNA were quantified using a spectrophotometer (NanoDrop, ND1000; Thermo Fisher Scientific, Ottawa, ON, Canada). Generation of cDNA was completed using qScript cDNA SuperMix (Quanta Biosciences, Beverly, MD, USA) in accordance with the manufacturer's instructions using a 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 (Bio-Rad, Mississauga,

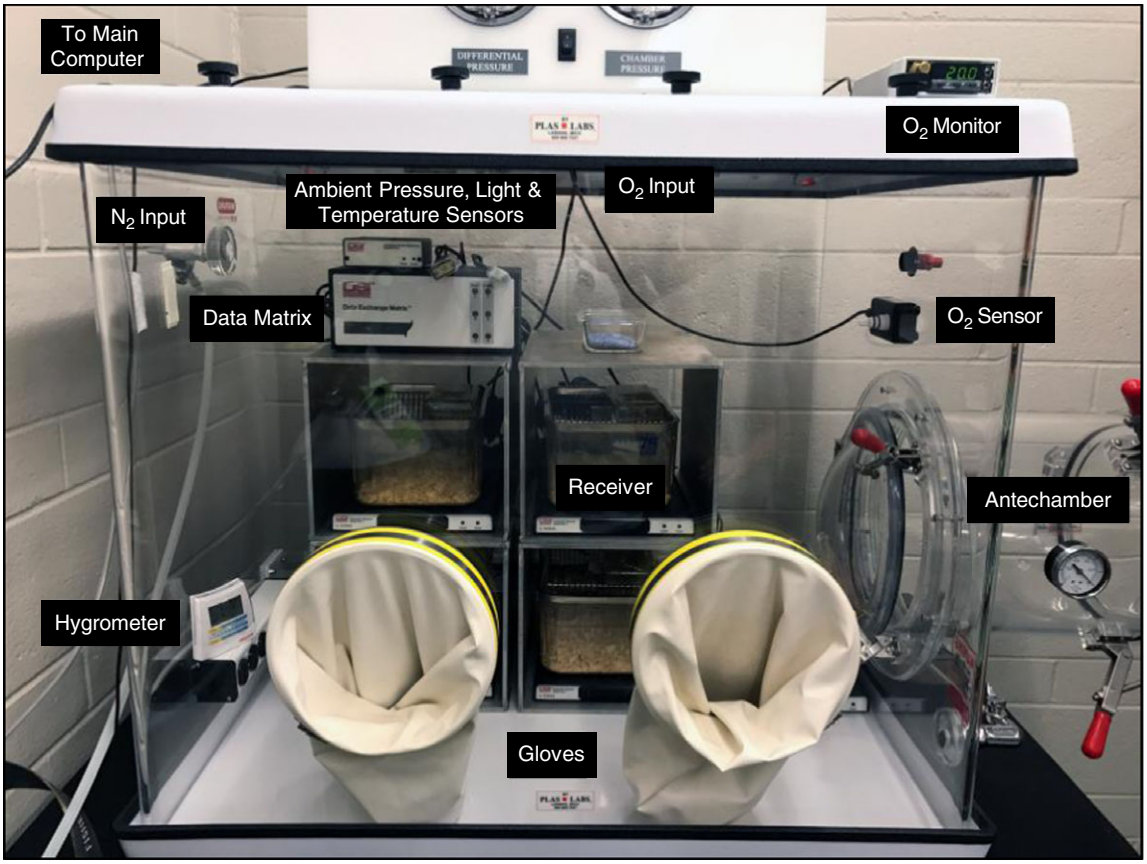

Figure 1. Experimental set-up
Hypoxia chamber and telemetry unit set-up. [Colour figure can be viewed at wileyonlinelibrary.com]

ON, Canada) and primers for HMOX1, EPO and GAPDH (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 the bicinchoninic acid assay as described previously (Foster *et al.* 2017). Briefly, samples were loaded onto a 4–20% Criterion TGX precast gel (Bio-Rad) alongside 10 μL of Precision Plus Protein Standards Kaleidoscope ladder (Bio-Rad) and then separated by SDS-PAGE followed by immunoblotting. Nitrocellulose membranes were rinsed in ddH_2O and then incubated in Pierce Reversible MemCode Stain (Thermo Fisher Scientific) for 5 min to confirm equal protein transfer. The blot was imaged using a ChemiDoc MP Imaging System (Bio-Rad) prior to stain removal (Pierce Stain Eraser; Thermo Fisher Scientific). Membranes were blocked (5% non-fat dry milk in 1 × Tris-buffered saline) and

Table 1. Sequence information

Gene	Sequence	GenBank accession number	T_m (°C)
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

incubated with a primary anti-HMOX1 antibody (dilution 1:1000) (catalogue number 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 (dilution 1:2000) (catalogue number 2054; Santa Cruz Biotechnology, Dallas, TX, USA). All antibody dilutions were completed in 1% non-fat dry milk and membrane washes were completed in 1 × Tris-buffered saline with 0.5% Tween. Signal was detected by chemiluminescence (Thermo Fisher Scientific), imaged (ChemiDoc; Bio-Rad) and then 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 (HRV) analysis was conducted using Kubios Heart Rate Variability Analysis software, version 2.2 (University of Kuopio, 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 s epoch taken at the beginning of each hour during a segment of the lights on period (10.00 to 18.00 h; corresponding to 2–10 and 122–130 h Zeitgeber time for baseline and normoxic recovery, respectively). The 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 confirm the absence of ectopic beats or signal artefact, 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 as 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).

Statistical 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 h periods and then averaged between all animals. For hypoxia and normoxic recovery, data means were averaged for the entirety between all animals. Each animal was recorded at baseline prior to hypoxic exposure, allowing them to serve as their own control. Circadian mesor (i.e. the mean value around which the wave oscillates), amplitude (i.e. difference between peak/trough and mean) and acrophase (i.e. time at which peak occurs) values were calculated and analysed using cosinor analysis as described previously (Munakata *et al.* 1990; Refinetti *et al.* 2007). For telemetry data, one-way repeated measures ANOVA were performed on ten 1 h averages from each animal during both light and dark cycles (i.e. excluding the four 1 h 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* test. A 5 × 2 (time × group) mixed model ANOVA was also performed on 10 h 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 HRV data was completed using a 2 × 2 (time × group) mixed model ANOVA and Holm–Sidak *post hoc* analysis was performed when appropriate. Baseline and 72 h post-hypoxia were chosen for HRV analysis because the latter showed the most divergent response in SBP. HRV analyses for all time points using a 5 × 2 (time × group) mixed model ANOVA are also presented. A 2 × 2 (time × group) mixed model ANOVA was performed for mRNA expression, except that 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 Prism, version 6 (GraphPad Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Body temperature and activity responses to moderate and severe hypoxia

To confirm that 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 (Fig. 2*A*, *C* and *D*). To confirm that 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 (Fig. 3 and Table 2). During moderate hypoxia, body temperature was slightly

decreased during lights off but not lights on (Fig. 2*B*, *E* and *F*); rhythm was unaffected (Fig. 3 and Table 2). To investigate whether hypoxia had any residual effects on physiological parameters, we continued our analysis after return to normoxia (Fig. 2*A*–*F* and Table 2). Although 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 h of recovery, with another modest increase compared to baseline at 72 h post-hypoxia. Normoxic recovery from severe

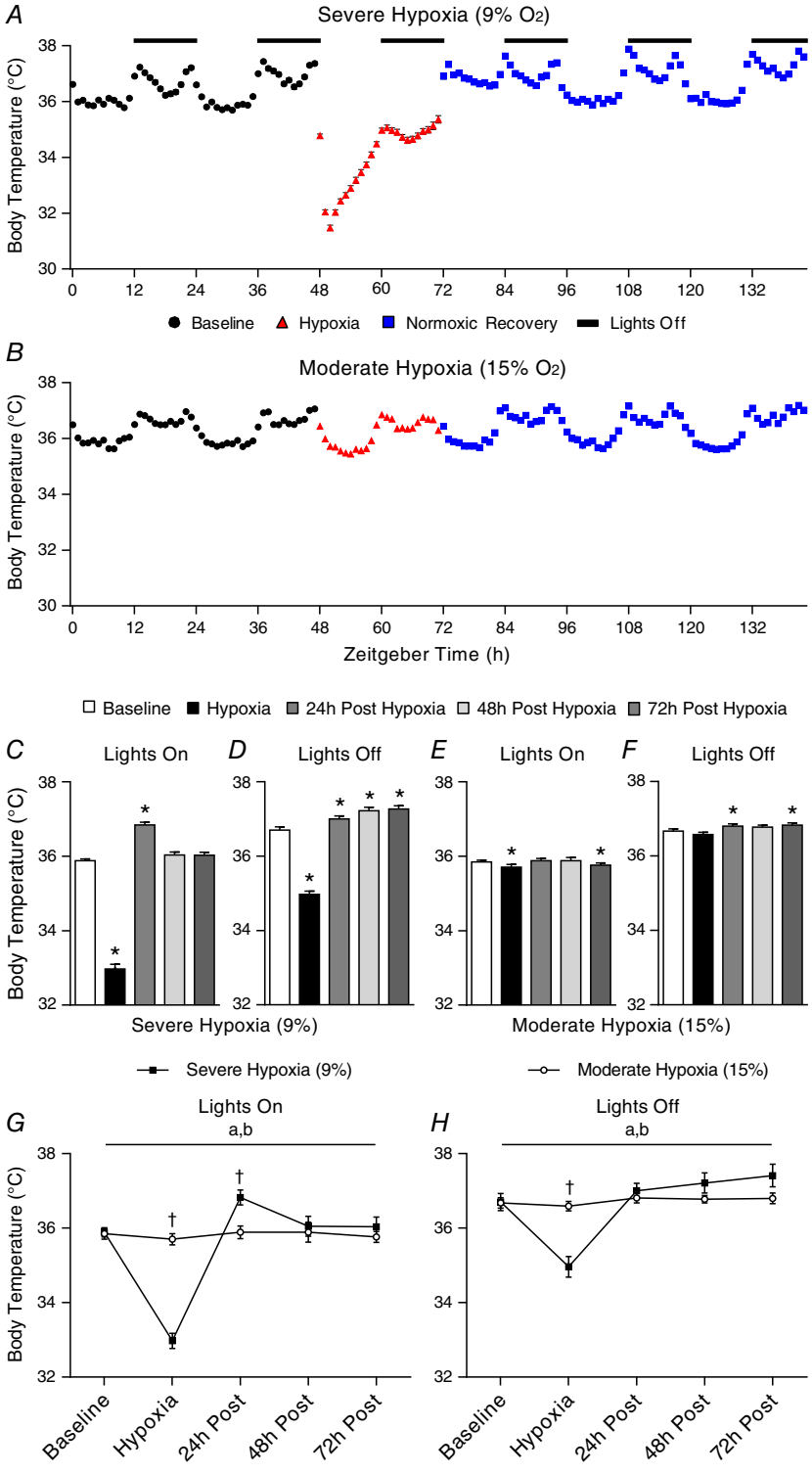

Figure 2. Physiological responses of body temperature during baseline, severe or moderate hypoxia, and 72 hours of normoxic recovery
Hourly averages for severe (*A*) or moderate (*B*) hypoxia experiments. Average body temperatures recorded following normoxia, severe hypoxia, 24 h post-hypoxia, 48 h post-hypoxia and 72 h post-hypoxia during lights on (*C*) ($\chi^2 = 235.3$, d.f. $= 4$, $P < 0.0001$) and lights off (*D*) ($F_{4,316} = 269.8$, $P < 0.0001$). Average body temperatures recorded following baseline, moderate hypoxia, 24 h post-hypoxia, 48 h post-hypoxia and 72 h post-hypoxia during lights on (*E*) ($\chi^2 = 23.4$, d.f. $= 4$, $P = 0.0001$) 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 $F_{1,14} = 0.1$, $P = 0.7579$). For panels (*C*) to (*F*): $^*P < 0.05$ compared to baseline. For panels (*G*) and (*H*): a, significant interaction; b, significant main effect of time; $^\dagger P < 0.05$ compared to moderate hypoxia at the same time point. Values expressed are the mean ± SEM ($n = 8$ per group). Note: *y*-axes for (*A*) and (*B*) are broader than (*C*) to (*H*) for visual clarity.
[Colour figure can be viewed at wileyonlinelibrary.com]

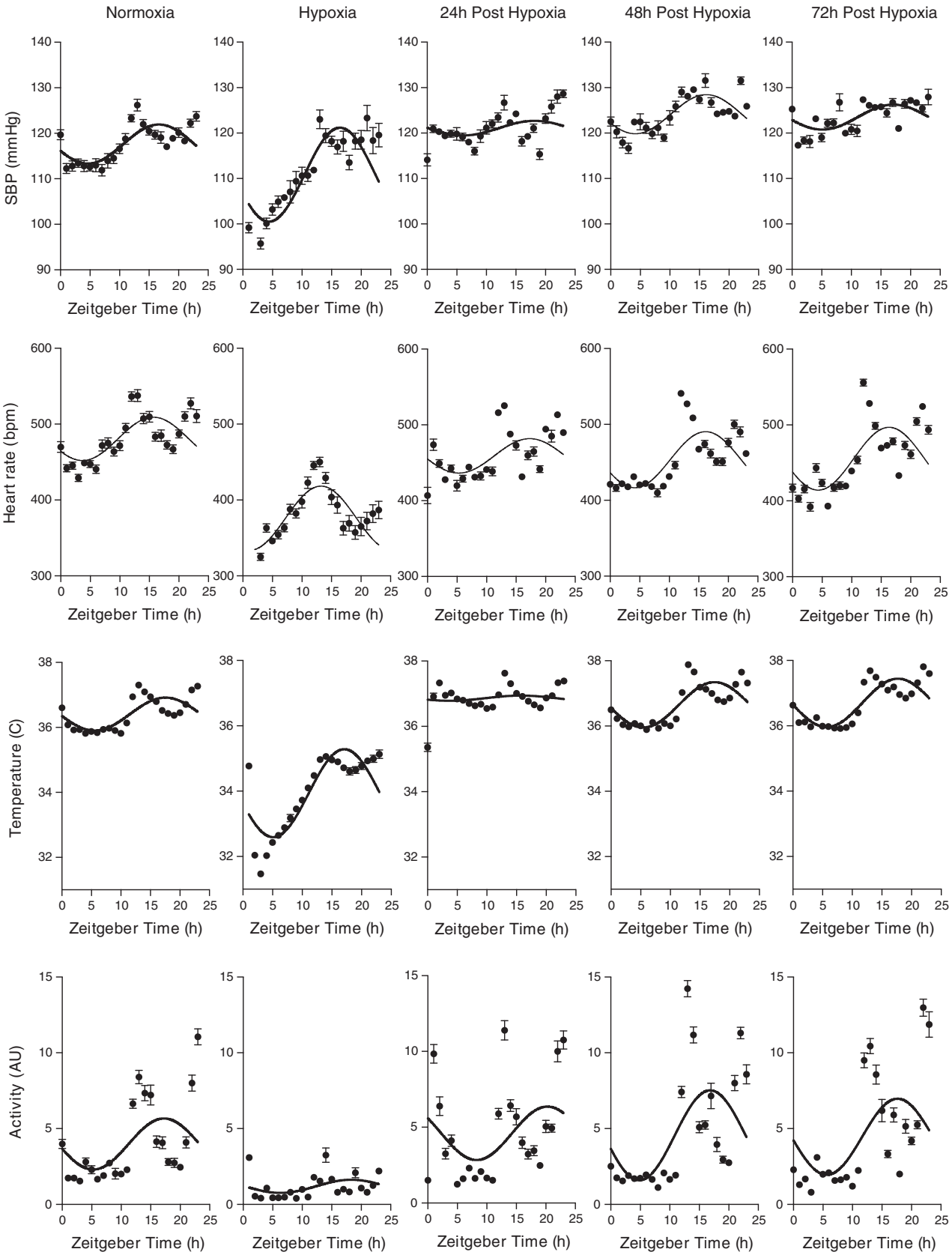

Figure 3. Cosinor analysis of severe hypoxia
Graphical representation of cosinor analysis of severe hypoxia (9% O_2).

Table 2. Cosinor analysis of physiological parameters

	Mesor	Amplitude	Acrophase (h)	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
24 h Post hypoxia	121.1 ± 0.8*	1.6 ± 1.1*	20.4 ± 0.7	0.09
48 h Post hypoxia	124.2 ± 0.6*	4.3 ± 0.8	14.6 ± 0.2	0.58
72 h Post hypoxia	123.5 ± 0.6*	2.8 ± 0.8	20.7 ± 0.3	0.35
HR (beats min^{-1})				
Baseline	481 ± 5	29 ± 7	15.0 ± 0.2	0.44
Hypoxia	376 ± 5*	42 ± 8	15.4 ± 0.2	0.62
24 h Post hypoxia	459 ± 6*	23 ± 9	14.3 ± 0.4	0.25
48 h Post hypoxia	454 ± 6*	37 ± 8	14.6 ± 0.3	0.46
72 h 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
24 h Post hypoxia	36.9 ± 0.1*	0.1 ± 0.1*	14.7 ± 1.6	0.02
48 h Post hypoxia	36.7 ± 0.1*	0.7 ± 0.1	14.3 ± 0.2	0.62
72 h 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
24 h Post hypoxia	4.6 ± 0.6	1.8 ± 0.9	13.5 ± 0.5	0.16
48 h Post hypoxia	4.6 ± 0.7	3.0 ± 0.9	20.7 ± 0.3	0.33
72 h 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
24 h Post hypoxia	114.6 ± 1.0	4.2 ± 1.4	14.0 ± 0.3	0.30
48 h Post hypoxia	110.6 ± 0.7*	4.6 ± 1.1	20.0 ± 0.2	0.47
72 h Post hypoxia	109.0 ± 0.9*	6.2 ± 1.3	20.3 ± 0.2	0.54
HR (beats min^{-1})				
Baseline	516 ± 4	33 ± 6	14.5 ± 0.2	0.59
Hypoxia	537 ± 6*	16 ± 8*	1.0 ± 0.5*	0.16
24 h Post hypoxia	491 ± 6*	34 ± 8	14.3 ± 0.2	0.48
48 h Post hypoxia	490 ± 6*	29 ± 8	14.4 ± 0.3	0.40
72 h 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
24 h Post hypoxia	36.4 ± 0.1	0.6 ± 0.1	20.4 ± 0.2	0.69
48 h Post hypoxia	36.4 ± 0.1	0.6 ± 0.1	14.1 ± 0.2	0.68
72 h 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
24 h Post hypoxia	4.9 ± 0.4	2.2 ± 0.6	20.3 ± 0.3	0.39
48 h Post hypoxia	4.4 ± 0.5	2.4 ± 0.6	20.2 ± 0.3	0.40
72 h Post hypoxia	5.5 ± 0.5*	3.6 ± 0.7*	20.1 ± 0.2	0.53

Data are the mean ± SEM. 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. *$P < 0.05$ compared to baseline.

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 h. Normoxic recovery from moderate hypoxia did not significantly affect rhythm. A two-way (time × group) ANOVA indicated a divergent response in body temperature to hypoxic severity (Fig. 2*G* and *H*). Severe hypoxia induced a rebound in body temperature that exceeded baseline levels, at 24 h post-hypoxia, whereas moderate hypoxia 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 (Fig. 5*A*, *C* and *D*). Rhythm was disrupted, as indicated by a strong disagreement in R^2 and a decreased mesor (Fig. 3 and Table 2). By contrast, moderate hypoxia had no effect on overall activity (Fig. 5*B*, *E* and *F*) or rhythm (Fig. 3 and Table 2). Severe hypoxia had no effects on activity during normoxic recovery, including rhythm. Interestingly, moderate hypoxia increased activity only at 72 h post-hypoxia during lights off (i.e. the last 12 h of recording). Rhythm was also disrupted at 72 h post-hypoxia because R^2 was in mild disagreement and mesor and amplitude were increased. A two-way (time × group) ANOVA confirmed a divergent response in activity to hypoxic severity (Fig. 5*G* 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 the arousal state was similar.

Systolic blood pressure responses to moderate and severe hypoxia

To determine the physiological risk for hypertension as a result of the severity of hypoxia, we assessed ambulatory SBP. Severe hypoxia decreased SBP during lights on but not lights off (Fig. 6*A*, *C* and *D*). Rhythm was disrupted during severe hypoxia with a mild disagreement of R^2, decreased mesor and increased amplitude, although with no change in acrophase (Fig. 3 and Table 2). By contrast, moderate hypoxia decreased SBP during lights off but not lights on (Fig. 6*B*, *E* and *F*); rhythm was disrupted with a mild disagreement of R^2 and decreased mesor, although with no change in amplitude or acrophase (Fig. 3 and Table 2). Surprisingly, although severe hypoxia had a rebound change in SBP, moderate hypoxia had a persistent change over 72 h. 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 h. Moderate hypoxia only had a persistent effect on mesor throughout 72 h. Furthermore, the after-effects of severe and moderate hypoxia were most salient in lights on or lights off, respectively. A two-way (time × group) ANOVA confirmed a divergent response in SBP to hypoxic severity (Fig. 6*G* and *H*) acutely and in recovery, thus indicating that 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 (Fig. 7*A*, *C* and *D*). Heart rate rhythm was also disrupted during severe hypoxia with a mild disagreement of R^2 and decreased mesor (Fig. 3 and Table 2). By contrast, moderate hypoxia increased heart rate during lights on but not lights off (Fig. 7*B*, *E* and *F*); rhythm was disrupted with a strong disagreement of R^2 and with increased mesor and decreased acrophase (Fig. 3 and Table 2). During normoxic recovery, heart rate rebounded initially following severe hypoxia with fluctuations during the 72 h period, then returned to baseline (Fig. 7*C* and *D*). There was a strong disagreement of R^2 at 24 h following severe hypoxia, although it returned to baseline by 72 h; mesor was decreased throughout. Conversely, moderate hypoxia decreased heart rate throughout the majority of the normoxic recovery period. Rhythm was disrupted following moderate hypoxia, as indicated by a mild decrease in R^2 and a sustained decrease in mesor. A two-way (time × group) ANOVA confirmed a divergent response in heart rate to hypoxic severity (Fig. 7*G* 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 HRV analysis 72 h post hypoxia because 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 (Fig. 8*A* and Table 3). Moderate hypoxia increased the SDs of normal R–R intervals and root mean square of successive normal R–R interval differences 72 h 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,

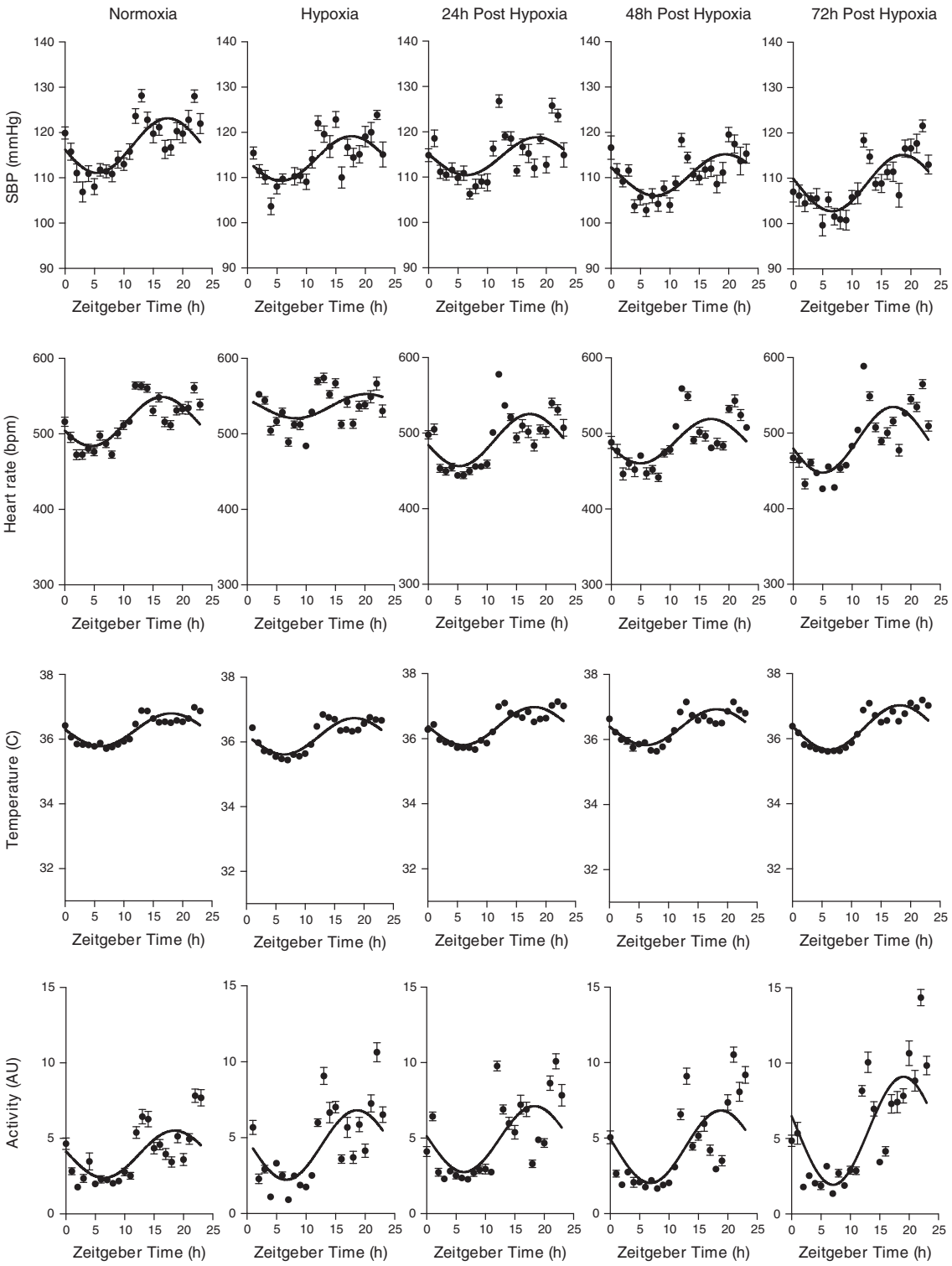

Figure 4. Cosinor analysis of moderate hypoxia
Graphical representation of cosinor analysis of moderate hypoxia (15% O_2).

whereas severe hypoxia 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 powers of the LF and HF bands were decreased and increased, respectively (Fig. 8*B* and *C* and Table 3). In addition, LF power was higher and HF power was lower 72 h following severe hypoxia compared to moderate hypoxia. This was also

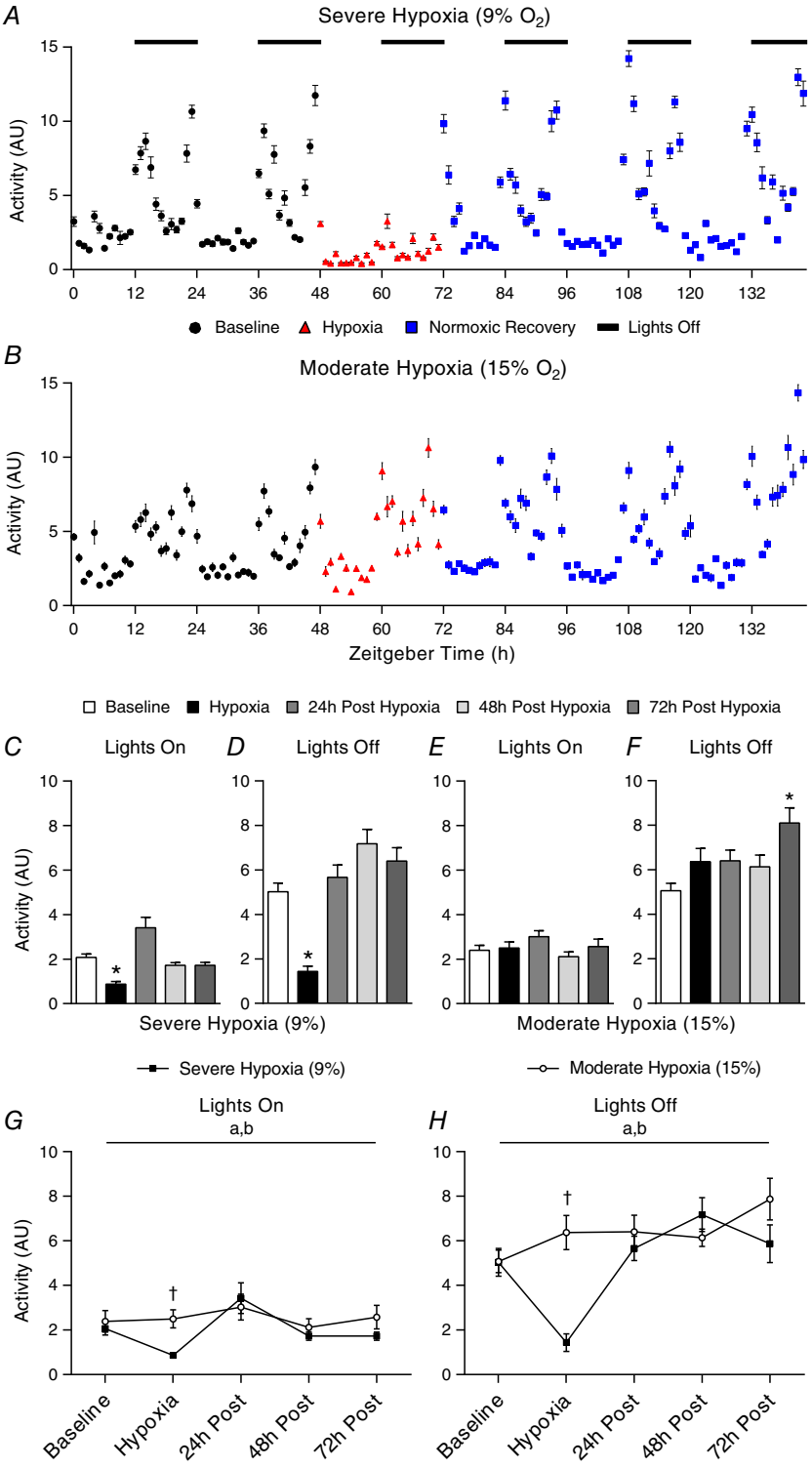

Figure 5. Physiological responses of activity during baseline, severe or moderate hypoxia, and 72 hours of normoxic recovery
Hourly averages for severe (*A*) and moderate (*B*) hypoxia experiments. Average activity recorded following baseline, severe hypoxia, 24 h post-hypoxia, 48 h post-hypoxia and 72 h post-hypoxia during lights on (*C*) ($\chi^2 = 80.7$, d.f. $= 4$, $P < 0.0001$) and lights off (*D*) ($\chi^2 = 112.8$, d.f. $= 4$, $P < 0.0001$). Average body temperatures recorded following baseline, moderate hypoxia, 24 h post-hypoxia, 48 h post-hypoxia and 72 h post-hypoxia during lights on (*E*) ($\chi^2 = 14.7$, d.f. $= 4$, $P = 0.0055$) and lights off (*F*) ($\chi^2 = 10.1$, d.f. $= 4$, $P = 0.0396$). 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 $F_{1,14} = 3.2$, $P = 0.0945$). For panels (*C*) to (*F*): $^*P < 0.05$ compared to baseline. For panels (*G*) and (*H*): a, significant interaction; b, significant main effect of time; $^\dagger P < 0.05$ compared to moderate hypoxia at the same time point. Values expressed are the mean ± SEM ($n = 8$ per group). Note: *y*-axes for (*A*) and (*B*) are broader than (*C*) to (*H*) for visual clarity. [Colour figure can be viewed at wileyonlinelibrary.com]

indicated by an increase in the LF/HF ratio following severe compared to moderate hypoxia (Fig. 8*D*). Thus, severe hypoxia induced a shift in sympathovagal balance towards sympathetic dominance, whereas 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

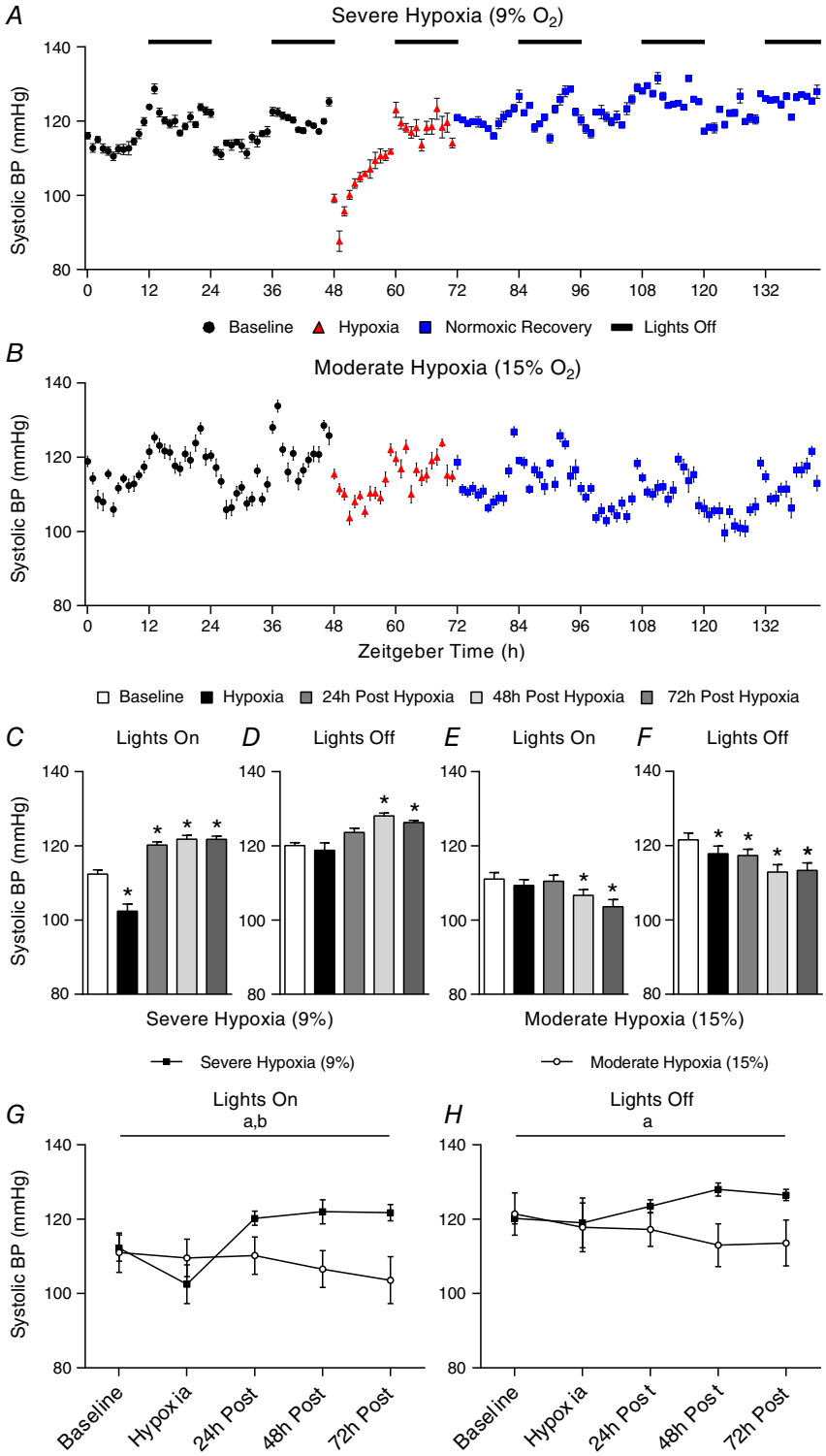

Figure 6. Physiological responses of systolic blood pressure (SBP) during baseline, severe or moderate hypoxia, and 72 hours of normoxic recovery
Hourly averages for severe (*A*) and moderate (*B*) hypoxia experiments. Average SBP recorded following baseline, severe hypoxia, 24 h post-hypoxia, 48 h post-hypoxia and 72 h post-hypoxia during lights on (*C*) ($\chi^2 = 85.4$, d.f. $= 4$, $P < 0.0001$) and lights off (*D*) ($\chi^2 = 41.2$, d.f. $= 4$, $P < 0.0001$). Average SBP recorded following baseline, moderate hypoxia, 24 h post-hypoxia, 48 h post-hypoxia and 72 h post-hypoxia during lights on (*E*) ($\chi^2 = 50.9$, d.f. $= 4$, $P < 0.0001$) and lights off (*F*) ($\chi^2 = 52.5$, d.f. $= 4$, $P < 0.0001$). 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 $F_{1,6} = 0.7$, $P = 0.4329$). For panels (*C*) to (*F*): $^*P < 0.05$ compared to baseline. For panels (*G*) and (*H*): a, significant interaction; b, significant main effect of time; $^\dagger P < 0.05$ compared to moderate hypoxia at the same time point. Values are the mean ± SEM (severe, $n = 4$; moderate, $n = 5$). [Colour figure can be viewed at wileyonlinelibrary.com]

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, whereas EPO was unchanged (Fig. 9*A* and *B*). HMOX1 protein levels (Fig. 9*C–E*) were in agreement with mRNA expression. This suggests that 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

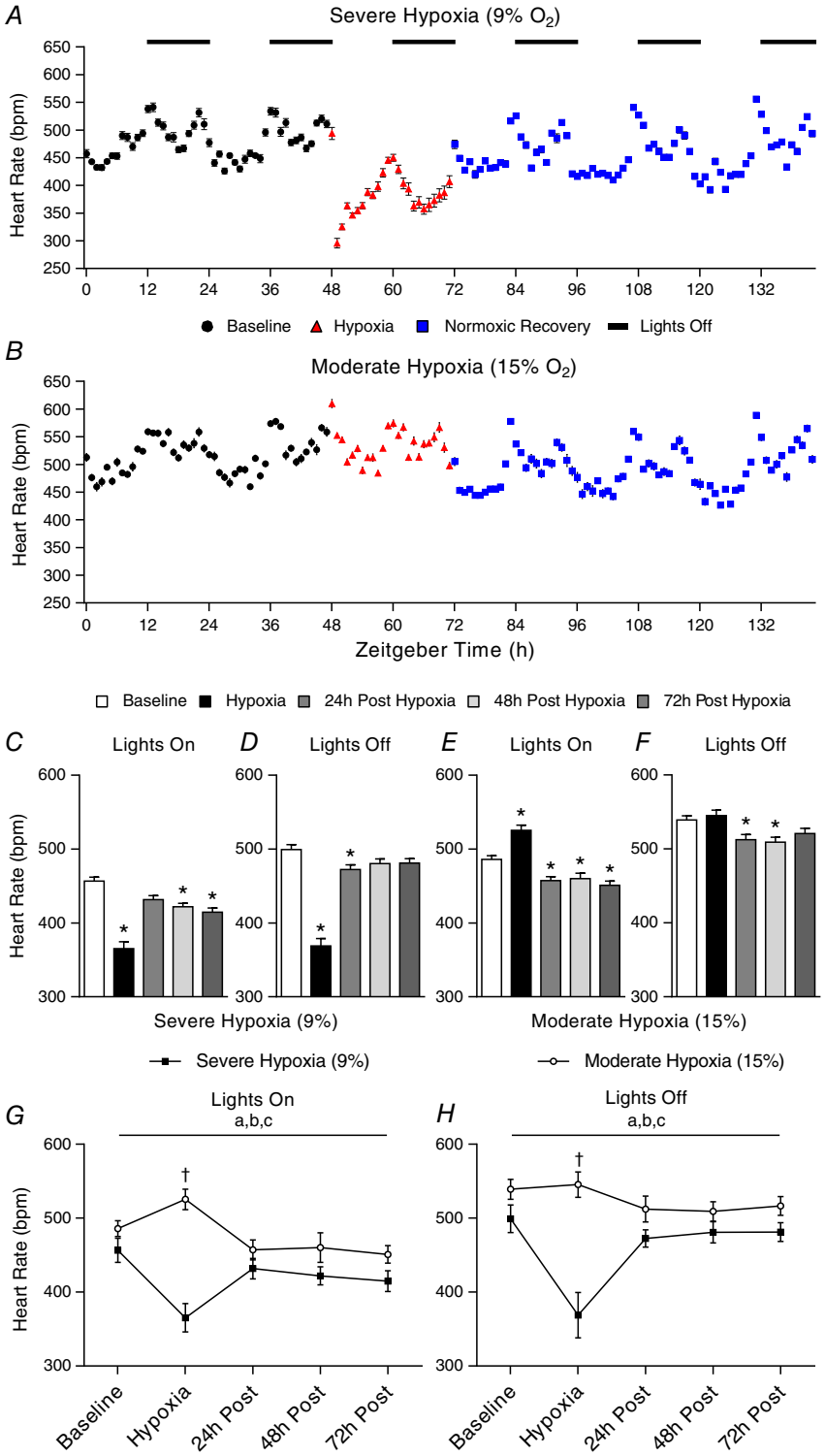

Figure 7. Physiological responses of heart rate during baseline, severe or moderate moderate hypoxia and 72 h of normoxic recovery
Hourly averages for severe (*A*) and moderate (*B*) hypoxia experiments. Average heart rate recorded following baseline, severe hypoxia, 24 h post-hypoxia, 48 h post-hypoxia and 72 h post-hypoxia during lights on (*C*) ($\chi^2 = 91.8$, d.f. $= 4$, $P < 0.0001$) and lights off (*D*) ($F_{4,276} = 78.2$, $P < 0.0001$). Average heart rate recorded following normoxia, moderate hypoxia, 24 h post-hypoxia, 48 h post-hypoxia and 72 h post-hypoxia during lights on (*E*) ($\chi^2 = 98.4$, d.f. $= 4$, $P < 0.0001$) and lights off (*F*) ($\chi^2 = 24.7$, d.f. $= 4$, $P < 0.0001$). 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 $F_{1,13} = 13.7$, $P = 0.0026$). For panels (*C*) to (*F*): $^*P < 0.05$ compared to baseline. For panels (*G*) and (*H*): a, significant interaction; b, significant main effect of time; c, significant main effect of group; $^\dagger P < 0.05$ compared to moderate hypoxia at the same time point. Values are the mean ± SEM (severe, $n = 7$; moderate, $n = 8$). Note: *y*-axes for (*A*) and (*B*) are broader than (*C*) to (*H*) for visual clarity. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3. Frequency and time domain heart rate variability analysis

	15% O_2		9% O_2	
	Baseline	72 h Post hypoxia	Baseline	72 h 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 the mean ± SD. SDNN, SD of normal R–R intervals; RMSSD, root mean square of successive normal R–R interval differences. [a]Significant interaction. [b]Significant main effect of time. [c]Significant main effect of group. *$P < 0.05$ compared to baseline. †$P < 0.05$ compared to moderate hypoxia at the same time point ($n = 7$ per group).

agreement in localized stress but disagreement in systemic sympathetic dominance.

Discussion

We demonstrate, for the first time, contrasting haemodynamic responses during normoxic recovery following moderate and severe hypoxia. These results highlight the importance of hypoxic severity with respect to mediating the physiological response. Moderate and severe hypoxia both decreased SBP during the hypoxic insult, whereas they induced divergent hypertensive and hypotensive responses, respectively, following normoxic recovery. Although 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 probably represent the net balance between the increased vasodilatory effects of HMOX1 and the opposing sympathetic vasoconstriction, secondary to chemoreflex activation. Furthermore, both moderate and severe hypoxia disrupted the circadian rhythm during the hypoxic insult and transiently so during normoxic recovery. Such observations have major implications for our understanding of basic physiology and the role of hypoxia in disease progression.

Although rare, severe reductions in P_aO_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 P_aO_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. Although 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 with respect to body temperature and activity. However, the effects on cardiovascular measures are less clear. Although we also report divergent responses in heart rate and SBP during the hypoxic insult, there is little support from the literature, which is largely attributed to the uniqueness and novelty of radiotelemetry methodology.

Circadian rhythms are fundamental to our homeostasis, occurring in almost every organ in the body, and, when disrupted, they 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 a 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). Furthermore, despite evidence of a hypoxic influence on circadian rhythm through interactions between clock genes Period1 and BMAL1 with HIF-1α, studies aiming to understand the effects of hypoxia on circadian rhythm are rare (Chilov *et al.* 2001; Peek *et al.* 2017). In the present study, we report that severe hypoxia suddenly and dramatically decreased SBP, whereas moderate hypoxia 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

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
24 h Post hypoxia	150.25 ± 16.82*	140.84 ± 15.05*
48 h Post hypoxia	155.77 ± 5.15*	141.72 ± 19.68*
72 h 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
24 h Post hypoxia	11.69 ± 3.29*	6.92 ± 3.24
48 h Post hypoxia	8.36 ± 2.25	6.32 ± 2.78
72 h 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
24 h Post hypoxia	14.60 ± 5.14*	8.71 ± 4.31
48 h Post hypoxia	9.50 ± 3.39	7.77 ± 3.82
72 h 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
24 h Post hypoxia	65.49 ± 13.86	60.75 ± 7.84
48 h Post hypoxia	72.91 ± 10.97*	60.93 ± 9.52
72 h 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
24 h Post hypoxia	34.50 ± 13.85	39.25 ± 7.84
48 h Post hypoxia	27.09 ± 10.97*	39.07 ± 9.52
72 h 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
24 h Post hypoxia	154.51 ± 86.83	50.97 ± 46.43
48 h Post hypoxia	70.33 ± 32.30	42.43 ± 29.83
72 h 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
24 h Post hypoxia	3.43 ± 3.00	2.11 ± 0.71
48 h Post hypoxia	4.10 ± 2.27*†	2.02 ± 1.01
72 h Post hypoxia	3.53 ± 0.79	1.79 ± 0.45

Data are the mean ± SD. SDNN, SD of normal R–R intervals; RMSSD, root mean square of successive normal R–R interval differences. [a]Significant interaction. [b]Significant main effect of time. [c]Significant main effect of group. *$P < 0.05$ compared to baseline. †$P < 0.05$ compared to moderate hypoxia at the same time point ($n = 7$).

of HIF-1α via BMAL1 (Peek *et al.* 2017). We also report that severe hypoxia disrupts the 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, although moderate hypoxia may not result in the 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 the other factors that play a crucial role in the aetiology of disease.

Heterogeneous activation of the HIF pathway occurs in response to different hypoxic severities following reductions in haemoglobin concentration (anaemic hypoxia) (Tsui *et al.* 2014) and F_IO_2 (hypoxic hypoxia) (Stroka *et al.* 2001). Furthermore, different severities of anaemia 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 anaemia in an organ-specific manner (Tsui *et al.* 2014). The results of the present study demonstrate that both moderate and severe hypoxia are associated with corresponding increases in HMOX1 mRNA and protein. HMOX1 is an inducible enzyme responsible for catabolizing haeme 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). Furthermore, 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 consider that the observed hypotension following moderate hypoxia is a result of alterations in local vascular tone because of the increased production of HMOX-derived carbon monoxide, despite potential reductions in endothelial NOS expression. However, following severe hypoxia, SBP is increased as a result of concomitant sympathetic activation, as demonstrated by the increased LF/HF ratio, probably because 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 partly a result of 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, because 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 that we did not observe during moderate hypoxia. By contrast, chronic exposure to severe hypoxia results in an 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 the present study is the absence of ventilation and arousal state (i.e. EEG) recordings for each animal, which may influence HRV. In addition, telemetry units were set to record only 30 s of data every 5 min. Although we acknowledge the limitation that our segment length is below the 1–3 min used in other studies, we found it easier to identify stationarity of the signal using shorter time lengths. To accommodate the shorter time length, we used nine 30 s segments. Indeed, the averaging of multiple 1 min segments produces comparable means to those of 3 min data segments (Thireau *et al.* 2008). Finally, although we observed disruptions to the circadian rhythm during and following hypoxia, longer durations of hypoxic stress and recovery should also be investigated. This could provide valuable insight regarding whether moderate hypoxia disrupts the circadian rhythm and contributes to diseases such as hypertension and mild COPD.

Impaired tissue oxygenation is present in numerous chronic diseases and is associated with a worse quality of life and clinical outcomes. Decades of research have almost exclusively focused on investigating the effects of severe hypoxia in pathophysiological states, whereas the same effects of moderate hypoxia remain uninvestigated. Furthermore, there is no standardization for the classification of hypoxic severities, with the same reduction in F_IO_2 being classified as mild,

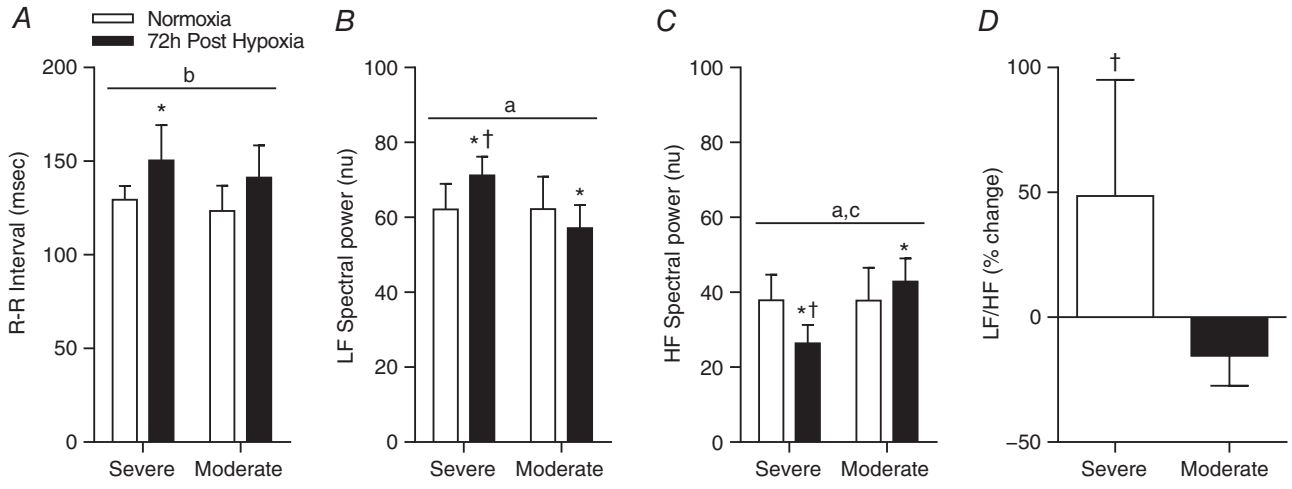

Figure 8. Effects of moderate or severe hypoxia on heart rate variability
Mean ± SD R–R interval of normal R–R intervals (SDNN) at baseline and 72 h post severe and moderate hypoxia (*A*) (interaction $F_{1,12} = 0.1$, $P = 0.7543$; main effect of time $F_{1,12} = 14.3$, $P = 0.0026$; main effect of group $F_{1,12} = 1.6$, $P = 0.2352$). 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 $F_{1,12} = 4.7$, $P = 0.0515$); 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 h 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 are the mean ± SD ($n = 7$ per group). [Correction made on 4 July 2018 after first online publication: y-axis label in D corrected from "LF/HF (Fold change)" to "LF/HF (% change)".]

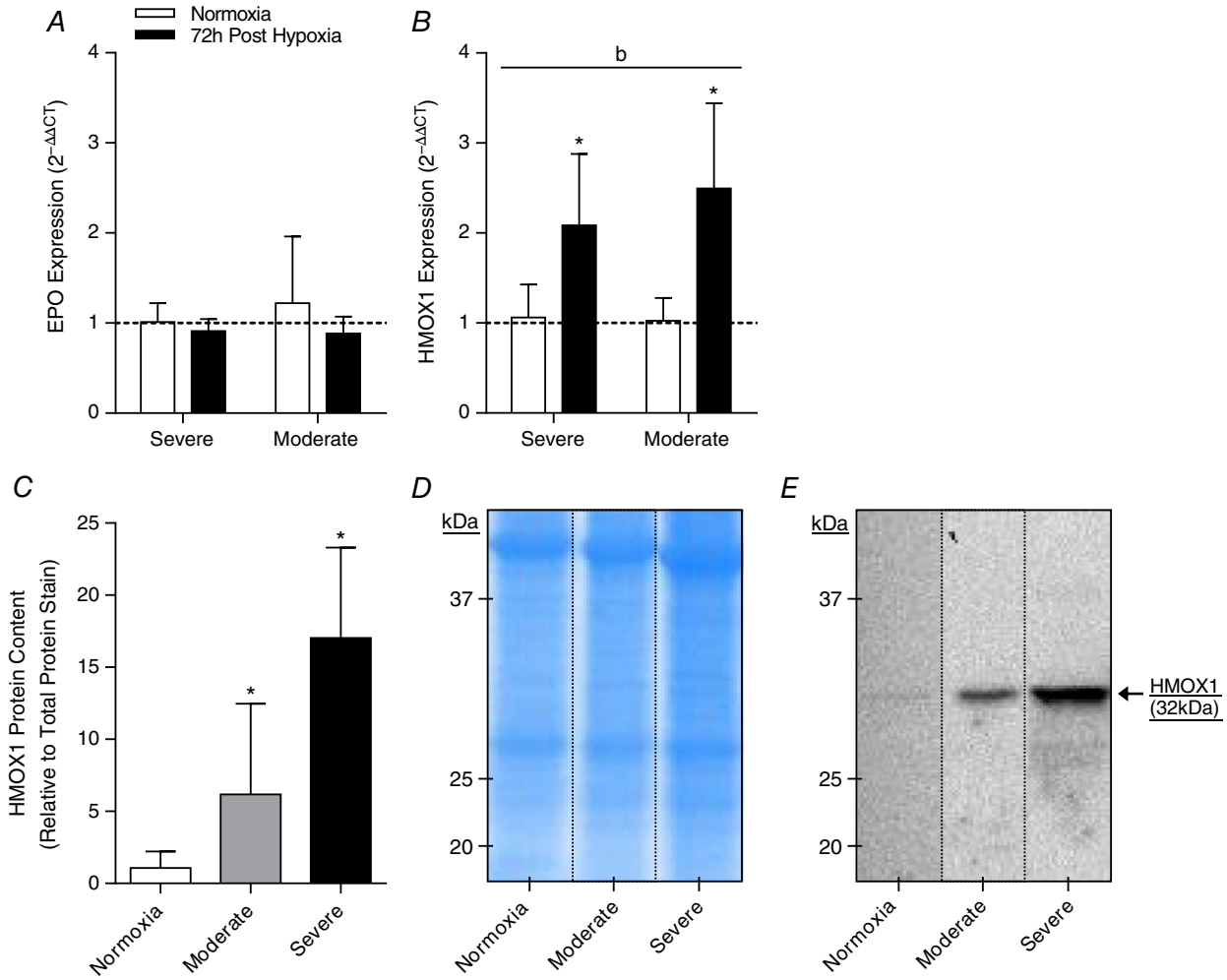

Figure 9. Effect of moderate or severe hypoxia on the expression of HIF targets in mesenteric resistance arteries
Mesenteric artery gene expression of erythropoietin (EPO) (*A*) (interaction $F_{1,15} = 0.3$, $P = 0.3227$; main effect of time $F_{1,15} = 1.1$, $P = 0.3085$; main effect of group $F_{1,15} = 0.2$, $P = 0.6631$) and haeme 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 h of severe or moderate hypoxia ($n = 4$–8 per group). b, significant main effect of time. HMOX1 protein levels 72 h 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 two animals were required per sample). $^*P < 0.05$ compared to normoxia. Values are the mean ± SD. Representative MemCode total protein stain (*D*) and HMOX1 immunoblot (*E*).
[Colour figure can be viewed at wileyonlinelibrary.com]

moderate and severe, depending on the study design. By contrast to hypoxic hypoxia, anaemic hypoxia has defined haemoglobin concentrations recommended by the World Health Organization for the classification of mild, moderate and severe anaemia. This lack of standardization represents a significant barrier in the interpretation and comparison of results obtained 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 the present study illustrate a critical need to revisit the basic pathophysiology of hypoxia to promote standardization, to reconcile our understanding of the literature and to improve clinical translation.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

MAA and JAS were responsible for the conception and design of the experiments. MAA, BAE, JSH, NR, AE, PJM, KRB and JAS were responsible for the collection, analysis and interpretation of data. MAA, BAE, JSH, NR, AE, PJM, KRB and JAS were responsible for drafting the article or revising it critically for important intellectual content. All authors have approved the final version of the manuscript submitted for publication 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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