

## RESEARCH ARTICLE

# Altitude matters: differences in cardiovascular and respiratory responses to hypoxia in bar-headed geese reared at high and low altitudes

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## ABSTRACT

Bar-headed geese (*Anser indicus*) fly at high altitudes during their migration across the Himalayas and Tibetan plateau. However, we know relatively little about whether rearing at high altitude (i.e. phenotypic plasticity) facilitates this impressive feat because most of what is known about their physiology comes from studies performed at sea level. To provide this information, a comprehensive analysis of metabolic, cardiovascular and ventilatory responses to progressive decreases in the equivalent fractional composition of inspired oxygen ( $F_{I_{O_2}}$ : 0.21, 0.12, 0.09, 0.07 and 0.05) was made on bar-headed geese reared at either high altitude (3200 m) or low altitude (0 m) and on barnacle geese (*Branta leucopsis*), a low-altitude migrating species, reared at low altitude (0 m). Bar-headed geese reared at high altitude exhibited lower metabolic rates and a modestly increased hypoxic ventilatory response compared with low-altitude-reared bar-headed geese. Although the *in vivo* oxygen equilibrium curves and blood-oxygen carrying capacity did not differ between the two bar-headed goose study groups, the blood-oxygen carrying capacity was higher than that of barnacle geese. Resting cardiac output also did not differ between groups and increased at least twofold during progressive hypoxia, initially as a result of increases in stroke volume. However, cardiac output increased at a higher  $F_{I_{O_2}}$  threshold in bar-headed geese raised at high altitude. Thus, bar-headed geese reared at high altitude exhibited a reduced oxygen demand at rest and a modest but significant increase in oxygen uptake and delivery during progressive hypoxia compared with bar-headed geese reared at low altitude.

**KEY WORDS:** *Anser indicus*, *Branta leucopsis*, Barnacle goose, Ventilation, Cardiac output, Metabolic rate, Oxygen uptake, Stroke volume

## INTRODUCTION

Birds exhibit enhancements at each step of their  $O_2$  transport cascade that help to support the high flux of  $O_2$  required for flight and endothermy (Scott, 2011). This cascade describes the flow of  $O_2$  from the atmosphere to the mitochondria in vertebrates and the steps include ventilation, pulmonary  $O_2$  diffusion, perfusion and tissue  $O_2$  diffusion. Bar-headed geese (*Anser indicus*), which migrate biannually over the Himalayan mountain range (Bishop

et al., 2015; Hawkes et al., 2011; Scott et al., 2015), exhibit further adaptations at each level of this transport cascade (Black and Tenney, 1980; Meir and Milsom, 2013; Scott and Milsom, 2006, 2007; Scott, 2011; Scott et al., 2015; Weber et al., 1993). While providing evidence of physiological differences between bar-headed geese and closely related low-altitude species, all studies to date have been conducted on groups of bar-headed geese born and raised for generations at sea level (Black and Tenney, 1980; Fedde et al., 1989; Hawkes et al., 2014; Scott and Milsom, 2007). Thus, relatively little is known about the influence of phenotypic plasticity (i.e. acclimatization) and developmental plasticity on the physiology of this species.

Hypoxic responses vary depending on when during an animal's development it is exposed to hypoxia and the duration of the exposure. In chickens, hypoxic exposure had no reported developmental effects when it occurred early in development or for an acute duration (Ferner and Mortola, 2009). When exposure to hypoxia occurred during the entire duration of incubation, or even during the final week, however, the hypoxic ventilatory response (HVR) of the chicks was blunted. This was a result of reduced ventilatory chemosensitivity (Ferner and Mortola, 2009; Mortola, 2011; Szdzyu and Mortola, 2007). Sustained hypoxic exposure in low-altitude birds also decreased whole animal oxygen consumption rate ( $\dot{V}_{O_2}$ ) and growth rate (Mortola, 2011). However, embryos of some birds successfully hatch with normal growth rates and  $\dot{V}_{O_2}$  at altitudes of 4000–6500 m (Carey et al., 1982; León-Velarde and Monge-C, 2004). For example, the high-altitude migrating bar-headed goose maintained  $\dot{V}_{O_2}$  as an embryo when exposed acutely to extreme ambient hypoxia (11.7 kPa) (Snyder et al., 1982). Thus, although hypoxia exposure during development can alter physiological responses in birds, some high-altitude bird species have adapted to mitigate these effects.

In adult animals, many rapid physiological changes occur minutes to hours after acute hypoxic exposure and can be modified during chronic acclimatization (Ivy and Scott, 2015; Powell et al., 1998). Black and Tenney measured changes in  $\dot{V}_{O_2}$ , total ventilation ( $\dot{V}_R$ ) and cardiac output ( $\dot{Q}$ ) during progressive hypoxic exposure in bar-headed geese following short-term (4 week) acclimation to simulated altitude (5640 m) (Black and Tenney, 1980). The acclimated bar-headed geese did not become polycythemic, a trait characteristic of other species endemic to high-altitude regions. They also displayed higher resting  $\dot{V}_{O_2}$ ,  $\dot{V}_R$  and  $\dot{Q}$  under ambient conditions, as well as greater increases in  $\dot{V}_R$  and  $\dot{Q}$  during exposure to progressive hypoxia (Black and Tenney, 1980). Thus, the HVR and hypoxic cardiovascular response of bar-headed geese can be affected by short-term high-altitude acclimation.

In the present study, we extended this work by examining the effects of high-altitude rearing and development on the physiological responses to hypoxia in bar-headed geese. This is

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**List of symbols and abbreviations**

BTPS	body temperature and pressure saturated
$Ca_{O_2}$	arterial oxygen content
$Cv_{O_2}$	venous oxygen content
$FE_{O_2}$	fractional oxygen composition of expired gas of the bird
$FE_{CO_2}$	fractional oxygen composition of expired gas of the chamber
$FI_{O_2}$	fractional oxygen composition of inspired gas
HAR	high-altitude-reared
Hb	hemoglobin
[Hb]	hemoglobin concentration
Hct	hematocrit
$[HCO_3^-]_a$	arterial bicarbonate ion concentration
HVR	hypoxic ventilatory response
LAR	low-altitude-reared
$Pa_{CO_2}$	arterial partial pressure of carbon dioxide
$Pa_{O_2}$	arterial partial pressure of oxygen
$pH_a$	arterial pH
$\dot{Q}$	cardiac output
RQ	respiratory quotient
STPD	standard temperature and pressure and dry
$\dot{V}_{O_2}$	whole animal oxygen consumption rate
$\dot{V}_C$	flow rate through chamber
$\dot{V}_R$	total ventilation

significant, considering that the effects of pre- or postnatal hypoxic exposure can differ significantly, and persist throughout adult life (Bavis, 2005; Ivy and Scott, 2015). Our primary objective, therefore, was to compare the changes in the convective steps in the  $O_2$  transport cascade, ventilation and circulation, of low-altitude-reared (LAR) bar-headed geese to a group of wild high-altitude-reared (HAR) bar-headed geese during short-term progressive hypoxic exposure. These responses were compared with those of LAR barnacle geese (*Branta leucopsis*), a member of a closely related genus that also migrates, but only at low altitude. We predicted that, because of lifelong exposure to high altitude, HAR bar-headed geese would show further enhancements in the overall magnitude of  $\dot{V}_R$  and  $\dot{Q}$  compared with those previously reported during short-term high altitude acclimation of this species (Black and Tenney, 1980). In addition, because bar-headed geese are known to be capable of large increases in  $\dot{V}_R$  during hypoxic exposure (Black and Tenney, 1980; Scott and Milsom, 2007), we predict that  $\dot{V}_R$  would be greatest in the HAR bar-headed geese and lowest in the barnacle geese.

**MATERIALS AND METHODS****Animals**

The experiments on the LAR geese were performed at the University of British Columbia, where the geese were housed at the Centre for Comparative Medicine. Cardiovascular measurements were made on 6 cannulated bar-headed geese (*Anser indicus* Latham 1790) (2.5±0.2 kg) and respiratory measurements were made on 5 non-cannulated bar-headed geese (2.4±0.1 kg). All cardiorespiratory measurements were made on 7 cannulated barnacle geese (*Branta leucopsis* Bechstein 1803) (2.5±0.2 kg). Cardiorespiratory measurements were also obtained from 5 cannulated HAR bar-headed geese (2.1±0.1 kg) that were born in the wild at 3200 m at Lake Qinghai, China and reared in captivity for at least 1 year at the lake. All experimental animals were fed similar diets, housed in outdoor pens under natural conditions and experienced similar levels of (in)activity. The HAR bar-headed geese, however, were born and reared in hypobaric hypoxia. All experimental procedures were conducted according to guidelines

approved by the Animal Care Committee at the University of British Columbia under the guidelines of the Canadian Council on Animal Care.

**Surgical procedures**

For cardiovascular measurements, surgery was conducted under general and local anesthesia 1 day before the hypoxic exposure. All geese were first weighed, gently restrained and induced with isoflurane (4%) supplemented with  $O_2$  (100%) by facemask prior to intubation. General anesthesia was maintained with isoflurane and  $O_2$ . The right brachial artery and vein were accessed via a small incision and blunt dissection and cannulated with polyurethane cannulae (PU-90; 0.102 cm internal diameter×0.410 cm outer diameter) filled with 1000 IU ml<sup>-1</sup> heparinized saline. Geese were recovered for at least 24 h prior to experimentation.

**Experimental protocol**

Each goose was placed in a flexible cradle that permitted unrestricted breathing. Its head was placed in an opaque Plexiglass chamber large enough to accommodate free movement of the neck and head, sealed around the neck with a flexible latex collar and supported by the cradle. Geese in the experimental apparatus were allowed 60–90 min to adjust to their surroundings. Then, air with varying equivalent fractional compositions of inspired  $O_2$  ( $FI_{O_2}$ ) was delivered at a flow rate ( $\dot{V}_C$ ) through the box ranging between 5 and 10 l min<sup>-1</sup>. Changes in  $FI_{O_2}$  were produced by mixing nitrogen and air through a series of calibrated rotameters. Birds were exposed to 25 min step reductions in equivalent  $FI_{O_2}$ : ambient [0.21 at 0 m or 0.134 at 3200 m], 0.12, 0.09 and 0.07. For the respiratory trials, birds were exposed further to 0.05  $FI_{O_2}$ . A 25 min recovery at ambient  $FI_{O_2}$  followed the hypoxic exposures.

**Measurements**

Whole animal  $\dot{V}_{O_2}$  was calculated from  $\dot{V}_C$ ,  $FI_{O_2}$  and the fractional  $O_2$  composition of expired gas from the chamber ( $FE_{CO_2}$ ), which were directly measured by a gas analyzer (Sable Systems, Las Vegas, NV, USA). Water vapour was removed from the gas prior to analysis. Tidal volume and breathing frequency were measured from the head mask outflow using a pneumotachograph connected to a differential pressure transducer (Validyne, Northridge, CA, USA).

Mean arterial pressure and all respiratory variables were recorded to a computer using PowerLab data acquisition software (ADInstruments, Colorado Springs, CO, USA). Arterial blood pressure was continuously monitored throughout using a pressure transducer (Deltran, Utah Medical Products, Midvale, USA) connected to the brachial artery cannula. Strategic sampling of arterial and venous blood (0.4 ml per sample) occurred 15 min after exposure to each  $FI_{O_2}$ , as well as after 5 and 25 min in recovery. Any blood remaining after analysis was returned to the bird. Blood samples were immediately analyzed for partial pressures of  $O_2$  and  $CO_2$ ,  $O_2$  content, Hb concentration ([Hb]), hematocrit (Hct), arterial pH ( $pH_a$ ) and plasma ions including  $HCO_3^-$ . Arterial  $O_2$  content ( $Ca_{O_2}$ ; mmol l<sup>-1</sup>) and venous  $O_2$  content ( $Cv_{O_2}$ ; mmol l<sup>-1</sup>) were determined at 41°C using the Tucker method (Tucker, 1967) with a FireSting  $O_2$  probe (PyroScience, Aachen, Germany). The  $O_2$  probe was calibrated with 0%  $O_2$  (3 g l<sup>-1</sup>  $Na_2SO_3$ ; Sigma-Aldrich) and water saturated with ambient air (21% at sea level and 12% at 3200 m) prior to each experiment. [Hb] (g dl<sup>-1</sup>), Hct (%), arterial bicarbonate ion concentration ( $[HCO_3^-]_a$ ; mmol l<sup>-1</sup>),  $Pa_{O_2}$  (kPa), arterial partial pressure of carbon dioxide ( $Pa_{CO_2}$ ; kPa), and arterial pH ( $pH_a$ ) were analyzed from arterial blood at 41°C using CG8+

cartridges with the i-STAT VetScan Analyzer (Abaxis, Union City, CA, USA). All i-STAT values were corrected according to Harter et al. (2015).  $[\text{HCO}_3^-]_a$  was calculated using the Henderson–Hasselbach equation, assuming a  $\text{pK}$  of 6.090 and a  $\text{CO}_2$  solubility coefficient of  $0.2117 \text{ mmol l}^{-1} \text{ kPa}^{-1}$  in plasma (Helbecka et al., 1964; Scott and Milsom, 2007).

### Data and statistical analysis

With the exception of the blood variables, all data were acquired and analyzed using the PowerLab data acquisition and analysis software (ADInstruments, Colorado Springs, CO, USA) at a sampling frequency of 1000 Hz per channel. Mean values were derived for each variable for a 1–2 min period before each blood sample (e.g. after 12–15 min of each hypoxic  $F_{\text{I}\text{O}_2}$  exposure, and after 3–5 min and 22–25 min of the normoxic recovery).  $\text{CaO}_2$  and  $\text{CvO}_2$  were acquired using software designed for the FireSting  $\text{O}_2$  probes.

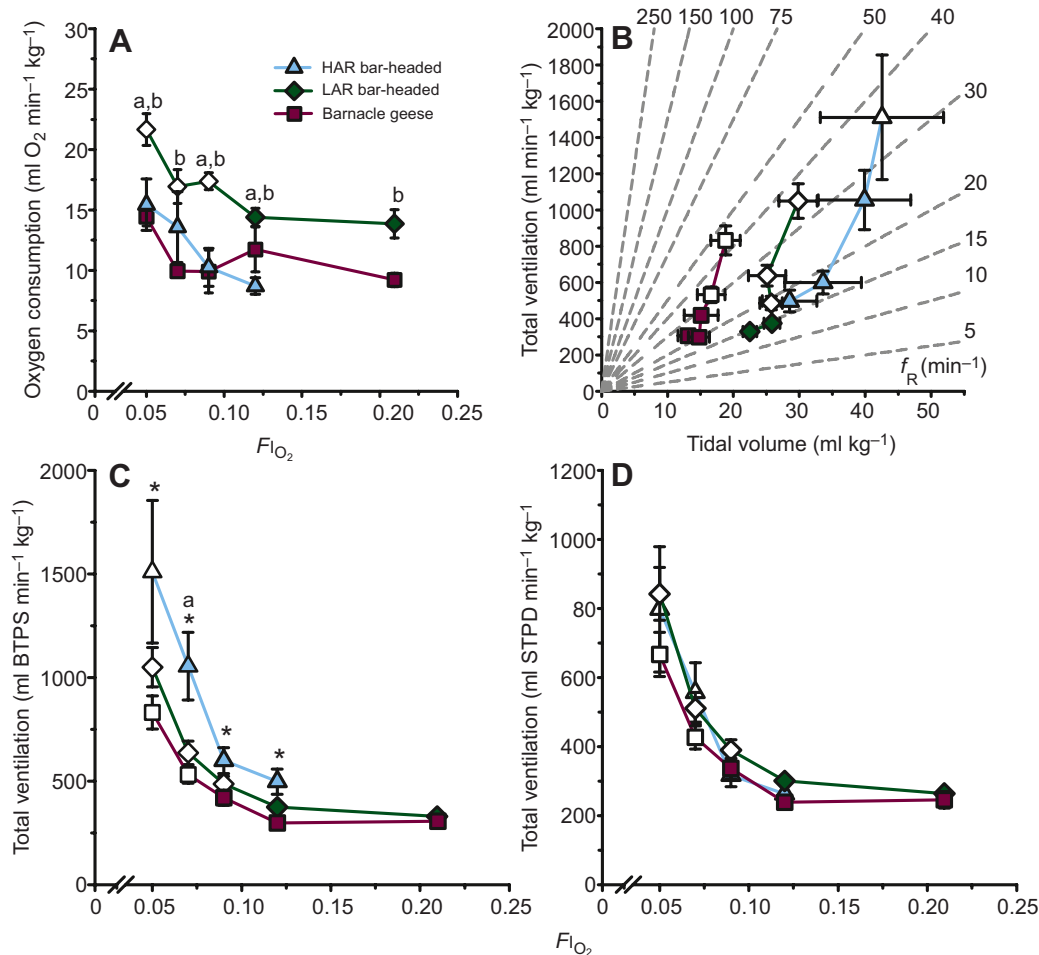
The following variables were either measured directly or used to calculate additional variables. Tidal volume ( $\text{ml kg}^{-1}$ ) and breathing frequency ( $\text{min}^{-1}$ ) were derived from the integrated differential pressure signal and  $\dot{V}_R$  ( $\text{ml min}^{-1} \text{ kg}^{-1}$ ) was calculated as their

product.  $\dot{V}_{\text{O}_2}$  ( $\text{ml min}^{-1} \text{ kg}^{-1}$ ) was calculated as:

$$\dot{V}_{\text{O}_2} = \frac{\dot{V}_C \times (F_{\text{I}\text{O}_2} - F_{\text{E}\text{CO}_2})}{M_b}, \quad (1)$$

where  $M_b$  is body mass (kg). Water vapour was removed prior to gas analysis (Withers, 1977) and the respiratory quotient (RQ) was assumed to be 1.0. It has been shown that RQ does not change with acute hypoxic exposure in bar-headed geese (Hawkes et al., 2014) and is unaffected by prolonged hypoxic exposure in deer mice (Cheviron et al., 2012; McClelland et al., 1998). Thus, while RQ in birds can range from  $<0.7$  to 1.0 and our calculations ignore this variation, they use the same value throughout, thus allowing for comparison without affecting our overall conclusions (Scott and Milsom, 2007). Air convection requirement was calculated as the quotient of  $\dot{V}_R$  and  $\dot{V}_{\text{O}_2}$ . Lung  $\text{O}_2$  extraction (%) was calculated as:

$$\text{Lung O}_2 \text{ extraction} = \left( \frac{F_{\text{I}\text{O}_2} - F_{\text{E}\text{O}_2}}{F_{\text{I}\text{O}_2}} \right) \times 100, \quad (2)$$



**Fig. 1. Oxygen consumption is maintained throughout hypoxia exposure by increases in total ventilation.** (A) Oxygen consumption (STPD) plotted as a function of the fractional oxygen composition of inspired gas ( $F_{\text{I}\text{O}_2}$ ) in all groups. (B) Hey plot depicting the breathing patterns of all groups. For any level of total ventilation, bar-headed geese had a higher tidal volume and lower breathing frequency (right-shifted curve). Dashed lines represent breathing frequency ( $f_R$ ) isopleths. (C) Total ventilation as a function of the fractional oxygen composition of inspired gas ( $F_{\text{I}\text{O}_2}$ ) in all groups in BTPS. (D) Total ventilation as a function of the fractional oxygen composition of inspired gas ( $F_{\text{I}\text{O}_2}$ ) in all groups in STPD. Values represent means  $\pm$  s.e.m. (blue: high-altitude-reared bar-headed geese,  $N=5$ ; green: low-altitude-reared bar-headed geese,  $N=5$ ; purple: barnacle geese,  $N=7$ ). Significant differences ( $P<0.05$ ) in the y-axis variable from values during exposure to ambient air within a species are indicated by open symbols and determined by one-way repeated measures ANOVA. Significant differences ( $P<0.05$ ) in the y-axis variable (A, C and D) between species are determined by two-way repeated measures ANOVA and indicated by different symbols: \*HAR bar-headed geese versus barnacle geese; <sup>a</sup>HAR bar-headed geese versus LAR bar-headed geese; and <sup>b</sup>LAR bar-headed geese versus barnacle geese.

where the calculated fractional expired level of  $O_2$  of the bird ( $F_{E_{O_2}}$ ) was calculated as:

$$F_{E_{O_2}} = \frac{(\dot{V}_R \times F_{I_{O_2}}) - \dot{V}_{O_2}}{\dot{V}_R} \quad (3)$$

$\dot{V}_{O_2}$ , air convection requirement and lung  $O_2$  extraction were reported in terms of standard temperature and pressure and dry (STPD). Tidal volume was reported in terms of body temperature and pressure saturated (BTSP), assuming a constant body temperature of  $41^\circ\text{C}$  and taking into account changes in barometric pressure and air density at altitude (Dejours, 1975).  $\dot{V}_R$  was reported in both BTSP and STPD for comparison.

Regarding cardiovascular measurements, heart rate ( $\text{min}^{-1}$ ) was calculated from the peaks in the pulsatile arterial blood pressure trace. Mean arterial pressure (kPa) was calculated as the sum of diastolic pressure plus 1/3 pulse pressure.  $\dot{Q}$  ( $\text{ml min}^{-1} \text{kg}^{-1}$ ) was calculated from the Fick equation given known values of  $\dot{V}_{O_2}$  and  $Ca_{O_2} - Cv_{O_2}$ . Stroke volume ( $\text{ml kg}^{-1}$ ) was calculated as the quotient of  $\dot{Q}$  and heart rate. Total peripheral resistance ( $\text{kPa min kg ml}^{-1}$ ) was calculated as the quotient of mean arterial pressure and  $\dot{Q}$  (Bech

and Nomoto, 1982). Blood convection requirement was calculated as the quotient of  $\dot{Q}$  and  $\dot{V}_{O_2}$ . Tissue  $O_2$  delivery was calculated as the product of  $\dot{Q}$  and  $Ca_{O_2}$  ( $\text{ml}^{-1} \text{min}^{-1} \text{kg}^{-1}$ ). Tissue  $O_2$  extraction (%) was calculated as:

$$\text{Tissue } O_2 \text{ extraction} = \frac{(Ca_{O_2} - Cv_{O_2})}{Ca_{O_2}} \times 100. \quad (4)$$

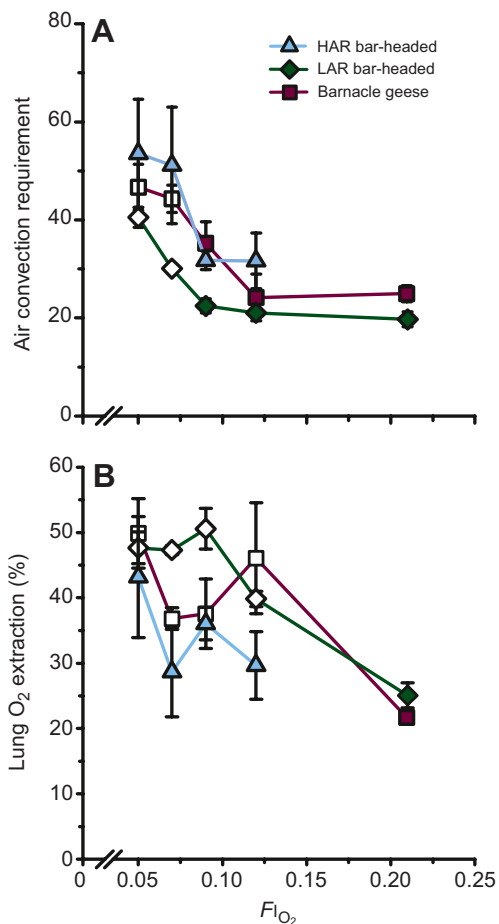
We corrected the Hct and [Hb] data collected by the i-STAT VetScan Analyzer for all groups based on the calibrations derived for bar-headed geese in Harter et al. (2015).

Data are presented as means  $\pm$  s.e.m. unless stated otherwise. Within each species, all data were analyzed using one-way repeated measures analysis of variance (ANOVA) and Holm–Šidák *post hoc* tests. Comparisons between each species were made using two-way (species and  $F_{I_{O_2}}$ ) repeated measures ANOVA and Holm–Šidák *post hoc* tests within each  $F_{I_{O_2}}$ . For statistical comparisons,  $P < 0.05$  was used to determine statistical significance. Variables analyzed with a one-way repeated measures ANOVA that did not meet assumptions for either normality or equal variance in barnacle geese were transformed with  $x' = \ln(x)$  for  $\dot{Q}$ , lung  $O_2$  extraction, and blood convection requirement and with  $x' = x^2$  for tidal volume. Similarly, variables were transformed when they did not meet assumptions for either normality or equal variance analyzed for a two-way repeated measures ANOVA (i.e.  $x' = \ln(x)$  and  $x' = 1/(1-x)$  for  $\dot{V}_R$ , tidal volume and  $Pa_{O_2}$ ). Student's *t*-tests were used to compare Hct and [Hb] prior to and following the experiment to ensure that no blood dilution had been incurred throughout the experiment. Statistical analyses were carried out using SigmaStat (version 3.0; Systat Software).

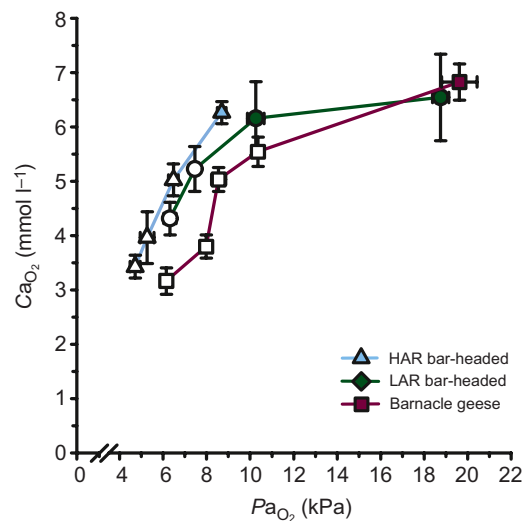
## RESULTS

### Metabolic response

Both bar-headed and barnacle geese maintained  $\dot{V}_{O_2}$  during progressive hypoxia (Fig. 1A), with  $\dot{V}_{O_2}$  increasing significantly during hypoxic exposure in LAR bar-headed geese ( $P < 0.001$ ),



**Fig. 2. Air convection requirement and lung oxygen extraction during progressive hypoxia.** (A) Air convection requirement plotted as a function of the fractional oxygen composition of inspired gas ( $F_{I_{O_2}}$ ) in all groups. (B) Changes in lung oxygen extraction in all groups exposed to progressive hypoxia. Values are reported in STPD and represent means  $\pm$  s.e.m. (blue: HAR bar-headed geese,  $N=5$ ; green: LAR bar-headed geese,  $N=5$ ; purple: barnacle geese,  $N=7$ ). Significant differences ( $P < 0.05$ ) in values from those during ambient exposure within a species are indicated by open symbols and determined by one-way repeated measured ANOVA.



**Fig. 3. Arterial oxygen content at a given level of arterial partial pressure differs between bar-headed geese and barnacle geese.** The relationship between arterial oxygen content ( $Ca_{O_2}$ ) and arterial partial pressure of oxygen ( $Pa_{O_2}$ ) was such that at any given  $Pa_{O_2}$  bar-headed geese carried a greater content of  $O_2$  in their arterial blood. Values are means  $\pm$  s.e.m. (blue: HAR bar-headed geese,  $N=5$ ; green: LAR bar-headed geese,  $N=6$ ; purple: barnacle geese,  $N=7$ ). Significant differences ( $P < 0.05$ ) in  $Ca_{O_2}$  from values obtained at ambient exposures within a species are indicated by open symbols and determined by one-way repeated measured ANOVA.

Table 1. Changes in cardiovascular variables during hypoxic exposure in bar-headed geese and barnacle geese

$F_{I_{O_2}}$	$P_{a_{O_2}}$ (kPa)	$P_{V_{O_2}}$ (kPa)	$Ca_{O_2}$ (mmol l <sup>-1</sup> )	$Cv_{O_2}$ (mmol l <sup>-1</sup> )	$pH_a$	$pH_v$	MAP (kPa)	TPR (kPa min kg ml <sup>-1</sup> )	Blood convection requirement	Tissue O <sub>2</sub> delivery (ml min <sup>-1</sup> kg <sup>-1</sup> )	Tissue O <sub>2</sub> extraction (%)
Bar-headed geese (0 m)											
(0.21)	18.8±0.4 <sup>a</sup>	7.6±0.8 <sup>a</sup>	6.5±0.8 <sup>a</sup>	4.0±0.4 <sup>a</sup>	7.57±0.02 <sup>a</sup>	7.54±0.03 <sup>a</sup>	26.4±0.8 <sup>a</sup>	0.08±0.01 <sup>a</sup>	24.4±6.8 <sup>a</sup>	52.8±11 <sup>a</sup>	36.8±6.8 <sup>a</sup>
0.12	10.3±0.4 <sup>b</sup>	—	6.2±0.7 <sup>a</sup>	2.9±0.6 <sup>a</sup>	7.66±0.02 <sup>b</sup>	—	25.1±0.9 <sup>a</sup>	0.11±0.04 <sup>a</sup>	16.2±3.8 <sup>a</sup>	33.0±9.2 <sup>a</sup>	53.3±8.2 <sup>a</sup>
0.09	7.5±0.1 <sup>c</sup>	—	5.2±0.4 <sup>b</sup>	3.1±0.7 <sup>a</sup>	7.72±0.03 <sup>b</sup>	—	24.5±0.8 <sup>b</sup>	0.11±0.02 <sup>a</sup>	22.0±5.1 <sup>a</sup>	28.4±6.5 <sup>a</sup>	44.2±10 <sup>a</sup>
0.07	6.3±0.3 <sup>d</sup>	4.7±0.3 <sup>b</sup>	4.3±0.3 <sup>b</sup>	2.9±0.4 <sup>a</sup>	7.76±0.03 <sup>b</sup>	7.69±0.03 <sup>b</sup>	23.4±0.8 <sup>b</sup>	0.05±0.00 <sup>a</sup>	32.0±3.6 <sup>a</sup>	49.3±0.6 <sup>a</sup>	34.1±4.5 <sup>a</sup>
Bar-headed geese (3200 m)											
0.12	8.7±0.2 <sup>a</sup>	—	6.3±0.2 <sup>a</sup>	3.7±0.4 <sup>a</sup>	7.72±0.01 <sup>a</sup>	—	20.8±1.2 <sup>a</sup>	0.09±0.01 <sup>a</sup>	20.7±3.5 <sup>a</sup>	24.8±5.8 <sup>a</sup>	38.7±7.0 <sup>a</sup>
0.09	6.5±0.2 <sup>b</sup>	—	5.0±0.3 <sup>b</sup>	3.9±0.2 <sup>a</sup>	7.78±0.02 <sup>a</sup>	—	21.2±0.8 <sup>a</sup>	0.06±0.01 <sup>a</sup>	41.2±5.7 <sup>a</sup>	44.1±3.4 <sup>a</sup>	23.1±2.5 <sup>a</sup>
0.07	5.3±0.3 <sup>b</sup>	—	4.0±0.5 <sup>c</sup>	2.7±0.6 <sup>b</sup>	7.79±0.04 <sup>a</sup>	—	19.5±0.6 <sup>a</sup>	0.04±0.01 <sup>a</sup>	35.4±6.7 <sup>a</sup>	50.6±8.6 <sup>a</sup>	32.3±7.7 <sup>a</sup>
0.05	4.7±0.2 <sup>b</sup>	3.4±0.4 <sup>a</sup>	3.4±0.2 <sup>c</sup>	1.8±0.4 <sup>b</sup>	7.72±0.06 <sup>a</sup>	7.65±0.06 <sup>a</sup>	19.5±0.9 <sup>a</sup>	0.05±0.01 <sup>a</sup>	29.3±4.4 <sup>a</sup>	33.1±4.5 <sup>a</sup>	48.4±7.9 <sup>a</sup>
Barnacle geese (0 m)											
(0.21)	19.6±0.8 <sup>a</sup>	—	6.8±0.3 <sup>a</sup>	5.0±0.4 <sup>a</sup>	7.62±0.01 <sup>a</sup>	—	21.3±0.9 <sup>a</sup>	0.10±0.01 <sup>a</sup>	25.8±3.0 <sup>a</sup>	36.4±5.3 <sup>a</sup>	27.5±3.4 <sup>a</sup>
0.12	10.4±0.2 <sup>b</sup>	—	5.5±0.3 <sup>b</sup>	3.5±0.2 <sup>b</sup>	7.69±0.02 <sup>a</sup>	—	19.0±0.9 <sup>ab</sup>	0.08±0.02 <sup>a</sup>	23.6±3.2 <sup>a</sup>	35.9±7.0 <sup>a</sup>	36.9±3.9 <sup>a</sup>
0.09	8.6±0.3 <sup>c</sup>	—	5.0±0.2 <sup>b</sup>	3.1±0.2 <sup>bc</sup>	7.77±0.03 <sup>b</sup>	—	17.7±1.1 <sup>ab</sup>	0.01±0.03 <sup>a</sup>	25.7±3.4 <sup>a</sup>	31.5±9.4 <sup>a</sup>	37.5±5.3 <sup>a</sup>
0.07	8.0±0.3 <sup>c</sup>	—	3.8±0.2 <sup>c</sup>	3.5±0.4 <sup>bc</sup>	7.71±0.05 <sup>c</sup>	—	17.4±1.4 <sup>b</sup>	0.05±0.01 <sup>a</sup>	36.1±7.7 <sup>a</sup>	32.9±7.5 <sup>a</sup>	37.8±10 <sup>a</sup>
0.05	6.2±0.2 <sup>d</sup>	—	3.2±0.2 <sup>c</sup>	1.9±0.1 <sup>c</sup>	7.8±0.04 <sup>d</sup>	—	19.8±1.9 <sup>ab</sup>	0.04±0.01 <sup>a</sup>	38.0±4.5 <sup>a</sup>	37.9±3.6 <sup>a</sup>	38.9±2.2 <sup>a</sup>

Changes in blood gases and cardiovascular variables with decreasing fractional inspired O<sub>2</sub> ( $F_{I_{O_2}}$ );  $P_{a_{O_2}}$ , arterial partial pressure of oxygen (kPa);  $P_{V_{O_2}}$ , venous partial pressure of oxygen (kPa);  $Ca_{O_2}$ , arterial content of oxygen (mmol l<sup>-1</sup>);  $Cv_{O_2}$ , venous content of oxygen (mmol l<sup>-1</sup>);  $pH_a$ , arterial pH;  $pH_v$ , venous pH; MAP, mean arterial pressure (kPa); TPR, total peripheral resistance (kPa); blood convection requirement; tissue O<sub>2</sub> delivery (ml min<sup>-1</sup> kg<sup>-1</sup>); and tissue O<sub>2</sub> extraction (%). Venous values were provided where available. <sup>a,b,c,d</sup>Significant differences ( $P<0.05$ ) of a variable within species from values at ambient  $F_{I_{O_2}}$  are indicated by different letters and determined by one-way repeated measured ANOVA.

almost significantly in HAR bar-headed geese ( $P=0.051$ ) and remaining unchanged in barnacle geese ( $P=0.72$ ). The  $\dot{V}_{O_2}$  of the HAR bar-headed geese was significantly lower than that of the LAR bar-headed geese at every level except 0.07  $F_{I_{O_2}}$  ( $P=0.004$ ).

### Hypoxic ventilatory response

The hypoxic ventilatory responses of each study group and the differences present in the relative contributions of breathing frequency and tidal volume to  $\dot{V}_R$  are depicted in a Hey plot

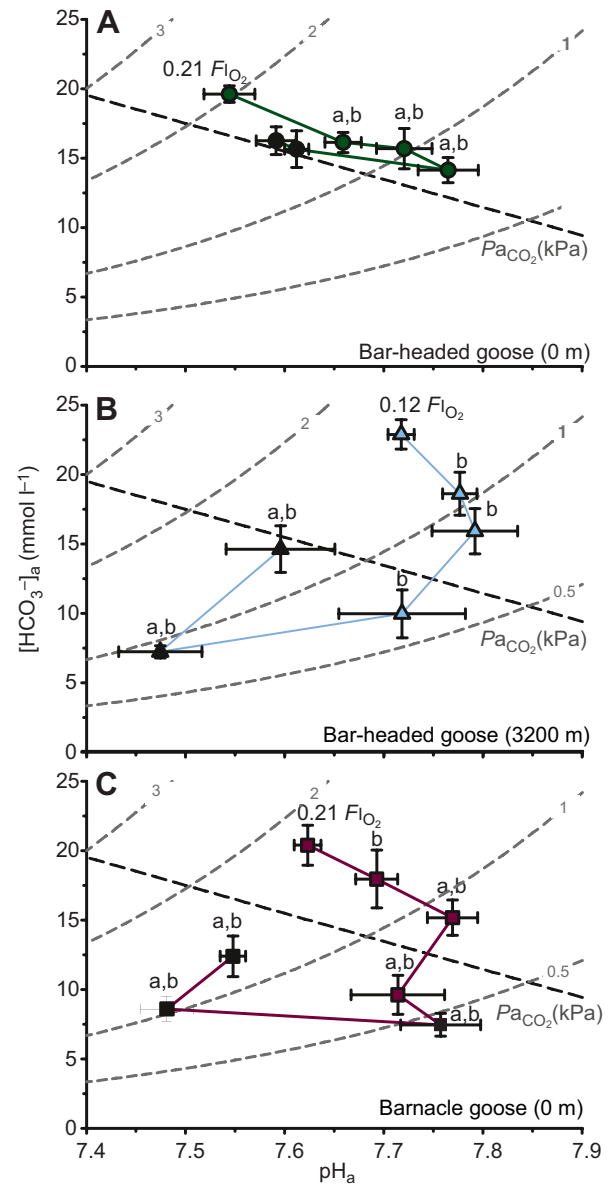


Fig. 4. Arterial acid-base status is altered during hypoxia exposure due to respiratory alkalosis and metabolic acidosis. These plots represent changes in arterial acid-base status (arterial pH,  $pH_a$ ; arterial bicarbonate ion concentration,  $[HCO_3^-]_a$ ) throughout the experimental exposures in (A) LAR bar-headed geese ( $N=6$ ), (B) HAR bar-headed geese ( $N=5$ ) and (C) barnacle geese ( $N=7$ ). The first shaded point represents the starting ambient exposure and is marked with the corresponding  $F_{I_{O_2}}$  (0.21 for low-altitude groups and 0.12 for the high-altitude group). Recovery in ambient  $F_{I_{O_2}}$  after 5 and 25 min is represented by the black symbols. The dotted isopleths represent arterial partial pressure of CO<sub>2</sub> ( $P_{a_{CO_2}}$ ; kPa). Significant differences ( $P<0.05$ ) in values from starting levels within a species are indicated by a for  $pH_a$  and b for  $[HCO_3^-]_a$  and determined by one-way repeated measured ANOVA.

(Fig. 1B), a graphical depiction of breathing patterns (tidal volume and breathing frequency) at different levels of  $\dot{V}_R$  (Guz and Widdicombe, 1970). At a given level of  $\dot{V}_R$ , bar-headed geese breathed at a slower rate with significantly larger tidal volumes (Fig. 1B, Fig. S1A,B) than the barnacle geese, and this difference in pattern was sustained in hypoxia. The increase in  $\dot{V}_R$  was greatest in the HAR bar-headed geese and was lowest for the barnacle geese (Fig. 1C).  $\dot{V}_R$  of HAR bar-headed geese was higher than that of barnacle geese during every exposure ( $P < 0.001$ ). LAR bar-headed geese trended towards having a larger  $\dot{V}_R$  than barnacle geese at 0.05  $F_{I_{O_2}}$ , but this did not reach statistical significance ( $P = 0.057$ ). The air convection requirement – the ratio of  $\dot{V}_R$  to  $\dot{V}_{O_2}$  – increased in hypoxia in all groups, significantly so in both LAR groups (Fig. 2A). Lung  $O_2$  extraction, which is the percentage of the inspired  $O_2$  extracted from inspired gas, increased initially in both LAR groups between 0.21 and 0.12  $F_{I_{O_2}}$  (Fig. 2B), and then remained constant at 30–50% beyond 0.12  $F_{I_{O_2}}$ . All differences in both the resting levels of  $\dot{V}_R$  between HAR and LAR bar-headed geese disappeared when our data were expressed as STPD rather than BTPS (Fig. 1D).

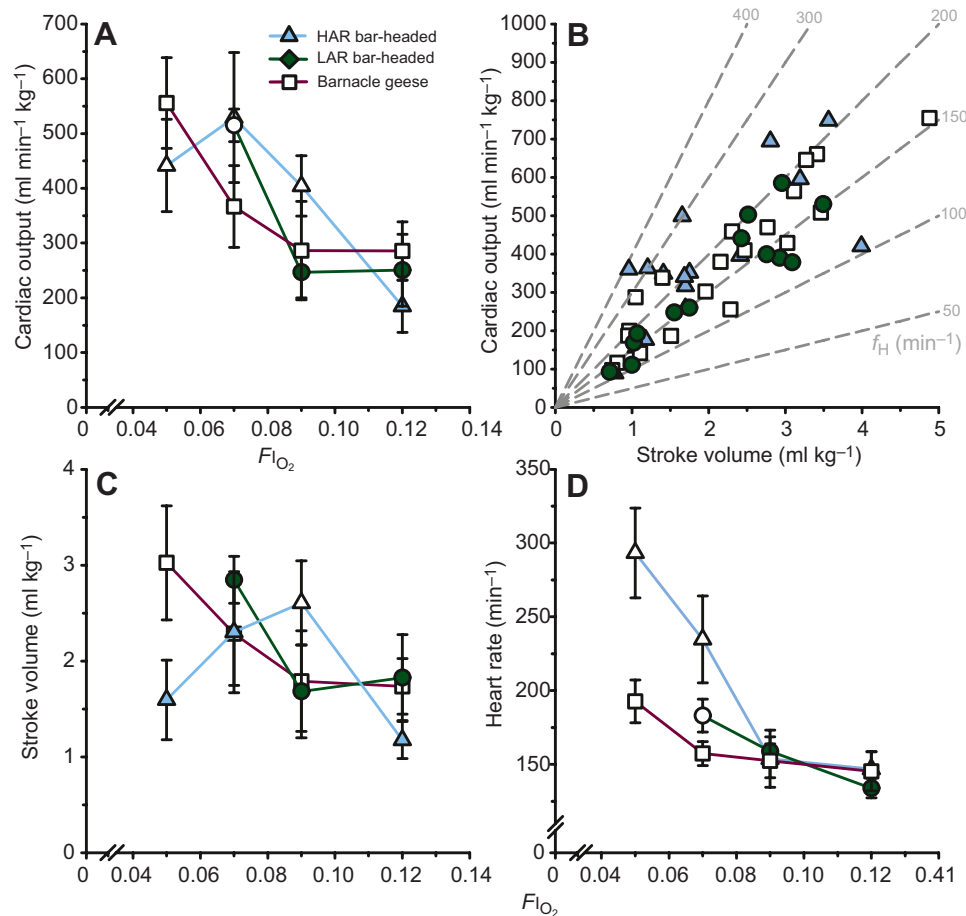
### Hypoxic cardiovascular response

#### Blood- $O_2$ carrying capacity and acid-base status

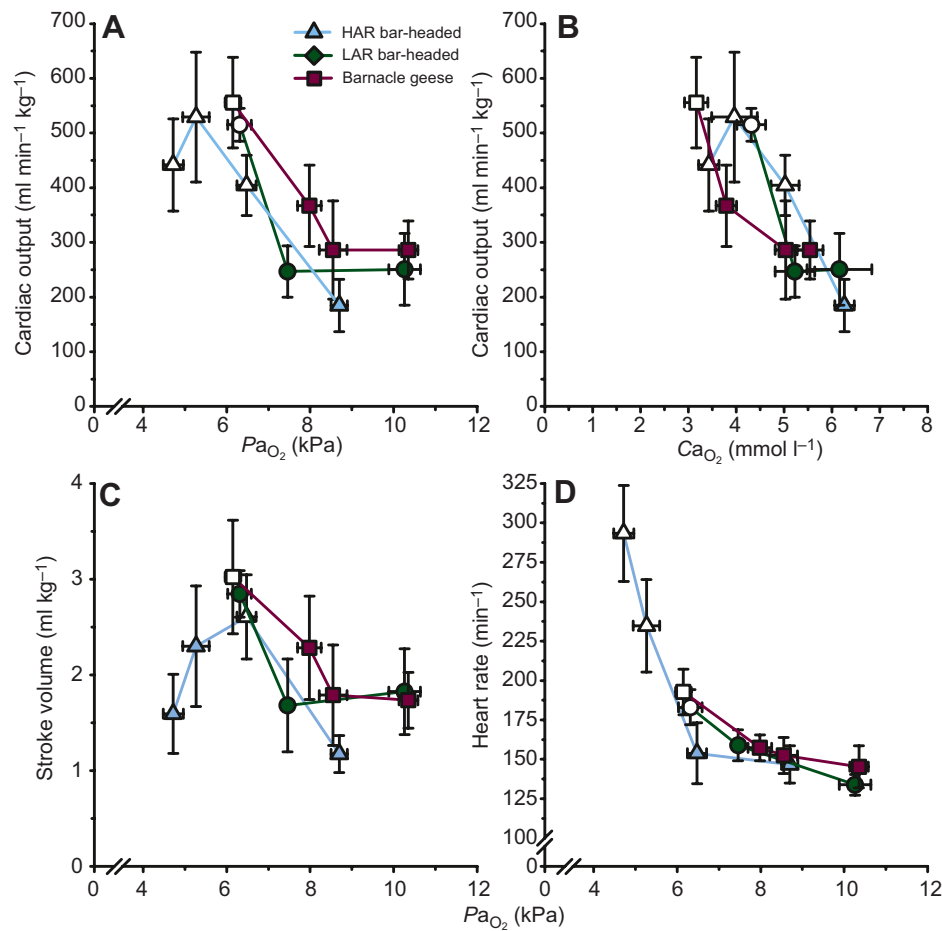
Hct and [Hb] were not significantly different among HAR bar-headed geese (Hct:  $38.8 \pm 2.8\%$ , [Hb]:  $117.3 \pm 7.0 \text{ g l}^{-1}$ ), LAR bar-headed geese (Hct:  $43.9 \pm 4.3\%$ , [Hb]:  $125.1 \pm 7.5 \text{ g l}^{-1}$ ), or LAR barnacle geese (Hct:  $43.1 \pm 1.9\%$ , [Hb]:  $112.6 \pm 4.0 \text{ g l}^{-1}$ ). In all three groups of geese, Hct and [Hb] were unchanged during progressive hypobaric hypoxia.

$P_{a_{O_2}}$  decreased with progressive decreases in  $F_{I_{O_2}}$ , and was lower ( $P < 0.001$ ) in the bar-headed geese than in barnacle geese at or below an  $F_{I_{O_2}}$  of 0.07 (Fig. S2A).  $Ca_{O_2}$  was similar between the groups of geese and decreased with hypoxia (Fig. S2B). Plotting  $Ca_{O_2}$  as a function of  $P_{a_{O_2}}$  generated *in vivo*  $O_2$  equilibrium curves (Fig. 3) that are representative of the arterial saturation given the prevailing acid-base conditions that accompanied hypobaric hypoxia (see Table 1). Differences in these  $O_2$  equilibrium curves reflect the higher  $O_2$  affinity of the bar-headed goose blood (Black and Tenney, 1980; Weber et al., 1993) compared with that of barnacle geese and differences in the  $pH_a$  at each  $F_{I_{O_2}}$  (Fig. 4).

The starting  $pH_a$  of the HAR bar-headed geese was higher than that of the LAR bar-headed geese ( $P < 0.001$ ) and was accompanied by higher starting  $[HCO_3^-]$  ( $P = 0.009$ ) (Fig. 4A,B).  $P_{a_{CO_2}}$  decreased ( $P < 0.001$ ) and  $pH_a$  increased ( $P < 0.001$ ) in all three groups of geese during progressive hypoxia (Fig. 4, Table 1). Both bar-headed goose study groups experienced a respiratory alkalosis during hypoxic exposure down to 0.07  $F_{I_{O_2}}$  (Fig. 4A,B). At that point, LAR bar-headed geese were recovered to normoxia (Fig. 4A), whereas HAR bar-headed geese were further exposed to 0.05  $F_{I_{O_2}}$  (Fig. 4B). Between 0.07 and 0.05  $F_{I_{O_2}}$  (Fig. 4B), the  $pH_a$  of HAR bar-headed geese remained unchanged, but  $[HCO_3^-]_a$  decreased significantly (Fig. 4B), indicative of a metabolic acidosis. This also occurred in the barnacle geese, but the metabolic acidosis was triggered at a less extreme level of hypoxia (0.07  $F_{I_{O_2}}$ ) (Fig. 4C). Intriguingly, only during ambient recovery did  $pH_a$  fall significantly in any group. LAR bar-headed geese recovered their  $pH_a$  within 5 min of normoxia after being exposed to 0.07  $F_{I_{O_2}}$ . The HAR bar-headed geese and barnacle



**Fig. 5. Changes in cardiac output and its contributing components as a function of  $F_{I_{O_2}}$ .** (A) Cardiac output plotted as a function of fractional oxygen composition of inspired gas ( $F_{I_{O_2}}$ ). (B) The hypoxic cardiovascular response of all groups is characterized generally by large increases in stroke volume with modest increases in heart rate. All points represent individual data. Dashed lines represent heart rate ( $f_H$ ) isopleths. (C) Stroke volume and (D) heart rate plotted as a function of  $F_{I_{O_2}}$ . Values are means  $\pm$  s.e.m. (blue: HAR bar-headed geese,  $N=5$ ; green: LAR bar-headed geese,  $N=6$ ; purple: barnacle geese,  $N=7$ ). Significant differences ( $P < 0.05$ ) from values obtained with ambient exposure within a species are indicated by open symbols and determined by one-way repeated measured ANOVA.



**Fig. 6. Changes in cardiac output and its contributing components as a function of  $P_{aO_2}$  or  $Ca_{O_2}$ .** (A) Cardiac output plotted as a function of arterial partial pressure of oxygen ( $P_{aO_2}$ ). (B) Cardiac output plotted as a function of arterial oxygen content ( $Ca_{O_2}$ ). (C) Stroke volume and (D) heart rate plotted as a function of  $P_{aO_2}$ . All variables increased initially with decreasing  $P_{aO_2}$  at a  $Ca_{O_2} \sim 6$  kPa. Values are means  $\pm$  s.e.m. (blue: HAR bar-headed geese,  $N=5$ ; green: LAR bar-headed geese,  $N=6$ ; purple: barnacle geese,  $N=7$ ). Significant differences ( $P < 0.05$ ) from values obtained with ambient exposure within a species are indicated by open symbols and determined by one-way repeated measured ANOVA.

geese that were exposed to  $0.05 F_{I_{O_2}}$  both had a persistent acidosis and low  $[HCO_3^-]_a$  even after 25 min of recovery (Fig. 4B,C).

### Cardiac output

All three groups of geese increased  $\dot{Q}$  by 2- to 3-fold, yielding similar maximum values during progressive hypoxia (Fig. 5A). The increase in  $\dot{Q}$  became significant at a different  $F_{I_{O_2}}$  between the groups: 0.09 in HAR bar-headed geese, 0.07 in LAR bar-headed geese and 0.05 in barnacle geese (Fig. 5A). The relative contributions of increases in heart rate and stroke volume to  $\dot{Q}$  are depicted in a cardiac equivalent of a Hey plot for individuals at all exposures (Fig. 5B). Increases in stroke volume accounted for most of the increase in  $\dot{Q}$  in barnacle geese and LAR bar-headed geese (Fig. 5B,C) - their heart rates increased only modestly (Fig. 5D) whereas stroke volume roughly doubled (Fig. 5C). This was also the case initially in the HAR bar-headed geese; however, at  $0.07 F_{I_{O_2}}$  heart rate increased substantially, with an associated decrease in stroke volume. The net overall result, however, was a trend for  $\dot{Q}$  to increase earlier and more rapidly in bar-headed geese than barnacle geese, and more so in the HAR bar-headed geese than in the LAR bar-headed geese (Fig. 5A).

While the increase in  $\dot{Q}$  of HAR bar-headed geese was triggered at a higher  $F_{I_{O_2}}$ , it is evident from Fig. 6A, which plots the changes in cardiac variables as a function of  $P_{aO_2}$ , that this response was associated with this group of geese having a lower  $P_{aO_2}$  at any given  $F_{I_{O_2}}$  during hypoxia (Fig. S2A). Significant increases in  $\dot{Q}$  occurred at the same  $P_{aO_2}$  in all three groups of geese ( $\sim 6$  kPa). That this reflects the differences in the Hb- $O_2$

equilibrium curves is clear from the extent of the overlap when  $\dot{Q}$  is plotted as a function of  $Ca_{O_2}$  (Fig. 6B). Similarly, significant changes in the contributions of stroke volume and heart rate to  $\dot{Q}$  occurred at  $\leq 6$  kPa (Fig. 6C,D).

### Tissue $O_2$ delivery and extraction

Neither blood convection requirement (the quotient of  $\dot{Q}$  and  $\dot{V}_{O_2}$ ) nor tissue  $O_2$  delivery (the product of  $\dot{Q}$  and  $Ca_{O_2}$ ) changed significantly in any of the three groups of geese during progressive hypoxia (Table 1). The percentage of the  $O_2$  extracted from arterial blood fluctuated between 30 and 50% and also did not change significantly either during progressive hypoxia or between any of the three groups of geese (Table 1).

### Blood pressure and total peripheral resistance

While  $\dot{Q}$  increased during hypoxic exposure, mean arterial pressure was generally maintained, decreasing minimally at  $0.07 F_{I_{O_2}}$  in the two LAR groups of geese (Table 1).

### DISCUSSION

In this study we comprehensively compared the metabolic, ventilatory and cardiovascular responses of HAR bar-headed geese with those of LAR bar-headed geese. In addition, we compared these responses to those of barnacle geese, a member of a closely related genus, to provide further insight into responses unique to bar-headed geese. We found that HAR bar-headed geese exhibited a reduced  $\dot{V}_{O_2}$  compared with LAR bar-headed geese. When exposed to progressive hypoxia, HAR bar-headed geese

exhibited a modestly increased HVR and initiated cardiac responses earlier than LAR bar-headed geese, supporting our initial hypothesis. However, the magnitude of these differences was not as large as those described for bar-headed geese during short-term acclimation to simulated high altitude (Black and Tenney, 1980). Explanations for these differences are discussed below.

While it cannot be determined with absolute certainty the extent to which the differences present between the HAR and LAR bar-headed geese can be attributed exclusively to differences in barometric pressure during rearing, many potentially confounding variables were controlled across study groups. All groups of birds were held in outdoor pens with access to indoor shelter during the winter. All groups were healthy, fed similar diets and had been housed for at least a year without flying. While body mass and body composition have been shown to vary seasonally in barnacle geese (Portugal et al., 2007), the relationship between heart rate and  $\dot{V}_{O_2}$ , when normalized for body mass, was unaffected in five of the six seasonal sampling periods and was also unaltered by molt (Portugal et al., 2009). Furthermore, many of the variables measured in this study have been previously measured on LAR bar-headed geese and barnacle geese, allowing us to compare results. Resting values for  $\dot{V}_{O_2}$  and  $\dot{V}_R$  in our LAR bar-headed geese in normoxia were comparable to those previously described in the literature (Black and Tenney, 1980; Fedde et al., 1989; Hawkes et al., 2014; Scott and Milsom, 2007). Our values for  $Ca_{O_2}$ ,  $Cv_{O_2}$ , Hct and [Hb] also fell within the range of values previously reported in the literature (Black and Tenney, 1980; Fedde et al., 1989; Hawkes et al., 2014; Scott and Milsom, 2007). Literature values for  $\dot{Q}$ , stroke volume and heart rate of LAR bar-headed geese in normoxia vary widely. Fedde et al. (1989) reported a high heart rate and low stroke volume, while Hawkes et al. (2014) reported a low heart rate and high stroke volume. Our values fall midway between the two.

$\dot{V}_{O_2}$  was significantly lower in the HAR bar-headed geese compared with LAR bar-headed geese, suggesting that rearing at altitude leads to a reduction in metabolism and in the demand for  $O_2$ . All groups maintained or increased  $\dot{V}_{O_2}$  when exposed to hypoxia (Fig. 1A). The small increases seen in  $\dot{V}_{O_2}$  in all groups may reflect an increased cost of ventilation and associated events. The net response suggests that the cardiorespiratory adjustments were sufficient to match  $O_2$  supply to  $O_2$  demand at all but the most severe levels of hypoxia.

There was evidence of a metabolic acidosis, indicative of recruitment of anaerobic metabolism, in barnacle geese at 0.07  $F_{I_{O_2}}$  and in HAR bar-headed geese at 0.05  $F_{I_{O_2}}$ . On return to control conditions, the HAR bar-headed geese recovered to control acid-base status faster (within 25 min) than the barnacle geese. Both groups exposed to 0.05  $F_{I_{O_2}}$  experienced a significant decrease in  $pH_a$  upon recovery to normoxia (Fig. 4B,C). This may reflect the sequestering of lactate and  $H^+$  during hypoxia that was rapidly released into the blood upon return to resting conditions. Because LAR bar-headed geese were recovered to ambient conditions after breathing 0.07  $F_{I_{O_2}}$  rather than 0.05  $F_{I_{O_2}}$ , we cannot ascertain whether the ability of bar-headed geese to avoid metabolic acidosis until more severe levels of hypobaric hypoxia is an adaptation or a consequence of high-altitude rearing. Nevertheless, the ability to recover quickly from severe hypoxia would be an asset during the high-altitude migration of bar-headed geese.

The magnitude by which  $\dot{V}_R$  increased during progressive hypoxia in the LAR bar-headed geese and barnacle geese of our study was within the range reported in previous studies (Black and Tenney, 1980; Fedde et al., 1989; Hawkes et al., 2014; Scott and Milsom,

2007). In addition, as previously reported, bar-headed geese exhibited a higher overall tidal volume and lower breathing frequency at any given  $\dot{V}_R$  than barnacle geese – a pattern hypothesized to be a more effective breathing pattern that reduces effective dead space ventilation (Scott and Milsom, 2007). The demonstration that this breathing pattern was common to LAR and HAR bar-headed geese supports the suggestion that this is an adaptation specific to bar-headed geese. Furthermore, we found that the increase in  $\dot{V}_R$  in hypoxia was greatest in magnitude in the HAR bar-headed geese and lowest in the barnacle geese ( $P < 0.001$ ; Fig. 1C). These findings support our hypothesis and are consistent with previous findings of a greater increase in  $\dot{V}_R$  in bar-headed geese following short-term acclimation to simulated altitude (Black and Tenney, 1980).

Differences in both the resting levels of ventilation and the HVR disappeared when our data were expressed as STPD rather than BTPS (Fig. 1D, Fig. S3B). Expressing volume as a function of STPD reveals the molar amount of air (and thus  $O_2$ ) moved. In this instance, STPD values are not significantly different for either LAR or HAR bar-headed geese, indicating that the differences in ventilation reported in BTPS were due to the thinner air.

Expressing volumes as a function of BTPS, however, is standard for showing how much gas an animal ventilates. Black and Tenney found that the resting ventilation in bar-headed geese acclimated under hypobaric conditions (equivalent to 5640 m) for 4 weeks was approximately double that of sea level-acclimated birds when measured at similar levels of  $Pa_{O_2}$  under normobaric conditions. In the present study, while the level of  $\dot{V}_R$  at an inspired partial pressure of  $O_2$  of 12 kPa ( $F_{I_{O_2}} = 0.21$  at 3200 m; sea level equivalent  $F_{I_{O_2}} = 0.12$ ) was roughly 30% higher in the HAR bar-headed geese relative to the LAR bar-headed geese, this difference was not significant. This suggests that despite apparent similarities, the changes seen following short-term acclimation (Black and Tenney, 1980) are more akin to ventilatory acclimatization to hypoxia, while those seen in the HAR bar-headed geese appear to reflect hypoxic desensitization (Powell et al., 1998).

Ventilatory acclimatization to hypoxia is defined as the further increase in ventilation, compared with the rapid initial response, which occurs over hours to days of acclimatization (Powell et al., 1998). This secondary increase has been ascribed to plasticity in  $O_2$  sensing by the carotid body chemoreceptors and in central integration of chemoreceptor input (Powell, 2007). Over many months at high altitude, however, this hypoxic ventilatory response can be gradually attenuated by hypoxic desensitization (Brutsaert, 2007; Powell et al., 1998). While the increases in breathing during ventilatory acclimatization to hypoxia improve  $O_2$  uptake, hypoxic desensitization could be representative of longer-term high-altitude exposure and the ability to effectively transport  $O_2$  without magnified convective transport. This would help reduce respiratory water loss, and reduce the metabolic cost of breathing (Powell, 2007; Storz et al., 2010). Despite the apparent hypoxic desensitization,  $\dot{V}_R$  remained elevated relative to  $\dot{V}_{O_2}$  in the HAR bar-headed geese, indicative of a reduction in lung  $O_2$  extraction (Fig. 2).

Differences between species in blood- $O_2$  carrying capacity were driven primarily by differences in intrinsic  $O_2$  affinity (i.e.  $P_{50}$  of Hb) and *in vivo* blood  $O_2$  affinity (i.e. blood  $O_2$  affinity subject to *in vivo* changes in temperature, pH and allosteric modulators). There were no inter- or intraspecies differences in [Hb] or Hct either in normoxia or with progressive hypoxia. The greater  $O_2$  affinity of the HbA isoform of bar-headed geese is well documented (Weber et al., 1993), although the properties of the HbD isoform have yet to be studied. Neither Hct nor [Hb] in bar-headed geese were affected by



rearing altitude, a finding also reported after short-term (4 weeks) acclimation of bar-headed geese to simulated altitude (5640 m) (Black and Tenney, 1980). This is also similar to patterns described in high-altitude-acclimatized Tibetan humans (Simonson et al., 2015) and deer mice (Lui et al., 2015). As a result of the differences in intrinsic and *in vivo* blood O<sub>2</sub> affinity, however, at any given P<sub>aO<sub>2</sub></sub>, bar-headed goose blood will be more saturated than that of barnacle geese (Fig. 3). Furthermore, the data suggest that at any given P<sub>aO<sub>2</sub></sub>, the blood of the HAR bar-headed geese would be slightly more saturated than that of the LAR bar-headed geese (Fig. 3). These small differences are most likely explained by the higher levels of [HCO<sub>3</sub><sup>-</sup>]<sub>a</sub> ( $P=0.009$ ) at 0.12 F<sub>I<sub>O<sub>2</sub></sub></sub>, and pH<sub>a</sub> ( $P<0.001$ ) at all levels of hypoxia in the high-altitude study group. Such an alkalosis would left-shift the O<sub>2</sub> equilibrium curve and enhance O<sub>2</sub> loading at the lung, which are possibly another features of high-altitude rearing. A respiratory alkalosis occurred in all groups during progressive hypoxic exposure due to heavy ventilation, further enhancing blood-O<sub>2</sub> carrying capacity (Fig. 4).

Based on the *in vivo* O<sub>2</sub> equilibrium curves, the blood P<sub>50</sub> of the two groups of bar-headed geese are unlikely to be appreciably different. While birds have organic phosphates (inositol pentophosphate) for altering the O<sub>2</sub> affinity of Hb, there is little evidence of an IPP-induced change in P<sub>50</sub> with high-altitude exposure (Weber, 2007). This suggests that isoHb switching did not occur in response to environmental hypoxia. Although large reversible changes in blood P<sub>50</sub> could be achieved by altering the expression levels of HbA and HbD, our data suggest that bar-headed geese do not do this. Similar results have been reported for high-versus low-altitude hummingbirds (Projecto-Garcia et al., 2013), sparrows (Cheviron et al., 2014), house wrens (Galen et al., 2015) and waterfowl (Natarajan et al., 2015).

All groups in the present study increased  $\dot{Q}$  2.0- to 2.5-fold during severe hypoxia. Previous reports of the magnitude and direction of change in  $\dot{Q}$  during severe hypoxia in bar-headed geese vary widely. Hawkes et al. (2014) reported that, in bar-headed geese breathing 0.07 F<sub>I<sub>O<sub>2</sub></sub></sub>,  $\dot{Q}$  decreased by ~20%, whereas Fedde et al. (1989) and Black and Tenney (1980) reported no change in  $\dot{Q}$  at this level of hypoxia. However, when Black and Tenney exposed their birds to a further reduction in O<sub>2</sub> to ~0.05 F<sub>I<sub>O<sub>2</sub></sub></sub>, corresponding to a P<sub>aO<sub>2</sub></sub> of ~3.5 kPa,  $\dot{Q}$  increased by a remarkable 7-fold (Black and Tenney, 1980). These differences most likely reflect the steepness of the exponential cardiovascular response curve beyond the inflection point and small differences in P<sub>aO<sub>2</sub></sub>. Analysis of the data based on F<sub>I<sub>O<sub>2</sub></sub></sub> suggests that increases in  $\dot{Q}$  in the present study were initiated first in the HAR bar-headed geese (0.09 F<sub>I<sub>O<sub>2</sub></sub></sub>), next in the LAR bar-headed geese (0.07 F<sub>I<sub>O<sub>2</sub></sub></sub>) and last in the barnacle geese (0.05 F<sub>I<sub>O<sub>2</sub></sub></sub>) (Fig. 5A). When expressed as a function of P<sub>aO<sub>2</sub></sub> (or C<sub>aO<sub>2</sub></sub>), however, all groups produced significant increases in  $\dot{Q}$  at a similar P<sub>aO<sub>2</sub></sub> of ~6 kPa (Fig. 6A, Fig. S3C), indicating that the differences in which  $\dot{Q}$  increases are initiated when plotted as a function of F<sub>I<sub>O<sub>2</sub></sub></sub> reflect differences in the blood O<sub>2</sub> affinity. Black and Tenney made a similar observation. They too noted that the differences they saw in the changes in  $\dot{Q}$  during progressive hypoxia following short-term acclimation in bar-headed geese could be accounted for by differences in C<sub>aO<sub>2</sub></sub> (Black and Tenney, 1980). Unlike Black and Tenney, however, we did not see an increase in the overall magnitude of  $\dot{Q}$  at a given P<sub>aO<sub>2</sub></sub> with lifelong exposure to high altitude. Under resting conditions,  $\dot{Q}$  does not differ in high- versus low-altitude native or domestic mammals either. Total blood flow has been found to be unaltered or slightly reduced in humans, alpaca, llama, rats and wild mice living at altitude (Banchero et al., 1971; Klausen, 1966; Monge et al., 1955; Sillau et al., 1976; Turek

et al., 1973). Thus, the differences present between the study of short-term acclimatization by Black and Tenney (1980) and of high-altitude rearing in our study not only suggest that both acclimatization to hypoxia and hypoxic desensitization occur with ventilatory responses, but also that similar acclimatization and desensitization to hypoxia occur with regard to blood flow in bar-headed geese, and may act to reduce the costs of convective transport of blood as they do for respiratory gases.

Previous studies on geese reported changes in heart rate as the primary contributor to changes in  $\dot{Q}$ , with stroke volume remaining largely unchanged (Faraci, 1986; Fedde et al., 1989; Smith et al., 2000). In the present study, this was true of the low-altitude groups down only to an F<sub>I<sub>O<sub>2</sub></sub></sub> of 0.12. Below this, all groups increased  $\dot{Q}$  during progressive hypoxia down to a P<sub>aO<sub>2</sub></sub> ~6 kPa (Fig. S3C) more by increasing stroke volume than heart rate (Fig. 5C and Fig. 6C). HAR bar-headed geese were the only group in which P<sub>aO<sub>2</sub></sub> fell <6 kPa, at which point heart rate increased substantially (Fig. 6D) associated with a decrease in stroke volume (Fig. 6C). The large increases in stroke volume seen in the present study could have been mediated either by extrinsic factors (circulating hormones or neurotransmitters) or intrinsic factors [cardiac muscle fiber contractile properties associated with the Frank–Starling response – the relationship between cardiac contractility and venous return (Smith et al., 2000)]. As outlined in Shiels and White (2008), limited information exists on the Frank–Starling response in avian cardiomyocytes, although it is known to facilitate large increases in stroke volume during hypoxia in fish (Farrell, 1991; Shiels and White, 2008). Further studies are required to determine the underlying mechanisms of this response.

## Conclusions

The primary differences present between the HAR and LAR bar-headed geese were ventilatory and metabolic in nature. But, at this point, we cannot discern the differential effects of phenotypic plasticity (i.e. acclimatization) from developmental plasticity on the physiology of this species. The reduction in resting  $\dot{V}_{O_2}$  was one of the most significant differences observed in the HAR bar-headed geese. We also observed an increase in resting  $\dot{V}_R$  and in the HVR that could be explained by the differences in barometric pressure at which the measurements were made. Even taking this into account, however, HAR bar-headed geese still exhibited a large air convection requirement (ratio of  $\dot{V}_R$  to  $\dot{V}_{O_2}$ ), compensating for a reduction in lung O<sub>2</sub> extraction (Fig. S3A). This may help to maintain blood acid–base balance at the expense of O<sub>2</sub> uptake.

All geese increased  $\dot{Q}$  by ~2-fold to a similar overall magnitude, but  $\dot{Q}$  increased earlier and more rapidly in bar-headed geese than barnacle geese as environmental O<sub>2</sub> fell, and more so in the HAR bar-headed geese than in LAR bar-headed geese. However, this could be explained by the differences in *in vivo* blood O<sub>2</sub> affinity. All groups increased perfusion at a similar P<sub>aO<sub>2</sub></sub> during hypoxic exposure. An unexpected finding was the prominent role of increases in stroke volume in increasing  $\dot{Q}$  in all groups, including the barnacle geese. Further studies are required to determine the underlying mechanisms of the differences reported here between HAR bar-headed geese and LAR bar-headed geese, and the extent to which these differences may also facilitate high-altitude flight.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

S.L.L. designed the study, carried out all lab work and field data collection, completed all data analysis, and drafted the manuscript. B.C. carried out all surgical procedures and helped collect field data. A.P.F. participated in study design and helped to draft the manuscript. Y.W. participated in study design, helped to carry out and coordinate field work. W.K.M. participated in study design, helped to carry out and coordinate field work, and helped to draft the manuscript. All authors edited the manuscript and gave final approval for publication.

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#### Supplementary information

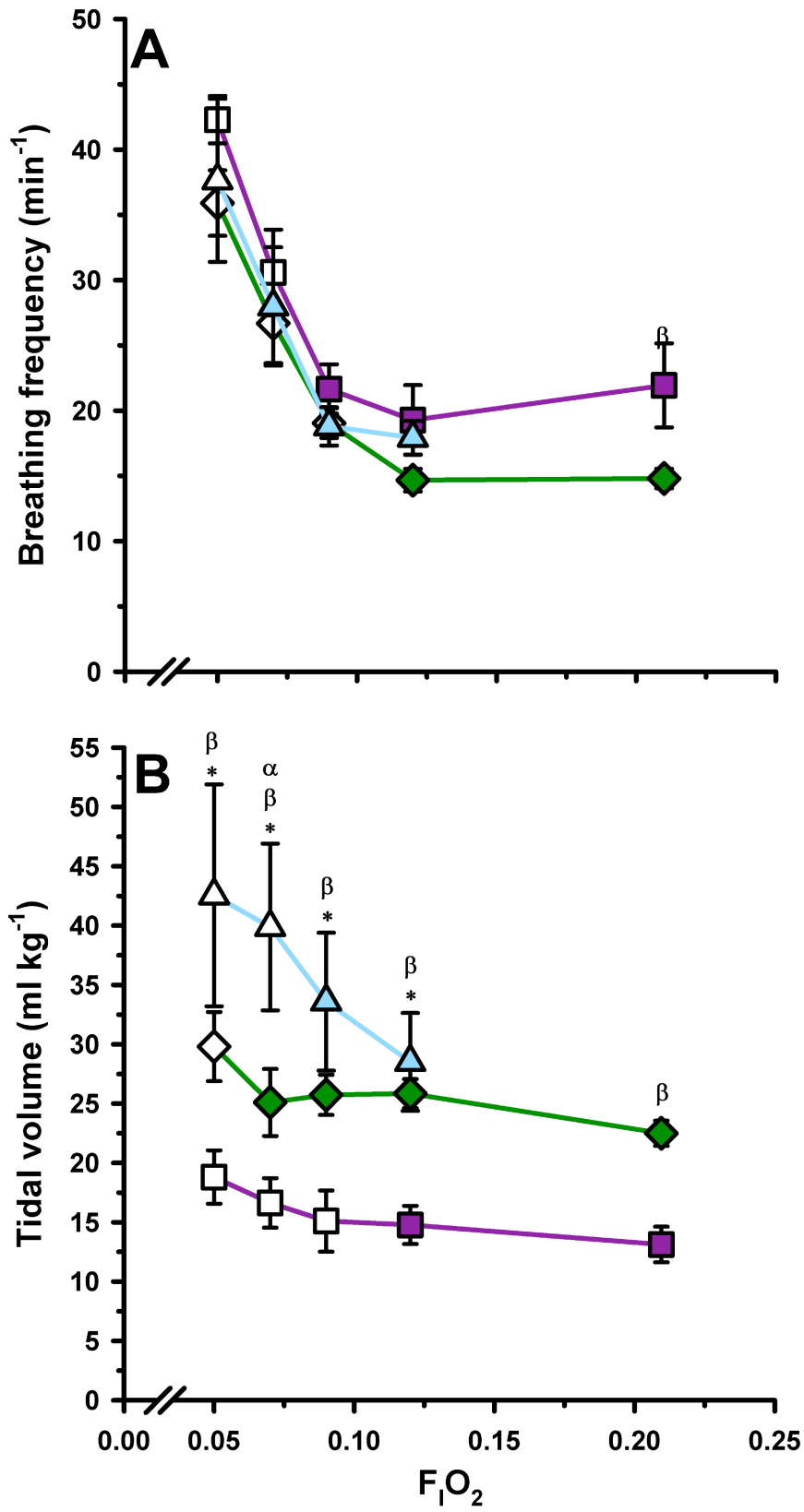
Supplementary information available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.132431/-DC1>

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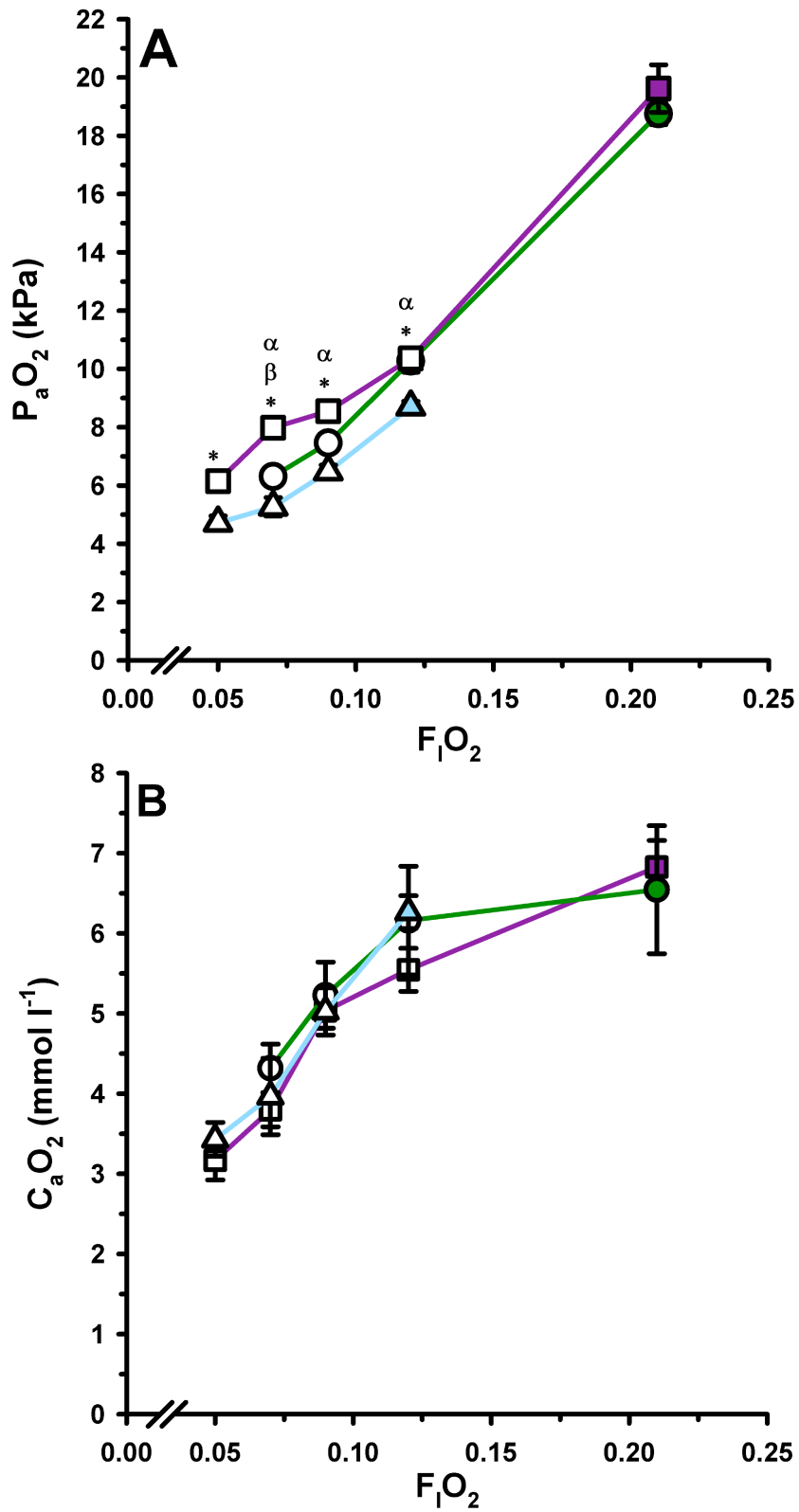
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### Supplementary Figures



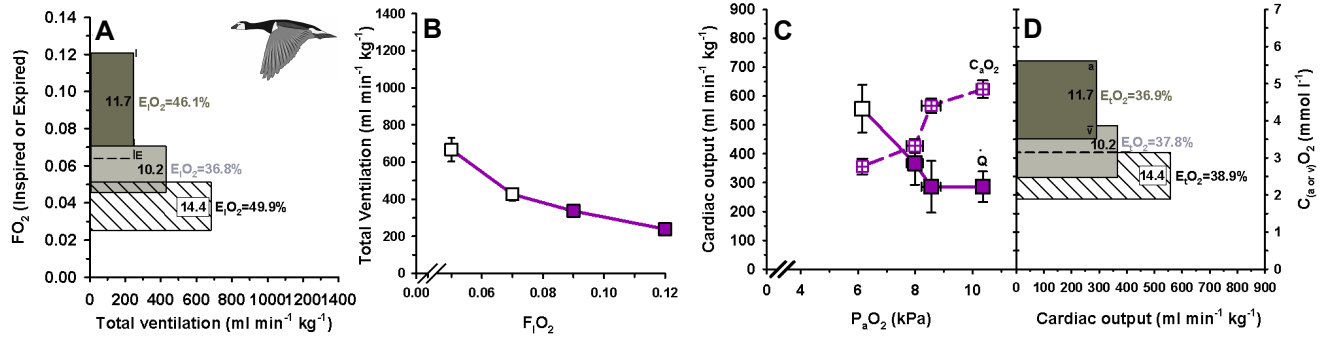
**Supplementary Figure S1: Changes in the components of total ventilation during progressive hypoxic exposure.** (A) Breathing frequency was initially higher in barnacle geese, but increased in all groups throughout hypoxia exposure. (B) Tidal volume (in BTPS) was greater in magnitude in bar-headed geese than barnacle geese, though it increased in all groups. Values represent means  $\pm$  s.e.m (blue triangle: high-altitude-reared bar-headed geese, N=5; green diamond: low-altitude-reared bar-headed geese, N=5; purple square: barnacle geese, N=7). ). Significant differences ( $P < 0.05$ ) from values obtained with ambient exposure within a species are indicated by open symbols and determined by one-way repeated measured ANOVA. Significant differences ( $P < 0.05$ ) between species are determined by two-way repeated measures ANOVA and indicated by different symbols: \*high-altitude-reared bar-headed geese versus barnacle geese, <sup>a</sup>high-altitude-reared bar-headed geese versus low-altitude-reared bar-headed geese, and <sup>b</sup>low-altitude-reared bar-headed geese versus barnacle geese.



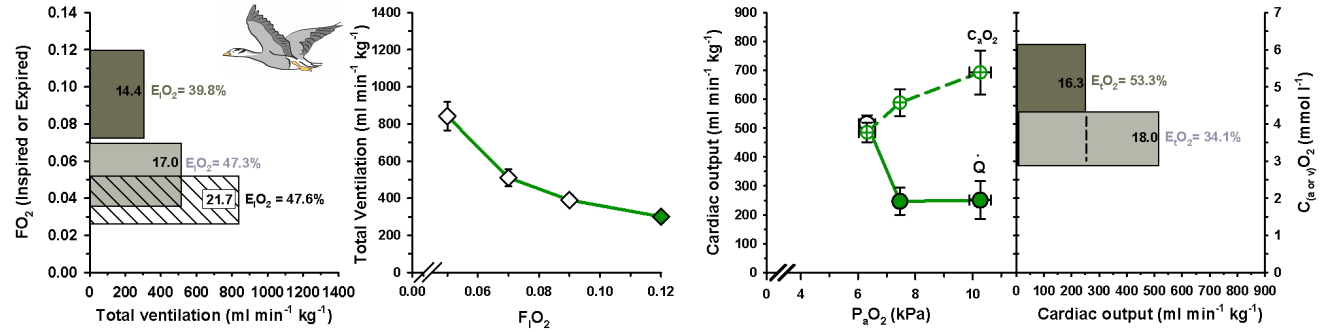
**Supplementary Figure S2: The relationship of arterial partial pressure of oxygen and arterial oxygen content with inspired oxygen level throughout hypoxic exposure.**

(A) Arterial partial pressure of oxygen ( $P_aO_2$ ) decreased with decreasing fractional oxygen composition of inspired gas ( $F_I O_2$ ) in all groups, though high-altitude-reared bar-headed geese had a lower  $P_aO_2$  at any given  $F_I O_2$  than either low-altitude-reared group. Significant differences ( $P < 0.05$ ) in  $P_aO_2$  between species are indicated by different symbols: \*high-altitude-reared bar-headed geese versus barnacle geese, <sup>a</sup>high-altitude-reared bar-headed geese versus low-altitude-reared bar-headed geese, and <sup>β</sup>low-altitude-reared bar-headed geese versus barnacle geese. (B) Arterial oxygen content ( $C_aO_2$ ) decreased with decreasing  $F_I O_2$ . Values are means  $\pm$  s.e.m (blue triangle: high-altitude-reared bar-headed geese, N=5; green diamond: low-altitude-reared bar-headed geese, N=6; purple square: barnacle geese, N=7). Significant differences ( $P < 0.05$ ) from ambient exposure performance within a species are indicated by open symbols and determined by one-way repeated measured ANOVA.

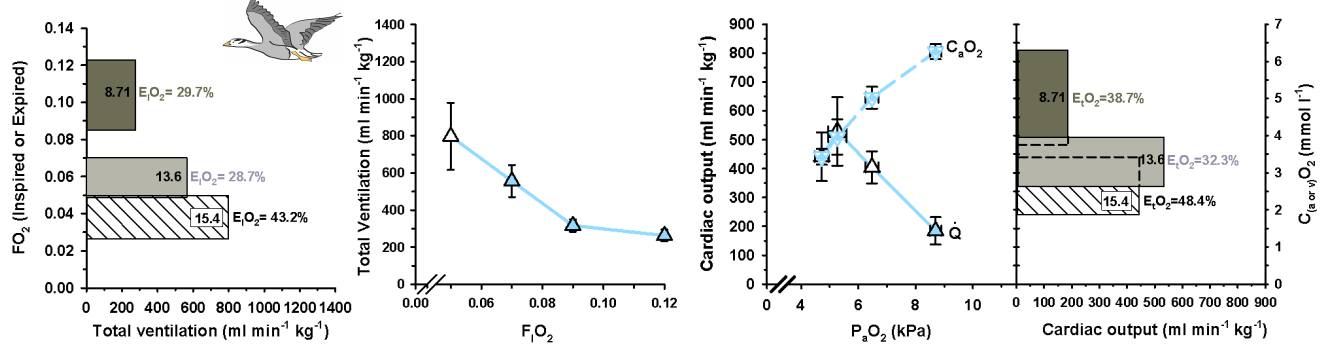
**Barnacle geese (0 m)**



**Bar-headed geese (0 m)**



**Bar-headed geese (3200 m)**





**Supplementary Figure S3: A summary of the trends in the hypoxic ventilatory response and hypoxic cardiovascular response of bar-headed geese and barnacle geese.** Graphs summarizing the hypoxic ventilatory responses and cardiovascular responses of barnacle geese (N=7), low-altitude-reared bar-headed geese (N=5), and high-altitude-reared bar-headed geese (N=5). The Y axis of the graphs in column A is the fractional composition of oxygen ( $F_{I}O_2$ ). The top of each box is the inspired  $F_{I}O_2$  while the bottom of each box is the expired  $F_{I}O_2$ . The difference between these two represents the amount of  $O_2$  extracted from the respired gas ( $E_{I}O_2$ ; %), while the number inside the box represents the area of box, which is equal to the oxygen consumption in  $ml O_2 \text{ min}^{-1} \text{ kg}^{-1}$  ( $\dot{V}_R \times F_{I}O_2 \times E_{I}O_2$ ). The shading of the boxes depicts different  $F_{I}O_2$  levels: dark grey = 0.12, light grey = 0.07, and striped = 0.05  $F_{I}O_2$ . The graphs in column B illustrate how total ventilation (in STPD) increased in each species with decreasing  $F_{I}O_2$ . The graphs in column C depict the relationship between cardiac output ( $\dot{Q}$ ; solid symbols, left axis) and arterial oxygen content ( $C_aO_2$ ; hatched symbols, right axis of column D) with arterial partial pressure of oxygen ( $P_aO_2$ ) in each species. The Y axis of the graphs in column D is the  $C_aO_2$ . The top of each box is the  $C_aO_2$ , while the bottom of each box is the venous oxygen content ( $C_vO_2$ ). The difference between these two represents the amount of  $O_2$  extracted from the blood by the tissues ( $E_tO_2$ ; %), while the number inside the box represents the area of box, which is equal to the oxygen consumption in  $ml O_2 \text{ min}^{-1} \text{ kg}^{-1}$  ( $\dot{Q} \times C_aO_2 \times E_tO_2$ ). The shading of the boxes depicts different  $F_{I}O_2$  levels: dark grey = 0.12, light grey = 0.07, and striped = 0.05  $F_{I}O_2$ . Significant differences ( $P < 0.05$ ) in values from those obtained at ambient exposure for  $\dot{V}_R$  and  $\dot{Q}$  within a species are indicated by open symbols in the graphs in columns B and C and determined by one-way repeated measured ANOVA.