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Title: The relationship between cardiopulmonary size and aerobic performance in adult deer mice at high altitude

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Summary

Deer mice (*Peromyscus maniculatus sonoriensis*) populations in the White Mountains of Eastern California are found across a substantial range of partial pressures of oxygen (pO_2). Reduction in pO_2 at high altitude can have a negative impact on aerobic performance. We studied plastic changes in organ mass and volume involved in aerobic respiration in response to acclimation to high altitude, and how those changes are matched with aerobic performance measured by $\dot{V}O_2$ max. Adult deer mice born and raised at 340 m were acclimated at either 340 m or 3800 m for a period of nine weeks. Lung volume increased by 9% in mice acclimated to high altitude. $\dot{V}O_2$ max was also significantly higher under hypoxic conditions after high altitude acclimation compared to controls. Body mass corrected residuals of $\dot{V}O_2$ max were significantly correlated with an index of cardiopulmonary size (summed standardized residuals of lung volume and heart mass) under both hypoxic and normoxic conditions. These data show that phenotypic plasticity in lung volume and heart mass plays an important role in maintaining aerobic performance under hypoxic conditions, and account for up to 55% of the variance in aerobic performance.

Introduction

Aerobic performance is an emergent trait that is dependent on a cascade of oxygen moving from the environment to the cells via a pathway that involves multiple organ systems (Weibel et al., 1981; Bassett and Howley, 2000). These organs must work together to support the

aerobic metabolism of the whole animal, and even small changes in the environment can impact the function of one or more of these systems resulting in a change in organismal performance. Populations of organisms living in areas of harsh abiotic conditions need to be equipped to deal with a variety of conditions. For example, animals living at high altitude must be able to survive and be active in particularly harsh conditions. Several biotic and abiotic factors vary with altitude including temperature, primary productivity, and UV exposure. However, perhaps the most important difference is the reduced ambient partial pressures of oxygen (pO_2) at high altitude.

Organisms that live at high altitudes generally must adapt to the lower levels of oxygen or face reduced aerobic performance as a result, either through evolutionary processes (genetic changes across generations) or phenotypic plasticity (physiological changes during an individual's lifespan) (Garland and Carter, 1994). Although some evolutionary changes have been documented in species that inhabit high altitudes, such as hemoglobin polymorphisms in deer mice (*Peromyscus maniculatus*) (Chappell and Synder, 1984; Storz et al., 2009; Storz et al., 2010a), phenotypic plasticity remains an important way to maintain aerobic performance in the face of environmental heterogeneity.

Because deer mice are widely distributed, both geographically throughout North America, and across a wide altitudinal range they have been a model system for the study of mammalian high altitude physiology. One subspecies, *P. maniculatus sonoriensis* is found across eastern California and has an altitudinal range that extends from below sea level in Death Valley, CA to over 4000 m in the Sierra Nevada and White Mountains (Sawin, 1970). These mice possess evolutionary adaptations to high altitude such as the aforementioned hemoglobin polymorphisms. Phenotypic plasticity also plays a major role in acclimation to high altitude both during development and adulthood. Wild caught mice at high altitude tended to have improved thermogenic performance relative to low altitude controls even when seasonal effects are taken into account (Hayes 1989). Likewise mice born at high altitude perform better during exercise tests than low altitude born mice acclimated to high altitude (Chappell et al. 2007).

The change in whole-animal aerobic capacity resulting from acclimation to high altitude is accompanied by increased hematocrit, hemoglobin concentration, and lung mass compared to animals acclimated to low altitude (Hammond et al., 1999; Hammond et al., 2001). Changes in splenic function have also been noted in response to high altitude acclimation, as it is generally

considered to be associated with storage of red blood cells (Baker and Remington, 1960; Boning et al., 2011). For example in thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), splenectomy results in a reduced hematocrit in response to low pO_2 (Mcglaghlin et al., 1972). These changes occur in systems that directly impact the oxygen cascade and it has been assumed they are at least partially responsible for maintenance of aerobic performance in high altitude natives.

More recently work has been conducted to determine if the phenotypic differences between altitude levels are related to performance. These studies have shown that while there is a genetic basis to many of the differences in thermogenic performance of deer mice from different altitudes (Cheviron et al. 2012), plasticity plays an important role as well (Cheviron et al. 2013). Furthermore, these studies have demonstrated a link between plasticity in thermogenic performance and subordinate changes in transcriptional profiles (Cheviron et al. 2014).

In this study we set out to build on recent efforts to illuminate the importance of plastic changes in the maintenance of organismal performance at high altitude. We set about to test the hypothesis that phenotypic changes, such as changes in hematocrit or lung volume, support aerobic performance in hypoxic conditions at high altitude. Our experimental design involved using a low altitude born captive colony of deer mice. The mice in this colony were all derived from a wild population of deer mice captured at high altitude in the White Mountains of eastern California. Mice from this colony were either maintained at low altitude (LA; vivarium at 380 m--no real acclimation; Control group) or moved to high altitude (HA; 3800 m--true acclimation) for nine weeks and then tested at both altitudes to challenge that acclimation (Fig. 1). We measured the mass of the spleen, heart, lung (lobar) volume, and aerobic performance ($\dot{V}O_2$ max) of all individuals. The aerobic performance was measured both before the experiments started and then at the end of the acclimation trials. We made three predictions in relation to our hypothesis:

1. Mice acclimated to high altitude will show improved aerobic performance ($\dot{V}O_2$ max during exercise) under both hypoxic and normoxic conditions.
2. Mice acclimated to high altitude will have greater heart mass, lung volume, and hematocrit.
3. Mice with higher cardiopulmonary residuals and hematocrit levels will have higher $\dot{V}O_2$ max residuals.

Results

Body mass

Body mass was not significantly different between HA and LA groups prior to the start of the acclimation process (HA = 24.44 ± 0.78 g, LA = 23.97 ± 0.74 g, $F_{1,17}$ = 0.18, P = 0.67), and at the time of sacrifice (Fig. 2A; Table 1) (HA = 23.52 ± 0.60 g, LA = 24.01 ± 0.76 g, $F_{1,17}$ = 0.27, P = 0.61).

Hematocrit and spleen measurements

Hematocrit was similar between the HA (46.9±1.55 %) and LA (47.04±0.83 %) groups at the time of sacrifice (Fig. 2B; Table 1) ($F_{1,17}$=0.007, P=0.93).

Dry spleen mass was also not significantly different ($F_{1,17}$=0.22, P=0.65) between HA (0.0076±0.0015 g) and LA (0.0069±0.0006 g) groups (Table 1). However, because past studies have consistently shown that deer mice acclimated to high altitude have increased hematocrit (8% higher) and hemoglobin concentrations (10% higher) compared to those at low altitude (Hammond et al., 2001; Hammond et al., 2002; Tufts et al., 2013), we also examined the relationship between spleen size and hematocrit in the HA mice. If red blood cells were sequestered in the spleen during their short time at low altitude, it might be expected that animals with a greater spleen mass would have lower values for hematocrit. To test this hypothesis we regressed hematocrit on dry spleen mass in the HA animals, and found a highly significant negative correlation of r = -0.771 ($F_{1,8}$ = 11.71, P = 0.0091).

Cardiopulmonary organs

Body mass was not a significant covariate for either wet heart mass ($F_{2,15}$=3.69, P=0.07) or dry heart mass ($F_{2,15}$=1.36, P=0.26), and heart mass did not vary significantly with acclimation altitude in either wet ($F_{2,15}$=2.64, P=0.13), or dry measurements ($F_{2,15}$=1.86, P=0.19). The body mass corrected mean dry heart mass was 8% higher in the HA group (0.0415±0.0015 g) compared to the LA group (0.0384±0.0016 g) (Fig. 2C) though this difference was not significant (T_{16} = 1.79, P = 0.091).

Mice acclimated to high altitude had 8% larger body mass corrected lung volume than low altitude controls (HA 0.91±0.019 ml, LA 0.84±0.017 ml; $F_{2,15}$=6.84, P=0.020). Likewise the summed lobar volume of the lung was 9% greater in the HA group (HA 0.80±0.017 ml, LA

0.73±0.025 ml; $F_{2,15}$=4.55, P=0.050) (Fig. 2D). In both cases body mass was a significant covariate.

Maximal oxygen consumption

All values of $\dot{V}O_2$ max were corrected for body mass using residuals of linear regression. Initial aerobic performance under normoxic conditions did not differ significantly between the treatment groups (HA=4.59±0.13 ml•min^{-1}, LA=4.42±0.20 ml•min^{-1}; $F_{2,15}$=1.02, P=0.383). A repeated measures ANOVA of post acclimation aerobic performance under normoxic and hypoxic conditions revealed a significant effect of acclimation altitude on $\dot{V}O_2$ max ($F_{1,16}$ = 8.86, P = 0.0089) such that HA mice performed better than LA controls. To determine if this was true in both hypoxic and normoxic conditions subsequent post-hoc tests were performed. These tests showed that HA mice performed significantly better than LA mice under hypoxia (z = 3.36, P = 0.0042), but not in normoxia (z = 1.58, P = 0.38) (Fig. 3). All mice did better in normoxia than in hypoxia regardless of their acclimation altitude ($F_{1,16}$ = 32.58, P < 0.0001), which was supported by subsequent post hoc analysis. However, HA mice experienced only a 9% reduction in $\dot{V}O_2$ max under hypoxic conditions versus normoxia compared to the 16.5% loss in performance observed in low acclimated mice. Under the pO_2 from their respective acclimation regimes, HA under hypoxia and LA under normoxia, mice showed no significant differences in aerobic performance (z = -0.78, P = 0.86), suggesting that HA mice are able to maintain the same level of performance as LA mice even when they are experiencing reduced pO_2.

Regression of cardiopulmonary size on $\dot{V}O_2$ max

The complete regression model used included either residuals of $\dot{V}O_2$ max under normoxic or hypoxic conditions from post-acclimation runs as the dependent variable and dry heart mass residuals, lobar volume residuals, and hematocrit as the independent variable. We used residuals of $\dot{V}O_2$ max, lobar volume, and heart mass to remove the effect of body mass.

Stepwise analysis showed that hematocrit was not a significant predictor of maximal aerobic capacity in either the hypoxia or normoxia, and thus was removed from subsequent analyses. Inclusion of both heart mass and lobar volume produced the best model fit in both the hypoxia run ($F_{2,15}$=9.29, P=0.0018) and the normoxia run ($F_{2,15}$=10.71, P=0.0013) with R^2 of 0.570 and 0.588 respectively. For hypoxia $\dot{V}O_2$ max both heart mass (t_{15}=3.55, P=0.0029) and

lobar volume (t_{15}=2.58, P=0.024) explained a significant proportion of the variance in $\dot{V}O_2$ max with squared semipartial correlation coefficients of $r^2_{Y(H_{\bullet}L)}$ = 0.361 and $r^2_{Y(L_{\bullet}H)}$ = 0.181 respectively. Both heart mass (t_{15}=4.06, P=0.0010) and lobar volume (t_{15}=2.02, P=0.061) were also important in explaining the variance in the normoxia run $\dot{V}O_2$ max with corresponding squared semipartial correlation coefficients of $r^2_{Y(H_{\bullet}L)}$ = 0.452 and $r^2_{Y(L_{\bullet}H)}$ = 0.112. Further reduction of the model to just heart mass resulted in a reduction of the R^2, and trimming the model to lobar volume alone produced a non-significant result.

Regression of $\dot{V}O_2$ max residuals on the summed standardized cardiopulmonary size gave similar results. The R^2 values for the regression of $\dot{V}O_2$ max under normoxia and hypoxia were 0.534 (Fig. 4A) and 0.555 (Fig. 4B) respectively. It was not possible to further partition the variance for lung volume and heart mass. However the summed cardiopulmonary size was a significant predictor of aerobic performance under both normoxia ($F_{1,16}$ = 18.33, P = 0.00057) and hypoxia ($F_{1,16}$ = 19.95, P = 0.00039).

Discussion

In this study we show a concrete link between phenotypic changes resulting from hypoxic exposure and whole organismal performance. We do this in the context of a novel experimental design that allows for direct comparison between acclimated and control animals by use of repeated testing at all altitudes. The power of this study thus comes from the ability to match the size of organs directly related with the oxygen cascade with organismal performance. By doing so we are able to show that the relative sizes of the heart and lungs are important predictors of aerobic performance in individuals at high altitude.

Our measurements on lung volume are consistent with our previous findings of significantly larger lung mass in high altitude acclimated deer mice (Hammond et al., 2001) and the 8-9% change in lung volume we found is consistent with the 9% increase in lung volume documented in guinea pigs (*Cavia porcellus*) developing at high altitude (Hsia et al., 2005). However, unlike other studies of mammals measured under similar protocols (Burri and Weibel, 1971; Lechner and Banchero, 1981; Hsia et al., 2005; Ravikumar et al., 2009) at high altitude, the mice used in this study were all well into adulthood, suggesting that deer mice retain the capacity for substantial morphological changes even after development has ended. It will be important to follow up this work with studies to document how those volume changes are

manifested at the alveolar and diffusive interfaces within the lungs, but a study of that magnitude was not the aim of this project.

Although the 8% difference in heart mass we found between acclimation altitudes is not statistically significant, small changes in the heart mass of HA animals has been noted previously in guinea pigs exposed to hypoxia (Van Bui & Banchero, 1980). Changes in heart mass due to hypoxia can be the result of right ventricular hypertrophy resulting from pulmonary hypertension (Rabinovitch et al., 1981; Reinke et al., 2011), which is generally considered maladaptive (Storz et al., 2010b), but could also potentially be the result of plasticity to improves cardiac output by hypertrophy of the left ventricle. It is also possible that the slightly lower (~6 ºC differential) temperature at the high site was sufficient to induce a small degree of cold acclimation which can also result in increased heart mass (Van Bui & Banchero, 1980; Hammond et al., 2001; Rezende et al., 2009), but the temperature difference observed in this paper is much smaller than the one induced in the aforementioned studies.

An unexpected result of this study was the lack of any difference in hematocrit between acclimation groups when measured at UCR at the end of the study. This was unexpected because in repeated published and unpublished work we have observed ~10% increase in hematocrit of high altitude acclimated mice from this same colony (Hammond et al., 1999; Hammond et al., 2001; Hammond et al., 2002). Because blood draws on deer mice can have a significant impact on aerobic performance up to two weeks after (Van Sant, 2012), we refrained from measuring hematocrit on HA animals while they were still at high altitude and waited until after their final normoxia run. Therefore, one possible explanation of the hematocrit results is that HA animals were able to sequester excess red blood cells into the spleen during the 1-2 days they were at UCR prior to hematocrit determination. The spleen acts as a reservoir for red blood cells in many mammals (Baker and Remington, 1960; Boning et al., 2011). Thus it is possible that mice acclimated to high altitude sequestered excess red blood cells in the spleen upon return to low altitude resulting in lower hematocrit than 1-2 days earlier at high altitude. The strong negative correlation between hematocrit and spleen mass strongly suggests that HA animals did exactly this after their return to low altitude and potentially explain why HA values of hematocrit approximated those of LA mice.

The reduction in aerobic performance between normoxic and hypoxic runs we observed in the HA group matches closely the difference reported between high and low altitude

acclimated mice in previous studies (e.g. Chappell et al., 2007). In fact, the aerobic performance of HA mice under hypoxic conditions was not significantly different from that of LA mice under normoxia. These results demonstrate once again the capacity of these animals to compensate aerobic capacity in spite of a reduction of alveolar pO_2 of up to 37% based on changes in barometric pressure and vapor pressure. Furthermore, the negligible difference in $\dot{V}O_2$ max observed between the HA group compared to the LA group at their respective acclimation pO_2 strongly suggests that the physiological changes resulting from acclimation to hypoxia are responsible for this improvement in performance.

Because aerobic performance is based on a cascade of oxygen throughout the body and is, therefore, dependent on multiple systems, all measured variables that could influence performance were included in a correlation analyses. However, due to the limitations of this study not all subordinate traits that are responsible for steps in the oxygen cascade are represented, for example, lung capillary density, muscle capillary density, or mitochondrial density. Thus it is important to acknowledge that it is impossible to determine the importance of each of those subordinate traits to aerobic performance. In spite of this limitation the results of this study still highlight the fact that the included measures are able to account for a significant portion of the variance in aerobic performance.

The lack of significance of hematocrit in the final model is probably explained by the sequestration of red blood cells by the spleen in high altitude mice. Still, it cannot be said for certain if hematocrit would have been significant in the final model had it had been measured while the animals were still at high altitude. The high altitude acclimated mice were brought to low altitude to ensure that all animals were processed in a consistent manner, and it was not possible to get hematocrit measurements prior to the completion of all metabolic testing.

The importance of heart mass in explaining variance in aerobic performance was expected, due to its connection with cardiac output. The delivery of oxygenated blood from the lungs to the rest of the body is key step in the cascade of oxygen and is dependent on bulk flow produced by contraction of the heart (stroke volume). Stroke volume is presumably greater in mice with relatively larger heart mass, and therefore for a given heart rate cardiac output should likewise be increased. At least in humans, that maximal cardiac output is likely the key limiting factor in aerobic performance (Bassett and Howley, 2000), thus the fact that heart mass explained up to 45% of the variance in our mice is unsurprising.

Perhaps more interesting is the fact that the significance of lobar volume as a predictor of aerobic performance was dependent on inclusion of heart mass as a predictor. Particularly in the case of the run in hypoxic conditions, the importance of the lung as a predictor of aerobic performance seems evident. By first principles it is reasonable to assume that any increase in lung volume may be accompanied by an increased surface for gas exchange, albeit not at the same rate, and hence a higher diffusive capacity for oxygen. Previous work has supported this observation that animals acclimated to high altitude develop increased surface area for diffusion (Hsia et al., 2005), but have also stated that diffusive capacity is also dependent on the rate of pulmonary blood flow (Yilmaz et al., 2008). Our results are consistent with the idea that large lungs cannot compensate for hypoxic limitations on aerobic performance unless coupled to enhanced cardiac output and hence a larger heart.

Furthermore, while classic work on the lungs suggests that diffusive capacity is in excess at sea level (Hsia et al., 2007; Weibel et al., 1981; Weibel et al. 1992), the significant correlation between lung volume and aerobic performance under hypoxia indicates that at low pO_2 the diffusive capacity of the lungs may indeed become limiting, and may explain why many different types of vertebrates, from fish to mammals, invest in the growth of gas exchange organs in the presence of hypoxia (Burggren and Mwalukoma, 1983; Burri and Weibel, 1971; Lomholt and Johansen, 1979). As the pO_2 gradient falls in the lung in response to hypoxia increasing the surface area should allow for greater diffusion and thus allow animals in hypoxic conditions to maintain aerobic performance. Thus, what has been sometimes characterized as an over-structuring of the lungs at sea level may be a form of evolutionary protection against hypoxia. If the excess capacity from sea level animals is completely used during acclimation to high altitude, then diffusive capacity of the lung should match aerobic performance under hypoxia. Tests such as this should be incorporated into future work to see if the phenotypic changes in lung volume are a part of ameliorating performance lost or not.

This study demonstrated a functional linkage between organ-level phenotypic changes and whole-animal aerobic performance. As with previous studies we found phenotypic changes (increased lung volume) in response to high altitude acclimation. We also showed that mice acclimated to high altitude have improved aerobic performance under hypoxic conditions compared to low acclimated mice. Lastly, we bridged the gap between organismal performance and subordinate traits, and showed that changes in organ size (lung) resulting from acclimation

are related to aerobic performance at the individual level. These results may have increasing significance as climate change continues, and other organisms seek cooler habitats by moving to higher elevations (Moritz et al., 2008). Past work has shown that low altitude natives do demonstrate an acclimation response to hypoxia (Beaudry and McClelland, 2010; Templeman et al., 2010). However, the question remains as to whether organisms that have evolved under the high pO_2 conditions of low altitude will demonstrate an acclimation response to hypoxia that matches the one exhibited by this population of deer mouse, which is originally native to high altitude, or if low pO_2 at high altitudes becomes a barrier for further movement. Future work might focus on an integrative approach that included measures from all steps in the oxygen cascade in order to attempt to discover the importance of each step to aerobic performance. Such work would make it possible to create a statistical model that accounts for changes at each step and allows us to answer questions about the importance of plasticity during acclimation. Additionally, it is important to compare the plastic responses of populations of *P. maniculatus* from low altitude to that of high altitude populations to answer questions regarding the evolution of plasticity. This could be expanded to test the plastic response of other species of *Peromyscus* that inhabit ranges that are much more restricted that *P. maniculatus*. Such studies would provide a better idea of how plastic response differ across population and species, and if our findings can be broadly generalized to other organisms.

Materials and Methods

Animals

We used 11 male and 7 female adult deer mice (*P. maniculatus sonoriensis*) for this study. Animals ranged from 382 days to 500 days in age and were captive bred at low altitude (340m) in a colony that was originally caught in the White Mountains of eastern California in 1995. We prevented mice from producing more than one generation a year so this colony has been reproducing for no more than 18 generations. Additionally most families produced offspring for 2-3 years with a quiescent phase in the winter so, on average mice are no more than 10-20 generations removed from the wild. Captive bred mice were used because of the risk of Hantavirus (which occurs in relatively high incidence in our study area) associated with trapping wild mice. While the colony has been removed from the wild for a substantial amount of time, and it is possible that the mice have been unintentionally selected for domestication, we have

done our best to maintain genetic variation. The same colony has been used extensively in the past (Chappell et al., 2007; Rezende et al., 2009; Russell et al., 2008; Van Sant and Hammond, 2008). Mice were acclimated to one of two conditions; high acclimated (n=10) or low acclimated (n=8), for a period of nine weeks. The high altitude study site was the Barcroft Laboratory at the University of California's White Mountain Research Center (Barcroft; 3800 m elevation) and the low altitude study site was the University of California at Riverside Campus (UCR; 340 m elevation).

Animals were housed as individuals or pairs in plastic shoebox cages (27 cm x 21 cm x 14 cm) with aspen shavings for bedding. They were given *ad libitum* food and water, and provided with approximately 1g of cotton for nesting. At Barcroft, cages were housed in a room with an average ambient temperature of 16ºC±2.27 SD, exposed to the natural photoperiod. Ambient temperature was recorded every 30 min with a Stowaway XTI data-logger (Onset Computer Corp, Bourne, MA, USA) placed in an empty cage filled with bedding. Low altitude animals were housed in a vivarium at a near constant ambient temperature of about 22ºC (range 21 – 23ºC). The lights in the vivarium were set to 14 h:10 h light:dark (L:D) photoperiod to approximate the natural photoperiod at Barcroft.

Aerobic performance by maximal oxygen consumption

Maximal oxygen consumption ($\dot{V}O_2$ max), as an estimate of aerobic performance, was measured on all mice on three separate occasions (Fig. 1). First, we measured aerobic performance on all mice at low altitude; Second aerobic performance was measured after the 9-week acclimation period at the site of the acclimation (high or low altitude; see below for details). The third time aerobic performance was measured was at the 'challenge site'; this was at high altitude for the low altitude group or at low altitude for the high-altitude group.

At the beginning of the experiment, both groups of animals were housed in the UCR Vivarium. Prior to acclimation we measured the initial low altitude aerobic performance (pre acclimation run; ambient pO_2~150mm Hg, T ~ 20ºC) of all 18 mice. Within a day of completing those initial measurements, 10 mice (high altitude acclimation treatment, HA) were moved to 3800 m for nine weeks and 8 mice (low altitude acclimation treatment, LA) remained in the UCR Vivarium. At the end of nine weeks, the final low altitude aerobic performance (normoxia run; ambient pO_2~150mm Hg, T ~ 20ºC) was again measured in the LA mice and within 48

hours that low altitude group was transported to Barcroft (3800 m) for twenty-four hours. At Barcroft we measured the final high altitude aerobic performance (hypoxia run; ambient pO_2~100mm Hg, T ~ 19ºC) for both treatment groups. Subsequently all mice were transported back down to UCR (low altitude). The final low altitude aerobic performance (normoxia run; ambient pO_2~150mm Hg, T ~ 20ºC) was measured in the HA group within 24 hours of arrival back at UCR. This design was chosen as it tests mice first at their acclimation pO_2 and then at a "challenge" pO_2 to prevent any possible deacclimation from occurring prior to completion of runs. Though mice performed a second bout of $\dot{V}O_2$ max within 48 hours of their first run there is no reason to believe that this was not sufficient time for recovery, as Belding's ground squirrels (*Spermophilus beldingi*) have demonstrated high repeatability in exercise $\dot{V}O_2$ max after only two hours (Chappell et al., 1995).

Although this experimental design involved the transport of mice in potentially stressful conditions (being in a vehicle 6 hours between Riverside CA and the high altitude site near Bishop) it allowed us to ensure that all animals were exposed to the same ambient and atmospheric conditions during the aerobic performance measurements. We have had a great deal of experience with transporting mice up and down the mountain in an air-conditioned vehicle and have found that with apples for hydration and food and adequate time to rehydrate upon arrival to a new site, they handle this disruption relatively well.

Maximal oxygen consumption was measured by open flow respirometry during forced treadmill exercise. Air was supplied either by outlet (UCR) or using a positive pressure pump (Barcroft). Incurrent air was dried by Drierite™ (Xenia, OH, USA) and scrubbed of carbon dioxide by soda lime. Flow rate was regulated by Porter mass flow controllers (Hatfeild, PA, USA) upstream of the treadmill. The treadmill's working section was enclosed by Plexiglas with dimensions of 6 cm x 7 cm x 13 cm. Flow rates of 2300 ml min^{-1} and 1550 ml min^{-1} standard temperature and pressure (STP) were used at UCR and Barcroft correspondingly. Approximately 150 ml min^{-1} of excurrent air was subsampled, then dried and scrubbed of CO_2 before being routed through the oxygen sensor. Oxygen concentration was analyzed with an Ametek/Applied Electrochemistry S-3A analyzers (Pittsburg, PA, USA) and then digitized by Sable Systems UI-2 (Las Vegas, NV, USA) A-D converters and recorded on a Macintosh computer running Warthog Lab Helper software (www.warthog.ucr.edu).

Body mass was measured on animals prior to all runs. Mice were then placed on the treadmill and allowed to adjust for a period of 2-4 minutes. During this time a reference reading of unbreathed air was obtained. The treadmill was then started at a low speed (approximately ~0.1m/s), and speed subsequently increased by increments of 0.1m/s every 30-45 seconds until the mouse could either no longer maintain position on the tread or $\dot{V}O_2$ did not increase with increasing speed. At this time the treadmill was stopped, but $\dot{V}O_2$ measurements continued for several minutes during the animal's recovery period before a second reference reading was recorded.

$\dot{V}O_2$ was calculated from O_2 concentrations using the mode 1 equation in Warthog Lab Analyst software (www.warthog.ucr.edu).

$$\dot{V}O_2 = \dot{V}\frac{(F_IO_2 - F_EO_2)}{(1 - F_EO_2)} \quad (1)$$

In equation (1) $\dot{V}$ is flow rate (ml•min^{-1}STP corrected), and F_IO_2 and F_EO_2 are incurrent (reference) and excurrent fractional O_2 concentrations respectively (F_IO_2 was assumed to be 0.2095, and F_EO_2 never fell bellow 0.2080). Due to the size of the treadmill the "instantaneous" correction was applied to account for mixing (Bartholomew et al., 1981) and better resolve short-term metabolic changes. $\dot{V}O_2$ max was calculated as the highest one-minute average during the running bout or post exercise recovery period.

Dissection and organ measurement

All dissections took place at UCR to ensure consistent processing. After post-acclimation metabolic measurements were completed, we euthanized mice by overdose of Euthasol (0.07 ml IP; Vibrac Animal Health, Fort Worth, TX, USA). High altitude acclimated mice were sacrificed within 48 hours of being returned to low altitude. We obtained blood samples by retro-orbital puncture using heparinized microhematocrit tubes. Hematocrit was calculated from centrifuged tubes as the proportion of packed cells over the total volume of blood in the tube. The heart was subsequently removed from the body, cleaned of any connective tissue, fat, and blood contained within and weighed separately (wet mass). The spleen was treated likewise and weighed for wet

mass. Organs were then placed in an oven at 70ºC for at least 72 hours and dried to a constant mass before being reweighed (dry mass).

The lungs were fixed by tracheal instillation of a 2.5% buffered gluteraldehyde solution at a constant airway pressure of 25 cm H_2O above the sternum for a period of 30 minutes. At the end of the 30 minutes the tubing leading to the trachea was tied off to maintain the pressure and the fixative in the lungs. The lungs and tubing were removed from the body and then transferred to a vial and submerged in the glutaraldehyde solution for a period of 24 hours at 4ºC. The fixed lungs were washed twice in 0.1 M cacodylate buffer (pH 7.4) before being placed in vials with the buffer and stored at 4ºC.

Lung volume was measured by immersion displacement directly after removal from the mouse using the method described by Sherle (1970), and again after being separated into lobes (lobar volume; right lung: 4 lobes, left lung: 1 lobe).

Statistical Analysis

We used a 2x2 factorial design with sex and altitude as the independent variables, and five dependent variables; dry heart mass, lung volume, hematocrit, and $\dot{V}O_2$ max (under normoxia and hypoxia). There were no differences between sex for any dependent variable, so we combined males and females for the final analysis. Differences between acclimation groups were determined by analysis of variance (ANOVA) and analysis of covariance (ANCOVA) with body mass as a covariate. We used repeated measures ANOVA to analyze mass corrected $\dot{V}O_2$ max data with acclimation altitude as the between subjects factor, and ambient pO_2 during run as the within subjects factor. A post-hoc Tukey HSD test was used for subsequent pairwise comparisons. An alpha of 0.05 was used for statistical significance, however we report all values that approached the threshold of significance. Treatment and error degrees of freedom are enumerated as subscripts to the F values, and unless otherwise stated all F values come from the aforementioned analyses. In all cases, means are reported with standard error of the means (SEM) and are corrected for body mass by adding least square residuals to the grand mean when appropriate. A list of means for all variables considered in this study can be found in Table 1.

In addition to ANOVA, we used a stepwise multiple regression analysis to explore the relationship between $\dot{V}O_2$ max and measures that might have an impact on maximal metabolic output including; lung volume, dry heart mass, and hematocrit. For measures that are typically

dependent on body mass ($\dot{V}O_2$ max, lobar volume, heart mass), we used mass residuals. The mass at sacrifice was used for this regression in all cases, including for regression on $\dot{V}O_2$ max. The body mass at sacrifice represented the fully hydrated state of the animals having been moved between sites, and was measured within five days of both the normoxic and hypoxic runs. In all cases except dry heart mass, body mass was a significant covariate, but the residuals of the dry heart mass regression with body mass were still used as it is known that body mass does have an effect on heart mass in general. We checked for multicollinearity by correlation matrix in all variables prior to adding them into the model, but correlations between the predictors were relatively low. Model fit was evaluated by F values and R^2, and individual regression coefficients were evaluated by t value and squared semipartial correlation coefficient.

As an alternative to multiple regression, residuals of dry heart mass and lobar volume were standardized as z-scores and added together to get a value of summed cardiopulmonary size. Residuals of $\dot{V}O_2$ max (for both normoxia and hypoxia) were then regressed on cardiopulmonary size and presented in graphically.

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Author Contributions

Both K.A.H. and N.J.S. were involved in the process of developing the project. N.J.S. was primarily responsible for conducting the experiment and subsequent data analysis. N.J.S. also was responsible for preparing the manuscript, with editing from K.A.H.

List of Abbreviations

HA – High altitude acclimated mice

LA – Low altitude acclimated mice

VO_2 max – Maximal oxygen consumption

IP – Intraperitoneal Injection

UCR – University of California Riverside

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Fig. 1. **Experimental design employed in this study.** All mice (black, N=18) were run at low altitude (150 mmHg) initially, and then acclimated either to 3800 m (blue, n=10) or 340 m (red, n=8) for a period of 9 weeks. At the end of the acclimation period all mice were run at low (150 mmHg) and high (100 mmHg) altitude before being brought to UCR for final processing.

Fig. 2. **Depicts the mean value of traits (A, body mass; B, hematocrit; C, heart mass; D, lobar volume) at time of sacrifice in deer mice from the two different acclimation altitudes.** Asterisks indicate statistically significant differences between acclimation groups. Values are means ±1 SEM. (*N* as in text).

Fig. 3. **Depicts $V\mathrm{O}_2$ max (ml/min) in both high acclimated (blue) and low acclimated (red) deer mice from the three $V\mathrm{O}_2$ max measurements.** Post hoc results are indicated. Letters that are different from each other indicate statistically significant differences within groups; asterisks indicate significant differences between groups. Values are least squares means of body mass ±1 SEM. (*N* as in text).

Fig. 4. **Depicts the regression of $V\mathrm{O}_2$ max residuals from normoxic (A) and hypoxic (B) conditions regressed against the summed standardized residuals of dry heart mass and lobar volume.** High acclimated (blue) and low acclimated (red) individuals are marked by color. Also shown are the line of best fit and the 95% confidence interval of the line.

Table 1	Low acclimated (n=8)		High acclimated (n=10)	
	Mean	±SEM	Mean	±SEM
Body mass (g)				
Pre-acclimation	23.97	0.74	24.44	0.78
Post acclimation	24.01	0.76	23.52	0.60
Hematocrit (%)	47.04	0.83	46.90	1.55
Dry spleen mass (g)	0.0069	0.0006	0.0076	0.0016
Heart mass (g)				
Wet	0.177	0.005	0.190	0.006
Dry	0.038	0.002	0.042	0.002
Lung volume (ml)				
Whole	0.840*	0.019	0.909*	0.017
Lobar	0.732*	0.025	0.797*	0.018
***V*O$_2$ max (ml•min^{-1})**				
Hypoxia	3.77*	0.19	4.38*	0.09
Normoxia	4.52	0.14	4.81	0.10

Values for heart mass, lung volume, and VO_2 max are mass corrected means.

* Asterisks indicate significant differences between groups

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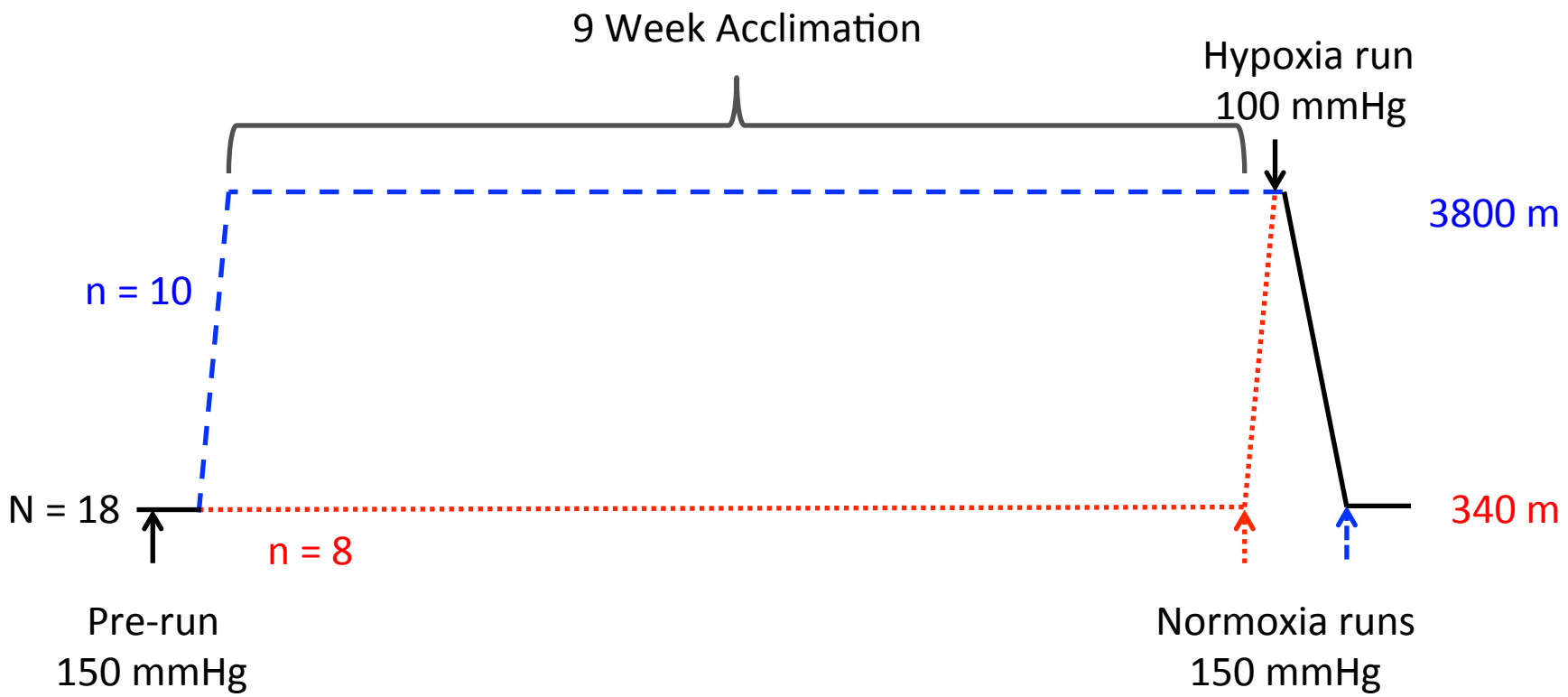
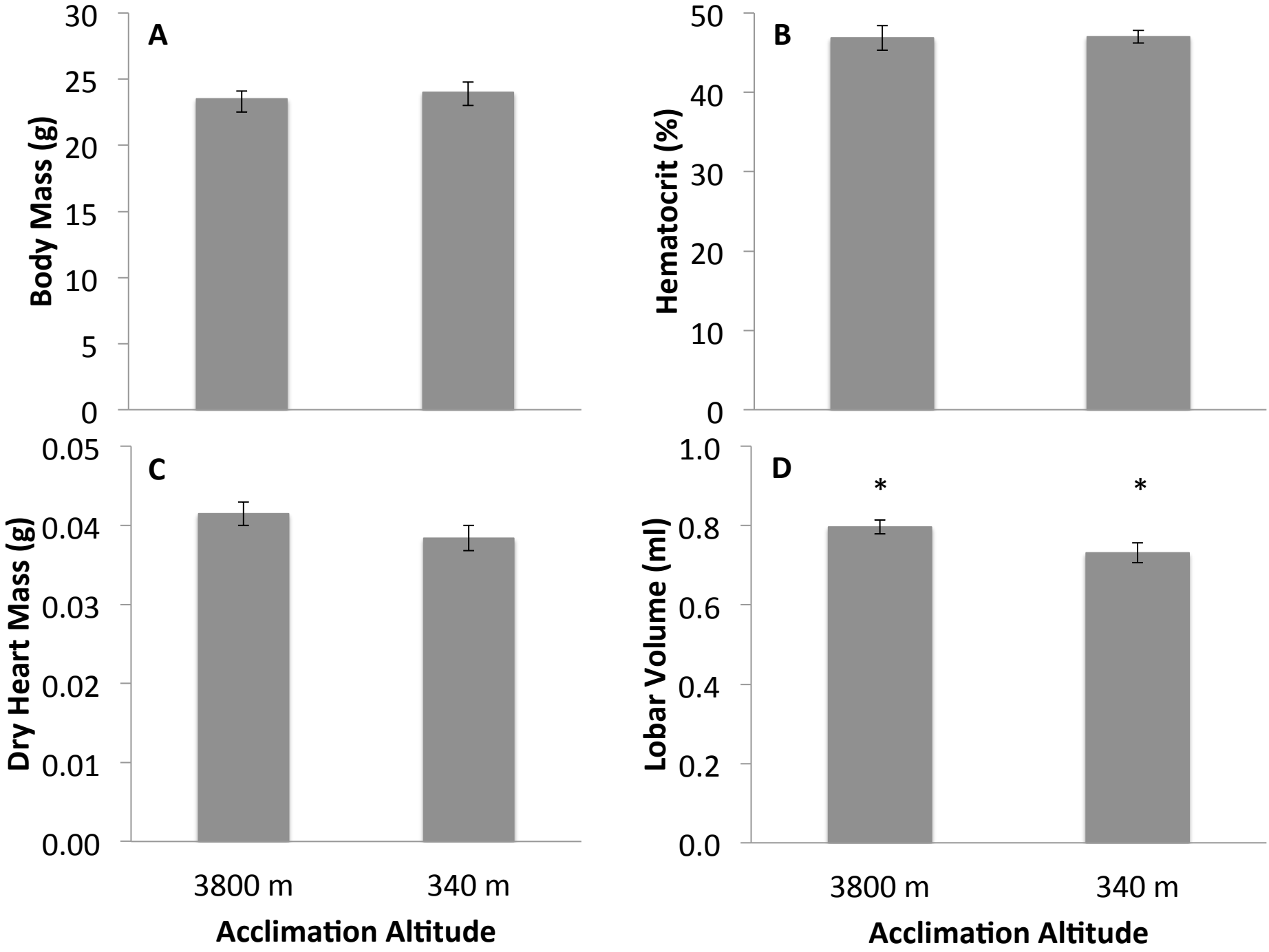
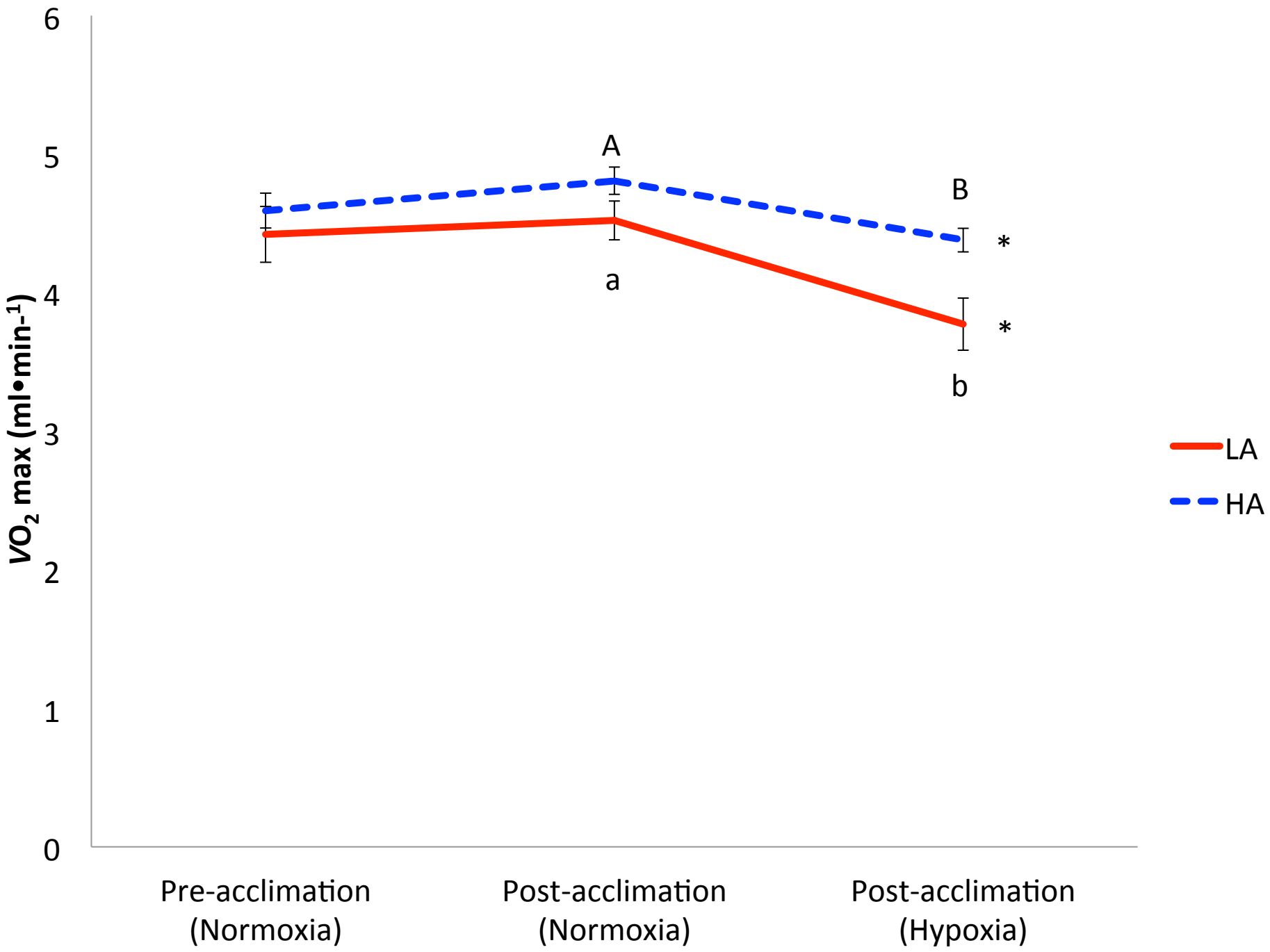
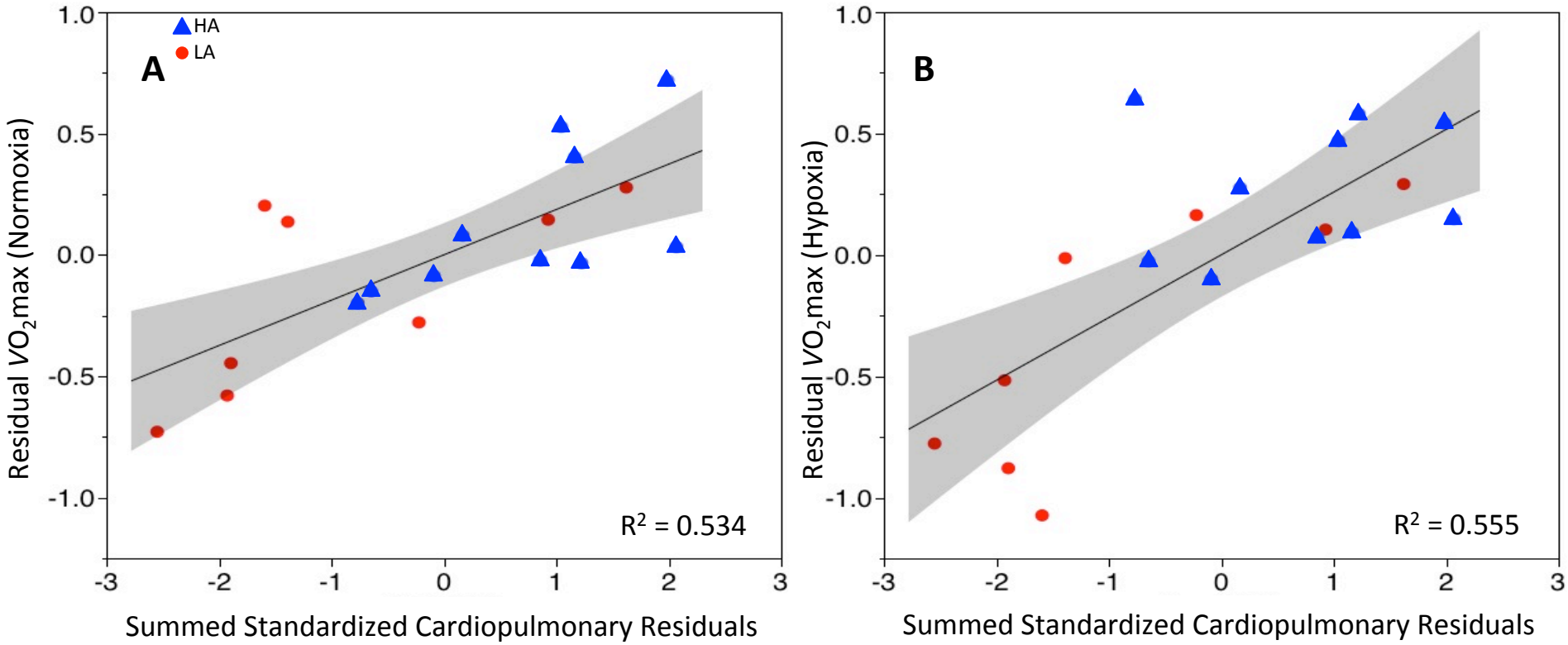

9 Week Acclimation
Hypoxia run
100 mmHg
3800 m
n = 10
N = 18
n = 8
340 m
Pre-run
150 mmHg
Normoxia runs
150 mmHg


A
Body Mass (g)
30
25
20
15
10
5
0
B
Hematocrit (%)
50
40
30
20
10
0
C
Dry Heart Mass (g)
0.05
0.04
0.03
0.02
0.01
0.00
D
Lobar Volume (ml)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
3800 m
340 m
Acclimation Altitude
3800 m
340 m
Acclimation Altitude


6
5
4
3
2
1
0
VO_2 max (ml•min^{-1})
A
B
a
b
*
*
LA
HA
Pre-acclimation (Normoxia)
Post-acclimation (Normoxia)
Post-acclimation (Hypoxia)


A
HA
LA
Residual VO_2max (Normoxia)
R^2 = 0.534
Summed Standardized Cardiopulmonary Residuals
B
Residual VO_2max (Hypoxia)
R^2 = 0.555
Summed Standardized Cardiopulmonary Residuals