CHANGES IN LUNG MORPHOLOGY IN HIGH AND LOW ALTITUDE DEER MICE

By

Melissa Sawaya

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APPROVED

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Dr. Kimberly Hammond
Department of Ecology, Evolution, and Organismal Biology

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Dr. Richard Cardullo, Howard H Hays Chair and Faculty Director, University Honors
Interim Vice Provost, Undergraduate Education

Abstract

Peromyscus maniculatus (deer mice) inhabit various altitudes, and studies have shown that different phenotypic changes occur in high altitude mice to compensate for hypoxic conditions (Hammond et al., 2001, Shirkey and Hammond 2014, Sortz et al., 2010). Of the phenotypic changes that are present, those in the lungs were the area of interest. In this study, the lungs of the low altitude deer mice (380m) and the high altitude deer mice (3800m) were compared, and lung mass of the high altitude group was larger than the 380m group (Shirkey and Hammond 2014). Many possibilities regarding lung morphology could explain what may be causing this increase in lung mass, but the exact plasticity is unknown. In this study, we first predicted that high altitude lung morphology would be different from low altitude mice. Second, lung morphology within the lobes of a mouse would not change, but lobes across the groups would change. Lastly, we predicted that due to hypoxic conditions, high altitude mice would have an increase in alveolar size compared to low altitude mice. A split plot design showed that there were no significant differences of lung morphology between the groups and across the mice' lobes.

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Introduction

In order for some species to survive when their original habitat is changed, they must find ways to thrive in their new environment without impeding their life's demands. In birds, metabolic processes are maintained when traveling from low altitude to high altitude (> 3500 m) due to phenotypic changes that occur, for example, in oxygen-hemoglobin binding and tissue's utilization of oxygen (Sortz et al., 2010). Similarly, this phenomenon is seen in many other animals and one such species is *Peromyscus maniculatus* (Deer mice). Deer mice inhabit various altitudes, and those living at high altitudes must compensate for the effects caused by low temperatures and depleted partial pressure of oxygen (Hammond et al., 2001). Such effects include an increase in metabolic rate since colder temperatures require more heat to be produced by the organism, and a decrease in maximum oxygen consumption due to a decrease in the partial pressure of oxygen at high altitudes. As a result to lower partial pressures of oxygen, oxygen delivery to the tissues is compromised due to less oxygen carried in the blood. Without any acclimation through plasticity, deer mice would not be able to overcome the stress caused by the high altitude environment leading to minimal chances of survival (Garland and Carter 1994). However, deer mice are able to thrive at high altitudes (> 3800m) indicating some sort of phenotypic plasticity.

Plasticity in deer mice has been seen in some organs such as the small intestine, but for this study we focused on understanding the plasticity that happens in the lungs (Hammond et al., 2001). A past study showed that high altitude (3800m) deer mice under hypoxic conditions have the same level of aerobic performance as low altitude (380m) deer mice under normoxic conditions. This is significant because even though there is a decrease in the partial pressure of

oxygen, phenotypic plasticity allows the high altitude mice to perform just as well as the low altitude mice (Shirkey and Hammond 2014). In addition, high altitude (> 2750m) native deer mice have higher hemoglobin-oxygen affinity in their lungs compared to low altitude (< 1750m) native deer mice allowing for the maintenance of aerobic respiration. This is due to allosteric regulators that are found in the former group of mice compared to the latter, but the sensitivity to these regulators are altered at high altitude as a result of acclimation. Thus there is less of an effect caused by the allosteric regulators due to the decrease in sensitivity resulting in higher hemoglobin-oxygen affinity. These findings are summarized in multiple publications (Sortz et al., 2009, Sortz et al., 2010, Sortz 2016). Similarly, high altitude mice (3800m) have a higher hematocrit level than mice at low altitude (380m) (Hammond et al., 2002). Past experiments have also shown that mice at high altitudes have larger lungs than mice at low altitudes under hypoxic conditions (c.f., Rezende et al., 2009, Chappell et al., 2007). In addition, a study used a rebreathing technique developed by Dr. Connie Hsia to show the same finding. In this study, the deer mice that were used were all born at low altitude. Before the study, maximal aerobic capacity was measured on all mice at 390m above sea level as a baseline measurement. Half of the mice were then moved to 3800m above sea level and half of the mice remained at 390m. To allow for acclimation to hypoxic or normoxic conditions, mice stayed in their assigned environment for a period of nine weeks. After this period of time, aerobic capacity was measured again. After the data had been collected, the heart and lungs were extracted and measured to obtain their mass (Shirkey and Hammond 2014, Russell et al., 2008), and the amount of oxygen transport across the alveoli (Hsia et al. *in preparation*). The results were consistent to past studies (Hammond et al., 2001, Rezende et al., 2009, Chappell et al., 2007). A larger lung

volume and greater oxygen transport across the alveoli were seen in high altitude mice in order to make up for the change in the partial pressure of oxygen.

Although these studies have suggested that these differences in lung size could result from an increase in alveolar size or number, increase in number of capillaries, surfactant-producing cells, and oxygen carrying cells, it has not been determined what exactly is changing in its plasticity. By observing these lungs microscopically, we will be able to deduce what factor of the anatomy of the lung contributes to the increase in lung mass of high altitude mice found in the previous studies (Shirkey and Hammond 2014). If this method supports that there are differences between the groups, it will provide further evidence supporting the idea that some characteristics of the lungs in mice have the capability to change as mice acclimate to high altitudes.

We hypothesized that lobes from mice within the same group will be unchanged, whereas lobes from the different groups, low altitude and high altitude mice, will vary. With this hypothesis, three predictions arose. First, we predicted that the distribution of lung tissue in all five lung lobes will be similar in each mouse. Second, we predicted that the distribution of lung tissue between the groups' lobes and whole lung section will be different. Lastly, we predicted that, due to the hypoxic conditions, high altitude mice would have an increase in alveolar size compared to low altitude mice.

Methods

Mice

The lung sections used for this study came from using eight different deer mice. Half of the deer mice were stationed at 380m and the other half were acclimated to 3800m (Shirkey and Hammond 2014); however, the sections were numbered and there was no information about the treatment for each animal so that there could be no bias in data collection. Deer mice have five different lung lobes: superior lobe, middle lobe, inferior lobe, left lobe, and cardiac lobe. Three random slices from each lobe were cut at different sites, and the second slice was used. Once the lung sections were obtained they were put into blocks to be prepared for further slicing. This totaled 40 blocks, each with one lung section.

Preparation of slides

Slicing: Every mouse had five blocks (16-mouse #-C2, I2, L2, M2, and S2). A lung section (C2, I2, L2, M2, and S2) was embedded in each block and sliced on a Sorvall microtome using a manual rotator to make slices. Glass knives were made by using a 7800 knife maker by LKG. Once the glass blade was made, which was replaced after two blocks due to knife dullness, it was put in the knife slot on the microtome. The block and the knife were both adjusted to ensure that the whole lung section was sliced. Once properly aligned, manual rotations resulted in thin (3.5um) slices, and each slice was then placed in a water bath to ensure proper unfolding. A slide was then cleaned with 70% ethanol, dipped in the water, and by using an eyelash brush the lung slice was moved above the slide. The slides were then dried on a hot plate for 10 minutes under a medium heat setting, allowing the lung slices to become fixed to the slide. To account for possible damage, two lung slices were placed on each slide.

Staining: After 24hrs, the lung slices were stained to visualize the potential differences in the anatomy of the lungs. This process required Permount, 1% Toluidine Blue, deionized water, and cover slips. The Toluidine Blue dye was used because it stains tissue and nuclei. This staining procedure resulted in clear visualization of the anatomical structures of interest. Permount was used to adhere the coverslip on top of

the lung slices, and deionized water was used as the wash to remove excess Toluidine Blue. The slides were allowed to dry for 20-30 minutes. Once completely dry, a cover slip was then quickly placed on the slide. Any air bubbles on the lung sample were gently removed by sliding them off the lung tissue with a plastic pipette or by adjusting the coverslip.

Imaging:

Each slide was imaged under a BZ-X700 series Keyence Fluorescence microscope using a brightfield, single-color setting and under 20x magnification. One of the lung sections was located using the 2x setting and ten random points on the lung section were chosen to eliminate any choice of tissue type bias. The random points were chosen while the image was still in the 2x magnification by simply dragging the lung section at ten different random spots and setting the positions through the BZ-X image program. The magnification was then switched to 20x to visualize lung structures, and ten images of each lobe of every mouse were captured (**see figure 1)**.

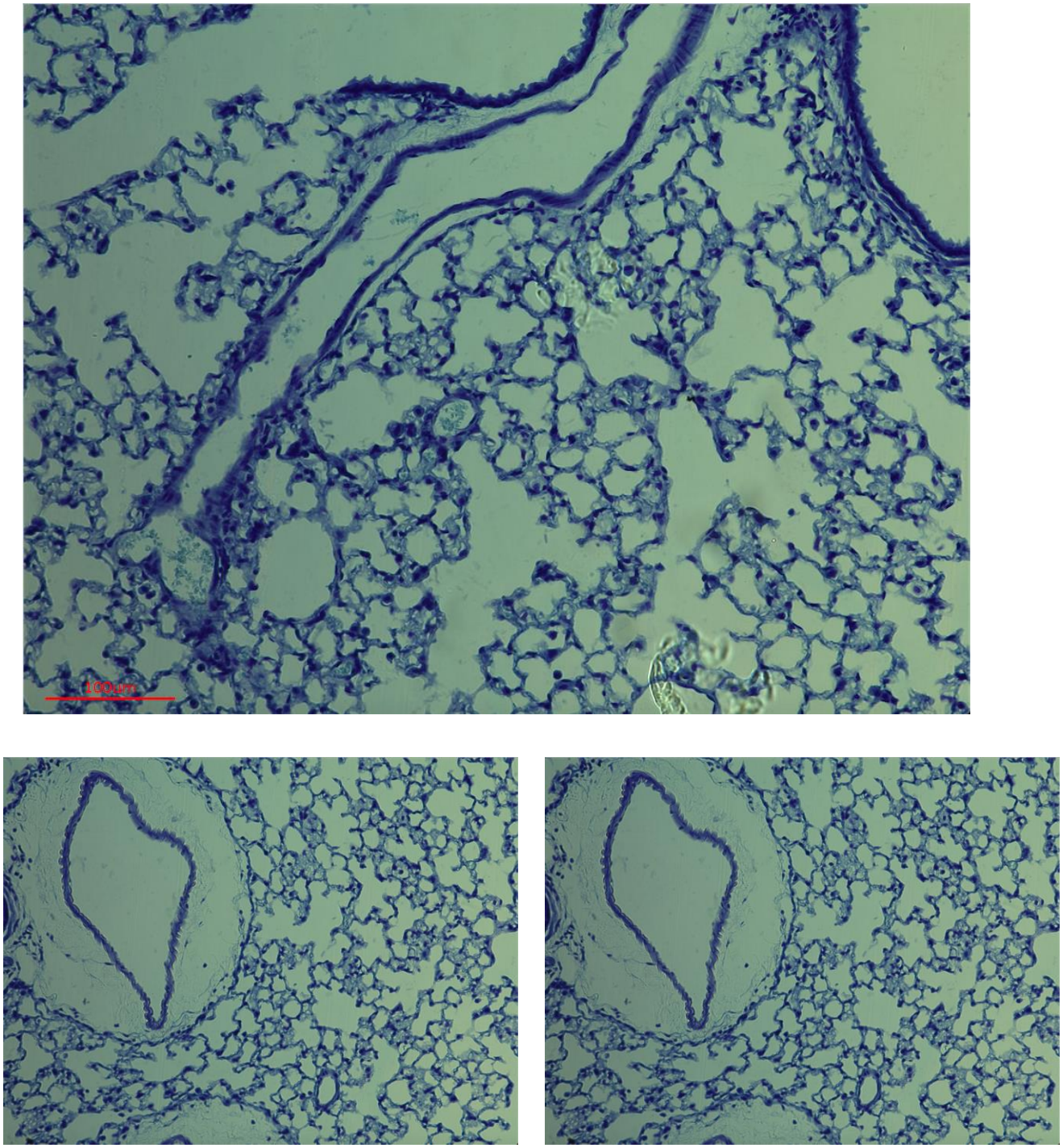

Figure 1 Three of the ten images of Mouse #1's left lobe taken using the BZ-X700 series Keyence Fluorescence microscope. The top image has a scale of 100um placed in its left corner.

Each slide was imaged under a BZ-X700 series Keyence Fluorescence microscope. This resulted in a total of 400 pictures (8 mice x 5 lobes x 10 pictures of each lobe). After one slide had all 10 of its images captured, a scale of 100um was added to the corner of only the first image. This scale was used to analyze the pictures through Image J.

Analysis and Accumulation of Data

For each image created, data on the number of alveoli, septa, ducts, sacs, and non-parenchyma (blood, large airways, and blood vessels) was collected at a setting of a 100um scale, a 250um^2 grid size, and a scale of 2.63 pixels/um **(see figure 2).**

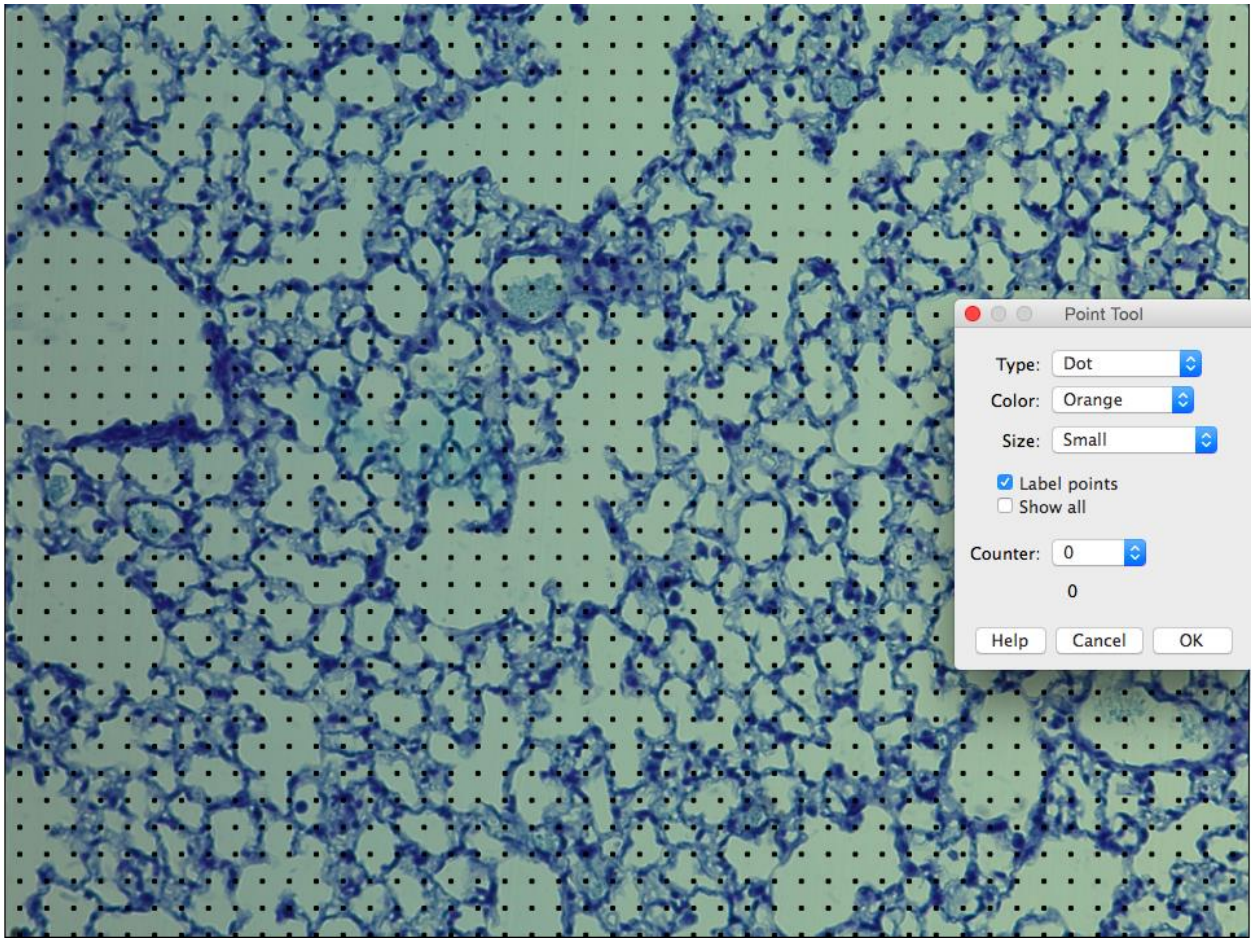

Figure 2 A scale of 100um was applied, a grid of 250um^2 was set to result in points on the lung slice (Black points). The point tool, also known as the cell counter, was used to keep track of the number of points that fell in every structure of interest.

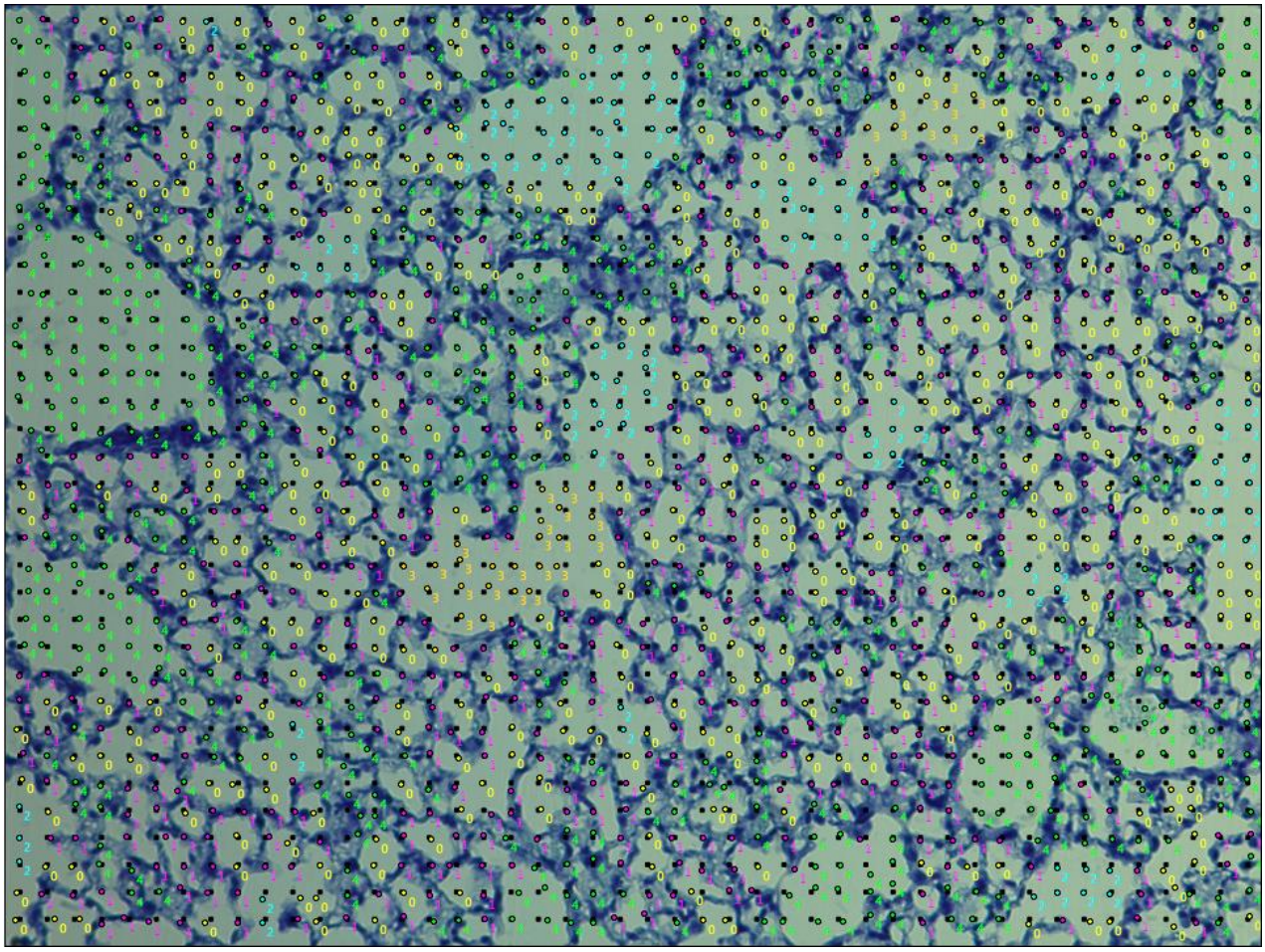

Figure 3. The end result of **Figure 2.**

The number of alveoli, alveolar septa, ducts, sacs, and non-parenchyma was obtained by clicking on the points that fell on each structure. A cell counter was used to keep count of the number of points that fell on each structure (Counter 0 = points that fell within alveoli, counter 1 = septa, counter 2 = sacs, counter 3 = ducts, and counter 4 = non-parenchyma, **see figure 3**). After the image was analyzed, the number of each of the structures was recorded along with the animal number, lobe, and section. This was done for 2 random pictures.

Summary Statistics:

We calculated the mean, standard error, and the coefficient of variation of diffusive tissue (alveoli, septa, sacs, ducts), non-diffusive tissue (non-parenchyma), and septal volume. Septal volume was computed as the ratio of septa to diffusive tissue (**see figure 7)**. The mean of each structure in the entire lung was compared between mice at high altitude and low altitude. The standard error was used as the value of the

error bars found on the histograms, and this enabled better visualization (**see figures 5-8**). To summarize the differences in lung structure within the different lobes, the mean of each structure in every lobe was compared between the groups, and standard error was used for the error bars (**see figures 9-14**).

Analyses: There were two types of statistical analysis used for this data. First, a simple two-sample multivariate Analysis of Variance (ANOVA) was used to determine the summed difference between the high and low altitude treatments. All structures (alveoli, sacs, ducts, septa, and non-parenchyma) in the 5 lobes were summed, and this analysis allowed a determination of the effect of altitude on the whole lung.

Second, a statistical analysis was used to determine the differences between the lobes of the mice at each altitude. This analysis required a Split Plot Design, and it allowed us to examine differences between the different lobes within a treatment group (high or low altitude) as well as between the high and low altitude groups.

For all statistical analyses we chose a p-value of 0.05.

Results

Differences Between High and Low Altitude Lungs

***Diffusive Structures*:** High altitude mice had 3% fewer alveoli (high mean: 367 low mean: 379), 8% more septa (high mean: 419 low mean: 385), 3% more sacs (high mean: 127 low mean: 123), and 4% fewer ducts (high mean: 33 low mean: 34) than low altitude mice. A p-value less than 5% was statistically significant ($p < 0.05$). These differences in diffusive structures between high and low altitude mice were not statistically significant ($p > 0.05$, **see figure 4**).

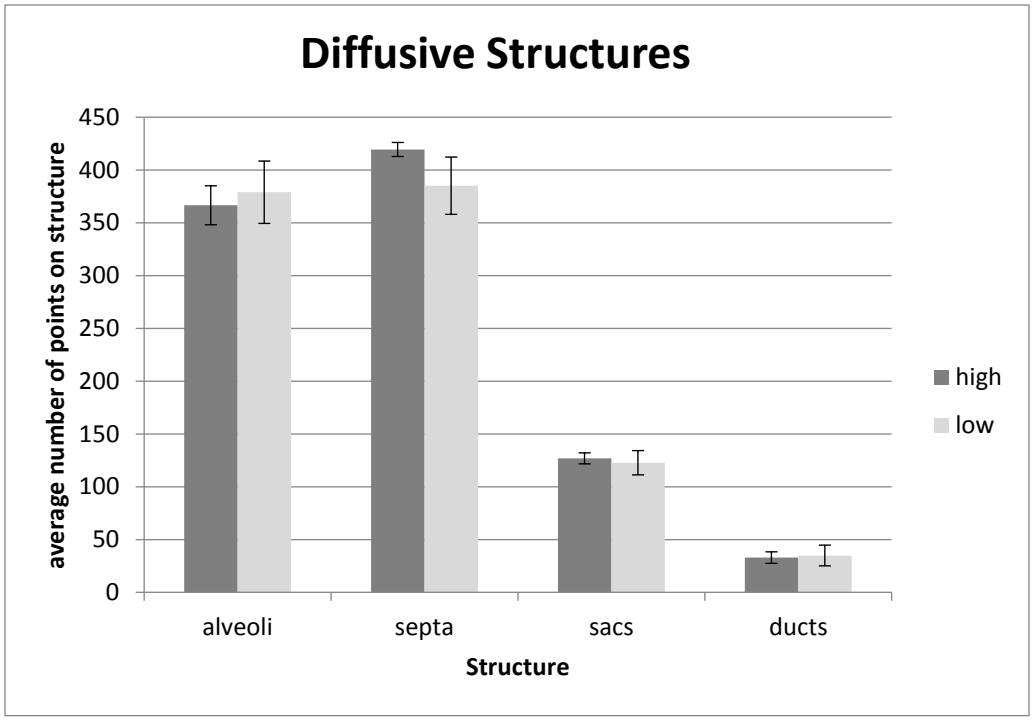

Figure 4. Average number of diffusive structures between the groups.

Airspace: There were no statistically significant differences seen in the number of alveoli, sacs, and ducts between high altitude and low altitude groups (**see figure 5**).

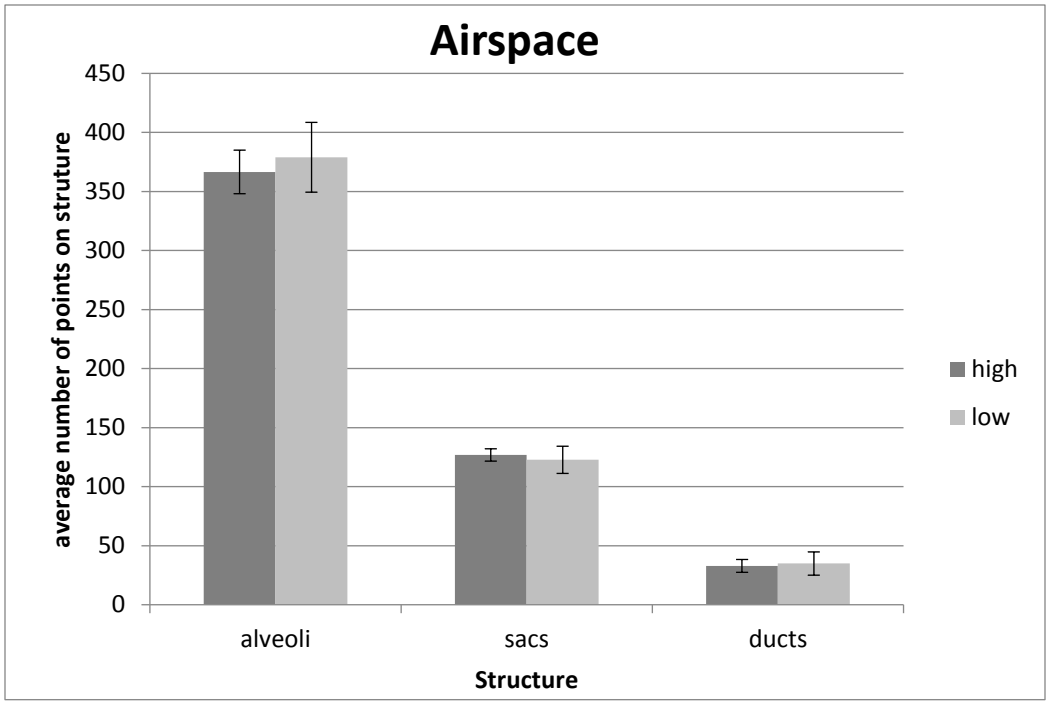

Figure 5. Average number of alveoli, sacs, and ducts between the groups.

***Transport Tissue*:** There was not a statistically significant difference in septa between high and low altitude groups (**see figure 6**).

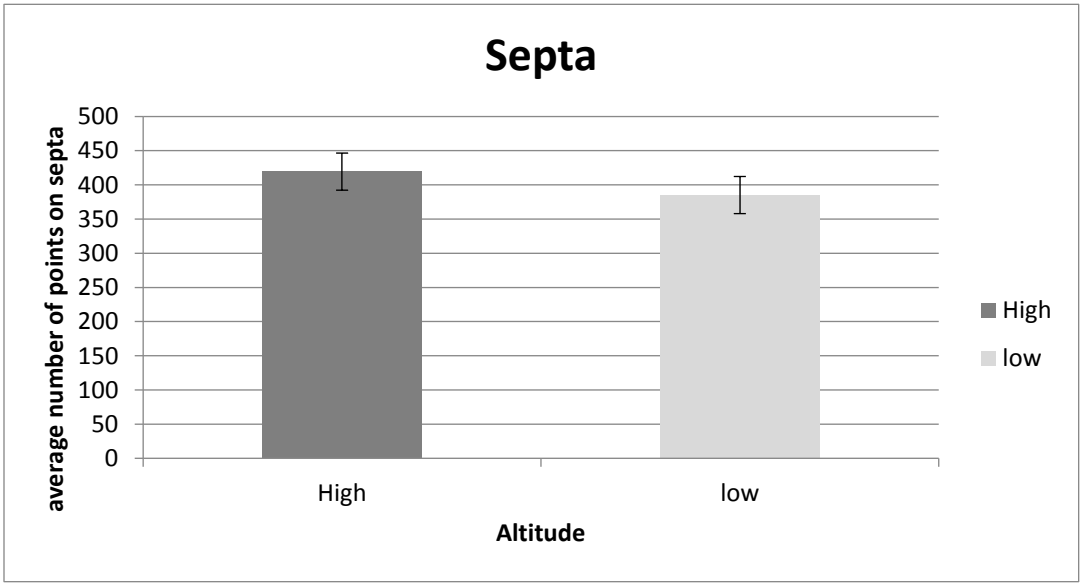

Figure 6. Differences in transport tissue between the groups.

Septal Volume: High altitude mice had 5% more septal volume (high mean: 0.4451 low mean: 0.4221) than low altitude mice. This result was not statistically significant (**see figure 7**).

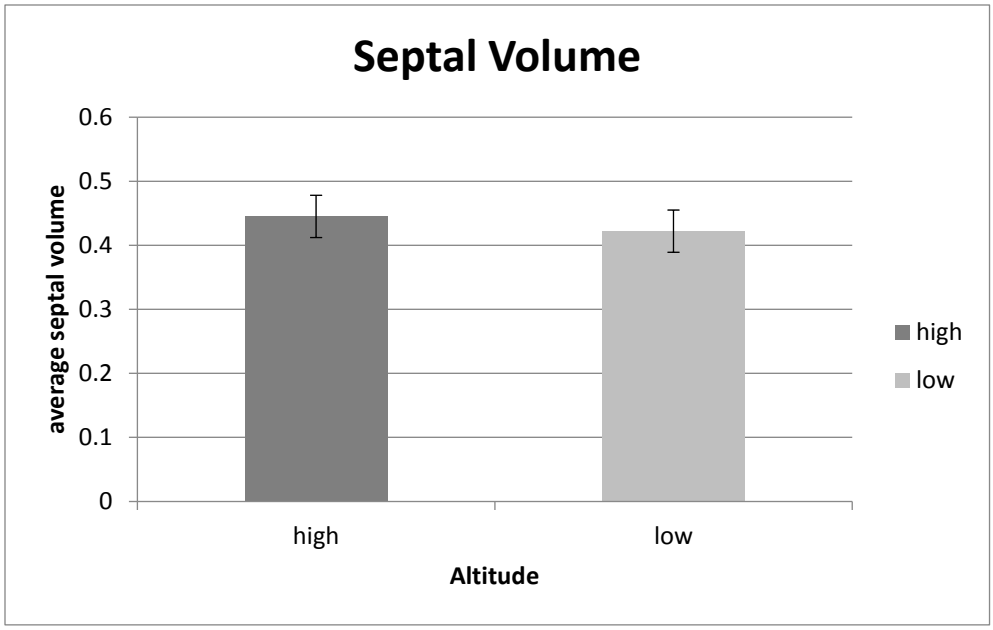

Figure 7. Difference in septal volume between the groups.

Non-Diffusive Structures: High altitude mice had 14% fewer non-diffusive structures (non-parenchyma) than low altitude mice (high mean: 398 low mean: 457). There was no statistically significant difference between the groups (**see figure 8**).

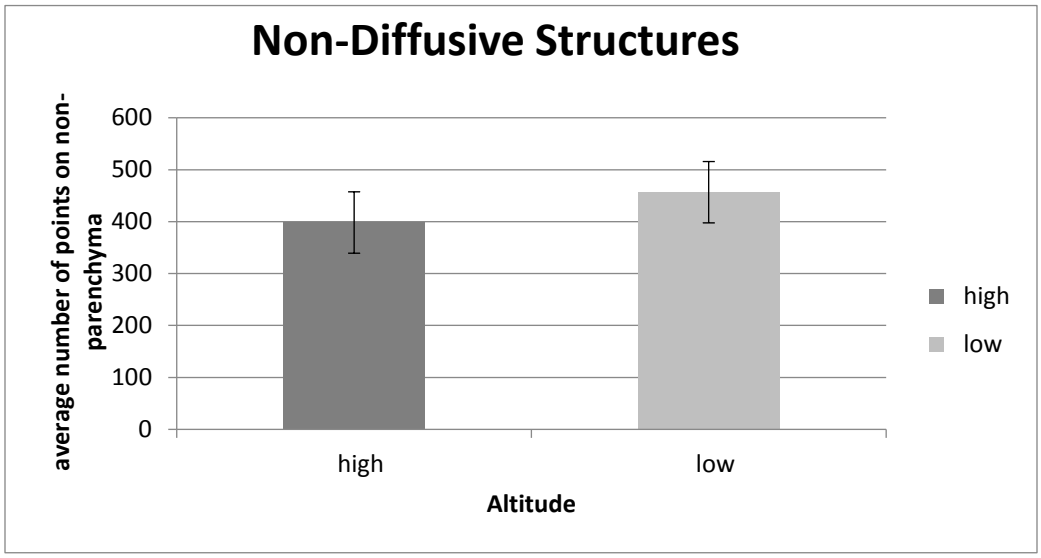

Figure 8. Differences in non-parenchyma between the groups.

Differences in lobes between the groups:

Alveoli: Average number of alveoli found: cardiac lobe (high mean: 338 low mean: 431), inferior lobe (high mean: 350 low mean: 351), left lung (high mean: 428 low mean: 343), middle lobe (high mean: 385 low mean: 357), and superior lobe (high mean: 325 low mean: 414). The number of alveoli in the lobes of high and low altitude mice was not statistically significant (**see figure** 9).

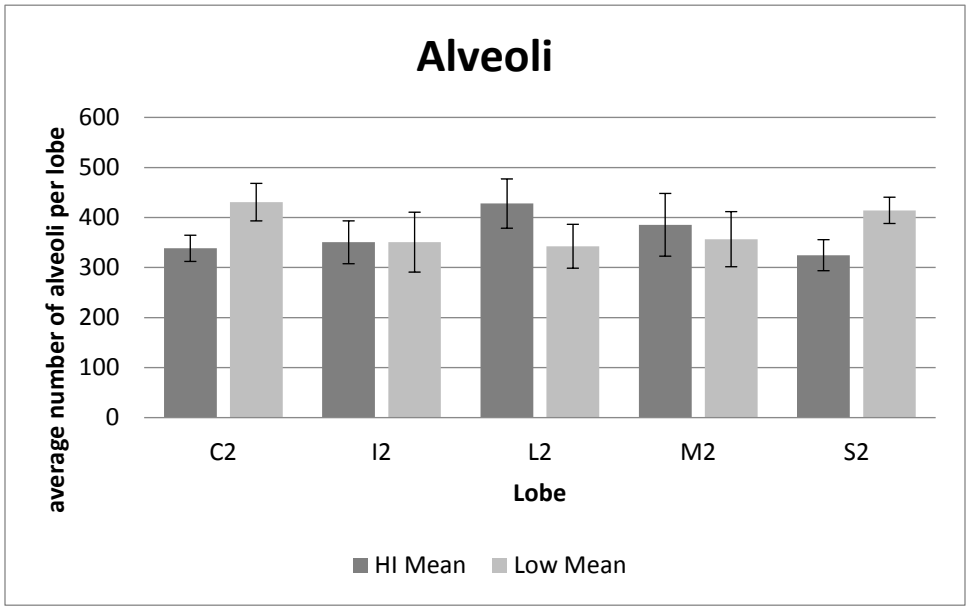

Figure 9. Differences in alveoli across the lobes.

Septa: Average number of septa found: cardiac lobe (high mean: 361 low mean: 434), inferior lobe (high mean: 385 low mean: 363), left lung (high mean: 515 low mean: 374), middle lobe (high mean: 471 low mean: 349), and superior lobe (high mean: 371 low mean: 405). No statistically significant differences were found in any of the lobes (**see figure 10**).

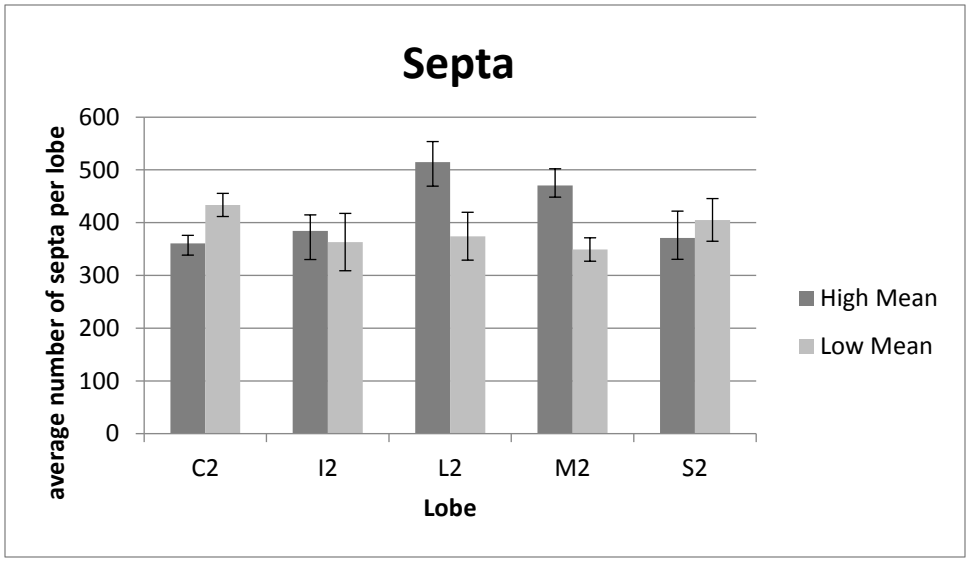

Figure 10. Differences in septa across the lobes.

Sacs: Average number of sacs found: cardiac lobe (high mean: 115 low mean: 162), inferior lobe (high mean: 119 low mean: 101), left lung (high mean: 140 low mean: 115), middle lobe (high mean: 153 low mean: 110), and superior lobe (high mean: 109 low mean: 126). There were no statistically significant differences in sac distribution between the lobes (**see figure 11**).

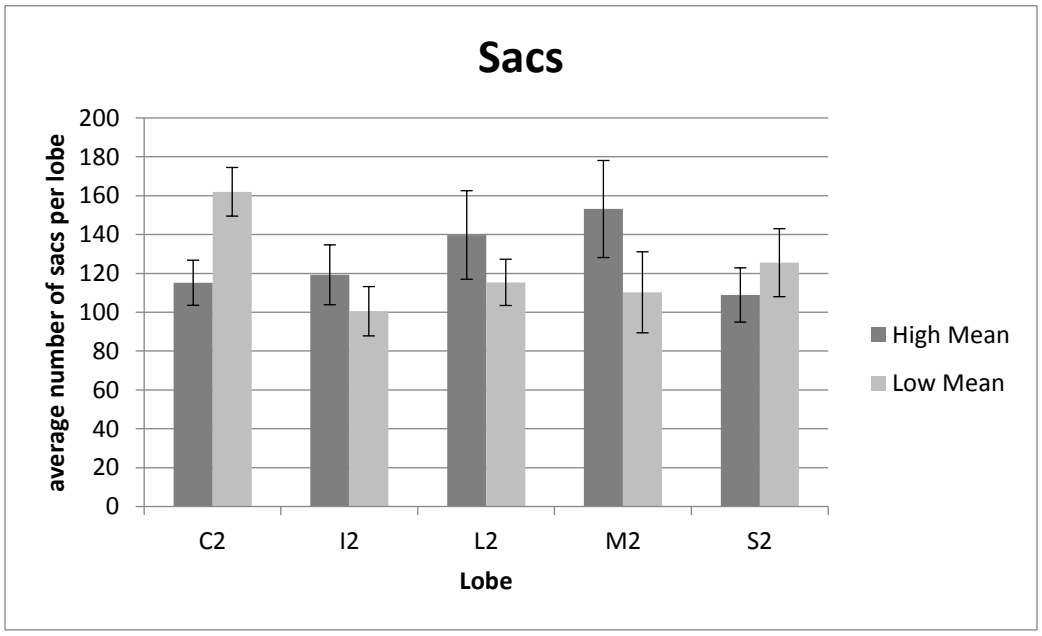

Figure 11. Differences in sacs across the lobes.

Ducts: Average number of ducts found: cardiac lobe (high mean: 57 low mean: 48), inferior lobe (high mean: 29 low mean: 47), left lung (high mean: 8 low mean: 24), middle lobe (high mean: 28 low mean: 33), and superior lobe (high mean: 41 low mean: 20). There were no statistically significant differences in ducts between the lobes (**see figure 12**).

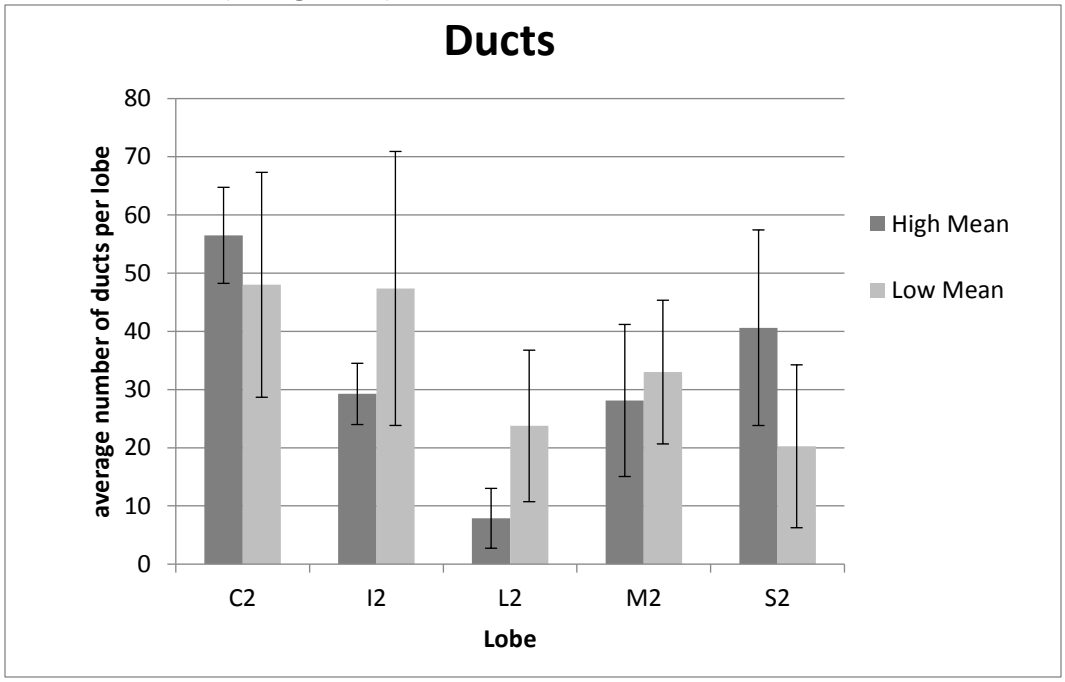

Figure 12. Differences in ducts across the lobes.

Non-Parenchyma: Average number of non-parenchyma found: cardiac lobe (high mean: 408 low mean: 371), inferior lobe (high mean: 335 low mean: 388), left lung (high mean: 382 low mean: 554), middle lobe (high mean: 419 low mean: 495), and superior lobe (high mean: 508 low mean: 476). There were no statistically significant differences in non-parenchyma between the lobes (**see figure 13**).

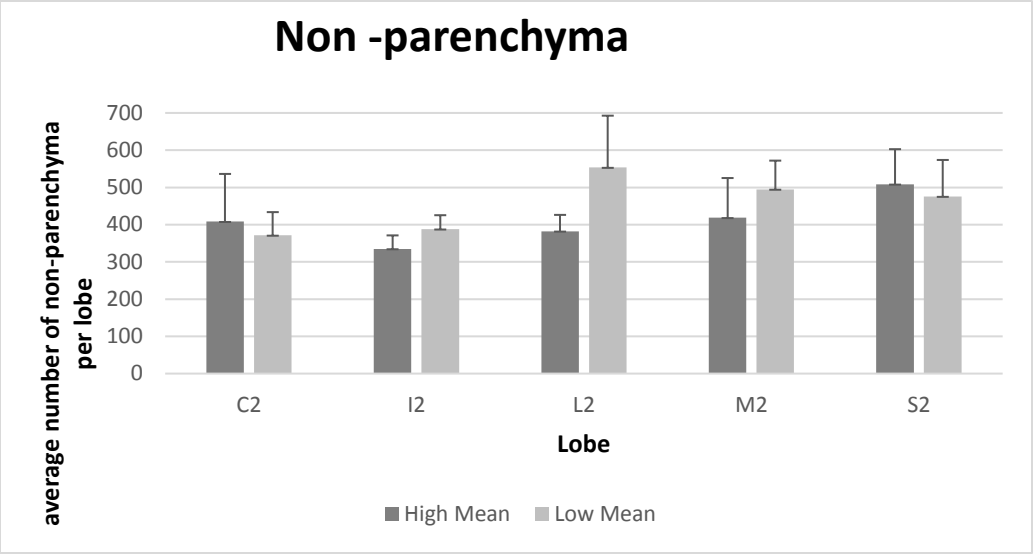

Figure 13. Differences in non-parenchyma across the lobes.

Septal Volume: Septal volume found: cardiac lobe (high mean: 0.4157 low mean: 0.4065), inferior lobe (high mean: 0.4368 low mean: 0.4241), left lung (high mean: 0.4769 low mean: 0.4360), middle lobe (high mean: 0.46 low mean: 0.4237), and superior lobe (high mean: 0.4382 low mean: 0.4204). There were no statistically significant differences in septal volume between the lobes (**see figure 14**).

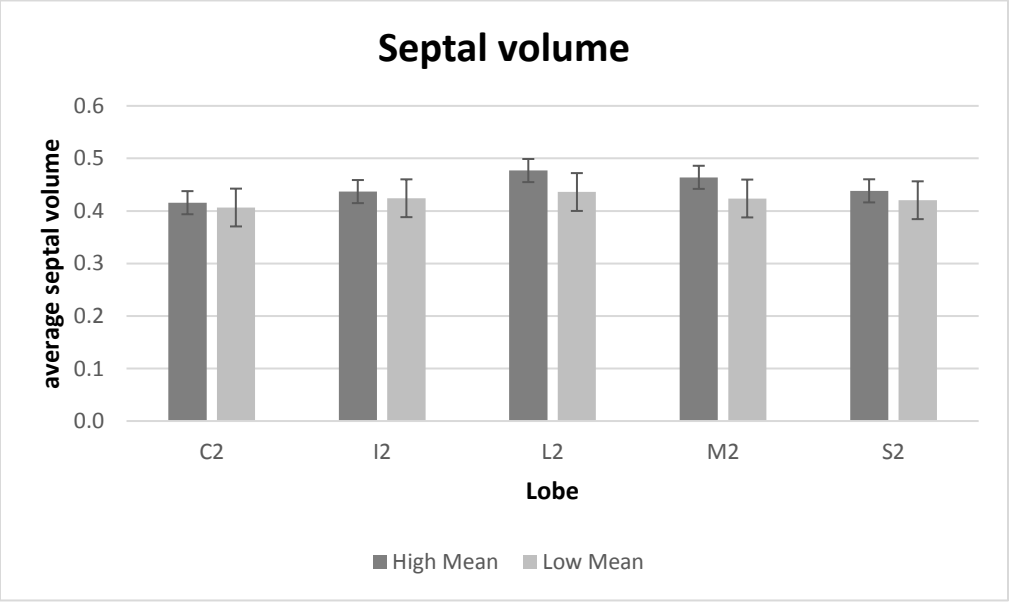

Figure 14. Differences in septal volume across the lobes.

When taken as a whole, and despite the consistent differences between the high and low altitude groups for some lobes, there were no statistically significant differences in alveoli, septa, sacs, ducts, non-parenchyma, septal volume, alveoli and sacs, and airspace between and within the groups (**see figure 15**).

ANOVA	***p-Value***
Alveoli	0.73407091
Septa	0.265824118
Sacs	0.75518012
Ducts	0.891439572
Non-parenchyma	0.434375708
Septa Volume	0.532219162
Alveoli and Sacs	0.847490871
Airspace	0.830962132

Figure 15. P-values for diffusive and non-diffusive tissue. ($p > 0.05$ was not statistically significant)

Discussion

Due to decreasing partial pressures of oxygen (pO2), it is reasonable to suggest that high altitude deer mice would have changes in their lung morphology not seen in low altitude deer mice. In addition, past studies showed that lung plasticity did occur when deer mice were acclimated to high altitude (Chappell et al., 2007, Rezende et al., 2009, Russell et al., 2008, Shirkey and Hammond 2014). Shirkey and Hammond (2014) showed that lung volume also increases in mice after they were acclimated to high altitude. Our current study tries to pinpoint what morphological character in the lungs is causing the increase in volume. However, our results indicated that there were no statistically significant differences in lung morphology that could be tied to lung volume between high and low altitude deer mice.

Although the results were not statistically significant, we found that high altitude mice had 3% fewer alveoli and 8% more transport tissue than low altitude mice. Since septa were scored as the border of the alveoli, more septa could correspond to larger alveoli surface area and therefore larger alveolar volume. If larger alveoli were prevalent in high altitude mice, then we would have to see more septa and a greater septal volume, which were represented by our data. As a result, larger alveoli may compensate for the decrease in pO2 and explain the increase in oxygen transport across the alveoli seen in high altitude mice (c.f., Rezende et al., 2009, Chappell et al., 2007). Further analysis of the data, such as measuring the size of alveoli, could provide us with more insight. In addition, a previous study (Shirkey and Hammond 2014) showed that despite the decrease in pO2, high altitude mice had a similar aerobic capacity to low altitude mice. This could possibly mean that one way lung morphology changes is to have more diffusive and transport tissue to aid in diffusion, allowing the mice to acclimate to the environmental conditions faced at 3800m. This was supported by our study showing that high altitude mice had less non-diffusive tissue than diffusive and transport tissue.

We predicted that lung tissue distribution in the lobes would be different between the groups, but they shouldn't vary within the group. In other words, if there was variation in lung morphology between the groups, this variation would be uniform across all five lobes. Therefore, it was unexpected to find that

the lobes differed within each group. Even though the results were not statistically significant, it is important to note this finding. We found it interesting that the cardiac and superior lobe were similar in terms of their plasticity (high altitude mice had more ducts, non-parenchyma, and greater septal volume than low altitude mice in both lobes). Similarly, the middle lobe and left lung both had more alveoli, septa, sacs, and greater septal volume in high altitude mice. More studies could be done to test the individual plasticity of the lobes, and if these future studies support individual plasticity it could lead us to the idea that the lobes in deer mice might not change in their morphology uniformly, but rather independently of the other lobes.

This study could be revisited with the availability of more data. Some of the images we analyzed may not be good representations of the lung section due to lung tissue only filling half of the area of analysis. Many of the lobes showed a large heterogeneity of tissue type, and the measurement in this study represented less that 20% of the overall tissue in any one lobe section. As a result, the analysis of all ten pictures representing each lobe of every mouse could possibly result in a more accurate representation of diffusive and non-diffusive structures among the lungs of the mice.

Despite not showing any statistically significant differences between the groups, our study opened the doors for more research questions. As mentioned, this study could have possibly been improved if all 400 pictures had been analyzed; however, even though we only analyzed 80 pictures our results were still interesting. Future studies should address the possibility of larger alveoli in high altitude mice by measuring the diameter of the alveoli, since we still don't know if the high altitude mice had a greater alveolar volume. Furthermore, we predicted that lobes within a group would be uniform in their plasticity, but this was not observed. As a result, it would be interesting to hypothesize why this observation was seen.

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