VARIATION IN CAPILLARY DENSITY BETWEEN LOW AND HIGH-ALTITUDE ACCLIMATED DEER MICE

By

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Abstract

Deer mice, Peromyscus maniculatus, from the White Mountains, can live at high and low altitudes. High altitude conditions include lower oxygen content and lower temperatures. Deer mice acclimating to high altitudes must adjust to the reduced oxygen content in order to survive and reproduce. While our laboratory has noted increased tissue content in the lungs of high-altitude acclimated mice relative to low altitude mice (Sawaya, Honors Thesis 2018), we have not measured changes in blood flow or capillary content. We hypothesize that capillary density and overall cell number in lung tissue is greater in high-altitude acclimated mice than in low altitude mice to allow for increased oxygen intake. To analyze capillary density and cell number, we stained lung sections from deer mice at high and low altitudes. Stained slides were then imaged using fluorescence microscopy. Overall structure of the lung tissue was observed using an autoflourescent signal from the cytoplasm in cells. Individual cells were identified using a fluorescent nuclear stain and capillaries were observed using a fluorescent probe directed at endothelial cells. All three fluorescent channels were subsequently evaluated using image processing software to quantify both cell number and capillary density. Understanding if capillary density plays a role in acclimation for deer mice will extend our knowledge of how this species adjusts to severe conditions. Final results show no statistically significant difference in capillary density between high-altitude acclimated and low altitude deer mice.

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I would like to acknowledge Dr. Kimberly Hammond, Dr. Richard Cardullo, and Dr. Catherine Thaler for their contributions. Thank you Dr. Thaler for teaching me how to carry out important lab procedures that were crucial in conducting this study. I am grateful for Dr. Cardullo who gave me this opportunity to join the lab and conduct my own research. I further want to thank Dr. Cardullo for not only helping me with my study but also for offering continuous guidance throughout my undergraduate education. I would additionally like to acknowledge Dr. Hammond for her faith in me as a researcher, her constant support, and for dedicating time to guide me throughout this project. All three of you have played a major role in my time as an undergraduate and I will continue to grow from the advice and lessons you have given me. Additionally, I would like to give thanks to my fellow lab member Sueann Lin who helped with coding the software used for image analysis.

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Introduction:

Acclimation is a gradual adjustment to changing environmental conditions. Many organisms must acclimate physiologically when they encounter a new habitat where they face unfamiliar and harsh physical characteristics. Deer mice are found at all elevations across North America and are distributed along a wide altitudinal gradient (Shirkey and Hammond, 2014). The White Mountains, which is location of the deer mice used for this study, has low altitude occurring at 380m while high altitude occurs at 3800m (Sawaya, Honors Thesis 2018). Compared to low altitudes, high altitudes have colder temperatures, reduced primary productivity, higher UV exposure, and lower oxygen content. Organisms living at this altitude face greater metabolic demands along with higher thermoregulation demands (Cheviron et al. 2012, Coronado, Honors Thesis 2017). Mice acclimating to high altitude must adjust to all these changes but accommodating for lower oxygen levels is most important.

A previous study indicated that high-altitude acclimated mice possess the same aerobic capacity at higher altitude as deer mice at low altitude (Shirkey and Hammond, 2014). The same study also found other morphological differences between low altitude and high-altitude acclimated mice. High-altitude acclimated mice have a larger mass of certain organs such as the heart and lungs. Although the mass of the heart was not significantly greater in high-altitude acclimated mice, the lung volume and mass difference in high-altitude acclimated mice is notably larger compared to low altitude mice (Shirkey and Hammond, 2014). The heart and lungs are important for oxygen transport throughout the body. A larger right ventricle in the heart allows a greater volume of deoxygenated blood to be pumped towards the lungs. Oxygen then leaves the lungs and enters the blood before moving to the left ventricle. A larger left ventricle

increases the amount of oxygenated blood received which subsequently reaches tissues all over the body. Furthermore, greater volume and mass of lungs allows for maximization of oxygen uptake. Such adjustments in cardiopulmonary organs are essential when dealing with hypoxia. Knowing that high-altitude acclimated mice perform roughly the same at high altitude as deer mice in low altitude, we can assume that the changes in heart and lung morphologies are responses to high altitude conditions.

This paper will focus on the specific morphological variations in lung size between low altitude and high-altitude acclimated mice. We believe the changes in lung physiology permit high-altitude acclimated mice to adjust to the lower oxygen content in their environment. Many aspects could be changing in the lungs to make them larger and heavier. Some potential factors include increase size or number of alveoli, capillaries, surfactant producing cells, and red blood cells. Alveoli in the lungs are responsible for rapid gas exchange. It was previously assumed that alveoli structures in the lungs of high-altitude acclimated mice increased, in turn allowing more oxygen uptake. A corresponding study conducted in our lab found that there is no significant difference in alveoli number between high-altitude acclimated and low altitude mice (Sawaya, Honors Thesis 2018). However, the same experiment found that high-altitude acclimated mice had an increased fine parenchymal tissue, which is the tissue associated with oxygen transport, compared to low altitude mice. The next possible morphological change is in the capillaries. Capillaries are cylindrical ducts in the lungs which are also used to exchange gases. Capillaries can change in two ways to allow more oxygen exchange. The capillary density can increase, or the capillary membrane can decrease. Increased capillary density would provide more room for oxygen to diffuse into the lungs. Decreasing the capillary membrane would increase the capillary diameter size and allow greater oxygen absorption. This paper will focus primarily on capillary

density as well as overall cell number in lung tissue between high-altitude acclimated and low altitude mice. We believe that high altitude acclimated mice have a greater capillary density and greater cell quantity in lung tissue compared to low altitude mice, which allows the former to accommodate for hypoxic conditions.

Materials and Methods:

General Experimental Design:

Eight deer mice (*P. maniculatus)* were used for this experiment. Four of the mice were born at low altitude (380m) and remained (*"acclimated"*) at that altitude for the duration of the experiment. The remaining four mice were born at low altitude and acclimated, as adults, to high altitude (3800m) for a period of nine weeks. Following the nine-week period, all mice were sacrificed at their respective acclimation altitude. Their lungs were subsequently inflated with 1% paraformaldehyde under 30 cm of pressure to reflect the inflation pressure of a live mouse and dissected.

Reagents

HEPES was purchased from Sigma-Aldrich and NaCl was purchased from Fischer Scientific. Isolectin B4 and Rhodamine were purchased from Vector Labs. Antifade was purchased from Invitrogen by Thermo Fisher Scientific.

Lung Imaging Preparation:

Deer mice lungs have five lobes.: the interior, cardiac, middle, left and superior. Three random 1 mm slices were cut from each lobe of every mouse. Of the three slices, the second one was placed inside an Immunobed plastic resin block. Sectioning of the lung tissues was completed previously by students in the laboratory (Coronado, 2017; Sawaya, 2018). Glass knives were made using a 7800 knife maker by LKG and then an individual glass knife was placed in the Sorvall microtome. A lung section enclosed in a block was then positioned on the microtome in front of the knife. The block and knife were both properly aligned to ensure that the knife would slice the entire lung section. Utilizing the manual rotator on the microtone, the

sections were cut to thickness of 3.5µm. After the sections were cut, they were placed on top of a water bath in order to unfold and then moved onto a microscope slide using an eyelash brush. The slides were then placed on a hot plate on medium setting for a minimum of ten minutes to ensure that the sections were fixed to the slides. The sections were subsequently placed on microscope slides and stained with various fluorescent probes as described below.

Stained slides were viewed using a Keyence BZ-X710 microscope under three different fluorescence channels to observe the overall structure of the cells, nuclei, and capillaries. Images were taken using 2x/NA 0.10, 20x/NA 0.75, and 40x/NA 0.95 objectives for each microscope slide. Once the images were digitally stored, the images were processed using ImageJ (NIH) and then quantified to determine nuclear and capillary densities for each section.

Once the sections were stuck onto the slides using the hotplate, they were stored in a slide container at room temperature for up to three days before images were collected on the microscope.

Fluorescent Labeling of Sections:

Prior to viewing slides under the fluorescence microscope, sections were stained in order to mark capillaries and nuclei. A 1 L solution of HEPES buffered saline (HBS) was prepared before each staining session to maintain the physiological pH of the samples and consisted of 10 mM HEPES (Sigma) and 120 mM NaCl (Fischer Scientific) at a pH of 8.2.

A PAP pen was used to draw a square around the sections on the slides and provided a thin hydrophobic film that localized the staining reagents on the tissue sections. Once the square was dried, slides were placed on a large petri dish with wet filter paper underneath to prevent sections from drying out.

A solution consisting of 2 mg/mL trypsin in HBS was placed on the sections to hydrolyze the extracellular matrix of endothelial cells, allowing fluorescently labeled reagents to reach their designated sites. The petri dish was covered to prevent dehydration of the tissue and the slides were incubated for one hour in the trypsin solution and then washed three times for ten minutes each in HBS.

A biotin blocking solution was added to the sections and the slides were incubated for fifteen minutes at room temperature. Afterwards, the slides were washed for five minutes in HBS and an avidin blocking solution was placed on the sections. Once again, the slides were incubated for fifteen minutes followed by a five-minute wash. The solutions blocked all endogenous biotin, biotin receptors, and avidin binding sites in the tissues. In turn, these steps minimized non-specific staining seen in the microscope from endogenous avidin, biotin, or biotin binding factors.

Following the washing steps, primary antibody isolectin B4 (Vector Labs) was added to the sections and diluted to a 1:50 ratio in HBS. Isolectin B4 recognizes glycoconjugates that are specific to endothelial cells in capillaries of non-primate mammals (Kirkeby and Moe, 2001). Two hundred microliters of the isolectin B4 reagents were used for each slide and were centrifuged at 10,000 rpm for ten minutes to remove particulates. The supernatant was then placed on the sections and the slides were incubated for two hours in a covered petri dish. The slides were then washed three times for ten minutes each.

Biotinylated-Rhodamine (Vector Labs), a fluorescent dye, was diluted 1:200 in HBS with 200 µL of this solution made for each slide. The solution was centrifuged at 10,000 rpm to remove particulates and the supernatant was placed onto the sections. The slides were incubated for thirty minutes. Biotinylated-rhodamine attaches to isolectin B4 which allowed the endothelial

cells of capillaries to be viewed using a fluorescence microscope. After incubation, the slides were washed three time for ten minutes each.

Finally, nuclei were labeled using the fluorescent DNA binding dye, Hoechst (Sigma). Hoechst was diluted 1:1,000 in HBS from a 10mg/ml stock solution and centrifuged at 10,000 rpm for ten minutes to remove particulates. The supernatant was placed on the sections and incubated for 1 minute. Following incubation, the slides were washed in HBS three times for ten minutes each and then dried on a paper towel. To minimize photo bleaching, Antifade component A (Invitrogen) was placed on top of the sections and coverslips were placed over the sections. The slides were stored in the dark at 4^{o}C until viewed with a microscope.

Imaging:

The labeled lung sections slides were viewed using a BZ-X700 series Keyence fluorescence microscope. The three channels we used were optimized for fluorescein (green channel), DAPI (blue channel), and Tetramethyl rhodamine isothiocyanate (TRITC; red channel). The green channel detected auto fluorescence from within the cytoplasm of the cells which allowed the overall structure of the lung sections to be observed. The blue channel was used to view the individual nuclei stained with Hoechst while the red channel was used to identify the capillaries labeled with the isolectin B (**Figure 1**). One image was taken per section using a 2x PlanApoλ objective while five were taken per section using a 20x PlanApoλ objective and three for each section using a 40x PlanApoλ objective. Each composite image consisted of four pictures, one from each channel and an overlay of all three. For the 2x magnification, images were taken of the whole section. For the larger magnifications, ten random points were chosen at 20x and five random points at 40x. After each spot was chosen, the image was focused and captured.

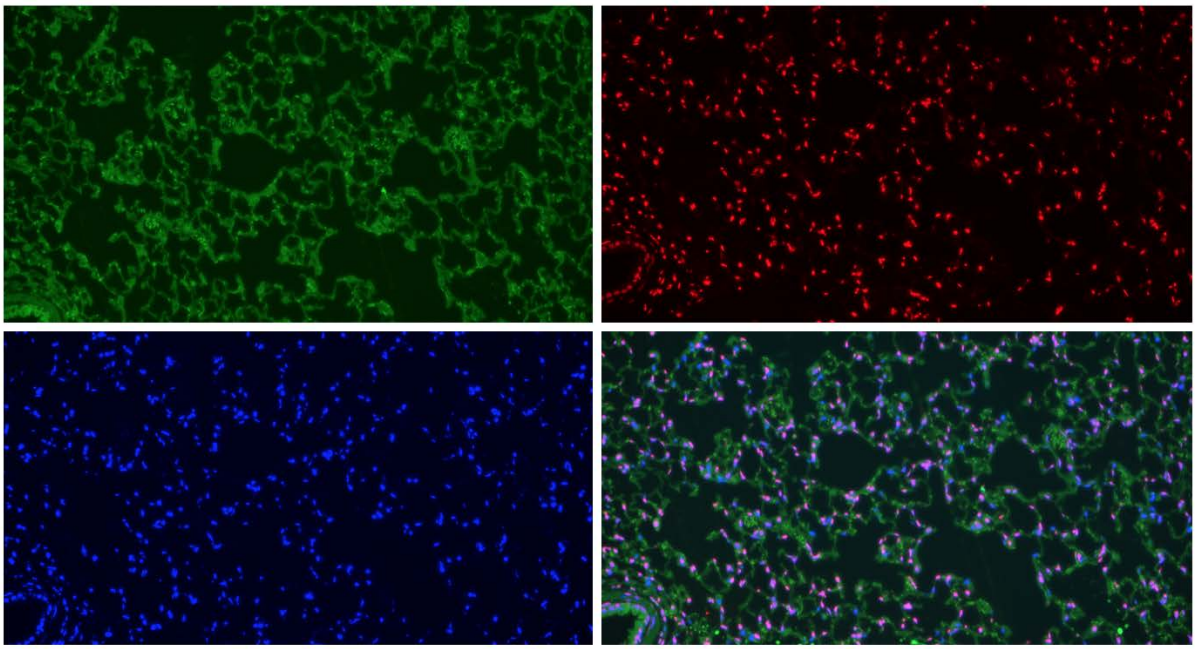

Figure 1: Image of a lung section at 20x magnification. The top row from left to right shows the Fluorescein and TRITC channels. The bottom row from left to right shows the DAPI and an overlay of all three images.

Image Processing and Analysis:

Following image capture on the Keyence microscope, the images were analyzed using the image processing software package ImageJ. Prior to analyzing the images to determine nuclear and cell densities, the images were processed to optimize the detection of discrete structures in all three fluorescence channels. First, each overlaid image was split it into its three component images, converted to 8-bit images, and the blue and red channels were thresholded resulting in a thin outline of individual nuclei and capillaries respectively. The blue and red channel images were then analyzed individually by the software yielding total cell and capillary number for the respective image (**Figure 2**) This process was repeated for all the images at 20x magnification at a scale of 1.15 µm/pixel. The process was automated by writing a script using

code within ImageJ that would automatically threshold the images and give the total cell and capillary count **(Figure 3)**. Data was downloaded to an Excel spreadsheet for each analyzed image and threshold minima and maxima were recorded. For images that were taken when the section was not entirely in focus, a tracing was made around the area in focus. For traced images, only the area in focus was used for analysis (**Figure 4**).

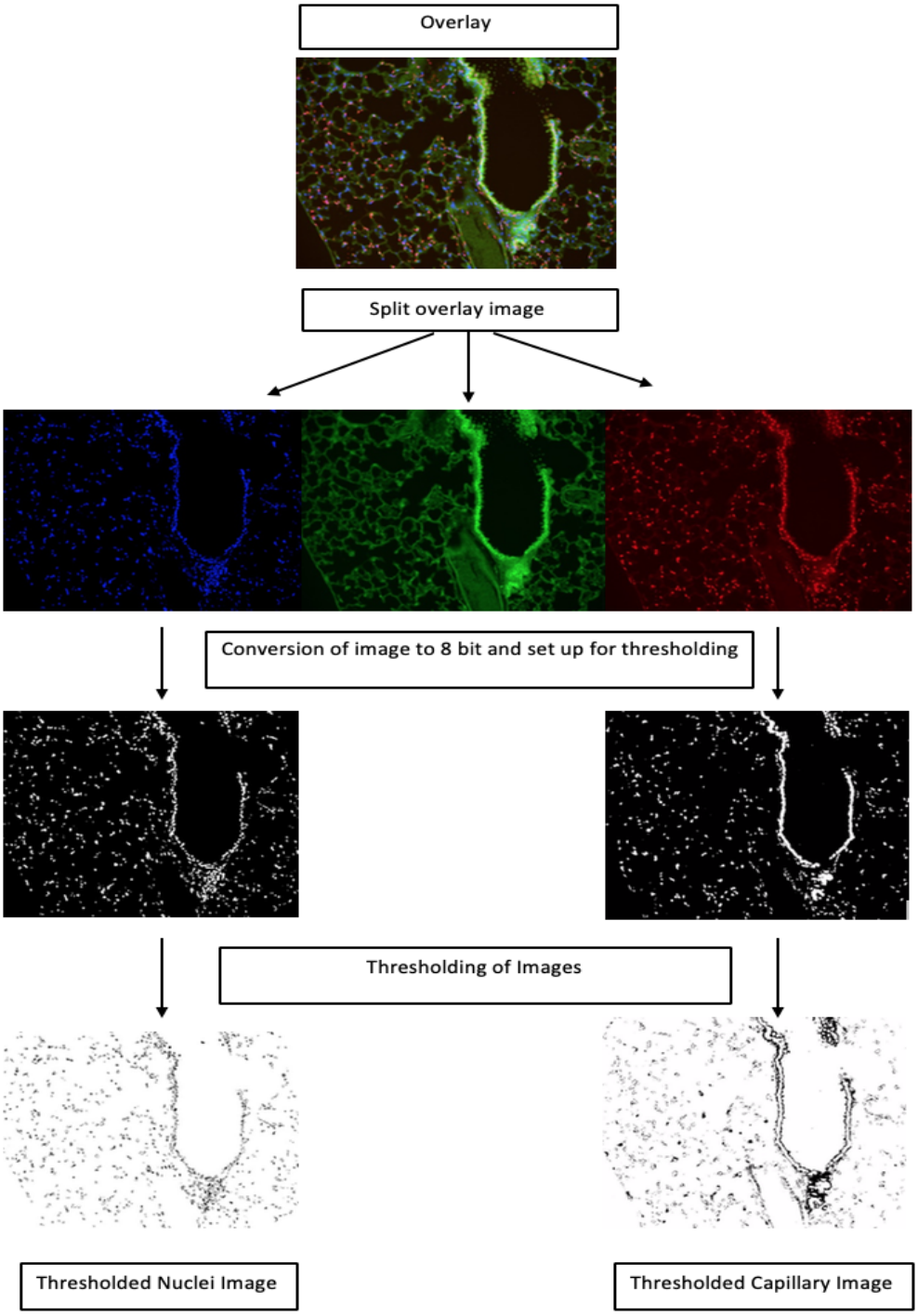

Figure 2: At the top is the three-channel overlay, which was split into the three individual images. The green fluorescence image was removed and the DAPI (blue) and TRITC (red) images were thresholded and analyzed to give the total cell and capillary number.

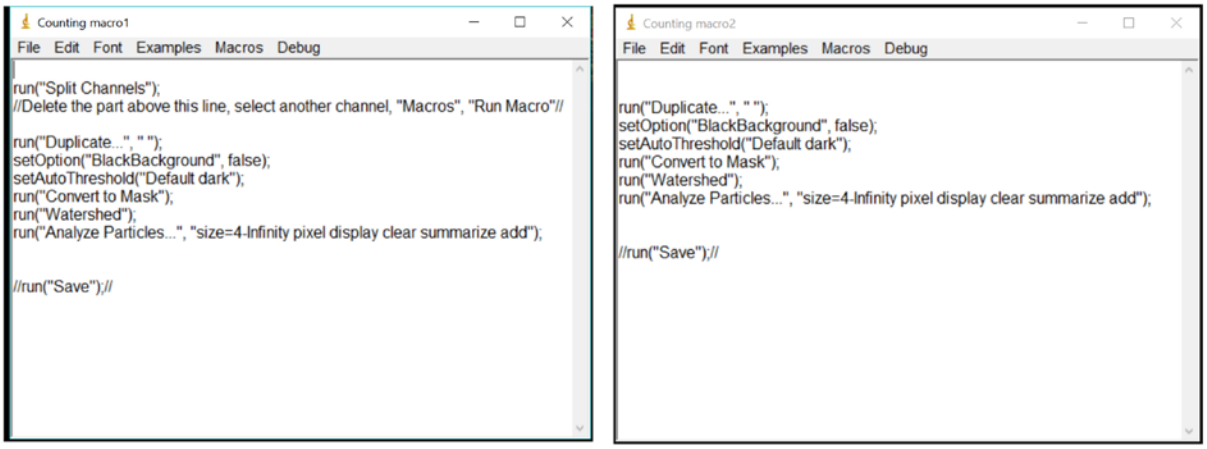

Figure 3: The script made in ImageJ to automatically threshold images and count objects. Counting macro 1 was used to threshold Hoechst (nuclei) images and counting macro 2 was used on isolectin B4 (capillaries) images.

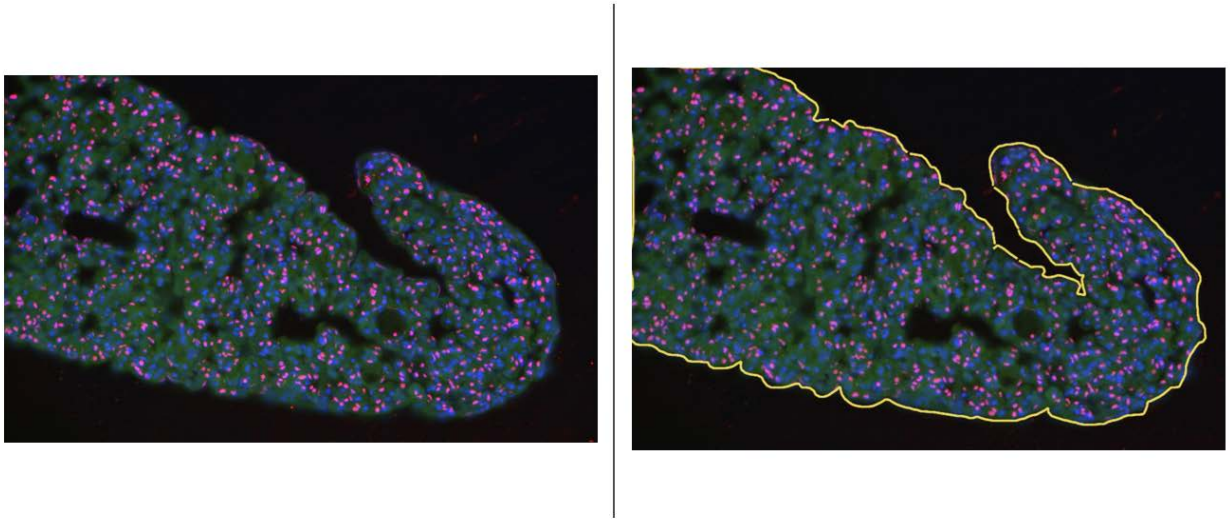

Figure 4: The image on the left shows the end of a lung tissue section. On the right, the area of the tissue was traced using ImageJ, allowing the enclosed area to be determined.

Results

After analyzing the data collected from the images, we found that there is no statistically significant difference in cell number between low and high-altitude acclimated deer mice. The mean nuclei density for high-altitude acclimated mice is 0.001226 nuclei/μm^2 while the mean nuclei density is for low altitude mice is 0.001256 nuclei/μm^2 (**Figure 5**).

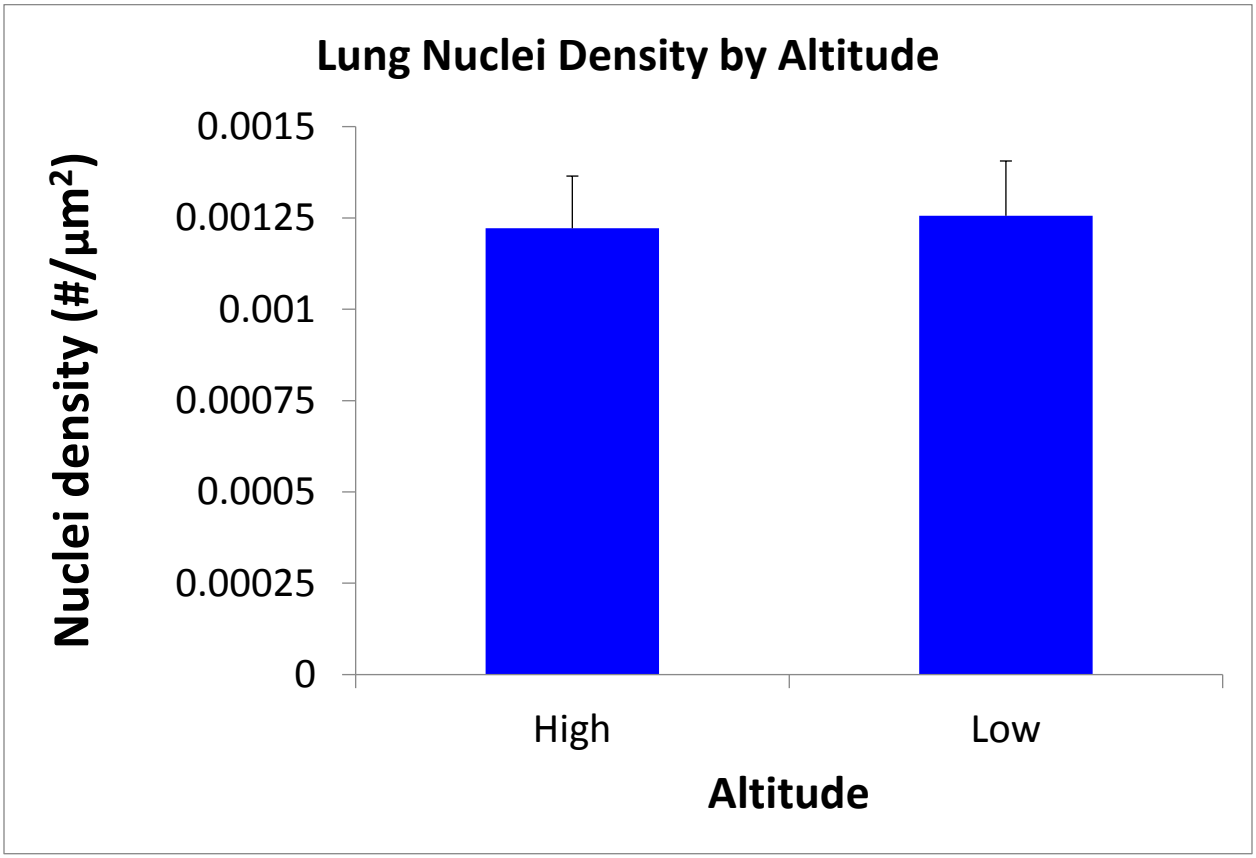

Figure 5: Relationship between Nuclei Density and Altitude of deer mice.

High altitude acclimated mice possess a greater capillary density than deer mice found at low altitude (**Figure 6**). For high altitude acclimated deer mice, the average capillary density is 0.001049 capillaries/μm^2 while low altitude deer mice have a mean capillary density of 0.00098 capillaries/ μm^2. Although high altitude acclimated mice possess a greater capillary density, the difference is not large enough to be statistically significant.

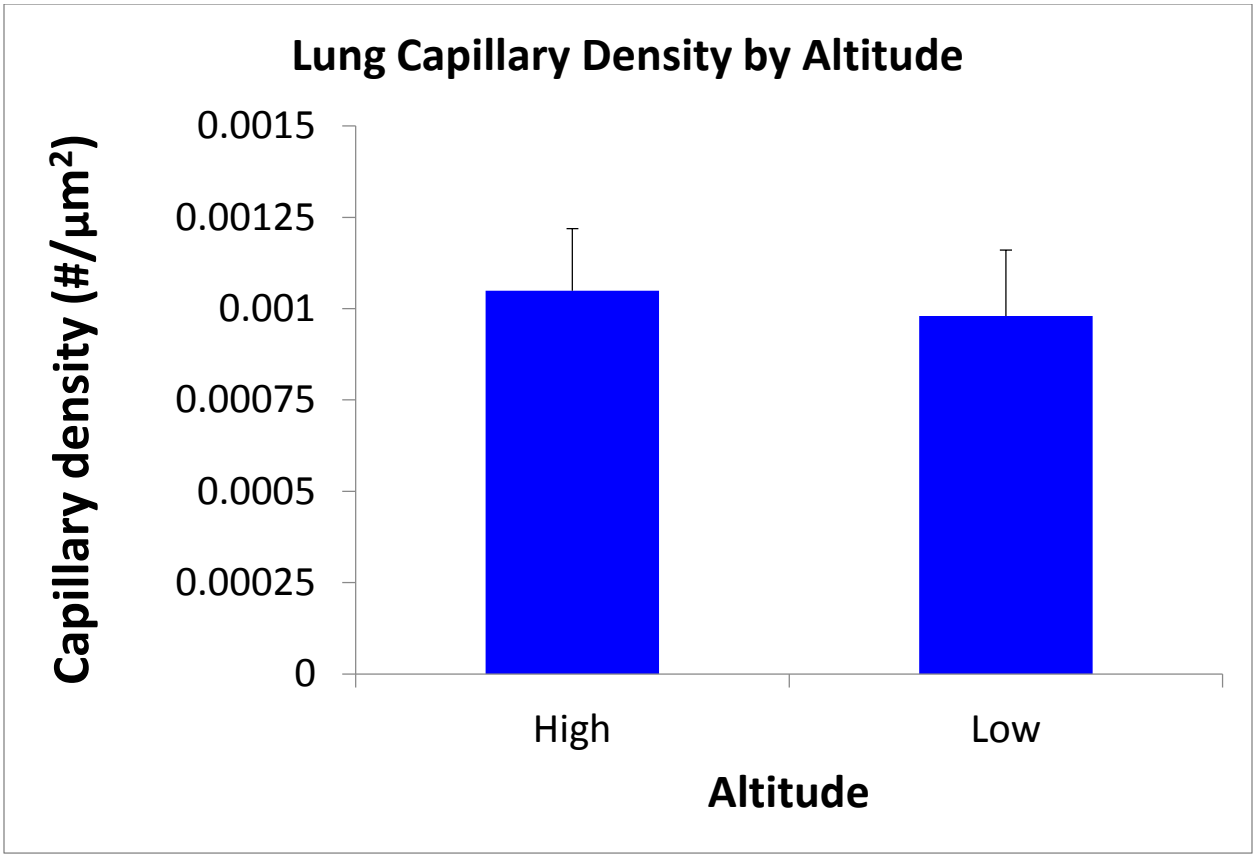

Figure 6: Relationship between capillary density and altitude of deer mice.

Nuclei density between lobes was analyzed for high-altitude acclimated and low altitude deer mice (**Figure 7**). At low altitude, the superior lobe has the greatest nuclei density followed by the left, cardiac, middle, and inferior lobes in order of decreasing nuclei density. For high altitude acclimated mice, the superior lobe possessed the largest nuclei density followed by the left, inferior, middle, and cardiac lobes in order of decreasing nuclei density. Based on the data, high-altitude acclimated mice possessed a greater cell density for the inferior and superior lobes and a lower cell density in the cardiac, middle, and left lobes compared to low altitude mice.

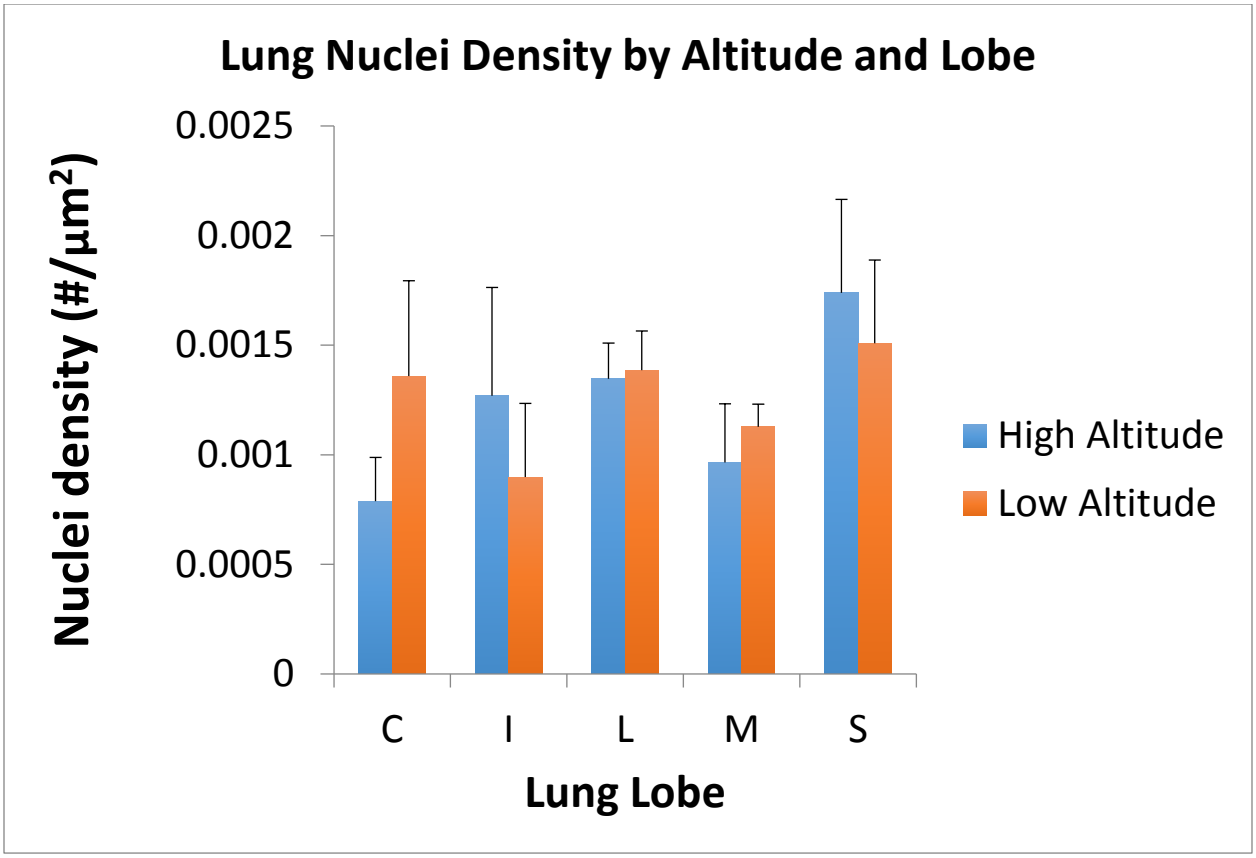

Figure 7: Differences in nuclei density between the cardiac (C), inferior (I), left (L), middle (M), and superior (S) lobes in high altitude acclimated and low altitude deer mice.

Capillary density between lobes was analyzed for high-altitude acclimated and low altitude deer mice (**Figure 8**). At low altitude, the cardiac lobe has the greatest capillary density followed by the superior, left, middle, and inferior lobes in order of decreasing density. For high altitude acclimated mice, the superior lobe possessed the largest capillary density followed by the left, inferior, middle, and cardiac lobes. In high-altitude acclimated mice, capillary density is greater for the superior, inferior, and left lobes but lower for the middle and cardiac lobes in comparison to low altitude mice.

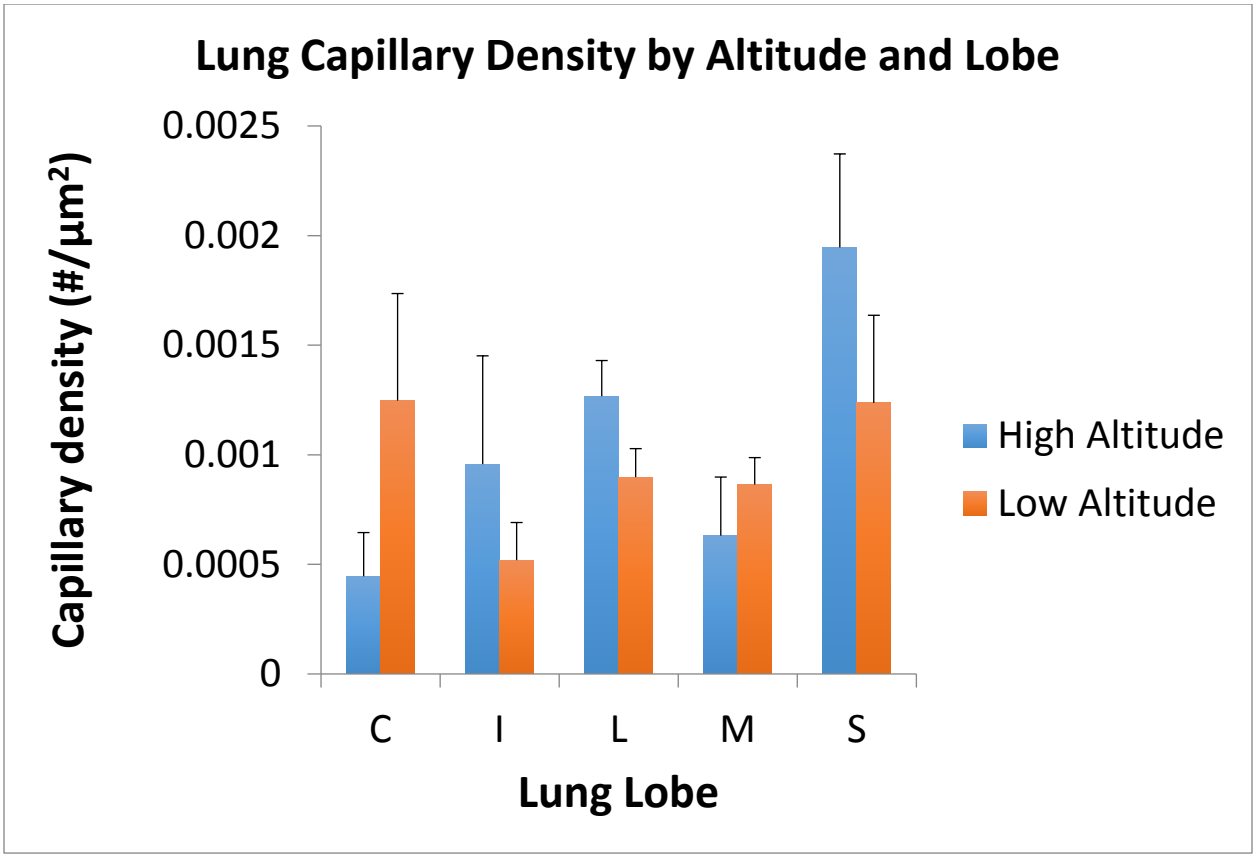

Figure 8: Differences in capillary density between the cardiac (C), inferior (I), left (L), middle (M), and superior (S) lobes in high altitude acclimated and low altitude deer mice.

The correlation coefficient for the nuclei and capillary density is R^2= 68.9%, indicating a positive relationship between both densities for low and high-altitude acclimated deer mice (**Figure 9**).

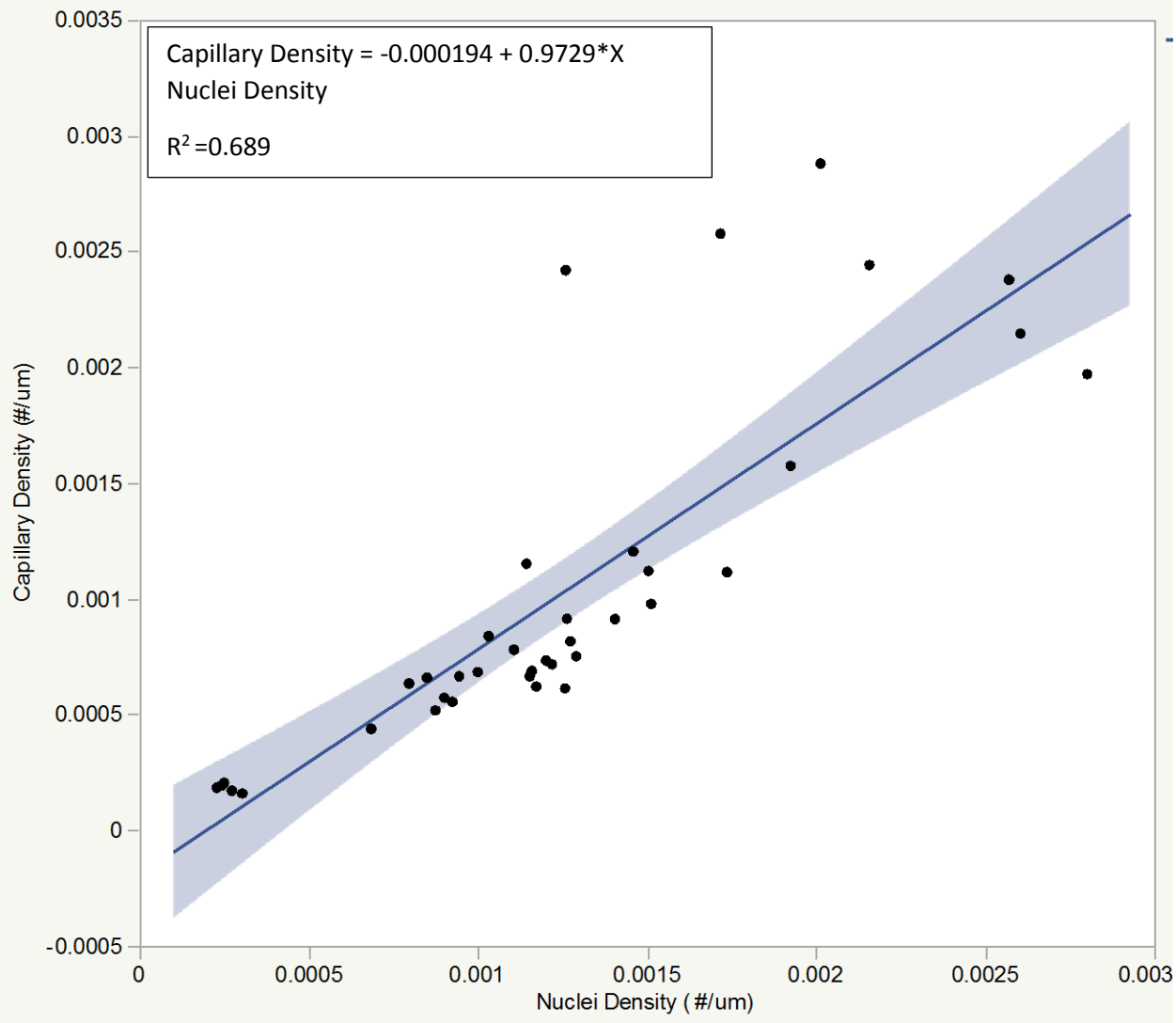

Figure 9: Linear regression line indicating a direct relationship between capillary and nuclei density.

Discussion:

The objective of this study was to measure and analyze capillary and cell density for high-altitude acclimated and low altitude deer mice lungs. The prediction was that high-altitude acclimated deer mice would possess a greater capillary and overall cell density compared to deer mice from low altitude. A greater capillary density would allow acclimated mice to compensate for the low oxygen content at high altitude. Past studies have indicated that deer mice acclimated to high altitude possess a greater lung volume and mass (Shirkey and Hammond, 2014). Further studies focusing on the alveoli of the lungs showed that alveolar density was not greater in high-altitude acclimated mice compared to low altitude mice. (Sawaya Honors Capstone, 2018). Results in this study indicate that high-altitude acclimated deer mice do not possess a statistically significant difference in capillary or cell density compared to low altitude deer mice.

Differences in capillary and cell density were also measured among the five lobes of the lungs between both high-altitude acclimated and low altitude deer mice. Of the five lobes the superior lobe possessed the greatest density of nuclei in both high-altitude acclimated and low altitude deer mice. Acclimated mice had a greater nuclei density in the inferior and superior lobes and a lower density in the cardiac, middle, and left lobes in comparison to low altitude mice. Capillary density was largest in the cardiac lobe for low altitude mice and superior lobe for high-altitude acclimated mice. In high-altitude acclimated mice, capillary density was greater in the superior, inferior, and left lobes and lower in the in the middle and cardiac lobes compared to deer mice at low altitude. The differences in lobes for both high-altitude acclimated and low altitude mice is not statistically significant.

To determine the capillary and cell density, the total number of labeled particles per image was divided by the overall area of the image. Densities were calculated separately so

particles for cell density were nuclei and particles for capillary density were capillaries. This method includes the area of structures that do not play a role in gas exchange, such as air spaces and ducts. To obtain more precise density values, future research will be directed towards measuring the total area of fine parenchymal tissue only. Fine parenchymal tissue in the lungs is used for oxygen exchange. After the area of fine parenchymal tissue is determined for each image, it will be substituted for the area of the image in previous density calculations. The new values will better reflect the densities of capillaries and cells between high-altitude acclimated and low altitude deer mice and give a more accurate explanation of how acclimation affects capillaries in lung tissues.

Learning how to utilize laboratory tools such as a microtone, pipette, pH barometer, and centrifuge was useful for creating the salines and solutions utilized in the staining process. Additionally, understanding the operations of the Keyence microscope and Image J allowed clear images to be taken and analyzed. Comprehending the manner in which lung tissue can escalate gas exchange by increasing capillary density was essential for developing the focus of this study. This project demonstrated the importance of planning and peer discussion. Furthermore, it taught me the impact that biological research has on explaining processes and observations in the natural world.

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