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DEVELOPMENT OF THE SECOND OSSIFICATION CENTER IN MICE CALCANEI

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ABSTRACT

The calcaneus, or the heel bone, is an extremely important bone that is located in the foot of most terrestrial vertebrates. It allows creatures to walk, run, dance, and move around. To fit such a specific function, the calcaneus has a unique shape and growth process that cause it to stand out from the rest of the bones in the foot. Unlike short bones, the calcaneus does possess a growth plate as seen in the long bones. Yet unlike a typical long bone, the calcaneus only possesses one growth plate, not two, which is visible at the posterior end of the bone. This one growth plate allows for two distinct centers of ossification to form on either side of it, yet the timing of this growth has not yet been mapped in mice. Here we study the stages of development in the calcaneus of mice, and then compare it to those found in similar studies previously conducted on the pisiform, by Kjosness and colleagues (2015), which is a bone located in the wrist and helps support a similar function in the forelimbs during quadrupedal locomotion. The research in this study discovered that the first center of ossification in the calcaneus is present at birth or P0, the growth plate emerges and becomes active by P4, the second center of ossification develops at P11, and the growth plate is no longer active by the age of P30. We observe generally similar process of ossification compared to the pisiform, however the timeline of these developments are not entirely synchronous for the calcaneus takes slightly longer to grow and mature than does the pisiform.

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ACKNOWLEDGEMENTS

"Tell me and I'll forget, show me and I might remember, involve me and I'll understand."
Chinese Proverb

The Schreyer Honor College is a truly amazing program, for it does exactly that- it motivates students to become involved not only in their area of study but also in our amazing University. It opens doors for us we did not even know were there. Therefore, I owe you a big thank you for giving me the chance to experience my academic career to the fullest.

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Chapter 1

Introduction

Bone growth occurs through different processes and at different paces depending on the bone and organism being discussed. These differences in growth allow for diverse shapes and structures to form that are specifically suited to support a unique function in the body. The shape of each and every bone is directly shaped in order for it to connect to the surrounding tissues and bones with which it articulates (Kronenberg 2003: 332). The calcaneus, which is the largest bone in the foot and forms the heel, is a perfect example of this. Its unique shape allows it to fit in between the other tarsals, or anklebones, that surround it along with serving as an important point for muscle attachment. Furthermore, the calcaneus' shape may differ from organism to organism depending upon their specific mode of locomotion that it uses. In humans, the calcaneus aids us in the process of walking, running or any other activity that relies upon the movement of ones' legs. For other animals that use quadrupedal locomotion, such as mice, the calcaneus is uniquely shaped in order to be conducive to walking on all four limbs. Therefore, it is an important part of the skeleton to study when examining the evolution of different methods of locomotion and their change throughout time (Gebo 2006).

The unusual growth process of the calcaneus is fitting given its important function in the hind limb. Most of the other short bones within the ankle or wrist joints are oddly shaped. However, the calcaneus is unique in the foot due to the fact that it forms one growth plate, at the posterior end, while the other end is formed primarily through direct ossification. The only other short bone that also displays a growth plate in most organisms is the pisiform (Kjosness et al.

2015). The pisiform is found in the wrist, and also serves as an important point of connection for tendons and skeletal articulation.

In most mammals, there are two ossification centers present with a growth plate between them present in each of these bones. Since the pisiform and the calcaneus play a similar functional role, particularly in quadrupedal mammals, it will be interesting to compare the growth of the two bones. Since the timing of the growth of the pisiform has been previously studied, I will use that research as a basis of comparison (Kjosness 2014: 227). I hypothesize that the growth of these bones will be rather similar because of the many commonalities that they share particularly in quadrupedal creatures like mice.

This process of the growth in the calcaneus has not been studied to the degree of depth that the pisiform has been, and the precise timing of the progression has not been suitably detailed in the mouse. Due to its similarity in terms of growth and formation to that of the pisiform, tracking the timing of ossification in the calcaneus could serve as an important point of comparison between the two bones.

In order to track the process of this formation, Safranin O staining of histological sections along with cleared and stained intact specimens were used to visualize the development of the calcaneus from birth until being fully-grown. Both of these methods were chosen because of the difficulty in identifying the secondary center of ossification based solely on the Safranin O stained sections. Although the histology of this bone is imperative to examine in order to truly understand the progression of growth that is occurring within the bone, it was not chosen as our sole method of examination because the secondary center of ossification is most likely going to develop at one particular area of the calcaneus and is, thus, very easy to overlook when examining the histology of a sectioned calcanei. However, in the cleared and stained mice where

the entire calcaneus can be observed and secondary center formation is readily identified under a microscope, it is much easier to see the moment that this feature begins to appear. In the end, I hope to have a clear timeline of calcaneal growth in order to compare it to the growth of the pisiform. Perhaps this study could even further our understanding of the evolutionary developments and differentiation of limbs during the growth process.

Chapter 2

Background Information

Calcaneus

With every step you take, you are utilizing a bone in your foot called the calcaneus; otherwise known as your heel bone. It is the largest bone in the foot and is uniquely shaped in order to articulate snugly with the talus and cuboid, making the calcaneus look like a small ice cream scoop. It serves a wide variety of functions for all mammals, including supporting any movement including walking, running, dancing, or playing sports. It is also the attachment site for many important muscles and tendons, perhaps most notably the Achilles tendon which attaches to the prominent tuberosity of the bone. Because of this, the calcaneus is crucial to forming the arch of the foot, which helps to support the weight of the body when standing. In return, changes to the calcaneus growth and structure can be important to determining the style of locomotion used by that specific organism. Therefore, it has become an extremely important bone to study for the discussion of evolution (Latimer 1989: 369).

Like most bones, the calcaneus is made up of both cortical and trabecular bone (Keener et al. 2005). Cortical, or compact bone, is found at the outer margins of a bone. It is a dense tissue that looks almost completely solid. This extra hard exterior surface serves to add support to the structure and protection of the bone (Leeson & Leeson 145). It also makes the bone much more rigid and resistant to bending (Maes et al 2012: 56). Much of the interior of the calcaneus is

made up of trabecular, or cancellous bones, which is a spongy-looking type of bone that forms a mesh-like network of cells surrounded by hollow pockets for bone marrow (Keener et al. 2005). This is especially true of the human calcaneus where the calcaneal tuberosity is inflated by an extensive network of trabecular bone (Latimer 1989: 373). This type of bone is found at the center of the calcaneus and serves to make the calcaneus more flexible and dissipate stress. Together these types of bone tissue allow the calcaneus to withstand the strain and pressure of bipedal and quadrupedal locomotion.

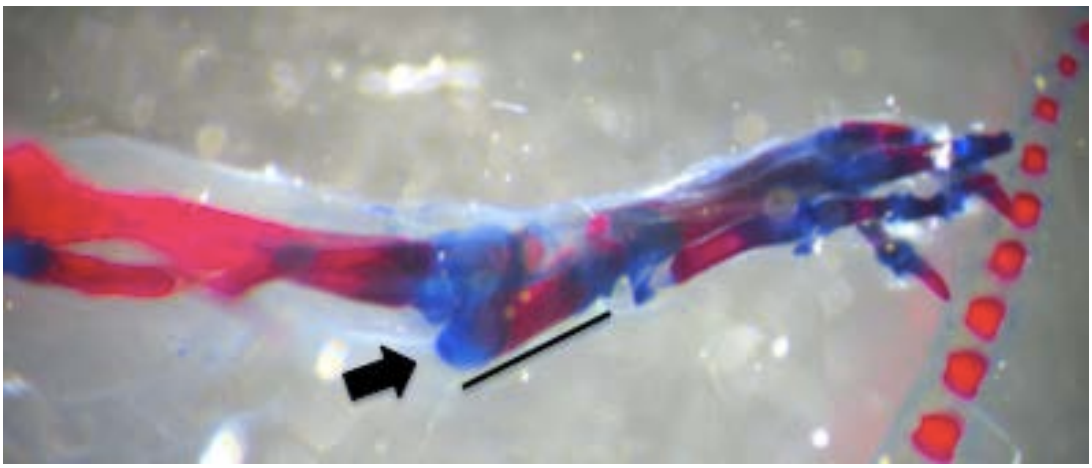

Figure 1. Cleared and stained hind limb of a P6 mouse. The calcaneus is indicated by the solid line and the arrow points to the calcaneal tuberosity

Bone Growth

Bones can grow through two different processes: endochondral and intramembranous ossification (Ornitz 2002: 1446). Intramembranous bone growth is primarily responsible for the formation and development of the flat bones in the skull and the clavicles. On the other hand, most of the other bones in the body form through the process of endochondral ossification, which is responsible for shaping the majority of the appendicular skeleton most notably the long bones. The calcaneus also grows through this process. This involves a cartilaginous model that is

eventually largely replaced by bone. It begins with mesenchymal cells differentiating into chondrocytes, which in turn produce the cartilaginous matrix. This matrix forms the hyaline cartilage base that expands as the chondrocytes continue to proliferate. This will serve as the model for the bone development. Next, the chondrocytes that have become trapped in the matrix begin to hypertrophy, or enlarge, and in the process absorb some of the surrounding cartilage matrix and cause the matrix to calcify (Maes et al 2012: 55).

The matrix is then mineralized and at this time the perichondrium, a type of connective tissue, forms and surrounds the cartilage. From the perichondrium chondroclasts, a type of osteoclast or multinucleated bone cell, along with blood cells and preosteoblasts invade this area where the hypertrophic chondrocytes are located (Maes et al 2012: 55). Through specific signaling pathways, the perichondrium begins to regulate the cartilage development. Some of the pre-osteoblasts that have entered this region mature into full osteoblasts and begin to lay down a mineralized collagen matrix around the cartilage mold. This will eventually develop into the thick, protective cortical bone that surrounds the spongier center (Maes et al 2012: 56).

Other preosteoblasts, which were introduced into the calcified cartilage matrix by the perichondrium, begin to mature into osteoblasts that now lay down a matrix called the primary spongiosa which will eventually develop into the second type of bone, trabecular bone. From this center point, the cartilage calcifies outward from the diaphysis towards the epiphyses. In the center of the diaphysis, a cavity forms and expands as the bone grows. This cavity is where the bone marrow can be found (Maes et al 2012: 56).

Subsequently, a second point of endochondral ossification initiates at the epiphysis in the heel. The same process occurs as the chondrocytes hypertrophy, and the periosteum invades that area, causing bone to replace of the cartilaginous mold. This is called the secondary center of

ossification, for it is not connected to the larger part of the bone that possesses the original primary center of ossification. In long bones, this occurs at both ends of the bone, yet due to the calcaneus's unique shape and structure, and the fact that it only contains one growth plate allows for only one secondary center to form (Maes et al 2012: 56).

This process preserves two types of cartilaginous regions: 1) joint surfaces such as the articulations with the talus and cuboid and 2) the space between the epiphysis and the diaphysis where the growth plate is located. The cartilage at the growth plate allows for continuous bone growth until the individual has matured and it too becomes completely ossified. (Leeson & Leeson 1981: 147-156)

Growth Plate Biology

As discussed above, the calcaneus only contains one growth plate that aides in calcaneus elongation in the posterior direction to form the heel of the creature. The growth plate, also known as the epiphyseal plate or physis, is a very specialized area of the cartilage where cells go through a series of differentiation steps to add length to the bone. The cells begin in the resting, or quiescent, zone located near the end of the bone where the secondary center of ossification is located. These cartilage cells, or chondrocytes, lie within a dense extracellular matrix and are responsible for storing some of the nutrients necessary for the continuation of growth in the area. These cells are typically believed to not proliferate, and appear to mostly serve as a storage unit for the other cells in the growth plate (Brighton 1978: 24-25). However, because there is no cellular marker that has yet been linked to the cells in this resting zone, recent studies suggest that this area may serve the zone of proliferation by enlarging as well as undergoing a substantial

amount of proliferation itself. In addition to supplying it with more chondrocytes, it is also believed that this region aides in the organization of the entire growth plate (Reno 2006:116). Yet, overall as the bone grows this zone experiences little growth overall and shortens as the ossification center expands (Leeson & Leeson 1981: 155). This phenomenon is probably due to the fact that this region is believed to be the origin of the other two zones, for when researchers removed the resting zone from the ulna of a rabbit- the resting zone was able to fully regenerate the zone of proliferation and the hypertrophic zone (Abad et al. 2002). Thus, the reason it experiences little growth is because it is converting its own chondrocytes into those that form the other zones.

The second zone in the growth plate is the zone of proliferation and is the location where most of the longitudinal growth in the bone takes place. The flattened cartilage cells in this region are constantly going through mitosis and dividing into two new cells (Leeson &Leeson 1981: 155). Because these cells proliferate in columns, the growth is mostly in length and not width. In addition, this zone's purpose is to also produce more cellular matrix for the cells to exist within (Brighton 1978: 25-26).

At the hypertrophic zone, the proliferation ceases and the flattened cells enlarge and become round in shape. Then at the zone of calcification, the lacunae, which are small cavities that encase a single cell within it, expand and fill with nutrients. The cell dies in the zone of retrogression, and the matrix absorbs the nutrients from it. The remaining structure consists of thick plates of matrix between the dead cells. Then osteoblasts invade and deposit bone tissue upon the remains of the calcified cartilaginous structure in the Zone of Ossification. Finally, in the zone of resorbtion, the calcified area is resorbed creating a large marrow cavity in the center, or diaphysis, of the calcaneus (Leeson & Leeson 1981: 155, 158).

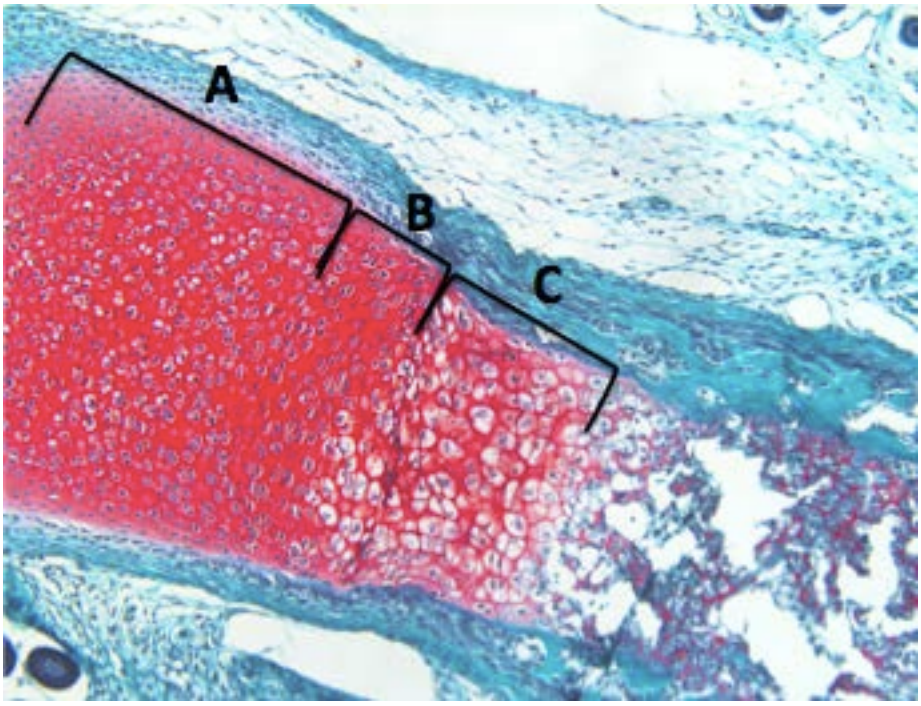

Figure 2. Growth Plate of a P5 calcaneus Area A: resting chondrocytes, Area B: proliferation zone, and Area C: hypertrophic zone

On the other the other side of the growth plate is the secondary center of ossification. This area also aides in the growth in a similar way, beginning as cartilage and then slowly ossifying into bone until the only remaining cartilage is a narrow plate in between the two centers of ossification called the epiphyseal disc (Leeson and Leeson 1981: 158-159). By this time, the growth plate has become inactive and is no longer present in the bone. Then the epiphyseal disc eventually disappears once the mouse reaches skeletal maturity and is considered full grown. Then the epiphysis, formed by the second center of ossification, can fuse to the diaphysis, or the location of the primary center of ossification, and create a single bone (Kilborn 2002: 21).

These two centers grow and mature at different times depending on the species in question. The purpose of this research is to investigate the timing of each of these phases,

particularly the creation and development of the first and second centers of ossification in the calcaneus of mice from the day they were born until they are eight weeks old, which is the age at which they are considered to be full grown. Similar research to this has been done on other bones, such as the pisiform. The research conducted by Kjosness et al. (2015) has mapped the course of development for the pisiform in mice by looking at Safranin O stained slides as well cleared and stained specimens. They uncovered that the primary center of ossification is visible very early on in the organism's life and clearly visible in cleared and stained mice by P4. This development was closely followed by the emergence of a secondary center of ossification at P7. Two days later the growth plate forms, until P19 when only a thin sliver of cartilage remains present between the two centers of ossification. By the third to fifth week of development, these centers fuse together to create a fully formed pisiform (Kjosness et al. 2014: 527-538). Following the example set by this previous study, my own research will closely examine the histology of the calcaneus by looking at sectioned specimens stained with Safranin O as well as cleared and stained complete mice.

Comparison to the Pisiform

Although the calcaneus is a unique bone in the hind limb, it has a similar equivalent seen in the forelimb. This bone is called the pisiform, and preforms similar function s to that of the calcaneus. Even though the pisiform is rather small in size, it has great importance for it serves as the insertion point for the extrinsic flexor in the hand. Previous research conducted by Kjosness et al. has revealed that this bone has two ossification centers and therefore a growth plate between them, very similar to that of the calcaneus. This appears to be true for most

mammals, except for humans perhaps due to our unique ability to make tools (Kjosness 2014: 527). Due to their similarities in function and form, I hypothesize that the calcaneus will develop at a similar rate to that of the pisiform, particularly in mice who crawl on all four limbs. In order to determine the validity of my hypothesis, I will examine the bone structure through the use of cleared and stained specimens as well as collect histological data in order to determine the rate of growth within the calcaneus. Then, I can compare that to the research already done on the pisiform in order to determine the extent of their similarities.

Chapter 3

Materials and Methods

Specimens

Mice from FVB/NJ and C57B/6 strains were examined for this study. All procedures involving the mice were conducted in accordance with protocols approved by the Penn State Institutional Animal Care and Use Committee (IACUC). They were collected after birth, every other post-natal day until P19, then weekly until from the third week (P21) to eight weeks old. The term P0 was used to delineate the day on which the specimen was born. Mice are essentially fully grown by eight weeks old, and the second center of ossification is fully formed and fused to the primary center prior to this age. Therefore, I did not investigate ages older than 8 weeks.

Two different methods were used to examine calcaneal ossification: cleared-and-stained whole mouse specimens and Safranin O staining of histological sections. It was hoped that the cleared and stained specimens would give a clear picture of when the second center of ossification began to form and how it developed until it was full grown. The histological slides were used to see more in-depth detail of the specific cartilage and bone formations at the different ages to give us a clearer picture of the two centers of ossification.

Cleared-and-stained Specimens

Our first method for examining formation and development of the calcaneus was to clear and stain a series of different ages in order to pinpoint the timing of the secondary center of ossification (Kjosness et al. 2014). First, the mice were euthanized. Then they were skinned and eviscerated, leaving as little tissue as possible on the mice while leaving the muscle and bones intact. Then they were washed in a 95% ethanol solution, followed by washes in acetone. They were placed in the staining solution that was a mix of 0.03% alcian blue and 0.01% alizarin red. The alcian blue stains cartilage, while the alizarin red stains bone. Afterwards, they were cleared in a gradient of glycerol and potassium hydroxide washes. Finally, the finished specimens were stored in a 100% glycerol solution.

Histology

Sectioned specimens were stained with Safranin O and Fast Green in order to highlight the cartilage and bone structures (Reno et al. 2006; Kjosness et al. 2014). To prepare the specimens for histology, the hind limbs were dissected from the mouse promptly after euthanasia and fixed in 4% paraformaldehyde. To decalcify the specimens, they were placed in a 10% EDTA solution with a pH of 7.5. They remained in the solution for one week if they were between the ages of P0 and P7 or two weeks if they were from P8 to P21. The specimens were then processed through a graded ethanol series and then in solutions of ethanol and Citrisolve. Then, they were thoroughly infiltrated with paraffin and embedded for sectioning. They were sliced using a microtome set to 6 um.

After the sections were mounted on Superfrost Plus slides, the specimens were placed in a series of Citrisolve washes to remove the paraffin. Then they were processed through another graded ethanol series in order to rehydrate the specimens. They were washed in Weigert's Iron Hematoxylin in order to make certain cell structures such as the nuclei and cytoplasm become more clearly defined as well as aid in the preservation of the specimen. Afterwards, the specimens were stained with the Safranin O, which stained the cartilage red. Then, they were quickly rinsed with fast green in order to stain the other tissues including bone greenish blue. Finally, the slides were dehydrated through a graded ethanol series and allowed to air dry before being cover slipped with DPX.

Data Collection

After all of the specimens were prepared, I was then able to examine them for data collection. The cleared and stained specimens were placed in a dish of 100% glycerol and placed under Leica Wild M3Z Stereozoom Microscope to be photographed at a 20X magnification.

As for the histological specimens, I used an Olympus BX50 microscope and used a 10X magnification. A Leica DFC450 camera was then used to photograph the different specimens under each of the microscopes. Finally, Leica Application Suite was used to sharpen the images and white balance the background.

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Chapter 4

Results

Early Development P0-P9

The following chronology of development and growth was charted through careful examination of the pictures taken of the histological and cleared and stained specimens. As you can see in Figure 3A, at P2 the bone is just in its infant stages of development. What appears red in these pictures is the cartilage tissue, and blue staining signifies bone tissue. Around the center of the calcaneus, the periosteum surrounds the hyaline cartilage model. Most of the structure appears to still be undifferentiated hyaline cartilage cells. However, in the center of the model the chondrocytes proceed to the hypertrophy stage and then to that of calcification.

Two day later at age P4 (Figure 3B), significant changes have already begun to take place. The calcified center has now ossified into the beginning of trabecular bone called primary spongiosa. This area has spread out considerably within the period. The epiphyses are still entirely composed of hyaline cartilage, while a bit of cortical bone has developed along the medial sides of the calcaneus forming the bone collar helping to create the shape of the bone. The only growth plate in the calcaneus is in its early stages of formation near the posterior end of the bone, which at this age is where the highest percentage of cartilage remains.

By P6 (Figure 3C), the spongy center has continued to expand, particularly at the anterior sections of the calcaneus. All epiphyses remain slightly cartilaginous yet definitely less so than the previous two ages. The primary ossification center continues to grow and

spread throughout the center of the calcaneus as more and more of the cartilaginous model hypertrophies, and becomes bone. This is extremely evident in the pictures seen in Figure 4, which show the progression of the calcified bone spreading further from the diaphysis to the epiphyses as the specimens' age.

During the development from P7 to P9, the distal end of the calcaneus (that does not possess a growth plate) appears to be almost completely ossified and shows very little staining for cartilage. There still remains thick area of cartilaginous cells making up the growth plate at the distal end of the calcanei. Yet these cells do appear to be growing increasingly smaller compared to earlier samples. This is due to the fact that the resting zone becomes progressively shorter as its cells are being pushed into the proliferation zone then through the rest of the growth plate stages. No sign of a second center of ossification can be seen in either the cleared-and-stained or the histology specimens.

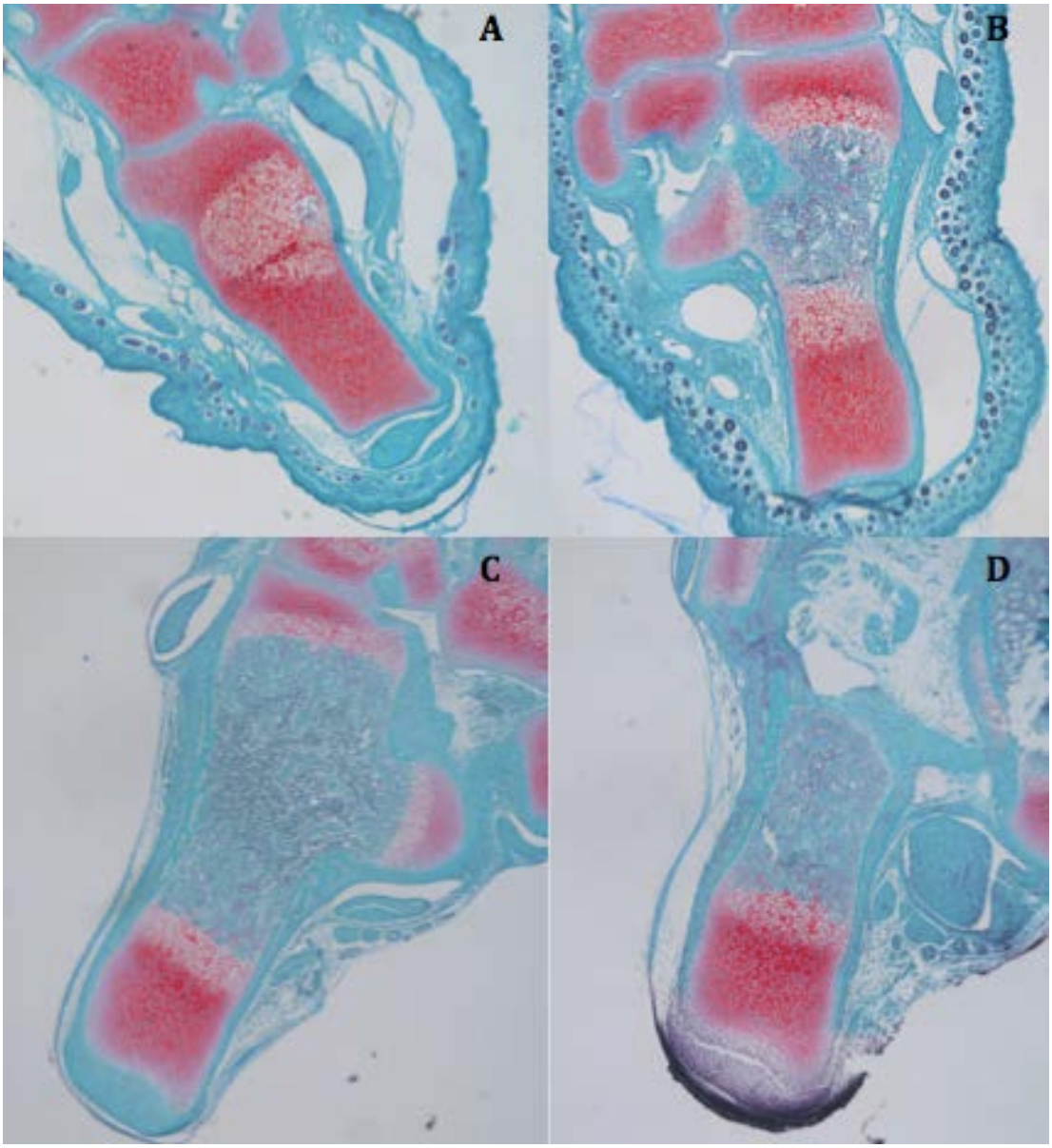

Figure 3. Safranin O staining of mouse calcanei (A) At P2, the entire calcaneus is still composed of red stained hyaline cartilage. However, the primary center of ossification is just beginning to take shape at the center where the chondrocytes are beginning to hypertrophy. (B) Two days later, at P4, the primary center has expanded and the ossification process has begun which is apparent by the blue color of the ossified chondrocytes. Furthermore, the growth plate has formed, with the resting, columnar, and hypertrophic zones all clearly evident (C) At P6, the resting zone begins to shrink in size as more chondrocytes become hypertrophic and ossify. (D) This progression continues at P7.

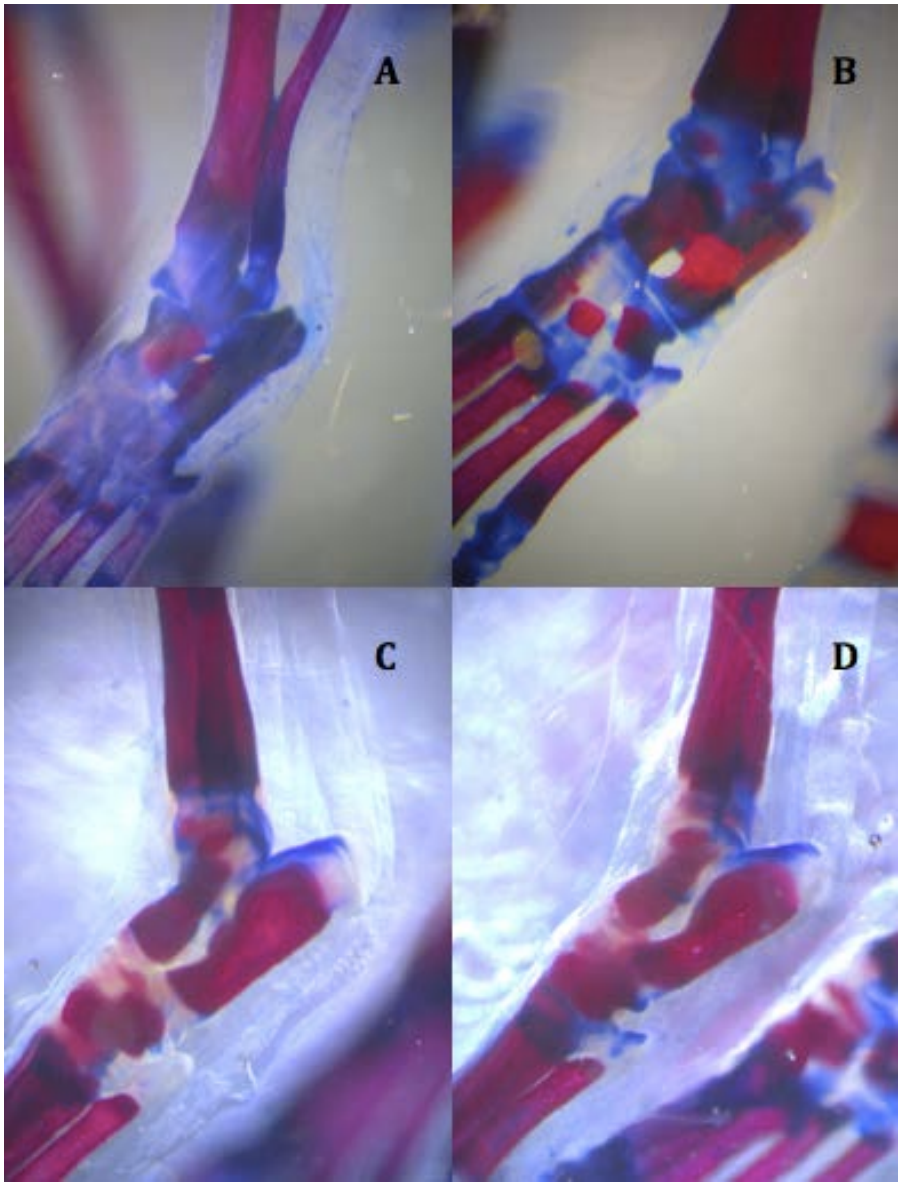

Figure 4. Cleared and stained specimens: (A) At P4, the bone is completely cartilaginous and shows no signs of ossification. (B) Two days later at P6, a considerable portion of the diaphysis has already undergone ossification which is evident by the region where the primary center of ossification is located has now stained red signifying a switch from cartilage to bone. In (C) at P7 and (D) P9, further expansion of the primary center is visible. Note that the region to the right at the posterior end of the bone appears clear with a bluish haze appearing on the left side. This is cartilage where the blue staining is incomplete.

Early Development of the Second Center of Ossification: P11-P1

Big changes begin to appear at age P11, which can be seen in Figures 3 and 4. The biggest progression seen in this period is that the secondary center of ossification has finally begun to form at the posterior epiphysis of the calcaneus. It is visible in the cleared and stained specimens beginning at age P11, which can be seen in Figure 6E. The small red dot that almost appears to be disconnected from the calcaneus in the picture signifies the second center of ossification since it is now appearing red after turning into bone. The clear section between that and the rest of the calcaneus is simply a result of insufficient staining of that section and in actuality should appear blue. However, the first appearance of the second center of ossification is only evident in the cleared and stained specimens, and there is no clear sign of this development in the histology slides yet. It is important to point of that while this second center is just beginning to form, it can only be seen at the bottom of the back end of the calcanei. As I sectioned the bones mostly along the long axis, I most likely missed it in my specimens. This is also a main reason as to why I chose to analyze both the cleared and stained specimens along with the histological ones. For the histology, slides are important to see the internal structure and cellular development within the calcaneus, but it is incredibly easy to miss an important development if your sections do not happen to cut through a specific part of the calcaneus. The cleared and stained specimens do not give an incredibly detailed look inside of the bone, however, because they allow you to glimpse the bone in its entirety, it allows small developments like the beginning of the second center to not be overlooked.

Another notable change is the formation of an epiphyseal plate, which is a slim segment of hyaline cartilage, which rests between the two centers of ossification in the metaphysis and allows the bone to continue to grow. This is the section that shows up clear in all of the cleared

and stained pictures located in Figure 6. You can see that as the specimen ages, the second center of ossification expands, thinning the epiphyseal plate. The second center of ossification appears to move upwards first, forming a thin disk at the very posterior end before it begins to thicken and shorten the hyaline cartilage section.

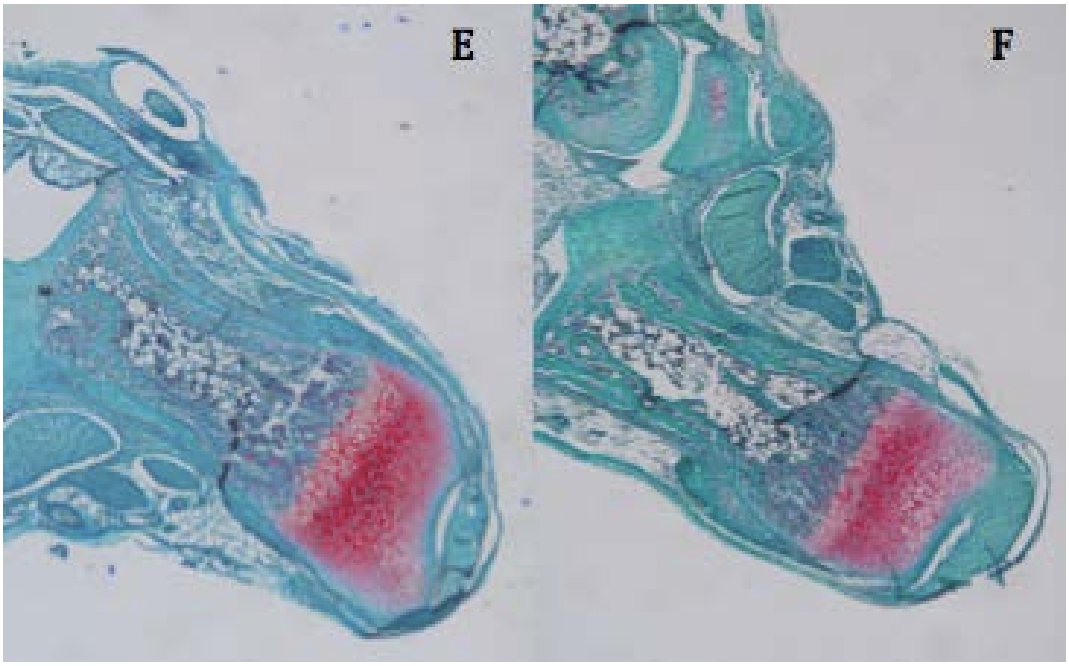

Figure 5. Histology specimens: (E) At P11 and (F) P13, the growth plate is still visible. However, it appears to be shrinking as ossification proceeds. Cartilage only remains at the posterior end of the calcaneus where the growth plate is located. In both of these pictures, the secondary center of ossification is not visible, although it is evident in the cleared and stained specimens showed in Figure 6. This is due to this section being located higher in the calcaneus where the secondary center has not yet reached.

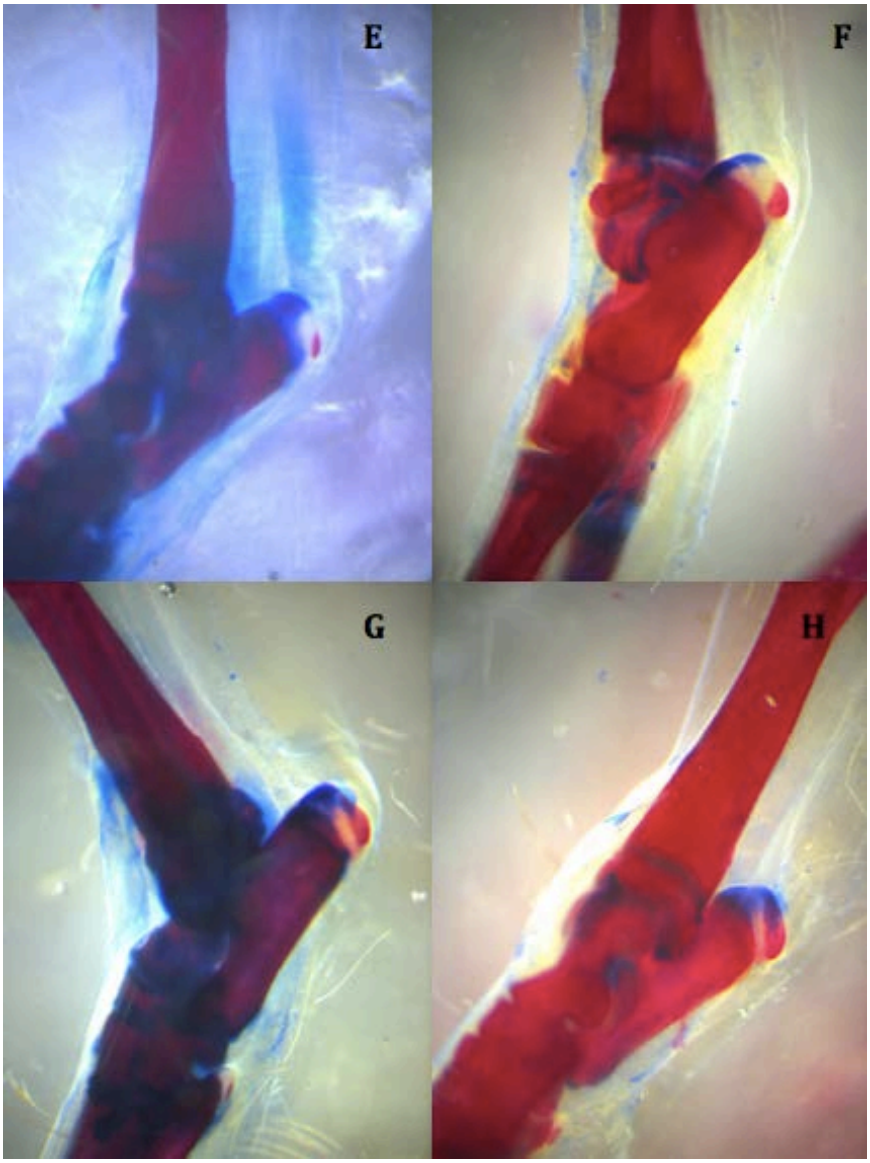

Figure 6. Cleared and stained specimens: (E) P11, (F) P13, (G) P15, and (H) P17. At P11, secondary center of ossification appears for the first time at the planter posterior end of the calcaneus. This small center grows throughout the following days in both length and width. By P17, only a small portion of cartilage remains at the top of the epiphysis. A disk of ossified bone has formed separated from the diaphysis only by a thin layer of cartilage that makes up the growth plate.

Maturation until Full Grown: P21 to 8 Weeks

From three weeks until the mouse is fully grown at eight weeks old, the two centers of ossification continue to expand and grow, leaving less and less cartilage remaining between them. In the middle of the first center of ossification, or the diaphysis, the trabecular bone is continuously absorbed making more room for the bone marrow cavity. By P30, the growth plate is no longer active and only a very thin line of cartilage remains that composes the epiphyseal disc. It is now the only section of cartilage left in the calcaneus that has not undergone ossification.

By week eight, the mouse is considered to be full-grown and through observation of the cleared and stained specimens there appears to be nearly no cartilage remaining. The epiphyseal disc has disappeared completely allowing both centers to become fully formed and fused together.

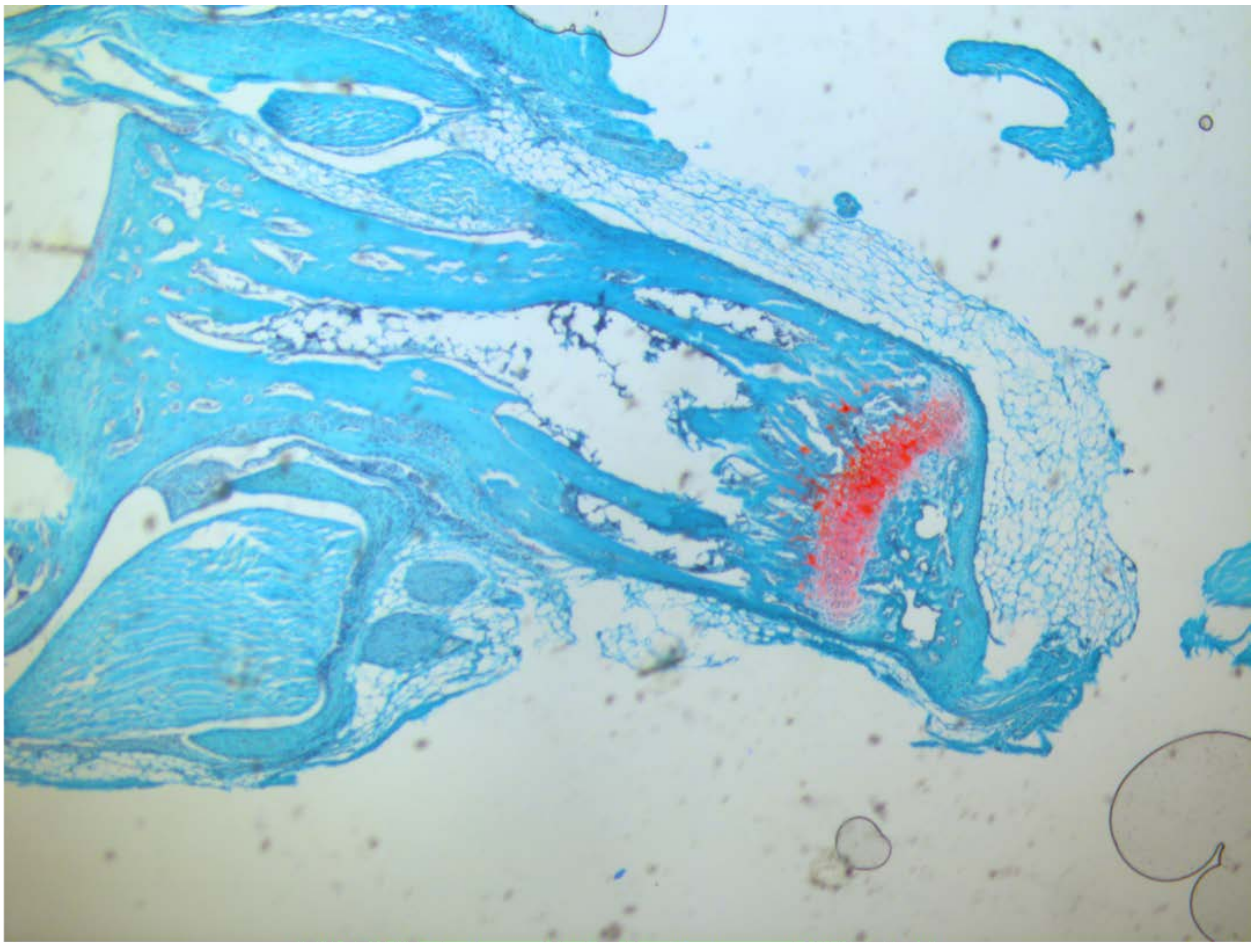

Figure 7. Histology specimen at P30 showing the inactive growth plate. A thin section of cartilage remains between the two centers of ossification forming an epiphyseal disc.

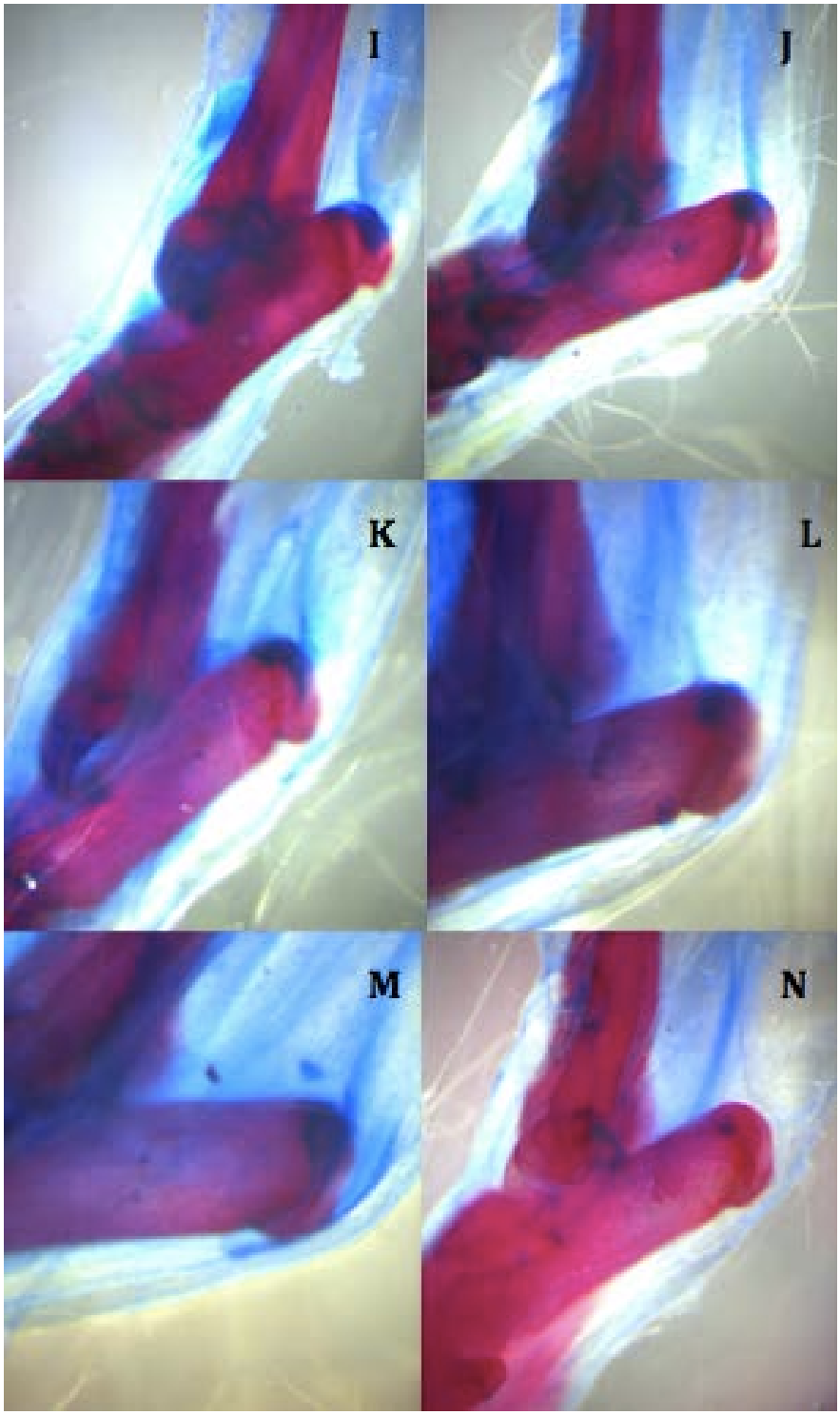

Figure 8. Cleared and stained specimens: (I) Week 3, (J) Week 4, (K) Week 5, (L) Week 6, (M) Week 7, and (M) is Week 8. This series demonstrates the final progression of the formation of the secondary center of ossification until it is fully fused with the diaphysis. The process of growth seems to slow compared to the rate of growth seen at ages prior to week 3. The epiphyseal disc remains present right up until the mouse reaches full sexual maturity at 8 weeks.

Chapter 5

Discussion

Through this research using the Safranin O stained slides and the cleared and stained specimens, a timeline for the calcaneus' growth and development was established. As I stated previously, we used these two different methods of data collection in order to ensure that nothing was missed in the examination and identification of the different processes occurring in the calcanei, especially in regards to the beginning of the secondary center of ossification. The secondary center of ossification proved to be exceedingly difficult to identify with the histology specimens due to the difficulties associated with sectioning where one is thus only examining a thin sliver of the calcanei at single time. Even though the cleared and stained specimens showed that the second center of ossification started around P11 at the bottom inferior end of the growth plate, it is easy to miss when looking at the Safranin O stained slides unless you are able to achieve perfect sections throughout the entirety of the specimen.

Ultimately, it appears that the pisiform does indeed have a very similar growth process to that of the calcaneus when compared to the timeline established by Kjosness et al. (2014). These two bones have similar structures and a similar progression of stages occurs throughout both of these bones' developments, such as the creation of two separate centers of ossification separated by a single growth plate. However, there are significant differences between them in regards to the rate of the development. It appears that the pisiform grows and matures at a faster rate than that of the calcaneus. From birth, the pisiform mostly consists of undifferentiated hyaline

cartilage cells, just like the calcaneus, with the development of the primary center of ossification occurring at P4. This is relatively close to the development of the calcaneus, whose primary center can begin to be observed taking shape at P2, and starting to ossify at least by P6. At age P7, the pisiform's primary center of ossification begins to turn from cartilage into trabecular bone in addition to the beginning signs of the second center of ossification becoming visible. Two days later, the growth plate is fully formed. Interestingly, this development of a second center of ossification occurs four days later in the calcaneus. Finally, the growth plate is no longer active by P17, drastically reducing any further growth from occurring within this bone. In comparison, we see this occurring at latest by P30 in the calcaneus. Only the thin epiphyseal disk that rests between the two centers of ossification remains as cartilage until the mouse is fully grown and the two distinct sections have fused together, which occurs between Week 3 to Week 5 in the pisiform and not until Week 8 in the calcaneus (Kjosness et al. 2014). Ultimately, both of these bones appear to possess similar growth and development patterns to that of the long bones within the skeleton, of course on a smaller scale and without another secondary center of ossification forming on the opposite end of the bone. This places the calcaneus and pisiform at an interesting position, for even though they are considered to be short bones due to the fact that they are located in the ankle and wrist respectively, they do not seem to fit into that category.

Perhaps these differences in timing between the growth of the pisiform and the calcaneus can be attributed to the fact that the calcaneus is significantly larger in mice than the pisiform. Thus, it may take longer for the cells to progress through the stages of the growth plate and ossify. It would be interesting to further this study and also compare the growth rates of larger long bones within the skeleton. Perhaps this information could further our knowledge of the evolution of limb development and its timing. This could be compared between animals that use

different styles of locomotion and investigate whether or not animals that do not walk quadrupedally have evolved different growth patterns in order to accommodate this difference. For instance, it has been noted that humans have lost the presence of a growth plate in their pisiform during their evolutionary history. Perhaps this change was influenced by the conversion from quadrupedal locomotion to bipedalism.

Chapter 6

Conclusion

The pisiform and the calcaneus are two relatively distinct and important bones within nearly all-terrestrial vertebrates due to the role they play in locomotion. By comparing the growth patterns of the pisiform and the calcaneus, we find a relatively similar development specifically in mice. Perhaps it is because these bones aide in the movement of the ankle and wrist that we see such similarities within their growth and development especially in a quadrupedal animal like a mouse. Although the pisiform developed at a faster rate than that of the calcaneus, the way they progressed through different developmental stages was exceedingly similar. From the unique formation of only one growth plate to the distinctive formation of the two centers of ossification, these two bones followed the same progression throughout their development.

This research raises an interesting question specifically for animals that move through alternative forms of locomotion, such as arboreal or bipedal locomotion. Since it appears that the similarities in the calcaneus and pisiform are due to the fact that both the hind limbs and the forelimbs are used for support, an animal that mainly uses one or the other may experience a different pattern of development and growth in these bones. Research has already been done to show that the pisiform in humans has changed and even lost its growth plate throughout its evolutionary development. Therefore, it would be interesting to see a comparison between that and its matching calcaneus and determine if there are any significant alterations in their growth from that of quadrupedal creatures. Perhaps other creatures also have developed differences in

the development of these bones as well throughout their evolutionary lineage and would show similar changes in the two bones as well but more suited to fit their form of locomotion instead.

In conclusion, I hope this work may serve as a stepping-stone for future research on this topic. Since the development of the mouse calcaneus has been established, more research can be done to build upon this knowledge and advance the understanding that the calcaneus and even the pisiform play in the development and evolution of the locomotion of diverse vertebrates.

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EDUCATION

The Pennsylvania State University University Park, PA
Schreyer Honors College Graduated: May 2017
B.A. Anthropology and B.A. Sociology Jewish Studies/Classic Ancient Mediterranean Studies Minors
Dean's List (7 semesters)

Tel Akko Total Archaeology Field School- Study Abroad Experience Akko, Israel
Participated in Zooarchaeological research as well as excavation at the Tel (Summer 2015)

- Interfacing with students from different ethnicities and backgrounds at the International Conservation Center

HONORS AND AWARDS

- *Enrolled in Penn State Schreyer Honors College- (Fall 2013 to Spring 2017)*
- *Schreyer Honors College Academic Excellence Scholarship- (Fall 2013 to Spring 2017)*
- *Markowitz Scholarship- (Fall 2016- Spring 2017)*
- *Dean List- (Fall 2013 to Spring 2017)*
- *The Virginia Todd Chapel Executive Internship Program - (Summer 2016)*
- *Liberal Arts University Scholarship- (Fall 2013 to Spring 2017)*
- *Eleanor Hoffer End Scholarship- (Spring 2017)*

RELEVANT EXPERIENCE

Stryker Sustainability Solutions Lakeland, FL
Human Resources Intern (Summer 2016)

- Developed and implemented a new employee onboarding program to welcome ten to twenty new employees every week and increase employee retention
- Coordinated events with local service agencies to engage employees in the community
- Completed on the job training and implemented skills learned throughout the internship to improve performance

Crafton Children's Corner Crafton, PA
Infant Room Caretaker (6/2014-8/2014)

- Worked with parents on daily goals, and informed them on progress and completion at the end of the day

Atria's Restaurant and Tavern Pittsburgh, PA
Assistant Server (5/2011 – 8/2013)

- Efficiently satisfy customer needs by utilizing communication skills

Carnegie Performing Arts Center **Carnegie, PA**
Dance *Teacher* (9/2011 – 6/2012)

- Planned and organized each class in advance for a class of ten children who were around five to seven years old

LEADERSHIP EXPERIENCE

Outdoor School at Shaver's Creek Petersburg, PA
Counselor (Fall 2016- Spring 2017)

- Served as a leader and role model overseeing a cabin of eight fifth grade campers throughout the week as well as teaching various lessons related to outdoor education and preservation

Sociology of the Family University Park, PA
Teaching Assistant *(Fall 2016)*

- Act as a source of knowledge and help for the students, assist in grading, and administering exams

Phi Sigma Pi National Honor Fraternity, **Alpha Pi Chapter** University Park, PA
THON Chair (6/2016–5/2017)

- Organize fundraising efforts for a team of sixty members to reach our goal of $10,000 benefiting the Four Diamonds Fund

Chapter Scholarship Chair (Spring 2016)

- Took initiative to create various fraternity events centered around learning and academics

Chapter Brother at Large (Spring 2016)

- Represent the brothers and voice their concerns and ideas during exec

Schreyer Honors College Student Council (StuCo) University Park, PA
Member (9/2013-5/2017)

Hospitality Committee for THON University Park, PA
Member/fundraiser

RESEARCH

EvoDevo Lab at The Penn State University Anthropology Department University Park, PA
Undergraduate Researcher (1/2015- 5/2017)

- Assist faculty researchers in collecting data on various projects related to evolution
- Investigated the growth development of the calcaneus and compared it to that of the pisiform for Honors Thesis