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# Comparing Digit Morphology of an Arboreal Salamander with Potential Competitors

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**Comparing Digit Morphology of an Arboreal Salamander with Potential Competitors**

**Thesis Submitted to the Graduate College of Marshall University**

**In partial fulfillment of the Requirements for the degree of**
**Master of Science**
**Biological Sciences**

**By**

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**Keywords:** ***Aneides aeneus, Plethodon kentucki, Plethodon glutinosus*****, arboreal, comparative zoology**

## Abstract

**Comparing Digit Morphology of an Arboreal Salamander with Potential Competitors**

**Eric Hugh Diefenbacher**

Green Salamanders (*Aneides aeneus*) are the only salamanders in West Virginia to exhibit an arboreal lifestyle. The focus of this study was to determine the cellular anatomy of the distal digit structures and how these structures may influence climbing ability. Comparative histology, comparative morphometrics, and comparative osteology of Green Salamanders, Cumberland Plateau Salamanders (*Plethodon kentucki*), and Slimy Salamanders (*Plethodon glutinosus*), were also used to determine if Cumberland Plateau Salamanders had the potential to compete with Green Salamanders for arboreal habitats. Histologically, Cumberland Plateau Salamanders had cell layer thickening similar to that of Green Salamanders. Morphometrically, Green Salamanders and Cumberland Plateau Salamanders had similar limb measurements; however Cumberland Plateau Salamanders were larger in trunk height and tail height. Osteologically, Cumberland Plateau Salamanders and Slimy Salamanders were virtually identical in carpal, tarsal, and terminal phalanx structure and arrangement.

## Acknowledgements

I would first like to thank my parents, Bonnie and Kevin Diefenbacher, for their unwavering support for my many adventures over the years. It didn't matter what the quest happened to be, whether it was catching critters in the backyard; or trekking through the jungles of Costa Rica and Madagascar, you were there for me. Everything I have pursued is a direct result of the curiosity, enthusiasm, and wonder you instilled in me for everything nature. After all, we didn't have to look at fish poop.

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Thank you to Dr. Thomas K. Pauley. His knowledge of everything reptile and amphibian in West Virginia is unending and without his insight into past research, this project would not have been possible. His generosity and trust in allowing me to take over a large portion of his lab for a project that was not traditionally done in natural history and conservation laboratory, made this project possible. I am grateful to have been a part of the legacy that is Dr. Pauley's Herpetology Lab.

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## Chapter One
## Significance of this Study

Adhesive properties of organisms have been a focal point of science for centuries. Arthropods are known to use several adhesive devices which are classified as either hairy or smooth type organs that cooperate with mechanical systems to produce a temporary hold on a wide variety of substrates (Gorb et al., 2002; Federle, 2002). Anurans use mucous glands in combination with columnar cells to form a meniscus (surface tension) between the flat plantar cell surface and different substrates (Ernst, 1973; Green, 1981; Hanna and Barnes 1991). Geckos are shown to employ scansors with rows of setae containing spatulae which create Van der Waals forces to adhere to various surfaces (Autumn and Peattie, 2002; Russell, 2002).

Studies of adhesion in urodeles have been neglected. The best-known group of urodeles to exhibit adhesive properties is the Neotropical genus *Bolitoglossa*. Some species of *Bolitoglossa* have an increase in the degree of interdigital webbing and a reduction in terminal phalangeal elements creating a paddlelike foot (Alberch, 1981; Green and Alberch 1981). This is accompanied by muscles that enable the plantar surface of the foot to be drawn away from the substrate. This creates a negative pressure between the plantar surface and substrate producing a "suction cup" like adhesive mechanism (Alberch, 1981; Green and Alberch, 1981).

Virtually no studies have been done on possible adhesive structures of North American arboreal salamanders, or how these structures may influence competitive interactions between species for particular habitats. Green Salamanders, *Aneides aeneus*, are known to climb on vertical surfaces such as trees and rock outcrops (Petranka, 1998; Waldron 2000; Waldron and Humphries 2005). Members of the genus *Aneides* are thought to be the only salamanders in temperate areas that utilize arboreal habitats (Green and Alberch, 1981). The Cumberland Plateau Salamander, *Plethodon kentucki*, has recently been found to be a major competitor in

behavioral experiments involving the Green Salamander, *Aneides aeneus* (Canterbury 1991). This evidence combined with field data suggests there may be a potential for *P. kentucki* to compete for rock crevices with *A. aeneus* where their respective ranges overlap in West Virginia (Thomas K. Pauley pers. comm.). If *P. kentucki* is competing with *A. aeneus* for arboreal habitats, then *P. kentucki* must have the morphology to exploit arboreal habitats. This study compares the cellular morphology of digit structures and morphometric data between a known arboreal salamander (*A. aeneus*), a known terrestrial salamander (*Plethodon glutinosus*) the Slimy Salamander which is also a sympatric species, and a terrestrial salamander (*P. kentucki*) which may be semi-arboreal.

The purpose of this study is to determine if field studies are needed to investigate if *P. kentucki* is actually competing with *A. aeneus* for rock crevices. Since *P. kentucki* is more aggressive and may be able to climb like *A. aeneus* (Canterbury 1991), they may be able to push *A. aeneus* out of their respective habitat effectively reducing population numbers of *A. aeneus* in areas where these two species overlap.

Investigating this possibility requires that each species be compared at the morphometric, histological, and osteological level. Morphometrics will help determine if *P. kentucki* is more similar in superficial physical characteristics to either an arboreal (*A. aeneus*) or terrestrial (*P. glutinosus*) salamander. Histological examination will determine if *P. kentucki* has a cellular morphology surrounding the digits more comparable to *A. aeneus* or *P. glutinosus* while examining the osteology of autopod structures will determine if the organization of the bone and cartilage structures in *P. kentucki* is more similar to arboreal or terrestrial salamanders.

## Chapter Two
## Cellular Morphology of Green Salamander Digits

Green Salamanders, *Aneides aeneus*, are known to climb on vertical surfaces such as trees and rock outcrops (Petranka, 1998; Waldron and Humphries, 2005). Members of the genus *Aneides* are the only salamanders in temperate areas that utilize arboreal habitats (Green and Alberch, 1981). While the osteology of the autopod shows the terminal phalanx is curved and "Y" shaped (Wake, 1963; Larson et. al., 1981), studies have not investigated the cellular structure of the digit itself or its potential role in the climbing ability of Green Salamanders. Before an investigation can be launched to determine if it is possible for *P. kentucki* to compete with an arboreal salamander, the cellular morphology of the digits of an arboreal must be realized first. The purpose of this chapter is to reveal this morphology and to set the stage for the remainder of the study.

### Materials and Methods:

*Scanning electron microscopy*- Digits (N=9) and whole autopods (N=2) were amputated from preserved adult *A. aeneus* from the West Virginia Reptile and Amphibian Museum at Marshall University. Digits and autopods were immersed in a dehydration series of ethyl alcohol, sputter coated in gold and pladium, and viewed under a Jeol JSM-S310LV scanning electron microscope at low and high power.

*Histology*- Digits (N=60) were amputated from preserved *A. aeneus* in the West Virginia Reptile and Amphibian Museum at Marshall University. Digits were decalcified in RDO rapid decalcifier for 3.5 hours and immersed in a dehydration series of ethyl alcohols each lasting one hour. This was followed by two 30-minute rinses in xylenes, immersion in melted paraplast at

60$^{o}$C overnight, and embedded in fresh melted paraplast in embeddeding cubes. Each digit was oriented in such a way as to produce sagittal, horizontal or cross sections. Digits were then sectioned at 10µm using a steel blade and a microtome. Sections were stained using Erhich's hematoxylin and Eosin Y stain. Stained sections were then viewed using an Olympus BX51 phase contrast light microscope and photographed using an Olympus CC12 camera and Olympus Microsuite Five Basic edition software.

**Results:**

*SEM*- Micrographs of digits (N=9) show a ridge formed around the outer edge of the distal tip of each digit (Fig. 2.1). This ridge is horseshoe shaped with the tips of each ridge terminating just anterior to the furrow of the terminal phalanx-secondary phalanx interface. Higher power micrographs show few surface pores both on the ridge and within the concavity adjacent the ridge (Fig. 2.1).

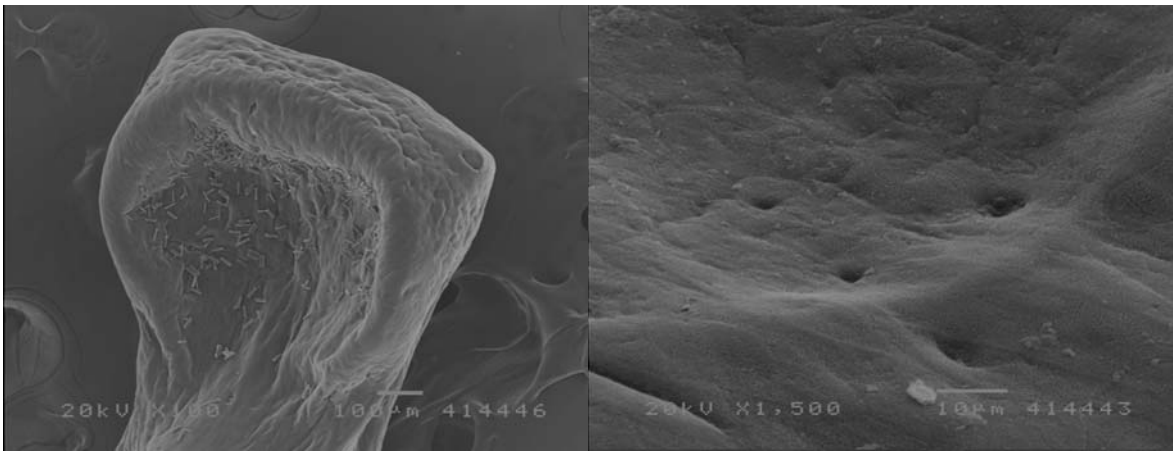



Fig. 2.1. Micrographs of digit showing "horseshoe" shaped ridge (left) and a higher power micrograph showing pores on the digital disc surface (right).

*Histology*- Dermal thickening was seen in sagittal section. Dermal cell layers increase from two at the crest of the terminal phalanx, to four or six cell layers thick at the distal most part of the

terminal phalanx (Fig. 2.2). Dermal thickening then tapers to two cell layers thick on the ventral surface of the digit. Dermal cells appear “raindrop” shaped with the tapered end positioned proximally and the rounded end positioned distally relative to the digit (Fig. 2.2).

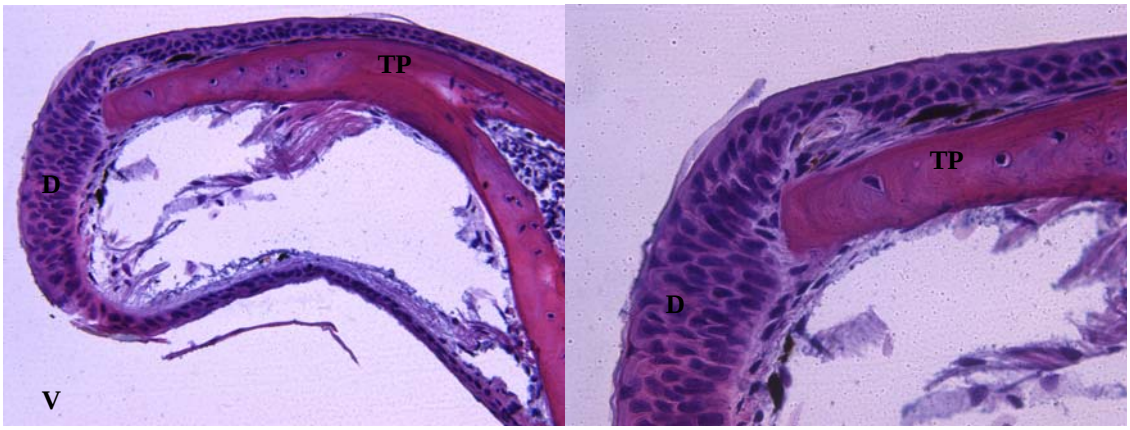



Fig. 2.2. Light micrographs of sagittal sections revealing overall anatomy of digital disc (left, 200x) and profile of dermal cells around the terminal phalanx (right, 400x). Artifacts and space were created by loss of tissue during histological preparation. Abbreviations: terminal phalanx (TP), dermis (D), ventral orientation (V).

Horizontal sections reveal glandular cells in clusters of 8 to 20 oval-shaped cells, lining the perimeter of each digit immediately following the inside dermal tissue layer (Fig. 2.3). Squamous tissue cells were also found to thicken, 4 or more layers, around the distal tips of the terminal phalanx and taper to 2 cell layers thick immediately after the tips of the terminal phalanx.

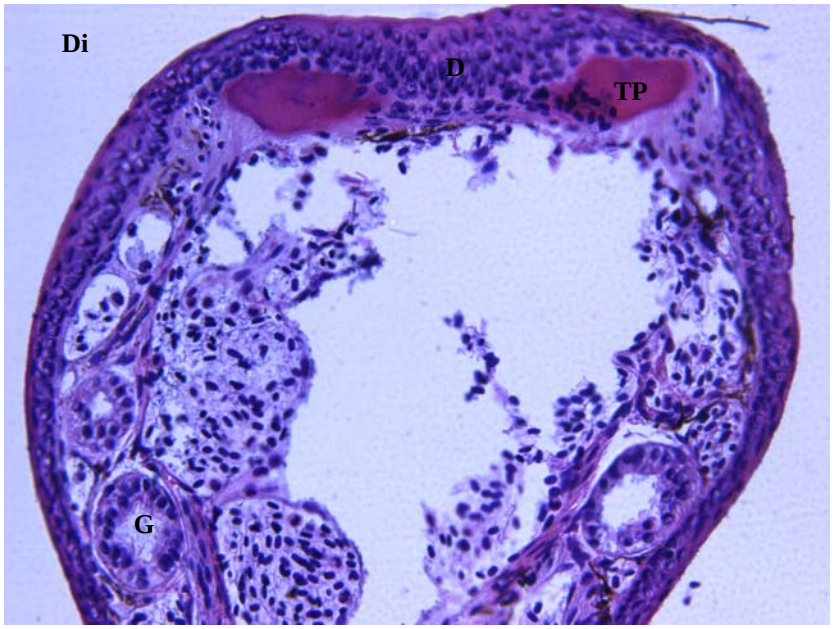


Fig. 2.3. Light micrograph of digital disc in horizontal section, 200x. Artifacts and space were created by loss of tissue during histological preparation. Abbreviations: terminal phalanx (TP), dermis (D), glandular cells (G), and distal orientation (Di).

**Discussion:**

There was some concern that the horseshoe shape of the digital disc observed under the SEM was a result of the dehydration and/or sputter coating process. I compared digital discs of *Aneides* with another species, *Eurycea lucifuga* which does not have digital discs, and found random wrinkling of the digit surface when viewed under the SEM. However, all 9 digital discs observed from *A. aeneus* had a consistent shape suggesting that the digit shape observed under the SEM was not a result of dehydration or the sputter coating process.

It is unknown how the terminal phalanx influences the climbing ability of Green Salamanders. Wake (1963) described the terminal phalanx as an adaptation to climbing since the phalanx was distally flattened, expanded and recurved with ventrally oriented projections. In addition there was a ventral projection on the proximal section of the phalanx enabling tendon attachment. However, there is no mention as to how the mechanism, friction or otherwise, aids in the ability of Green Salamanders to climb. With the degree of dermal thickening and possible

glandular cells associated with the terminal phalanx; it is possible that these structures act as a simple friction based mechanism. The thick dermis and glandular structures could lend to the disc shape while allowing the disc to be flexible enough to grip irregularities on the substrate surface. However, surface tension created between the substrate and ventral surface of the salamander may aid in climbing performance even on rough surfaces such as rocks and bark. This has yet to be explored with this species.

Also left unknown is the degree to which mucous is secreted from the glandular cells and its effects on the digital disc surface. This can be invaluable in determining whether or not digits or autopods exhibit any adhesive strength which may aid in adhering salamanders to different substrates. It is known that *Bolitoglossa* (Green and Alberch, 1981) and trees frogs (Green, 1981; Hanna and Barnes 1991) exhibit mucous secretions to keep the autopod surface moist, conversely playing a crucial role in adhesive capabilities. However since *A. aeneus* are easily plucked off of their respected substrate with very little effort, it is doubtful that there remains little, if any, mucous secreted that plays a vital role in climbing performance.

## Chapter Three
## Comparative Morphometrics

Vernier calipers were used to measure physical characteristics deemed crucial to an arboreal lifestyle. All measurements were done on preserved *A. aeneus* (N=44), *P. kentucki* (N=45), and *P. glutinosus* (N=45) from the reptile and amphibian museum at Marshall University. Snout-vent length (SVL) was measured as the length from the tip of the snout to the posterior most point of the vent. Trunk height (TRH) was measured as the vertical distance between the ventral and dorsal part of the pectoral region posterior to the axilla. Tail height (TLH) was measured as the vertical distance between the ventral and dorsal part of the pelvic region posterior to the insertion of the posterior limbs. Cranial depth (CD) was considered the vertical height of the head at articulation of the jaw. Cranial width (CW) was measured as the width of the head at articulation of jaw. Cranial length (CL) was measured as the length from the tip of the snout to the fold posterior to articulation of the jaw. Humerus, femur, radio-ulna, and tibio-fibula length were measured externally. Humerus length (HUL) was considered the distance from the axilla to knee of anterior limb. Femur length (FL) was the distance from the groin to knee of posterior limb. Radio-ulna length (RUL) was measured from the elbow to the tip of the longest digit and tibio-fibula length (TFL) was measured from the knee to the tip of the longest digit. Humerus and radio-ulna lengths were later combined to determine the total length of the anterior limbs while femur and tibio-fibula lengths were combined to determine total lengths of posterior limbs. Appendix A shows a summary of the measurements made for this part of the study. After all measurements were taken, data were recorded in a spreadsheet and analyzed using SPSS software.

**Results:**

*Aneides aeneus* was significantly different from *P. glutinosus* in snout-vent length (figure 3.1, table 3.2), cranial length, cranial depth, and cranial width (figure 3.2, table 3.2), left and right radio-ulna length (figure 3.3, table 3.2), left and right humerus length (figure 3.4, table 3.2), left and right tibio-fibula length (figure 3.5, table 3.2), left and right femur length (figure 3.6, table 3.2), trunk height (figure 3.7, table 3.2), and tail height (figure 3.8, table 3.2), left and right anterior limb length (figure 3.9, table 3.2), and left and right posterior limb length (figure 3.10 table 3.2).

*Plethodon kentucki* was found to be similar to *A. aeneus* in cranial width (figure 3.2, table 3.1), left and right radio-ulna length (figure 3.3, table 3.1), left and right humerus length (figure 3.4, table 3.1), left and right tibio-fibula length (figure 3.5, table 3.1), left and right femur length (figure 3.6, table 3.1), left and right anterior limb length (figure 3.9, table 3.1), and left and right posterior limb length (figure 3.10, table 3.1). *Plethodon kentucki* was significantly different from *A. aeneus* in snout-vent length (figure 3.1, table 3.1), cranial length and cranial depth (figure 3.2, table 3.1), trunk height (figure 3.7, table 3.1), and tail height (figure 3.8 table 3.1).

*Plethodon glutinosus* was significantly different from *P. kentucki* in snout-vent length (figure 3.1, table 3.3), cranial length, cranial depth, and cranial width (figure 3.2, table 3.3), left and right radio-ulna length (figure 3.3, table 3.3), left and right humerus length (figure 3.4, table 3.3), left and right tibio-fibula length (figure 3.5, table 3.3), left and right femur length (figure 3.6, table 3.3), trunk height (figure 3.7, table 3.3), and tail height (figure 3.8, table 3.3), left and right anterior limb length (figure 3.9, table 3.3), and left and right posterior limb length (figure 3.10, table 3.3).

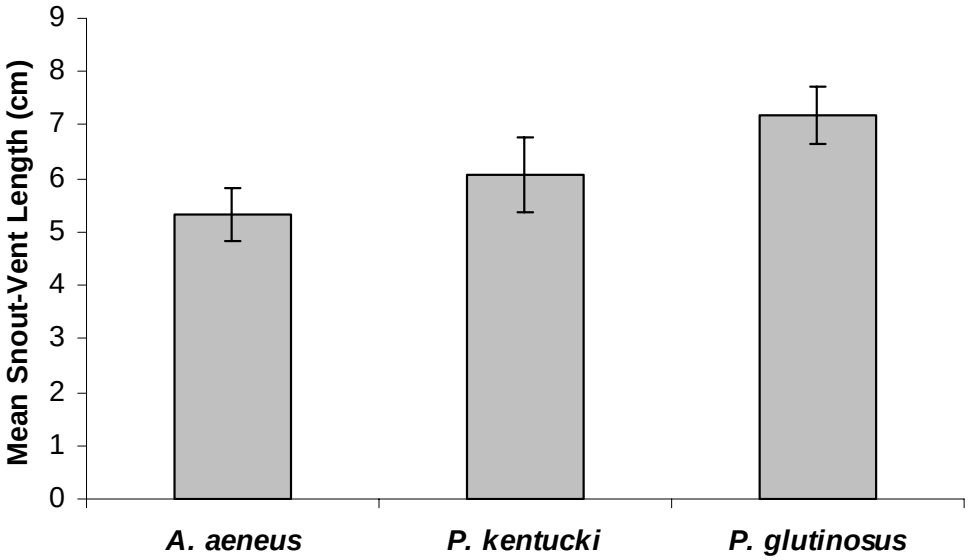



Figure 3.1. Comparisons of mean snout-vent length between *A. aeneus*, *P. kentucki*, and *P. glutinosus*.

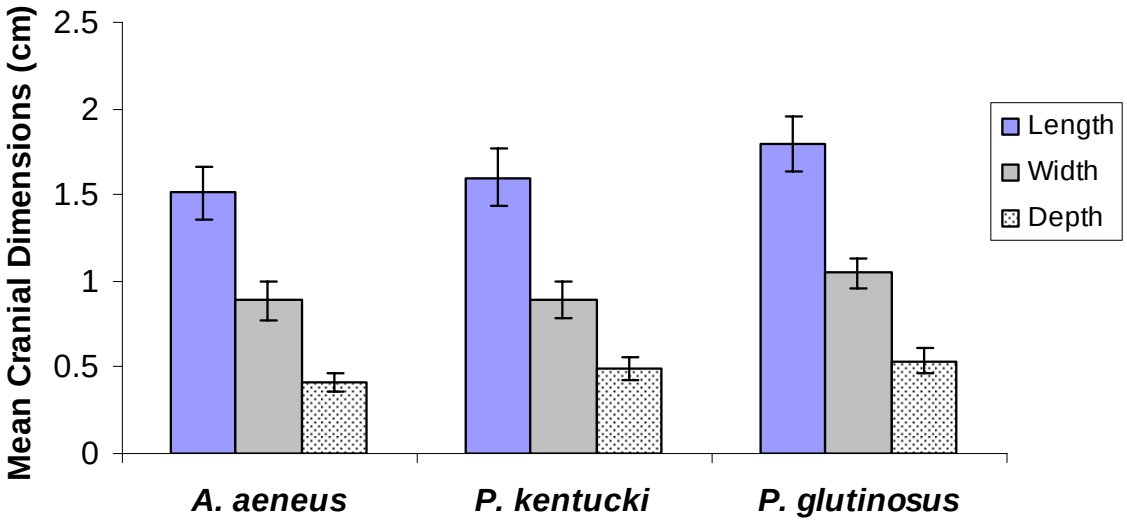



Figure 3.2. Comparisons of mean cranial dimensions cranial length (blue), cranial width (grey), and cranial depth (dotted) between *A. aeneus*, *P. kentucki*, and *P. glutinosus.*

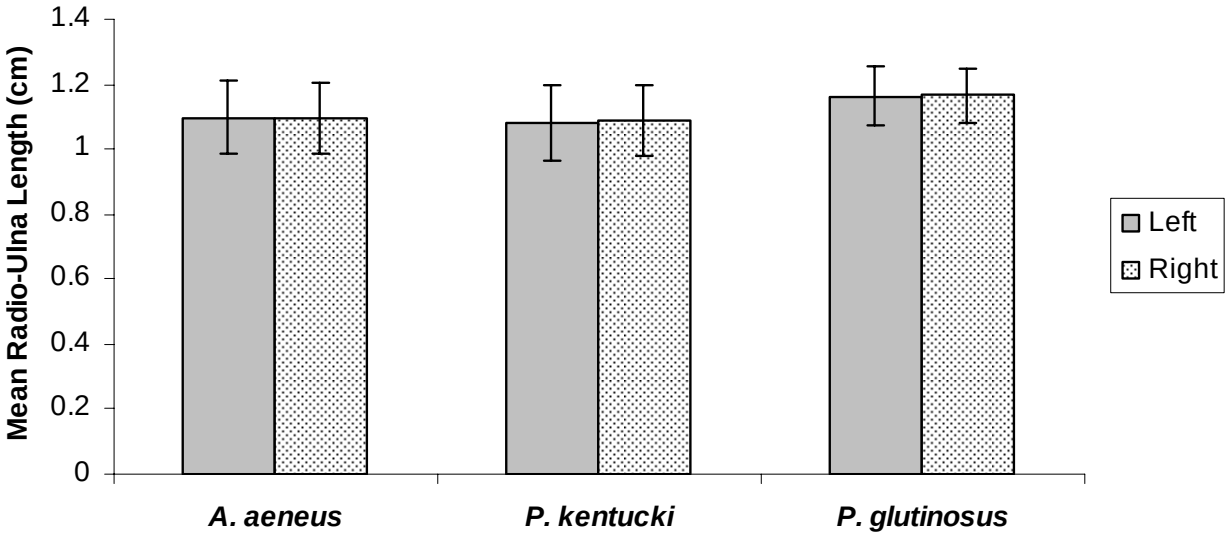



Figure 3.3. Comparisons of mean left radio-ulna length (grey) and right radio-ulna length (dotted) between *A. aeneus, P. kentucki,* and *P. glutinosus.*

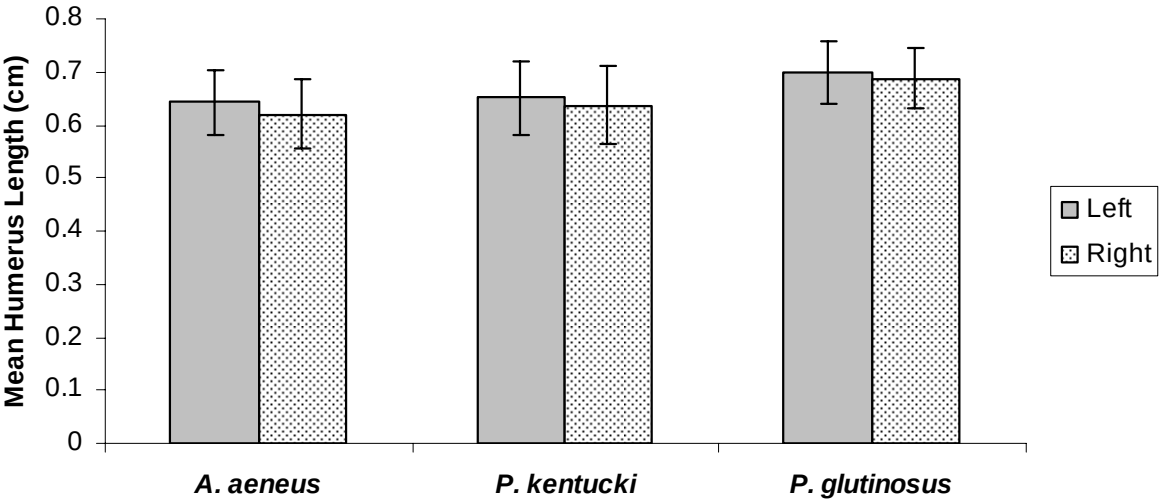



Figure 3.4. Comparisons of mean left humerus length (grey) and right humerus length (dotted) between *A. aeneus, P. kentucki,* and *P. glutinosus.*

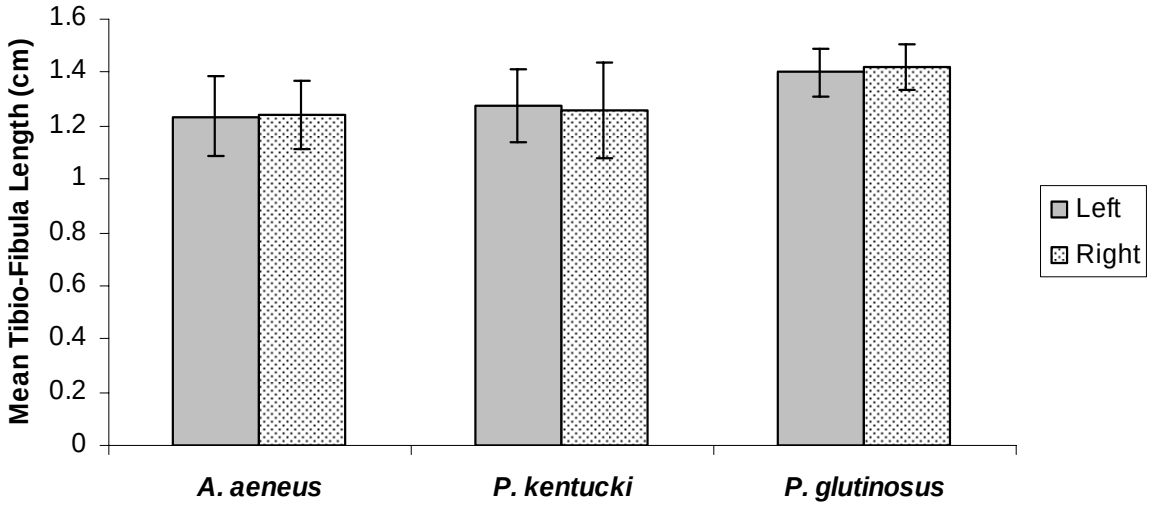



Figure 3.5. Comparisons of mean left tibio-fibula length (grey) and right tibio-fibula length (dotted) between *A. aeneus*, *P. kentucki*, and *P. glutinosus.*

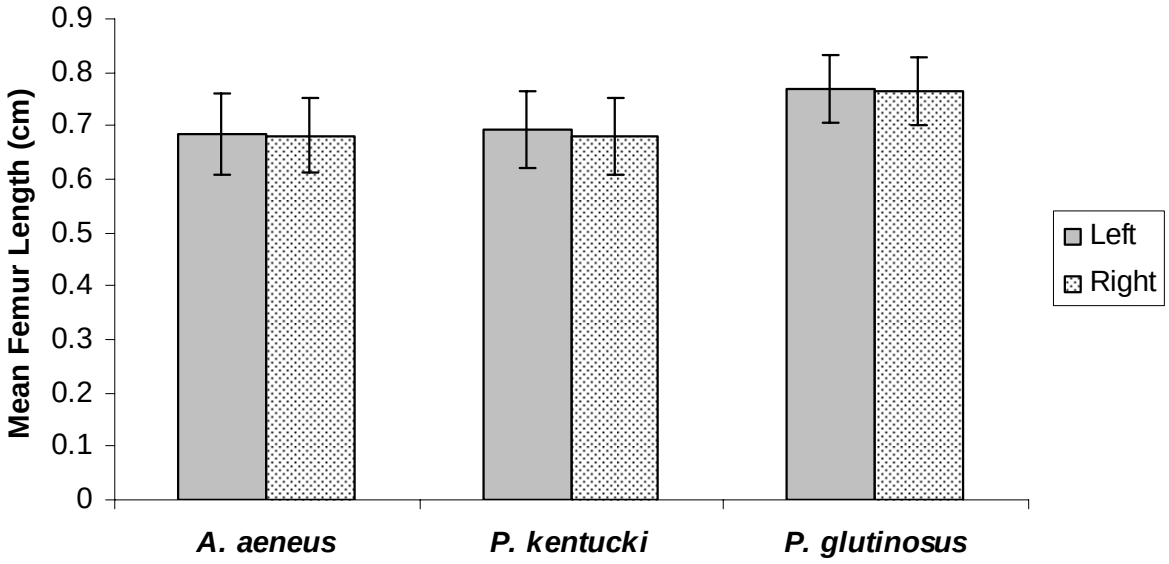



Figure 3.6. Comparisons of mean left femur length (grey) and right femur length (dotted) between *A. aeneus*, *P. kentucki*, and *P. glutinosus.*

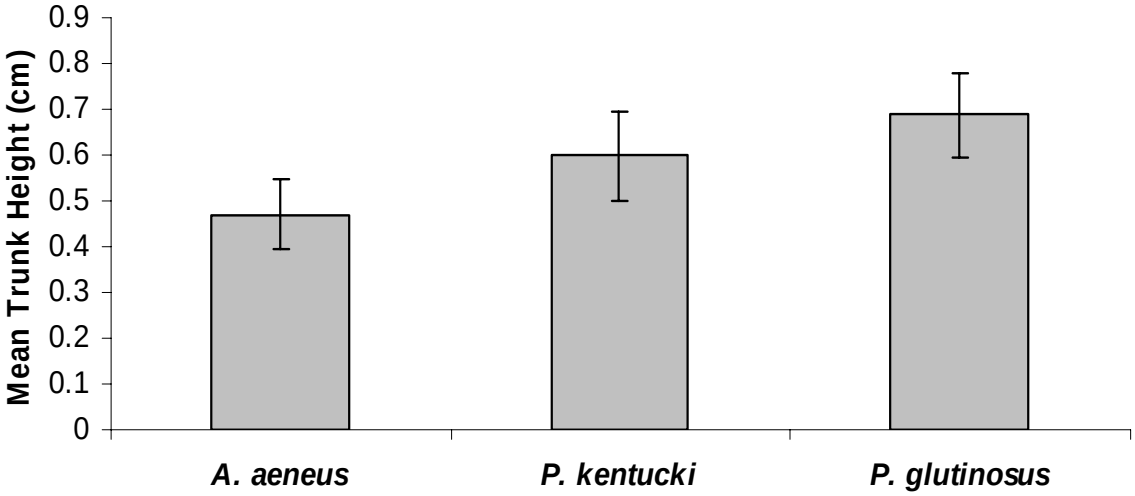



Figure 3.7. Comparisons of mean trunk height between *A. aeneus*, *P. kentucki*, and *P. glutinosus.*

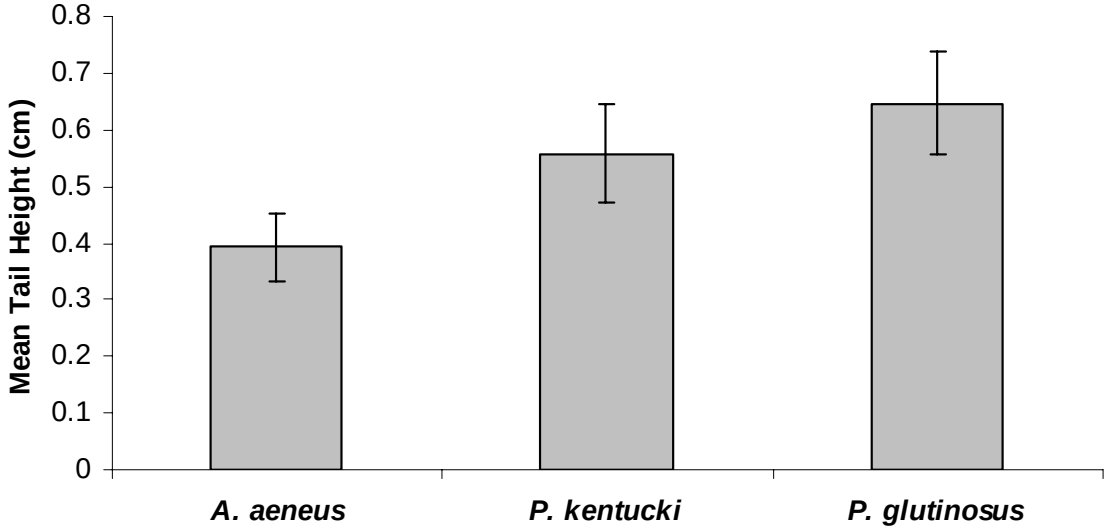



Figure 3.8. Comparisons of mean tail height between *A. aeneus*, *P. kentucki*, and *P. glutinosus.*

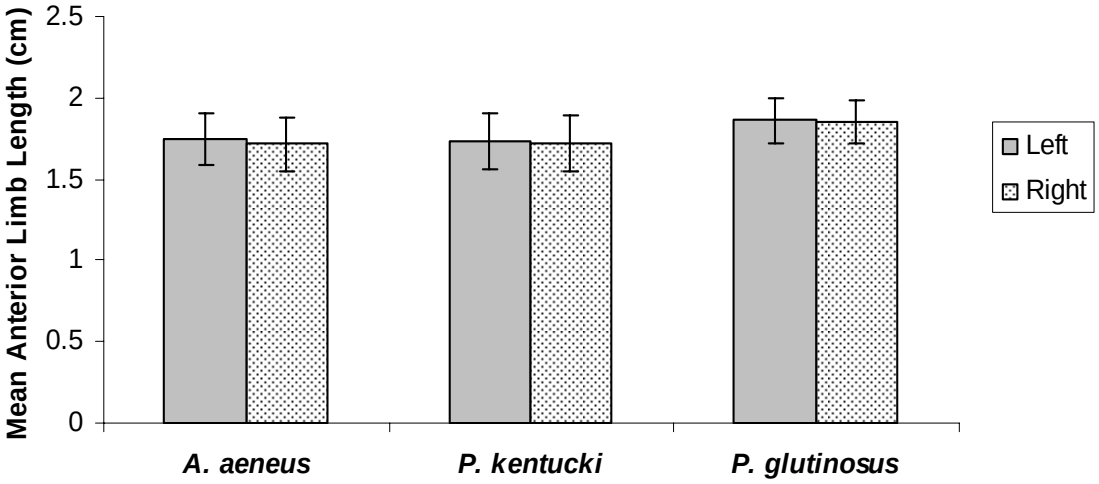



Figure 3.9. Comparisons of mean left anterior limb total length (grey) and right anterior limb total length (dotted) between *A. aeneus*, *P. kentucki*, and *P. glutinosus.*

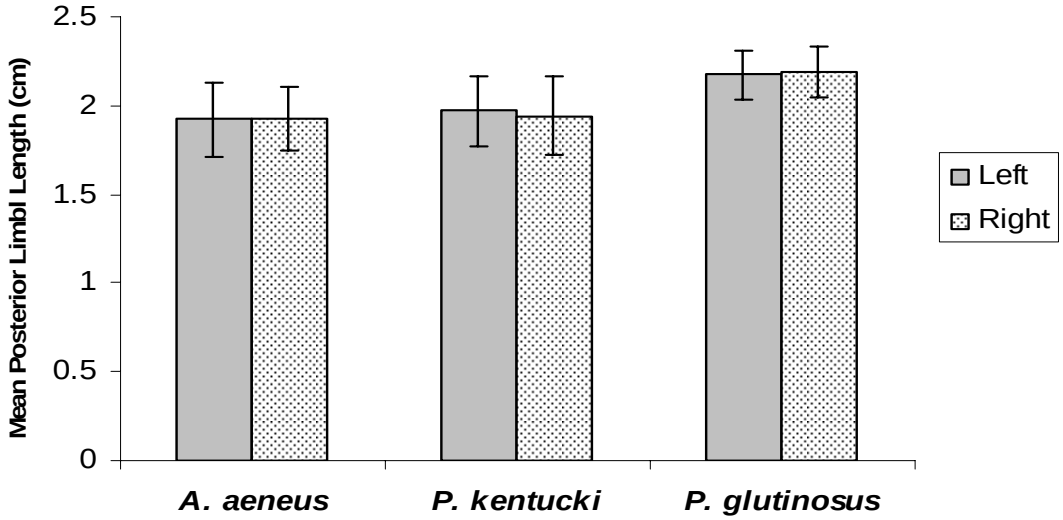



Figure 3.10. Comparisons of mean left posterior limb total length (grey) and right posterior limb total length (dotted) between *A. aeneus*, *P. kentucki* and *P. glutinosus.*

Table 3.1. Summary table comparing mean values (cm) of morphological characteristics between *A. aeneus* and *P. kentucki* (*t*-test, df=87).

| **Characteristic** | ***A. aeneus*** | ***P. kentucki*** | **P-value** |
|---|---|---|---|
| Cranial Length | 1.51 | 1.60 | P= 0.012 |
| Cranial Width | 0.88 | 0.89 | P= 0.812 |
| Cranial Depth | 0.41 | 0.48 | P<0.05 |
| Anterior Left Limb Length | 1.74 | 1.73 | P= 0.832 |
| Anterior Right Limb Length | 1.71 | 1.72 | P= 0.862 |
| Posterior Left Limb Length | 1.92 | 1.96 | P= 0.250 |
| Posterior Right Limb Length | 1.92 | 1.93 | P= 0.728 |
| Trunk Height | 0.47 | 0.59 | P<0.05 |
| Tail Height | 0.39 | 0.55 | P<0.05 |
| Left Radio-Ulna Length | 1.09 | 1.08 | P= 0.483 |
| Right Radio-Ulna Length | 1.09 | 1.08 | P= 0.664 |
| Left Humerus Length | 0.64 | 0.65 | P= 0.499 |
| Right Humerus Length | 0.62 | 0.63 | P= 0.279 |
| Left Tibio-Fibula Length | 1.23 | 1.27 | P= 0.181 |
| Right Tibio-Fibula Length | 1.24 | 1.25 | P= 0.608 |
| Left Femur Length | 0.68 | 0.69 | P= 0.578 |
| Right Femur Length | 0.68 | 0.68 | P= 0.891 |
| Snout-Vent Length | 5.32 | 6.06 | P<0.05 |

Table 3.2. Summary table comparing mean values (cm) of morphological characteristics between *A. aeneus* and *P. glutinosus* (*t*-test, df=87).

| **Characteristic** | ***A. aeneus*** | ***P. glutinosus*** | **P-value** |
|---|---|---|---|
| Cranial Length | 1.51 | 1.79 | P<0.05 |
| Cranial Width | 0.88 | 1.04 | P<0.05 |
| Cranial Depth | 0.41 | 0.53 | P<0.05 |
| Anterior Left Limb Length | 1.74 | 1.86 | P<0.05 |
| Anterior Right Limb Length | 1.71 | 1.85 | P<0.05 |
| Posterior Left Limb Length | 1.92 | 2.17 | P<0.05 |
| Posterior Right Limb Length | 1.92 | 2.18 | P<0.05 |
| Trunk Height | 0.47 | 0.68 | P<0.05 |
| Tail Height | 0.39 | 0.64 | P<0.05 |
| Left Radio-Ulna Length | 1.09 | 1.16 | P= 0.003 |
| Right Radio-Ulna Length | 1.09 | 1.16 | P= 0.001 |
| Left Humerus Length | 0.64 | 0.69 | P<0.05 |
| Right Humerus Length | 0.62 | 0.68 | P<0.05 |
| Left Tibio-Fibula Length | 1.23 | 1.40 | P<0.05 |
| Right Tibio-Fibula Length | 1.24 | 1.42 | P<0.05 |
| Left Femur Length | 0.68 | 0.76 | P<0.05 |
| Right Femur Length | 0.68 | 0.76 | P<0.05 |
| Snout-Vent Length | 5.32 | 7.18 | P<0.05 |

Table 3.3. Summary table comparing mean values (cm) of morphological characteristics between *P. kentucki* and *P. glutinosus* (*t*-test, df=87).

| **Characteristic** | ***P. kentucki*** | ***P. glutinosus*** | **P-value** |
|---|---|---|---|
| Cranial Length | 1.60 | 1.79 | P<0.05 |
| Cranial Width | 0.89 | 1.04 | P<0.05 |
| Cranial Depth | 0.48 | 0.53 | P= 0.002 |
| Anterior Left Limb Length | 1.73 | 1.86 | P<0.05 |
| Anterior Right Limb Length | 1.72 | 1.85 | P<0.05 |
| Posterior Left Limb Length | 1.96 | 2.17 | P<0.05 |
| Posterior Right Limb Length | 1.93 | 2.18 | P<0.05 |
| Trunk Height | 0.59 | 0.68 | P<0.05 |
| Tail Height | 0.55 | 0.64 | P<0.05 |
| Left Radio-Ulna Length | 1.08 | 1.16 | P<0.05 |
| Right Radio-Ulna Length | 1.08 | 1.16 | P<0.05 |
| Left Humerus Length | 0.65 | 0.69 | P= 0.001 |
| Right Humerus Length | 0.63 | 0.68 | P<0.05 |
| Left Tibio-Fibula Length | 1.27 | 1.40 | P<0.05 |
| Right Tibio-Fibula Length | 1.25 | 1.42 | P<0.05 |
| Left Femur Length | 0.69 | 0.76 | P<0.05 |
| Right Femur Length | 0.68 | 0.76 | P<0.05 |
| Snout-Vent Length | 6.06 | 7.18 | P<0.05 |

**Discussion:** Characteristics such as radio-ulna length, humerus length, tibio-fibula length, femur length, anterior limb length, and posterior limb length were found to be of similar size between both *A. aeneus* and *P. kentucki*. This suggests *P. kentucki* may be able to climb similar surfaces as *A. aeneus* since its limb measurements are similar to those of an arboreal salamander. Limbs of a certain length may help individuals climb better than if their limbs were shorter or longer by comparison. Short limbs may not allow the individual to reach areas needed to secure a firm hold on vertical surfaces, while longer limbs may be too cumbersome to use while climbing

vertical surfaces.

However, *P. kentucki* has a greater cranial depth, trunk height, and tail height than *A. aeneus*. This suggests that even if *P. kentucki* could climb as well as *A. aeneus,* it would not be able to exploit the same size rock crevices as *A. aeneus* once an adult. Since *A. aeneus* only exploits rock crevices of a certain size (Canterbury 1991, Waldron 2000) there should be no threat of *P. kentucki* competing for these rock crevices. However, what these data and previous competition studies in the lab (Canterbury 1991) fail to determine is if the aggressiveness of these salamanders persists or changes as individuals age. While only adults were measured for this study, there is no evidence to suggest that juveniles could not compete for rock crevices. Juveniles and smaller adults of *P. kentucki* could climb and compete for rock crevices and ultimately out-compete *A. aeneus*. What may drive *P. kentucki* to climb for habitat or foraging opportunities may be competition on the ground with other *P. kentucki* or, in areas where their ranges overlap, *P. glutinosus*. It has been noted in other species that habitat stratification does occur where one species out-competes another for territory and/or food (Bailey 1992) essentially forcing the other species into a different niche.

All measurements indicated that *P. glutinosus* was the largest species. Why *P. glutinosus* does not climb to the degree of *A. aeneus* or *P. kentucki*, could be due to its aggressiveness (Bailey 1992, Canterbury 1991). If *P. glutinosus* out-competes or intimidates other species for territories or food items, then there would be no reason for *P. glutinosus* to climb since it could exploit all of the food on the forest floor and defend its territory effectively against intruders.

## Chapter Four
## Comparative Histology

Digits (N=12) were amputated from formalin-fixed specimens in the reptile and amphibian museum at Marshall University. Three digits were amputated from the anterior autopod and posterior autopod on the same side of the specimen when possible. Digits were chosen based on distance of spread between digits and flatness of digits.

Amputated digits were then decalcified in RDO rapid decalcifying agent for 3.5 hours and immersed in a graded series of ethyl alcohols each lasting 1 hour. Digits were placed in two rinsings of xylene each lasting 30 minutes then immersed in melted paraplast at 60$^{o}$C for overnight. Digits were then embedded in fresh melted paraplast in embedding molds and oriented to obtain sagittal and horizontal sections. Molds were left to cool and harden overnight. Appendix B has a thorough step-by-step protocol of the above procedure.

Embedded specimens were then sectioned at 10µm using a microtome and steel knife. Ribbons of sections from the microtome were placed in a 30$^{o}$C water bath to flatten out, placed on subbed slides (Appendix C), and left to cure on a 30$^{o}$C hot plate overnight. Sections were stained using Ehrlich's hematoxylin and eosin Y stain. Appendix D explains the procedure used for hematoxylin and eosin Y staining. Histological preparations were then viewed with an Olympus BX51 phase contrast light microscope and photographed using an Olympus CC12 camera and Olympus Microsuite Five Basic edition software.

**Results:** The distal end of the digits of *A. aeneus* show a highly curved terminal phalanx with an increase in dermal cell layers toward the distal tip of the terminal phalanx (Fig. 4.1). The amount of dermal cell layers changes from two cell layers on the dorsal portion of the terminal phalanx, increasing to three cell layers mid-way through the dorsal portion of the terminal phalanx

near the distal tip; to five cell layers directly adjacent the distal tip of the terminal phalanx (Fig. 4.1). Dermal cell layers taper towards the ventral portion of the distal tip of the digit to two cell layers and continue across the ventral part of the digit for the remainder of the digit.

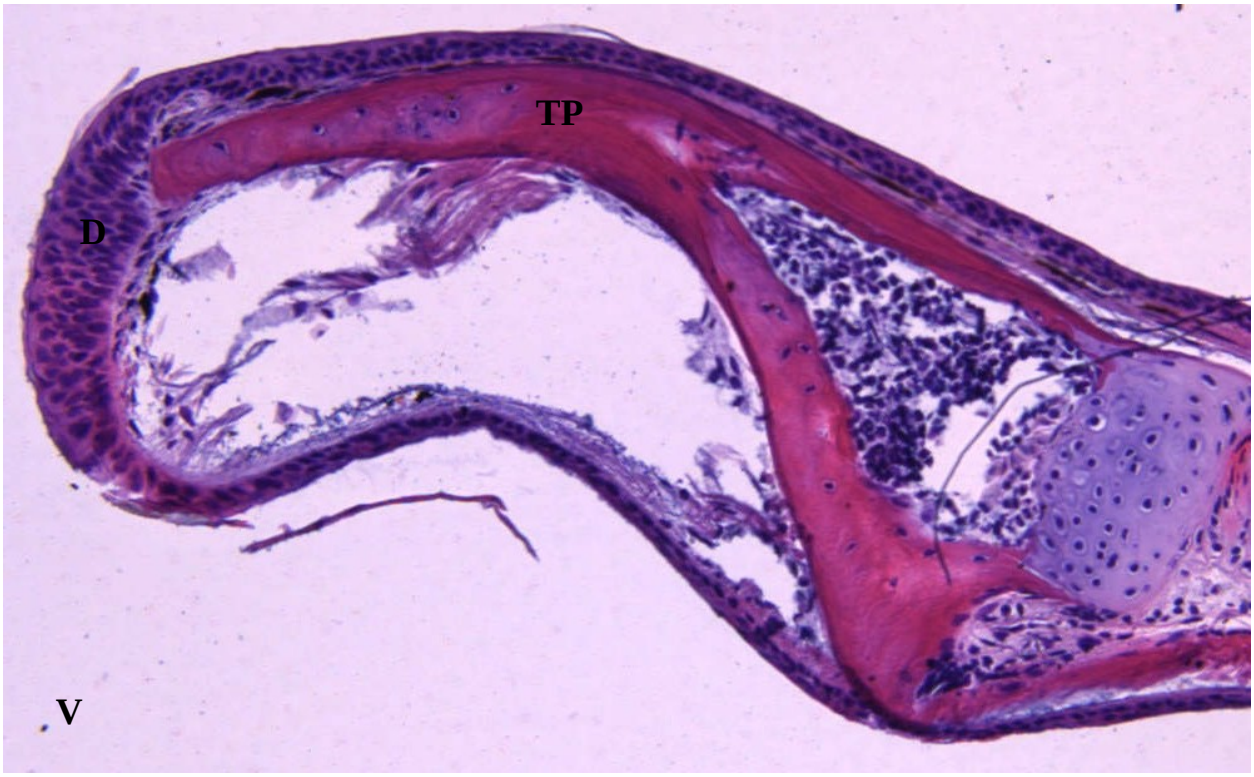



Figure 4.1. Sagittal section of *Aneides aeneus* terminal phalanx, (200x). Artifacts and space created by loss of tissue during histological preparation. Abbreviations: terminal phalanx (TP), dermis (D), ventral orientation (V).

Histological preparations of *P. kentucki* show a slightly curved terminal phalanx as well as thickening in the amount of dermal cell layers progressing from the proximal to distal tip of the digit (Fig. 4.2). The curvature of the terminal phalanx begins at the ventral proximal portion of the terminal phalanx at the projection where the tendon attaches and proceeds to slant upwards in a dorsal distal direction. Just past the mid point of the terminal phalanx the tip curves downward toward the ventral portion of the digit. Dermal cell layers begin at two cell layers thick for the majority of the dorsal part of the digit until approximately mid-way through the

terminal phalanx. Dermal cell layers increase at this point form two, to five cell layers directly adjacent to the distal tip of the terminal phalanx. Four to five cell layers persist through the distal and ventral portion of the digit only to taper at the terminal phalanx/ secondary phalanx joint back to two or three cell layers for the remainder of the digit.

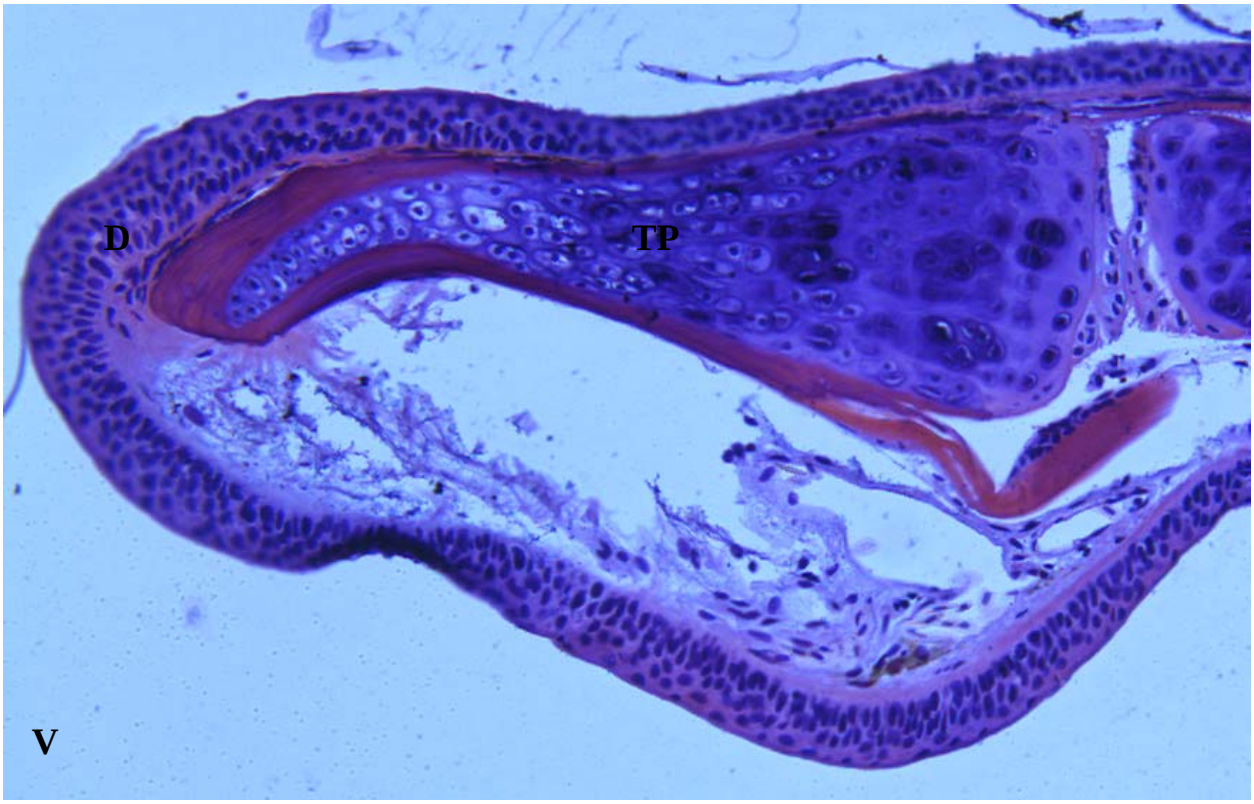



Figure 4.2. Sagittal section of *Plethodon kentucki* digit, (100x). Artifacts and space created by loss of tissue during histological preparation. Abbreviations: terminal phalanx (TP), dermis (D), ventral orientation (V).

Digits of *P. glutinosus* show a flat terminal phalanx with no curvature and barely any increase in dermal cell layers (Fig. 4.3). Two or three cell layers comprise the dermal layer which remains this thickness from the dorsal portion continuing on around the distal tip adjacent the terminal phalanx proceeding ventrally for the rest of the digit.

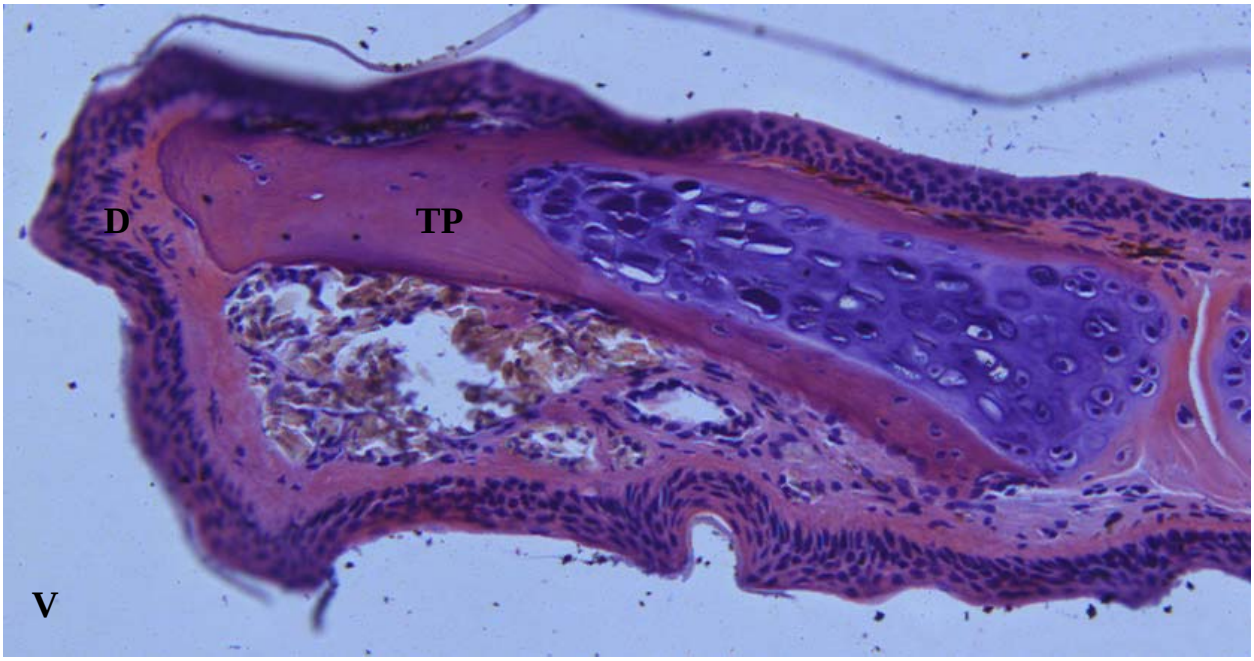


Figure 4.3. Sagittal section of *Plethodon glutinosus*, (100x). Artifacts and space created by loss of tissue during histological preparation. Abbreviations: terminal phalanx (TP), dermis (D), ventral orientation (V).

Table 4.1. Summary table of histological observations

| **Species** | **Curvature of Terminal Phalanx** | **Dermal Cell Layers** | | |
|---|---|---|---|---|
| | | **Dorsal** | **Distal** | **Ventral** |
| *A. aeneus* | Heavily Curved | 2 | 4 - 5 | 2 |
| *P. kentucki* | Curved | 2 | 4 - 5 | 4 - 5 |
| *P. glutinosus* | Slightly Curved | 2 - 3 | 2 - 3 | 2 - 3 |

**Discussion:** The presence of increased dermal cell layers is not nearly as important as the locality of thickening. *Aneides aeneus* digits showed thickening around the distal tip of the terminal phalanx but cell layers tapered rapidly to two cell layers along the ventral part of each digit. *Plethodon kentucki* digits showed thickening around the distal tip of each digit, however this thickening remained around the distal tip and remained so throughout the ventral portion of the digit only tapering at the joint between the terminal phalanx and secondary phalanx. The

significance of the location of this thickening could lend to the location on the digit that experiences the most friction when the organism is traveling along different substrates and the location on the digit that under goes the most stress. This thickening may be similar to how calluses develop on hands or feet which protect areas of increased wear from cuts and infections. More layers results in more padding and therefore more protection.

In *A. aeneus,* thickening indicates that the majority of the stress is applied to the distal tip of the terminal phalanx. This suggests *A. aeneus* uses the distal tip of the digit to climb on substrates rather than the ventral part of the digit. This is not to say that *A. aeneus* never uses the ventral portion of the digits, it has been noted in some populations that *A. aeneus* travels between trees and rocks and must travel along the horizontal surfaces to do so (Waldron and Humphries 2005) which would require use of the ventral surface of the digit. Not only does this indicate that the terminal phalanx is specifically adapted for a climbing lifestyle (Wake 1963) but the cellular morphology around the terminal phalanx may also be indicative of a climbing lifestyle.

*Plethodon kentucki* digits show a similar thickening around the terminal phalanx as in *A. aeneus*, however the number of cell layers around the terminal phalanx is consistent through the ventral portion of the digit. There is also a slight curve to the terminal phalanx as in *A. aeneus.* While the terminal phalanx of *P. kentucki* is not nearly as curved as *A. aeneus,* the small degree to which the terminal phalanx is curved may be just enough to aid *P. kentucki* in climbing vertical or nearly vertical surfaces. The cellular morphology surrounding the terminal phalanx suggests *P. kentucki* may exploit several regions of the digit to traverse different substrates. Thickening around the distal tip of the terminal phalanx may aid in climbing vertical substrates, while continuation of this thickening across the ventral part of the digit would help to protect ventral portion of the digit while traveling along horizontal surfaces. Since dermal thickening

in *P. kentucki* digits is not as heavily localized as it is in *A. aeneus*, this thickness continues across the ventral portion of the digit, and there is a small curvature of the terminal phalanx, evidence suggests that *P. kentucki* may remain on horizontal surfaces for the majority of its life processes but may climb on vertical surfaces more than previously thought. Reasons for climbing vertical surfaces include breeding habitat, shelter, and foraging.

*Plethodon glutinosus* digits show a uniformity of cell layers along the dorsal, distal, and ventral portion with only a slight curvature to the terminal phalanx. This suggests *P. glutinosus* remains terrestrial and climbs very little, if at all, during any aspect of its life processes.

## Chapter Five
## Comparative Osteology

Specimens used for osteological examination were obtained from the reptile and amphibian museum at Marshall University. The dermis and all internal organs minus spinal and cranial tissue were removed before processing. Specimens were then stained with Alcian Blue cartilage stain and Alizarin Red S to stain bone. The protocol used to clear and stain specimens can be found in appendix E. Specimens were then viewed with a Leica MZ6 stereo microscope, Volpi NCL150 light source, and photographed using an Olympus CC12 camera and Olympus Microsuite Five Basic edition software.

**Results:**

***Aneides aeneus***

The manus of *A. aeneus* consists of the carpus with 8 cartilaginous elements; the radiale, ulnare, intermedium, 2 centrales and 3 carpals (Fig 5.1). The radiale, intermedium, and ulnare constitute the proximal most elements with centrale 1 articulating proximally with the intermedium, preaxial with the radiale and centrale 2, postaxial with the ulnare and carpal 3, and distally with carpals 1 and 2. The ulnare is articulated proximally with the ulna, preaxial with the intermedium, and distally with carpal 3. The radiale is articulated proximally with the radius, postaxial with the intermedium, and distally with centrale 2. Centrale 2 articulates proximally with the radiale, postaxial with centrale 1, and distally with carpal 1. Carpal 1 articulates proximally with centrales 1 and 2, and distally with metacarpals 1 and 2. Carpal 2 articulates preaxial with carpal 1, distally with metacarpal 3, and postaxial with carpal 3. Carpal 3 articulates preaxial with carpal 2 and centrale 1, distally with metacarpal 4, and proximally with the ulnare. Metacarpals 1 and 2 articulate to the distal portion of carpal 1, metacarpal 3 to carpal 2, and metacarpal 4 articulates proximally with carpal 3 (Fig 5.1).

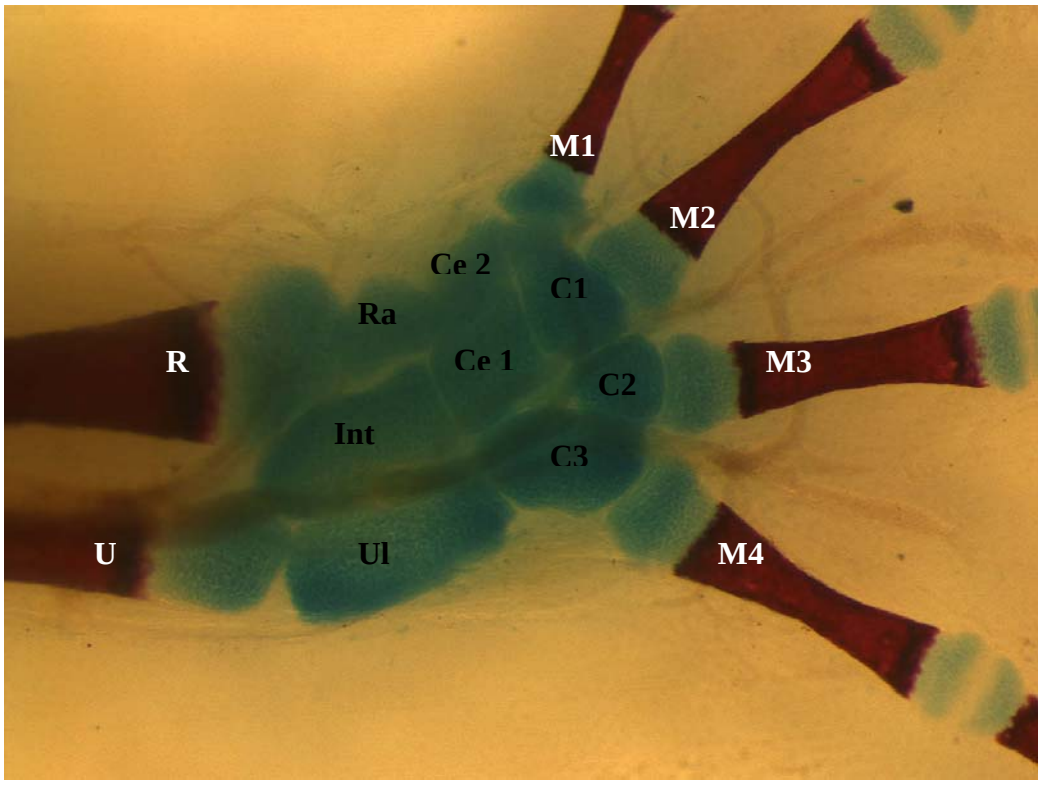



Figure 5.1 Cleared and stained manus of *A. aeneus* showing bone (red) and cartilage (blue). Abbreviations: R= radius; U= ulna; Int= intermedium; Ul= ulnare; Ra= radiale; Ce 1-2= centrale 1-2; C1-3= carpal 1-3 ; M1-4= metacarpal 1-4.

The pes of *A. aeneus* is represented by 9 cartilaginous elements, a tibiale, intermedium, fibulare, 2 centrales, and 4 tarsals (Fig. 5.2). The fibulare is articulated proximally with the fibula, preaxial with the intermedium and centrale 1, and distally with tarsal 4. The intermedium is articulated preaxial by the tibiale and postaxial by the fibulare and articulated distally with centrale 1. The tibiale is articulated postaxial with the intermedium and distally with centrale 2. Centrale 1 is articulated proximally with the intermedium and a portion of the fibulare, preaxial with centrale 2, postaxial to tarsal 4, and distally with tarsals 1 and 2. Centrale 2 is articulated proximally by the tibiale, postaxial with centrale 1, and distal with tarsal 1. Tarsals 1, 2, 3, and 4, all articulate proximally with centrale 1 with tarsal 1 slightly articulating preaxial with centrale 2 and tarsal 4 articulating proximally with the fibulare. Metatarsals 1 and 2 connect

proximally with tarsal 1, metatarsal 3 proximally with tarsal 2, metatarsal 4 proximally with tarsal 3, and metatarsal 5 proximally with tarsal 4 (Fig. 5.2).

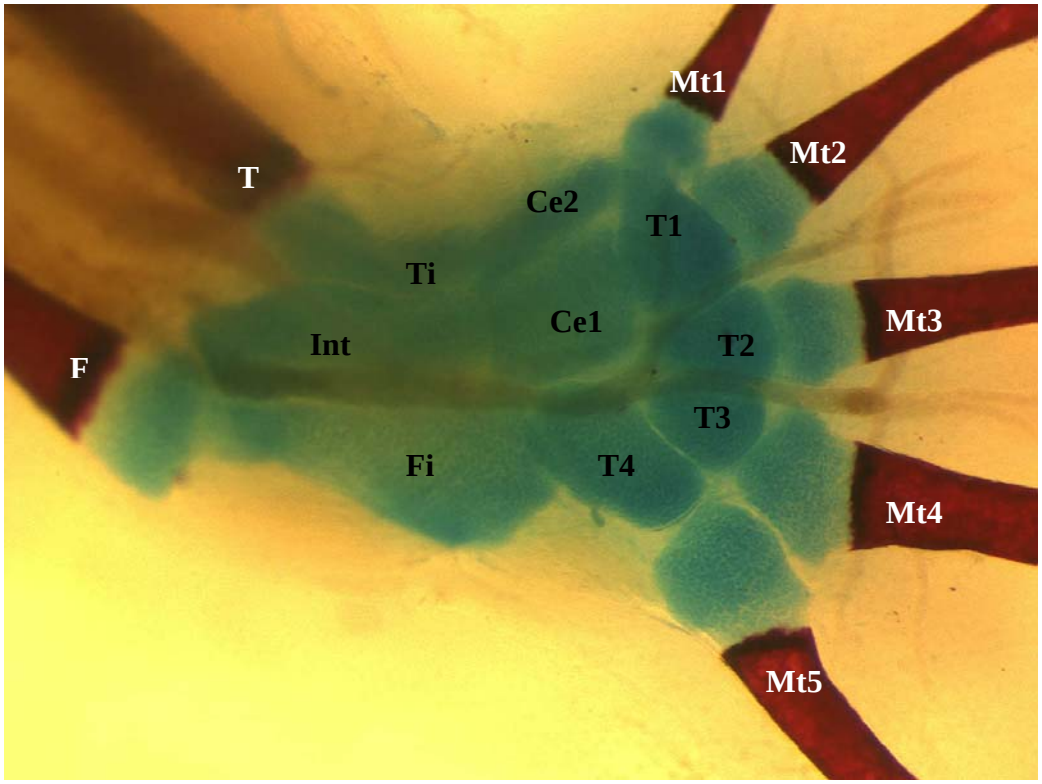



Figure 5.2 Cleared and stained pes of *A. aeneus* showing bone (red) and cartilage (blue). Abbreviations: T= tibia; F= fibula; Int= intermedium; Fi= fibulare; Ti= Tibiale; Ce1-2= centrale 1-2; T1-4= tarsal 1-4; Mt1-5= metatarsal 1-5.

The terminal phalanx of *A. aeneus* is distally flattened, recurved, and expanded into a Y-Shape tip at the distal end (Fig 5.3). There is also a projection extending ventrally from the proximal portion of the terminal phalanx used for tendon attachment.

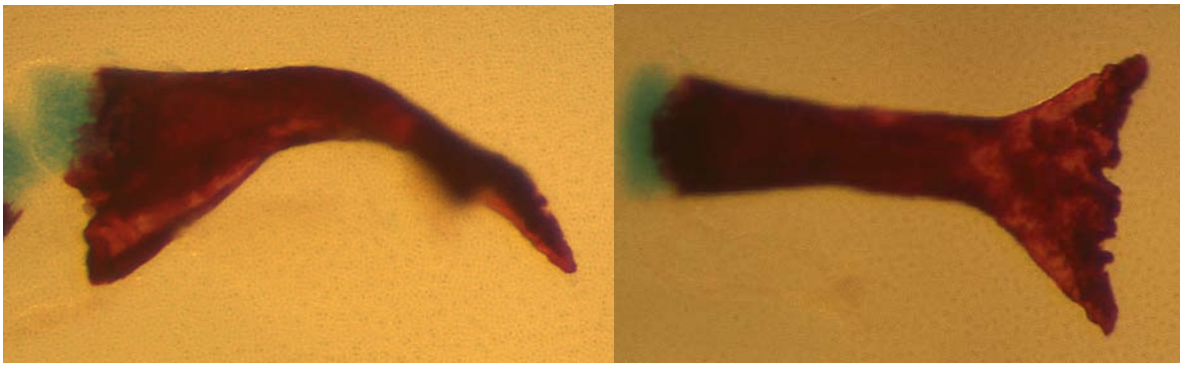


Figure 5.3 Cleared and stained images of an *A. aeneus* terminal phalanx showing bone (red) and cartilage (blue). Lateral view (left) and ventral view (right).

***Plethodon kentucki***

The manus of *P. kentucki* consists of the carpus with 8 carpal elements; an ulnare, intermedium, radiale, 2 centrales, and 3 carpals (Fig 5.4). The ulnare articulates proximally with the ulna, preaxial with the intermedium, and distally with carpal 3. The intermedium articulates postaxial with the ulnare and preaxial with the radiale, proximally with both the ulna and radius, and distally with centrale 1. The radiale articulates proximally with the radius, postaxial with the intermedium and centrale 1, and distally with centrale 2. Centrale 2 articulates proximally with the radiale, postaxial with centrale 1, and distally with carpal 1. Carpal 1 articulates proximally with centrales 1 and 2, postaxial with carpal 2, and distal with metacarpals 1 and 2. Carpal 2 articulates proximally with centrale 1, preaxial with carpal 1, postaxial with carpal 3, and distally with metacarpal 3. Carpal 3 articulates preaxial with carpal 2 and centrale 1, proximally with the intermedium and ulnare, and distally with metacarpal 4.

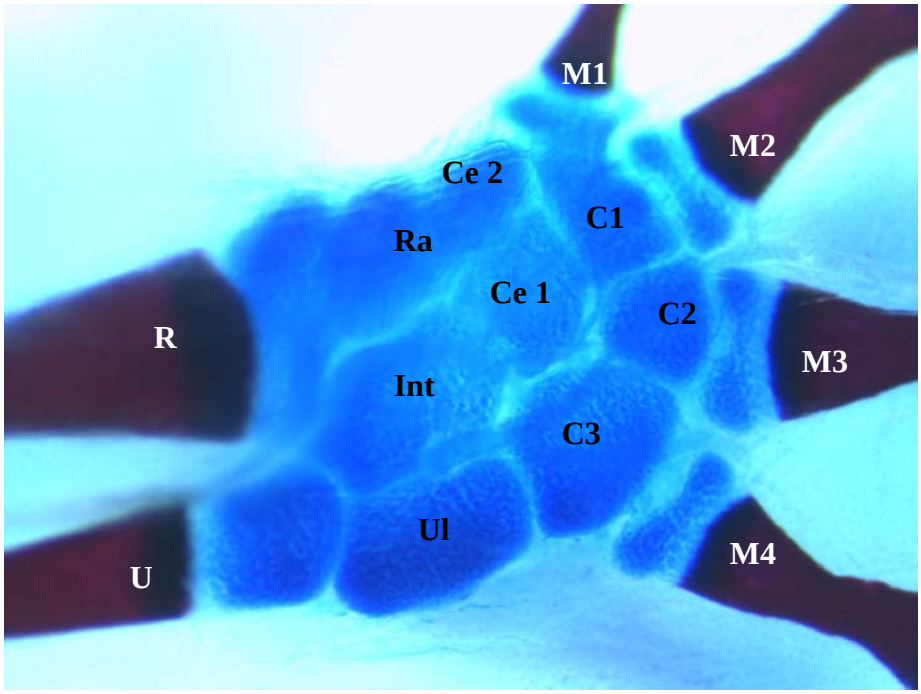



Figure 5.4 Cleared and stained manus of *P. kentucki* showing bone (red) and cartilage (blue). Abbreviations: R= radius; U= ulna; Int= intermedium; Ul= ulnare; Ra= radiale; Ce 1-2= centrale 1-2; C1-3= carpal 1-3 ; M1-4= metacarpal 1-4.

The pes of *P. kentucki* consists of the tarsus with 9 cartilaginous tarsal elements; the fibulare, intermedium, tibiale, 2 centrales, and 4 tarsals (Fig 5.5). The fibulare is articulated proximally with the fibula, preaxial with the intermedium and centrale 1, and distally with tarsals 3 and 4. The intermedium is articulated proximally with the tibia, postaxial with the fibulare, distally with centrale 1, and preaxial with the tibia and tibiale. The tibiale articulates proximally with the tibia, postaxial with centrale 1 and the intermedium, and distally with centrale 2. Centrale 1 articulates proximally with the intermedium, postaxial with the fibulare and tarsal 3, preaxial with centrale 2 and the tibiale, and distally with tarsals 1 and 2. Centrale 2 articulates proximally with the tibiale, postaxial with centrale 1, and distally with tarsal 1. Tarsal 1 articulates proximally with centrales 1 and 2, and distally with metactarsals 1 and 2. Tarsal 2 articulates proximally with centrale 1, preaxial with tarsal 1, and postaxial with tarsal 3. Tarsal 3 articulates proximally with centrale 1 and the fibulare, distally with metatarsal 4, preaxial

with tarsal 2, and postaxial with tarsal 4. Tarsal 4 articulates proximally with the fibulare, preaxial with tarsal 3, and distally with metatarsal 5 (Fig. 5.5).

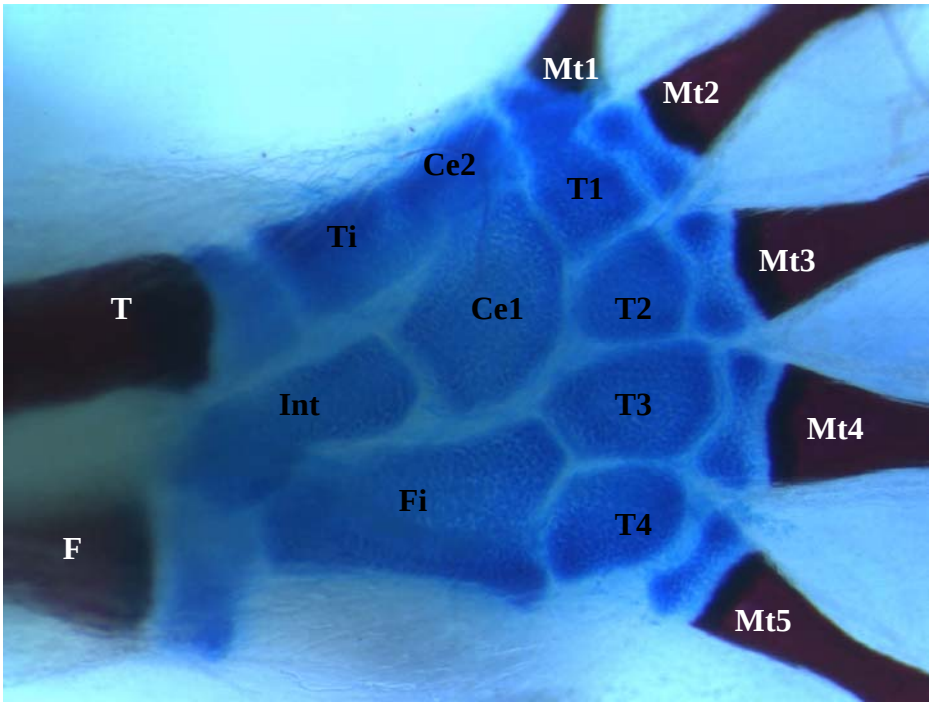



Figure 5.5 Cleared and Stained pes of *P. kentucki* showing bone (red) and cartilage (blue). Abreviations: T= tibia; F= fibula; Int= intermedium; Fi= fibulare; Ti= Tibiale; Ce1-2= centrale 1-2; T1-4= tarsal 1-4; Mt1-5= metatarsal 1-5.

The terminal phalanx of *P. kentucki* is wide at the proximal end and tapers towards the distal end in a triangular shape with a flattened and spread out “Y” shaped tip. There is also a small curve to the terminal phalanx and that reaches its peak at the point of constriction just before the distal tip flattens and spreads. There appears to be no pronounced ventral projection for tendon attachment (Fig 5.6).

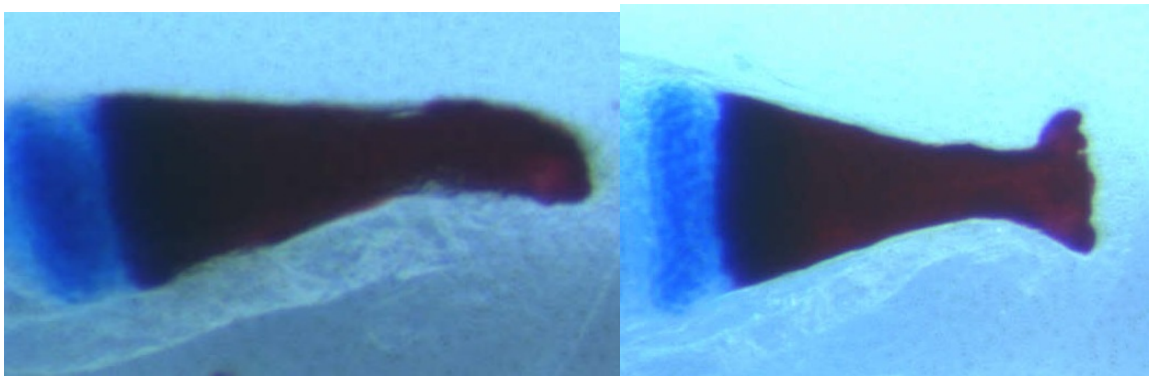


Figure 5.6 Cleared and stained terminal phalanx of *P. kentucki* showing bone (red) and cartilage (blue). Lateral view (left) and ventral view (right).

***Plethodon glutinosus***

The manus of *P. glutinosus* consists of the carpus with 8 cartilaginous elements; the radiale, ulnare, intermedium, 2 centrales, and 3 carpals (Fig. 5.7). The radiale articulates proximally with the radius, postaxial with the intermedium and centrale 1, and distally with centrale 2. The intermedium articulates preaxial with the radius and radiale, distally with centrale1, postaxial with carpal 3 and ulnare, and proximally with the ulna. The ulnare articulates proximally with the ulna, preaxial with the intermedium, and distally with carpal 3. Centrale 2 articulates proximally with the radiale, postaxial with centrale 1, and distally with carpal 1. Centrale 1 articulates preaxial with the radiale and centrale 2, distally with carpal 1, postaxial with carpal 3, and proximally with the intermedium. Carpal 1 articulates preaxial with centrale 2, distally with metacarpals 1 and 2, proximally to centrale 1, and postaxial with carpal 2. Carpal 2 articulates preaxial with carpal 1, distally with metacarpal 3, and postaxial with carpal 3. Carpal 3 articulates proximally with the ulnare, preaxial with the intermedium and centrale 1, distal with carpal 2, and postaxial with metacarpal 4.

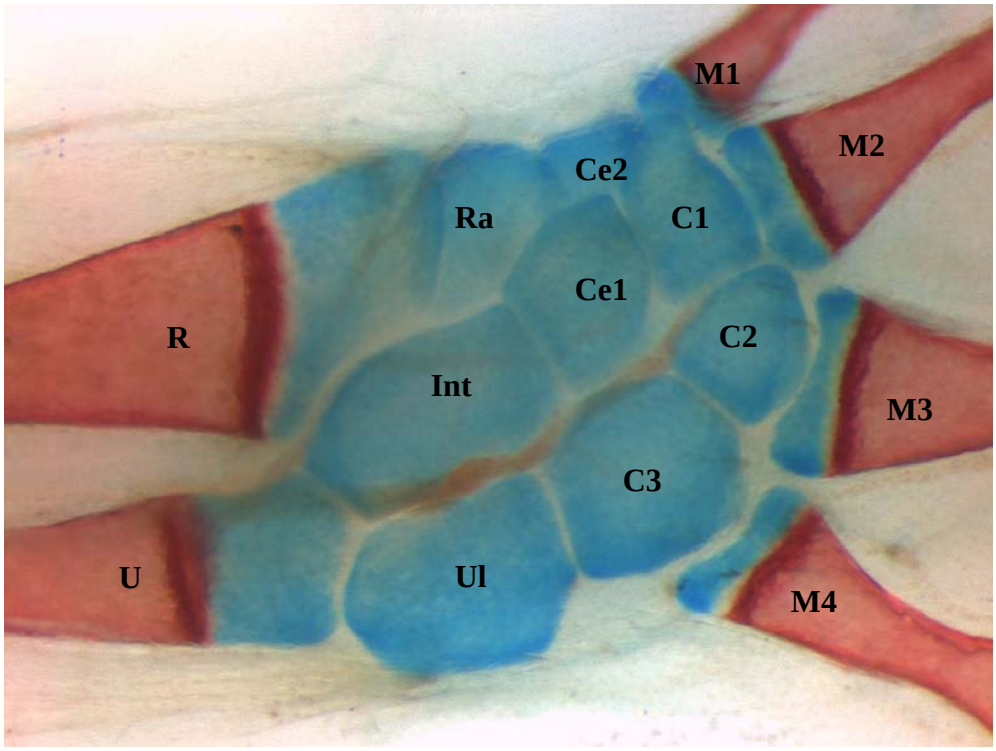


Figure 5.7 Cleared and stained manus of *P. glutinosus* showing bone (red) and cartilage (blue). Abbreviations: R= radius; U= ulna; Int= intermedium; Ul= ulnare; Ra= radiale; Ce1-2= centrale 1-2; C1-3= carpal 1-3 ; M1-4= metacarpal 1-4.

The pes of *P. glutinosus* consists of the tarsus with 9 cartilaginous elements; the tibiale, fibulare, intermedium, 2 centrales, and 4 tarsals (Fig. 5.8). The tibiale articulates proximally with the tibia, postaxial with the intermedium and centrale 1, and distally with centrale 2. The intermedium articulates preaxial with the tibia and tibiale, distally with centrale 1, postaxial with the fibulare, and proximally with the fibula. The fibulare articulates proximally with the fibula, preaxial with the intermedium and centrale 1, and distally with tarsals 3 and 4. Centrale 2 is articulated proximally with the tibiale, postaxial to centrale 1 and tarsal 1, and distally with metatarsal 1. Centrale 1 articulates preaxial with the tibiale and centrale 2, proximal with the intermedium, distal with tarsals 1, and postaxial with the fibulare and tarsals 2 and 3. Tarsal 1 articulated preaxial by centrale 2, proximally by centrale 1, postaxial by tarsal 2, and distally by

metatarsals 1 and 2. Tarsal 2 articulates preaxial with tarsal 1, distal with metatarsal 3, proximally with centrale 1, and postaxial with tarsal 3. Tarsal 3 articulates preaxial with tarsal 2, distal with metatarsal 4, proximal with the fibulare, and postaxial with tarsal 4. Tarsal 4 articulates preaxial with tarsal 3, distal with metatarsal 5, and proximally with the fibulare.

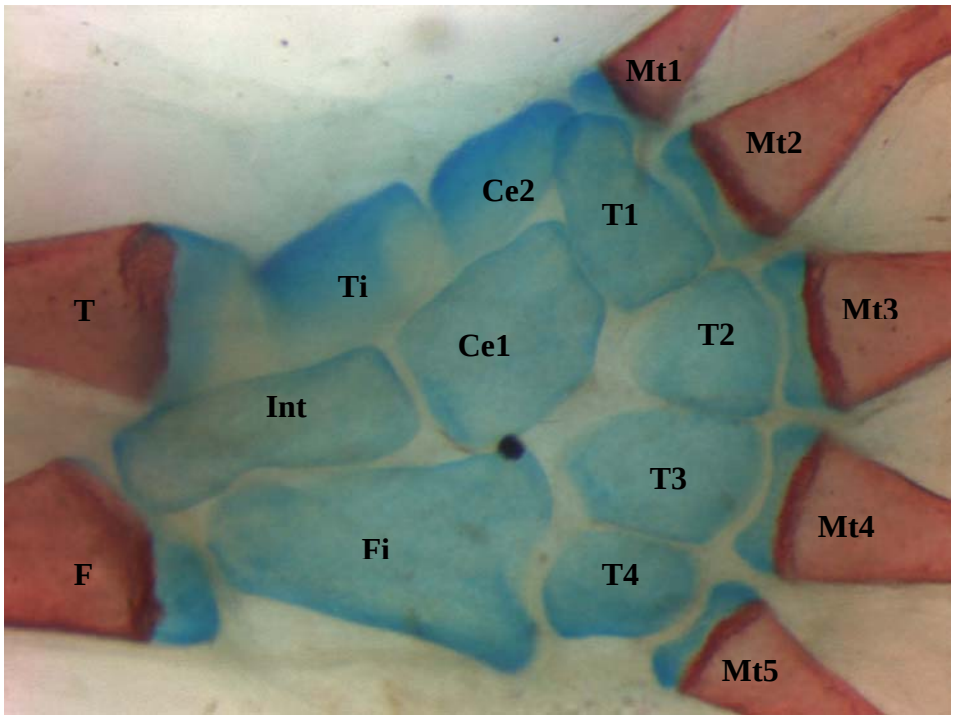



Figure 5.8 Cleared and Stained pes of *P. glutinosus* showing bone (red) and cartilage (blue). Abreviations: T= tibia; F= fibula; Int= intermedium; Fi= fibulare; Ti= Tibiale; Ce1-2= centrale 1-2; T1-4= tarsal 1-4; Mt1-5= metatarsal 1-5.

The terminal phalanx of *P. glutinosus* is slightly curved with no ventrally oriented projection. The proximal portion of the phalanx is wide, tapers slightly progressing distally only to again expand into a small “Y” shaped tip (Fig. 5.9).

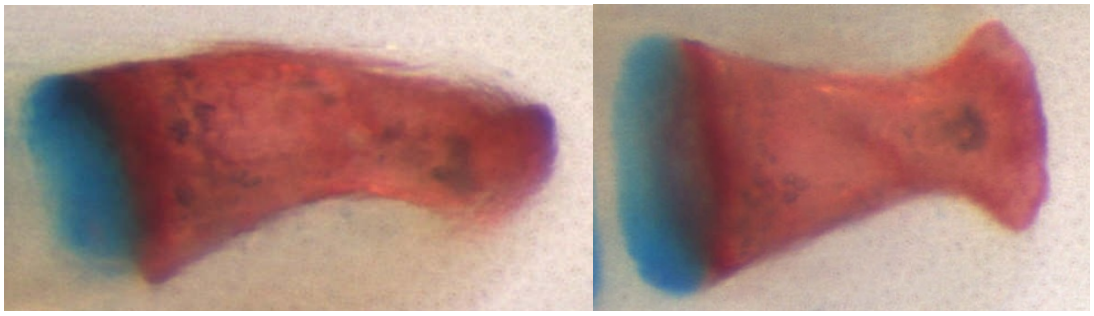


Figure 5.9 Cleared and stained images of a *P. glutinosus* terminal phalanx showing bone (red) and cartilage (blue). Lateral view (left) and ventral view (right).

**Discussion:** All three species in this study showed no difference in the number of cartilaginous elements in either the manus (8 elements) or pes (9 elements). However, there were subtle differences that set the manus and pes of *A. aeneus* apart from *P. kentucki* and *P. glutinosus*. As seen in the manus of *A. aeneus*, all cartilaginous elements were articulated to centrale 1 including the ulnare. In *P. kentucki* and *P. glutinosus*, all cartilaginous elements are articulated to centrale 1 except for the ulnare. This is consistent with Wake's (1963) conclusion that these characteristics are unique to the *Aneides* genus. In reference to the pes, the relative size of distal tarsal 4 was enlarged in *A. aeneus* compared to tarsal 4 in *P. kentucki* and *P. glutinosus* where tarsal 3 was enlarged more than tarsal 4. This has been found to be due to a macro-evolutionary event whereby a tarsal element found in Paleozoic amphibians fused to tarsal 4, in the case of *Aneides*, rather than tarsal 3 and therefore considered an adaptive syndrome related to arboreality (Wake 1980). Finally, there is a large projection extending postaxial from the distal portion of the fibulare shown in *P. kentucki* and *P. glutinosus* that is absent in *A. aeneus*. This projection allows the fibulare of *P. kentucki* and *P. glutinosus* to articulate with distal tarsal 3 and 4 simultaneously while only distal tarsal 4 articulates with the fibulare in *A. aeneus*. The absence of this projection may aid in climbing mobility by allowing for a greater range of motion and pivoting ability in the pes of *A. aeneus* while climbing vertical services.

In regards to the terminal phalanx, both bones of *P. kentucki* and *P. glutinosus* are virtually identical to one another. Both have wide proximal bases with no conspicuous boney projection oriented ventrally for tendon attachment unlike the terminal phalanx of *A. aeneus* where the projection is obvious. This more distinct projection would allow for better tendon attachment to the terminal phalanx, allowing for greater force to be applied to irregularities on the vertical surface. Greater force means *A. aeneus* could gain a better grip and for a longer period than other terrestrial salamanders that attempt to climb. The shaft of *A. aeneus* terminal phalanx is also long and slender whereas the shaft of the terminal phalanx of *P. kentucki* and *P. glutinosus* is shorter and more robust in shape. However *P. kentucki*, while still with a wide base, appears to be narrower with a longer constriction area just before the expansion in to the distal tip than *P. glutinosus*. This was seen in both lateral and ventral view. Whether this is a documented evolutionary shift or simply an artifact of the species has yet to be determined. All three species showed a curve in the terminal phalanx and a Y-shaped distal tip with the greatest of both seen in *A. aeneus* which has been presumed to be an adaptation to an arboreal lifestyle (Larson et. al. 1981, Wake 1963). While there is some curving of the terminal phalanx of both *P. kentucki* and *P. glutinosus* and an expansion of the distal tip, it is unclear that this small degree to which they are curved and expanded would aid in climbing performance.

It should be noted that in the genus *Bolitoglossa*, arboreality is correlated with an increase in interdigital webbing, reduction or modification in terminal phalangeal elements, and fusion of tarsal elements (Alberch 1981). Interdigital webbing aids in adhesion through the cooperation of mucous glands and the ability of the plantar surface of the autopod to be lifted creating negative pressure between the plantar surface and substrate similar to how a suction cup operates (Alberch 1981). While this mode of adhesion works well for tropical salamanders

that live in moist habitats where many tropical plants have smooth waxy surfaces, this mode of adhesion and corresponding morphology are absent in North American temperate salamanders, even in the *Aneides* genus which contains some species considered to be the most arboreal in temperate forests of North America (Spickler 2006, Wake 1963, Petranka 1998). Temperate forests in North America lack flora with smooth waxy surfaces and are replaced by plants with heavily contoured surfaces, bark and rock outcrops for example. In this case it would not be adaptive to develop a "suction cup" adhesive mechanism since a firm seal could not be created on heavily contoured surfaces. Instead a shift may occur where it is more advantageous to have long, slender, well defined digits with an elaborate terminal phalanx with which to grip highly contoured surfaces. In regards to fusion of tarsal elements, with the exception of the tarsal rearrangement presented by Wake (1980), there have been no other fusions noted for any of the species in this study.

How the arrangement of the carpals and tarsals affects climbing ability is still widely unknown. It could be that having all cartilaginous elements in the manus articulated to centrale 1 in *A. aeneus* helps climbing mobility by allowing the salamander to pivot while being suspended or allow for more flexibility of the manus to move in a lateral plane parallel to the surface while in a vertical position. Arboreal locomotion typically has two forms, scansorial which is climbing in trees using claws, and brachiation which is locomotion via arm swinging under branches with gripping hands (Kardong 2002). Brachiation is obviously out of the question however, *A. aeneus* may be using its specialized terminal phalanx as a type of padded claw with which to grip creating a similar situation to scansorial movement. Even though salamanders exhibit a lateral-sequence gait, where three of the four limbs are in contact with the substrate at the same time (Kardong 2002), *A. aeneus* could be using combination of scansorial locomotion with a

lateral-sequence gait to move around on vertical surfaces. Unfortunately, investigating the effects of carpal and tarsal arrangements and the role of the terminal phalanx of these three species on climbing performance was beyond the scope of this project.

## Chapter Six
## Summary Discussion

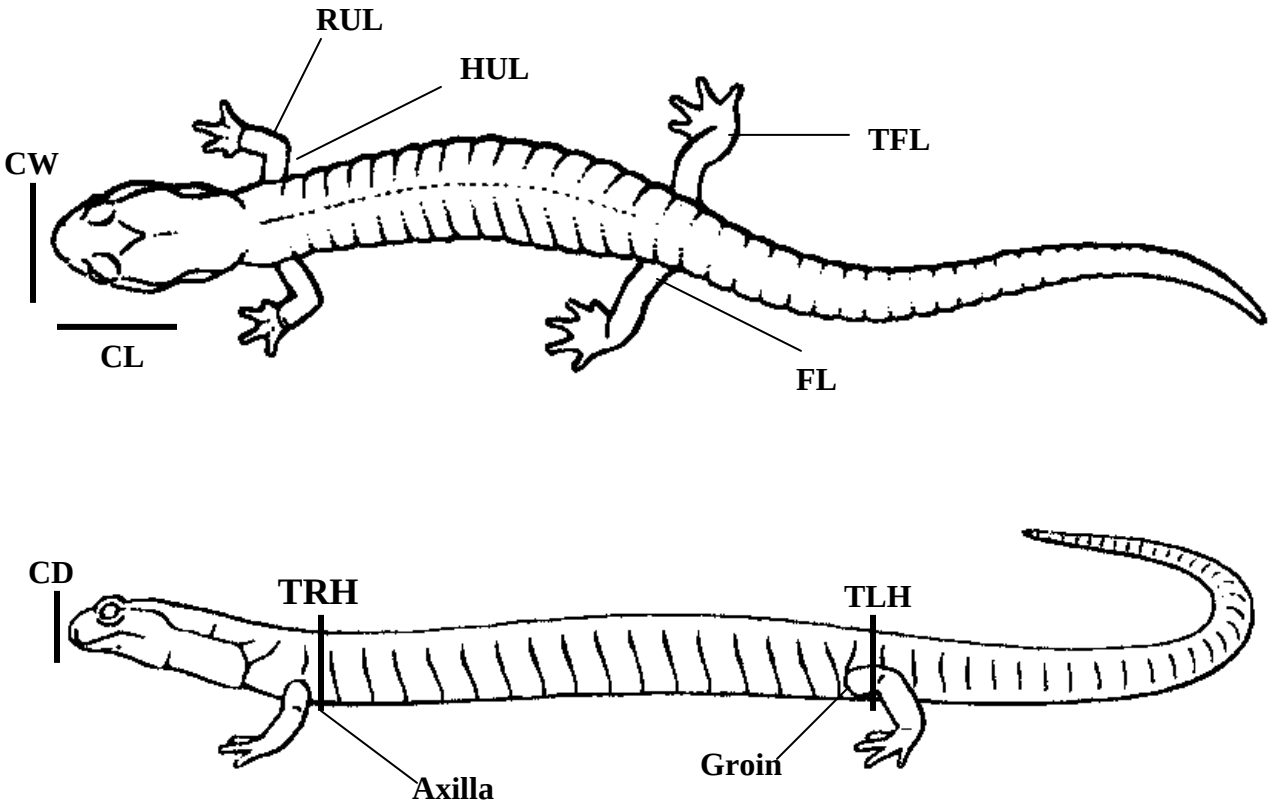
Coming back to the objective of this study, determining if field studies are needed to investigate if *P. kentucki* is competing with *A. aeneus* for rock crevices, we must first summarize what has been found. Morphometrically, *A. aeneus* and *P. kentucki* are similar in two important characteristics, snout-vent length and limb measurements. However, *P. kentucki* has a greater trunk and tail height compared to *A. aeneus* and is more comparable in size to *P. glutinosus* in this respect. Histologically, all three species showed curvature of the terminal phalanx with the greatest amount seen in *A. aeneus* which has been noted as an adaptation for climbing, followed by *P. kentucki* then *P. glutinosus. Aneides aeneus* also had the greatest amount of dermal cell thickening concentrated around the tip of the terminal phalanx, followed by *P. kentucki* with cell thickening around the tip of the terminal phalanx that continued down across the ventral of the digit surface, and *P. glutinosus* with no cell thickening. Osteologically, all three species were virtually identical with the exception of *A. aeneus* having all manus elements articulated to centrale 1 and having an enlarged distal tarsal 4 rather than an enlarged tarsal 3 as in *P. kentucki* and *P. glutinosus*. The terminal phalanx of *P. kentucki* and *P. glutinosus* are identical in curvature and amount a distal expansion even though the terminal phalanx of *P. kentucki* is slightly more slender in the shaft. However, neither phalanx is as specialized as that of *A. aeneus*.

Research following this study should include field studies to determine the extent to which *P. kentucki* inhabits rock crevices once occupied by *A. aeneus* and how severe this threat is at sites where both *P. kentucki* and *A. aeneus* reside. Climbing performance studies are also needed to quantify the climbing ability of these three species. This study should incorporate a

platform attached to an adjustable incline with a surface similar in texture to the substrate surface on which these salamanders climb. Lastly cellular development of digits should be viewed, possibly using anti-BrdU immuno-cytochemistry, to determine differences in these developing structures. These studies, combined with the data presented here, should provide a better understanding of the interactions between these three species.

# APPENDIX A

**Diagram of Salamander Measurements**




**Abbreviations:**

**CW** (Cranial width) width of the head at articulation of jaw

**CL** (Cranial Length) the length from the tip of the snout to the fold posterior to articulation of the jaw

**CD** (Cranial Depth) vertical height of the head at articulation of the jaw

**RUL** (Radius-Ulna Length) elbow to the tip of the longest digit

**HUL** (Humerus Length) distance from the axilla to knee of anterior limb

**TFL** (Tibio-Fibula Length) the knee to the tip of the longest digit

**FL** (Femur Length) distance from the groin to knee of posterior limb

**TRH** (Trunk Height) vertical distance between the ventral and dorsal part of the pectoral region posterior to the axilla.

**TLH** (Tail Height) vertical distance between the ventral and dorsal part of the pelvic region posterior to the insertion of the posterior limbs

# APPENDIX B

## Decalcification and Embedding Protocol for Paraffin Wax Histology

1) Soak in decalcifying agent, 3.5 hours minimum (or overnight)
2) 70% Ethanol for 1 hour to overnight (or longer)
3) 85% for at least 1 hour
4) 95% for at least 1 hour
5) 100% ethanol, 3 changes, 1 hour each
6) (optional) 1:1 mixture of 100% ethanol and xylene, 30min
7) 100% xylene, 30min
8) 100% xylene, 30min
9) (optional) 1:1 mixture of xylene and melted paraplast embedding wax, 1 hour
10) Fresh melted Paraplast, 60 degrees C, let sit overnight
11) Embed in embedding mold of fresh melted paraplast

# APPENDIX C

## Protocol for Subbing Slides

1) Clean slides by agitating in 95% ethanol to which drops of glacial acetic acid have been added.

2) Rinse slides in distilled water (3 changes).

3) Dip slides into subbing solution:

    Subbing Solution:

    0.1% gelatin (1.0g in 1.0 liter glass distilled water)
    Dissolved gelatin by heating water
    Cool
    Add 0.01% Chromium potassium sulfate (0.0g in 1.0 liter)

4) Drain the slides and dry in a rack for at least 1 hour or more in a 60$^{o}$C oven

5) Subbed slides can be stored indefinitely in a slide box

# APPENDIX D

## Protocol for Staining Paraffin Sections with Hematoxylin and Eosin Y

A. Deparaffinize sections:
1. Xylene: 5 minutes
2. Xylene: 5 minutes
3. 100% ethanol: 2 minutes
4. 100% ethanol: 2 minutes
5. 95% ethanol: 1 minute

B. Stain with hematoxylin:
7. Ehrlich's hematoxylin: 15-20 minutes
8. 95% ethanol: dip up and down to remove excess stain
9. 70% ethanol: 1 minute
10. 0.1% sodium bicarbonate: until sections turn blue (<5 minutes)
11. 70% ethanol: 1 minute

C. Stain with Eosin:
12. 95% ethanol: 1 minute
13. Eosin Y (0.5% in 95% ethanol): 15-30 seconds
14. 95% ethanol: dip up and down to remove excess stain
15. 100% ethanol: 1 minute
16. 100% ethanol: 1 minute

D. Make permanent (mount):
17. Xylene: 5 minutes
18. Xylene: 5 minutes
19. Check staining under microscope, repeat if necessary
20. Cover slip with permanent mounting medium (Permount)
21. Cure overnight on 45$^{o}$C hotplate

# APPENDIX E

**Protocol for Clearing and Staining for the Demonstration of Cartilage and Bone**

1) Fix specimens in 10% buffered formalin (at least 24 hours)
2) Rinse for at least one day in water, with several changes
3) Postfix in 70% ethanol…can store indefinitely
4) Skin and eviscerate specimen (remove all skin, even on toes)
5) Place specimen in solution of Alcian Blue cartilage stain (20mg alcian blue in ethanol/glacial acetic acid= solution of 70mls 100% ethanol: 30mls glacial acetic acid)… one day.
6) Transfer to plain ethanol/glacial acetic acid (1 hour), then to 100% ethanol (1-24 hr)
7) soak specimen in tap water to remove ethanol (several hours to overnight)
8) Transfer to 1.0% tripsin in 30% saturated sodium borate…check after 16 hours or so (overnight)…if limp and blue cartilage can be clearly seen, go to next step. Otherwise, repeat with fresh trypsin.
9) Use perforated plastic spoon to transfer to 0.5% KOH to which enough saturated Alizarin Red-S has ben added to turn the solution dark, dark purple…1 day
10) Transfer to plain 0.5% KOH to rinse out Alizarin Red
11) Treat with graded series of 0.5% KOH: glycerin: 2:1, then 1:1, then 1:2, then 100% glycerin, at least 24 hours for each step, until specimen becomes crystal clear. Store specimen in 100% glycerin plus one crystal of thymol in a screw top jar

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**Education**

MS., Biology, Marshall University, 2008.
BA., Biology, Hartwick College, 2006.

**Professional Experience**

-2006-2008: Teaching Assistant, Marshall University, Department of Biological Sciences, Huntington, WV 25755.
-June, 2006-August 2006: Laboratory Technician, Food additives research department, Kerry BioScience, Norwich, NY 13815.
-2002-2006: Assistant Laboratory Technician, Biology Department, Hartwick College, Oneonta, NY 13820
-2002-2006: Laboratory Assistant, Amphibian Research Laboratory, Hartwick College, Oneonta, NY 13820.
-2002-2006: Reptile/Amphibian Curator, Hartwick College, Oneonta, NY 13820.

**Courses Taught**

- BSC 104- Principles of Biology Lab (for non-majors)
- BSC 120- Principles of Biology Lab (for majors)
- BSC 324- Principles of Genetics Lab
- West Virginia Department of Natural Resources Master Naturalist Instructor (Presented herpetological related talks to the general public regarding the natural history, conservation, and identification of native amphibian and reptile species)

-Marshall Herpetology Lab Outreach Program Coordinator

**Awards and Grants**

-Hartwick College, Department of Biology Hellbender Award, 2006
-West Virginia Division of Natural Resources Research Grant ($6,208), 2007
-Marshall University Summer Thesis Research Grant ($500), 2007

**Professional Memberships**

-BBB Biological Honor Society: Hartwick College 2003-2006
-Society for the Study of Amphibians and Reptiles (SSAR)

**Research Interests**

Herpetology: developmental biology, genetics, cytogenetics, ecology, conservation, morphology.
Tropical Herpetology: Costa Rica, Madagascar.

**Relevant Research Experience**

-An analysis of cellular growth in developing limbs (undergraduate thesis)

Principle investigator: Analyzed cellular growth, patter formation, and morphogenesis in developing *Ambystoma maculatum* larvae limb and brain structures using histology, anti-BrdU immuno-cytochemistry, and hematoxylin and eosin staining.

-Comparative embryology: *Triturus* and *Ambystoma*

Co-investigator: Investigated the relationship between cell size and rate of development among related species within each genus using cleared and stained staged developing embryos.

-Cytogenetics: *Ambystoma* and *Necturus*

-*Ambystoma*: Co-investigator- Processed specimens of *A. laterale* and *A. jeffersonianum* to investigate the role and distribution of polyploidy among members of the *jeffersonianum* complex.

-*Necturus*: Co-investigator- Aided in collecting and processing of *N. maculosus* to investigate the role of sex chromosome linkage in males.

-Amphibian/Reptile Biodiversity of Hartwick College Pine Lake Environmental Campus

Principle investigator: Developed and conducted quadrate searches in three distinct habitats to determine herpetofaunal biodiversity to better facilitate the college in future land management decisions.

-Rapid new protocol for clearing and staining specimens for osteological examination.

Principle investigator: Project focused on modifying previous technique set forth by Hanken and Wassersug 1981 to produce less macerated specimens, especially small specimens, in a timelier manner.

-Ecology, natural history, and distribution of the Eastern Hellbender, *Cryptobranchus a. alleganiensis*, in West Virginia.

Field Technician: Assisted in field surveys and the acquisition of morphological and environmental data.

-Habitat preference and ecology of the Rough Greensnake, *Opheodrys a. aestivus*, and Smooth Greensnake, *Ophedrys vernalis*, in West Virginia.

Field Technician: Assisted in field surveys and the acquisition of morphological and environmental data.

-Natural History and Distribution of the Eastern Worm Snake, *Carphophis amoenus amoenus*, in West Virginia.

Principle investigator: Investigated current distribution by searching historical collection sites as well as taking morphological, climatological, and topographical data which was statistically analyzed and geographically analyzed using GIS software.

-Cellular morphology of Green Salamander, *Aniedes aeneus*, digits and comparison with possible competitors.

Principle investigator: Investigated the topographical, skeletal, and cellular morphology of digits using histology, hematoxylin and eosin staining, and scanning electron microscopy. Also compared digit morphology of other caudates in sympatry with Green Salamanders to determine the role, if any, digit morphology may play in possible microhabitat competition.

-Iris Pattern Identification (IPID): a new technique for identifying individuals during field studies.

Principle investigator: Used high magnification photographs of *Bufo americanus* irises and identified pattern aberrations unique to each individual to serve as a "finger print." This technique was developed to serve as an alternative to toe clipping and passive integrative transponder (PIT) tags.

**Research Abroad**

-January 2004 Hartwick College Off-Campus Program: Natural History of Costa Rica.
Assisted Projects: aposemitism of snakes, *Ctenosaura* behavior (principle investigator), tropical stream ecology, arthropod biodiversity.

-January 2006 Hartwick College Off-Campus Program: Culture, Conservation, and Natural History of Madagascar.
Assisted Projects: herpetofaunal biodiversity, floral biodiversity, tidal pool ecology, *Napenthese* pitcher plant natural history.

**Acquired Research Techniques**

-Electron Microscopy (TEM and SEM)
-Histology (paraffin embedding)
-Hematoxylin and Eosin staining
-Anti-BrdU immuno-cytochemistry
-Specimen preservation and tagging
-Amphibian anesthesia and surgery
-Clearing and staining specimens for osteological examination
-Field Techniques (drift fences, cover boards, pitfalls, trapping, etc…)
-Laboratory Techniques (chemical preparation, data analysis, etc…)
-Cytogenetic techniques
-Tissue Culture
-PCR and Electrophoresis
-GIS software
-Webmaster (Marshall University Herpetology Lab)

**Invited Talks**

-Lead Herpetological Educator: Herpetological Education Demonstration, Hartwick College, Oneonta, NY, 2005.
-Guest Speaker on Herpetology: Greater Plains Elementary School, Oneonta, NY, 2006.
-Herpetological Educator: BBB Science Day, Hartwick College, Oneonta, NY, 2006.
-Assistant Herpetological Educator: Highlawn Church, Huntington, WV, 2006.
-Guest Speaker on Herpetology: Milton Elementary School, Milton, WV, 2006.
-Guest Speaker on Herpetology: Meadows Elementary School, Huntington, WV, 2007.
-Guest Speaker on Herpetology: Barnett Child Care Center, Huntington, WV, 2007.
-Guest Speaker on Herpetology: Milton Elementary School, Milton, WV, 2007.
-Guest Speaker on Herpetology: Kiwanis Dare Care Center, Huntington, WV, 2008.
-Guest Speaker on Herpetology: Highlawn Elementary School, Huntington, WV, 2008.
-Lead Herpetological Educator: West Virginia Wildlife Diversity Day, Charleston, WV 2008.
-Speaker on Amphibians and Reptiles of WV: Master Naturalist, Cross Lanes, WV 2008.

**Presentations**

-Diefenbacher, Eric H., K. R. Pawlik, and T. K. Pauley. 2007. Morphological examination of Green Salamander (*Aneides aeneus*) digital discs. *82nd meeting of the West Virginia Academy of Sciences.* (Poster).

-Diefenbacher, Eric H., K. R. Pawlik, and T. K. Pauley. 2007. Morphological examination of Green Salamander (*Aneides aeneus*) digital discs. *Association of Southeastern Biologists 68th Annual Meeting.* Columbia, South Carolina. (Poster).

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*Papers*

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-Diefenbacher, E.H. and T.K. Pauley. 2007. *Carphophis amoenus helenae.* Defensive Behavior. *Herpetol. Rev.* (in review).

-Diefenbacher, E.H. and T.K. Pauley. 2007. Iris Pattern Identification (IPID): A new technique for identifying individuals during field studies. *Herpetol. Rev.* (in review).

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