

## ABSTRACT

LEONARD, KAITLYN CARLENE. Physiological and Preservational Influences on Muscle Architecture. (Under the direction of Dr. Adam Hartstone-Rose).

The function of a muscle is directly conferred by its architectural properties which has been correlated to a wide variety of functions including feeding and locomotor adaptations. Although widely applied, there are still some questions and assumptions about consistency throughout ontogeny, anatomical region, and preservation that have yet to be investigated, and the research objective of this dissertation is to elucidate elements of all the aforementioned. I evaluated the ontogeny of the masticatory muscle architecture within a large sample of *Microcebus murinus* and observed that muscle mass and PCSA increased rapidly and plateaued into adulthood and throughout senescence. Fascicle lengths reached their maximal length early in development and remained relatively constant thereafter. The effects of age and anatomical region on muscle density within a sample of New Zealand White rabbits (*Oryctolagus cuniculus*) were evaluated and found significant differences influenced by both. Rather than the universal density constant used in calculating PCSA, the findings indicate that muscle density should be determined on a muscle by muscle basis. However, this is not always feasible, so from the findings values were calculated for density that can be more accurately applied: for instance, densities of 1.0558 g/cm<sup>3</sup> and 1.0502 g/cm<sup>3</sup> would be appropriate for general adult and juvenile muscles, respectively. Preventing post-mortem deterioration in cadaveric specimens is essential in anatomical research and therefore, various preservational methods are employed to prevent this decay of biological tissue. To investigate the effects of short-term preservation, a sample of fresh rabbits was obtained, and various treatments applied which included formalin fixation, formalin fixation followed by subsequent ethanol storage for 1,3 and 6 months, and repetitive freeze/thaw cycling. A variety of muscles were selected excised individually to obtain a

representative sample. Muscle mass, volume and density were then measured. The formalin fixation group exhibited approximately a 24% reduction in mass and volume and less than a 1% reduction in density. The ethanol storage treatment groups all experienced a reduction in mass and volume of approximately 40% and approximately a 3% reduction in density. Freezing and thawing had much less severe effects with freezing once leading to approximately a 3% reduction in mass and volume and freezing twice leading to approximately a 9% reduction in mass and volume. For both of the freezing treatment groups, muscle density remained virtually unchanged. These preservational practices are relatively common, and as demonstrated, have rather significant effects on the architectural properties of muscle. Therefore, based on these findings, the following correction multipliers were derived for each variable: *Mass*: ethanol 1.69; 10% formalin 1.32; frozen once 1.03; frozen twice 1.09; *Volume*: ethanol 1.64; 10% formalin 1.32 ; frozen once 1.03 ; frozen twice 1.10 ; *Density*: ethanol 1.03; 10% formalin 1.00; frozen once 1.00; frozen twice 1.00. Furthermore, another challenge in conducting anatomical research is obtaining specimens, especially fresh cadavers, and therefore, anatomists often obtain specimens from the fluid collections of museums some of which have been in ethanol for over a century. Therefore, to evaluate the effects of *long-term* preservation a sample of mice, *Mus musculus*, was obtained from museum collections some of which dated back to 1889. Within this sample the muscle mass, volume and density decreased by 62%, 60% and 5% respectively and the associated correction multipliers are as follows: 2.64, 2.49 and 1.054 respectively. Muscle fascicles did not shrink lengthwise, and histological analyses demonstrated that the average cross-section of a muscle fiber correlates strongly with muscle mass and volume (i.e., muscle fibers shrank in diameter). Overall, the findings thoroughly demonstrate that muscle architecture is dynamic and changes based on ontogeny, anatomy and preservation.

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Physiological and Preservational Influences on Muscle Architecture

by  
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## BIOGRAPHY

Kaitlyn Carlene Leonard was born on April 20th, 1993 to Mike and Donna Leonard in Greensboro, North Carolina. She grew up in Welcome, NC a quaint but adorable little town. She comes from two generations of nurses, and from this background sprouted an interest in medicine, but ultimately biology. She attended Wingate University where she studied Biology and graduated in May of 2015 with a Bachelor's of Science. After a misplaced year at a rival graduate school, one which shall not be named here, she transferred to the University of South Carolina School of Medicine as a Biomedical PhD student. She completed multiple laboratory rotations before finding the lab. She officially joined Dr. Adam Hartstone-Rose's lab in February of 2017. The following September, Dr. Hartstone-Rose accepted a position at North Carolina State University and Kaitlyn transferred to NC State in January of 2018 (Go Pack!). While here at NC State, Kaitlyn has enjoyed her active role as mentor to undergraduate students in their research endeavors in Dr. Hartstone-Rose's lab. As for her own research, she enjoys studying the architectural properties of muscle and functional correlates thereof as well as how these properties are influenced inherently (i.e., physiologically) as well as experimentally through researcher introduced variables. Kaitlyn has a passion for teaching and mentoring students and has accepted a tenure-track position in the area.

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## **CHAPTER 1: Introduction-Physiological and Preservational Influences on Muscle Architecture**

### *Muscle Architecture and Functional Significance*

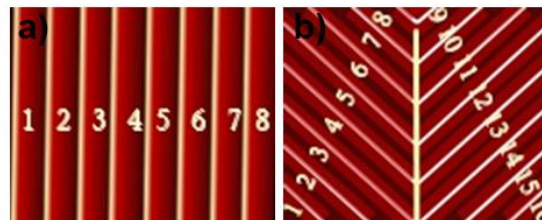
A muscle's architecture ó its gross anatomy, mass, volume, fascicle lengths, physiological cross-sectional area ó have been correlated with the species's functional adaptations including locomotor style (Oishi et al., 2008; Michilsens et al., 2009; Kikuchi, 2010; Huq et al., 2015; Yang et al., 2015), substrate selection (Leischner et al., 2018; Lowie et al., 2018; Marchi et al., 2018), dietary adaptations (Taylor et al., 2006; Perry and Wall, 2008; Dumont et al., 2009; Eng et al., 2009; Taylor and Vinyard, 2009; Perry and Hartstone-Rose, 2010; Perry et al., 2011; Hartstone-Rose et al., 2012; Perry et al., 2014; Santana and Cheung, 2016; Fabre et al., 2017; Curtis and Santana, 2018; Hartstone-Rose et al., 2018; Prufrock and Perry, 2018; Deutsch et al., 2019; Hartstone-Rose et al., 2019) and overall anatomical scaling (Anapol et al., 2008; Dick and Clemente, 2016). Despite the fact that these architectural variables are so widely utilized to infer these important aspects of functionality, there are still assumptions about their consistency during development, anatomical region, and specimen preservation that have yet to be tested. This body of work evaluates both physiological and preservational factors that may be influential on the architectural properties of muscle.

### *Muscle Fiber Architecture*

The strength of a muscle is directly proportional to its cross-sectional area (Knuttgén, 1976; Maughan et al., 1983) ó something that can be achieved either by increasing the overall mass, or through pennation (discussed below). The excursion potential (i.e., stretching capabilities) and contraction speed is reliant upon longer muscle fibers. Based on these characteristics, a muscle can be optimally adapted for stretch and speed or strength capabilities.

This optimization is facilitated by the configuration of the individual fibers located therein (Gans and Bock, 1965; Gans, 1982; Anapol and Barry, 1996; Lieber and Fridén, 2001).

In the simplest configuration, the muscle fibers are oriented parallelly and span the entirety of the muscle from origin to insertion (Figure 1.1a). Thus, the length of the fibers within this configuration is maximized (Gans and Bock, 1965; Gans and De Vree, 1987). Muscle fibers themselves are comprised of small contractile units (sarcomeres) arranged serially, with each sarcomere being capable of stretching ~30% beyond its resting length (Williams and Goldspink, 1978). As vertebrate sarcomeres are all theoretically the same length (Williams and Goldspink, 1978), longer muscle fibers are made up of more sarcomeres and therefore, have greater inherent stretch capabilities. Longer fibers are also faster because, upon muscle contraction, each sarcomere shortens simultaneously and thus, since longer fibers shorten in the same amount of time as shorter fibers, they will close a greater distance per unit of time (i.e., by definition greater speed (Bodine et al., 1982; Sacks and Roy, 1982; Gans and De Vree, 1987).



**Figure 1.1.** Configurations of muscle fibers within a) a parallel arrangement; notice that there are fewer fibers than is observed in the pennate muscle but they are much longer b) a pennate arrangement; notice that there are twice as many muscle fibers within the same amount of volume but these fibers are shorter. This exemplifies the physiological trade-off between fiber length (stretch and speed) and physiological cross-sectional area (strength).

Alternatively, muscle fibers can be arranged such that they attach to a tendon at an angle to the muscle's line of action ó so called "pennate" or "pennate" muscles (Figure 1.1b). The fibers in this arrangement are by definition shorter, but this configuration allows for more fibers to be packed into a given volume (Gans and Bock, 1965). Every muscle fiber can theoretically pull with specific amount of force ó variously quantified as  $3\text{kg}/\text{cm}^2$  (Close, 1972) of cross-sectional area, but the cross-section that matters is not the cross-section perpendicular to the whole muscle (except in non-pennate muscles), but the cross-section of all of the fibers combined ó the so called "physiological cross-sectional area" (PCSA). Muscles with larger physiological cross-sectional areas have more muscle fibers and therefore, can generate more force (Knuttgen, 1976; Maughan et al., 1983).

If a muscle needs to be both forceful and stretchy or speedy (e.g., the mandibular adductors of a hyena need to be both strong enough to crack bones, and stretchy enough to provide sufficient gape for large prey), then they must have relatively many long fibers, and thus they must be larger than a muscle adapted for merely *either* maximal stretch or force. Although these principles act physiologically on the cellular level ó i.e., these are the contractile principles of muscle *fibers* ó whereas here they are studied on the macroscopic level, as measures of *bundles* of muscle fibers or "fascicles" which function equivalently. Thus, it is noted that there is a fundamental physiological trade-off between fascicle length and PCSA such that muscles *of a given volume* (é mass) with relatively longer fascicles are therefore faster and stretchier, but have a reduced PCSA and are therefore less forceful whereas muscles with high PCSA have relatively shorter fascicle lengths and therefore contract with greater force, but less speed or excursion. To accomplish both great force and speed or stretch, muscles therefore must be relatively larger ó that is, with both longer *and* more numerous fibers.

*Ontogenetic Changes in Architecture (Chapters 2 & 3)*

While studies of muscle architecture are generally quite numerous, how architectural properties develop and change within the masticatory musculature with ontogeny has not been well studied (but see Langenbach and Weijs, 1990; Linuma et al., 1991; Pfaller et al., 2009; Pfaller et al., 2011) especially within primates (but see Carlson, 1983; Cachel, 1984; Dickinson et al., 2018; Prufrock and Perry, 2018).

One explicitly ontogenetic study, albeit limited, was conducted by Cachel (1984), who evaluated the growth and allometry of the masticatory muscles across an interspecific sample of primates using only dry weights, not incorporating any of the other architectural properties of muscle (i.e., fascicle length or PCSA), and observed that muscle mass scaled isometrically with body mass throughout ontogeny. In another study, Carlson (1983) used radiopaque markers embedded only into the masseter of juvenile macaques and repetitive radiographs taken overtime to track development and observed that based on the migration of the markers, the masseter underwent elongation throughout ontogeny. In a more comprehensive study conducted by Dickinson and colleagues (2018) of the masticatory muscle architecture across ontogeny within an intraspecific study of the crab-eating macaque (*Macaca fascicularis*) they observed that muscle mass, PCSA and fascicle length scaled with positive allometry relative to jaw length and condyle-molar length across ontogeny.

A study conducted by Perry and colleagues (2014) did not intentionally investigate the muscle architecture of the masticatory muscles throughout ontogeny within aye-ayes (*Daubentonia madagascariensis*) however, the authors compared the muscle architecture of an adult individual with a juvenile specimen that had been previously dissected and excluded from a previous study (Perry et al., 2011). They observed that both the adult and juvenile aye-ayes had

relatively long fascicles (compared to other lemurs) but the juvenile more so. They also noted that the adult aye-aye had relatively large muscles and PCSA but not as much in the juvenile which could indicate that due to the functional demands of being able to forage independently of parental assistance, the masticatory musculature of the aye-aye must develop to have large PCSA (Perry et al., 2014).

The comparative nature of these studies provides evidence that the muscle architecture of the masticatory apparatus is dynamic as the functional demands placed on the system change and highlights the need for studies that capture the architectural properties within the system across developmental stages.

The sparse number of ontogenetic studies investigating the muscle architecture in the masticatory apparatus in primates is likely due to some inherent challenges associated with this type of study. One of these challenges is that primates have relatively long lifespans, and therefore, obtaining an ontogenetic sample could take decades in many cases. Another challenge associated with this type of study is not just in obtaining the sample, but in obtaining a sample for which exact ages are known. However, as primates are of fundamental interest to many of the experts on fiber architecture, it is clear that a comprehensive systematic study of the effects of ontogeny of primate architecture has been warranted. To address this deficit, in Chapter 2 the ontogenetic trends in the mandibular adductors of a small, Malagasy primate, the grey mouse lemur (*Microcebus murinus*) will be investigated and is the largest intraspecific sample of primates of known ages, ranging from 6 days to 8 years, to date to do so.

While the mouse lemur masticatory ontogeny chapter addresses general changes in the architecture of muscles across ontogeny in primates, it did so only in one constrained anatomical system. However, it is also important to understand how muscle architecture develops across the

whole organism ó the task accomplished in Chapter 3 which evaluated the differences in muscle mass, volume and density based on age and anatomical and histological regionality in a highly controlled sample of New Zealand White rabbits.

*Assumptions About Density as a Constant*

Physiological cross-sectional area (PCSA) as an estimator of force production capabilities is calculated by dividing muscle mass by the product of average fascicle length and muscle density (Schumacher, 1961). While muscle mass and fascicle length are determined directly, though there is some disagreement within the field about the best way to determine average fascicle length (see Hartstone-Rose et al., 2018), the prevailing practice is to use a previously determined ðconstantö (often taken from Méndez and Keys, 1960; Murphy and Beardsley, 1974) for muscle density.

This practice is potentially problematic as the studies from which these constants are taken were not actually focused on establishing variables that should be universally applied. In fact, in both of the most predominantly cited papers (Méndez and Keys, 1960; Murphy and Beardsley, 1974) the density constant was established simply as a step in solving broader functional questions, and it was done so for the very specific taxa and systems under investigation. For instance, both of these studies calculated densities using tissue from adult individuals, with Méndez and Keys (1960) using tissue from rabbits and dogs (1960) while Murphy and Beardsley (1974) used cats.

The use of these constants derived from solely adult tissue makes the assumption that muscle density is resistant to ontogenetic influence. Density is defined as mass per unit of volume and in order for it to remain constant throughout an animal's life, muscle mass and volume would have to change proportionally and in tandem. However, it has been demonstrated

that muscle mass decreases with senescence a process known as sarcopenia (Rosenberg, 1997; Janssen et al., 2000; Narici et al., 2003; Cruz-Jentoft et al., 2010). Furthermore, other studies directly evaluating muscle density found age-related changes which included an increase in muscle density through middle-age and a subsequent decrease (Imamura et al., 1983; Newton et al., 1993).

Another assumption associated with the use of a density constant is that the anatomical region does not affect muscle density. Méndez and Keys (1960) sampled the quadriceps cruralis, gastrocnemius, tibialis, and the psoas, a hip flexor all predominantly from the lower limb while Murphy and Beardsley (1974) only evaluated the soleus. To date, no study has comprehensively examined the differences in muscle density of a broad sample of muscles from anatomical regions throughout the body. Chapter 3 does exactly this, by evaluating muscle density not only between multiple age cohorts (to evaluate the ontogenetic assumptions discussed in the last section) and anatomical regions within a larger sample of adult New Zealand White rabbits.

#### *The General Influence of Preservation on Muscle Architecture (Chapters 4 & 5)*

While chapters 2 & 3 address assumptions about the effects of ontogeny and anatomical region on muscle architecture, there is another potentially even more important influencer on muscle architecture that has yet to be systematically or histologically evaluated: the effects of preservation (the subject of chapters 4 & 5). Tissue preservation, most often through freezing, chemical infusion, or some combination thereof is critical to the practice of anatomical research in order to prevent the rotting effects of microbial activity and cell lysis (Jones, 1976; Eltoun et al., 2001; Simmons, 2014). However, the methods used have different (and underappreciated) effects on the properties of anatomical specimens and how true of a representation they are of living specimens. These are important considerations for not only selecting the least detrimental

methods for preserving anatomical specimens, but also for increasing the utility for myological study of existing preserved specimens housed both within the collections of individual researchers as well as the vast museum collections.

However, in order to use preserved specimens as proxies for fresh specimens (the state in which physiological parameters have been established) it is important to systematically study the effects of preservation and provide correction factors for them ó something that has never been done before, and the subject of Chapters 4 and 5. Chapter 4 addresses the acute effects of preservation that occur with both short-term repetitive freezing and fluid preservation through a series of controlled experiments. Specifically, in that chapter, both repetitive freeze-thaw cycling (frozen at  $-20^{\circ}\text{C}$ ) and fluid preservation on the muscle mass, volume and density of a sample of New Zealand White rabbits (*Oryctolagus cuniculus*) investigated. This replicates the handling, within the first weeks and months, of tissues acquired by an anatomist prior to study. Chapter 5 seeks to elucidate the effects of fluid preservation occurring over a large time period in a sample of the common house mouse (*Mus musculus*) comparing fresh specimens to those historically archived in museum collections spanning specimens preserved for more than 100 years to evaluate the impact of long-term preservation techniques on tissue integrity. In both chapters, correction factors are calculated, for the first time, for the key variables of interest to functional morphology researchers. This allows the first real combination of samples collected in different states and will allow colleagues to go back and reevaluate findings that may have been unintentionally influenced by these effects.

#### *Specific Theoretical Effects of Freezing on Muscle Properties*

Freezing is probably the most commonly used method of preservation because it does not require a significant investment of time or materials and is available nearly universally at any

animal facility, while, the chemicals needed for fluid preservation are not always immediately available. Even when specimens are ultimately chemically preserved freezing may be a necessary intermediate stage. However, the effects of freezing on muscle architecture have not been well studied by functional morphologists, though it has been a focus within the meat sciences as it impacts meat quality and value (Callow, 1952; Añón and Calvelo, 1980; Carroll et al., 1981; Grujić et al., 1993; Sen and Sharma, 2004; Xia et al., 2009; Leygonie et al., 2012; Qi et al., 2012; Kim et al., 2018). For instance, the food industry has long known that freezing leads to the formation of ice crystals within the tissue that disrupt the internal structure of the muscle (Birdseye, 1929; Callow, 1952; Mills, 1975; Añón and Calvelo, 1980; Carroll et al., 1981; Scott and Aquino-Shuster, 1989; Grujić et al., 1993; Sen and Sharma, 2004; Xia et al., 2009; Leygonie et al., 2012; Qi et al., 2012; Kim et al., 2018). Freezing also leads to loss of fluid as an exudate (Ramsbottom and Koonz, 1939; Añón and Calvelo, 1980; Gonzalez Sanguinetti et al., 1985; Strange, 1987) which would ultimately be reflected in the physical properties of the tissue through loss of mass and volume and potentially with changes in density if the fraction of loss is more or less dense than what remains.

Fluid preservation practices have evolved over time, but the premise remains the same: submerging or injecting preservatives into organic material to prevent the detrimental effects of cellular lysis and microbial activity (Jones, 1976). Early preservation practices were more focused on preserving food rather than anatomical specimens and often involved storing food in vinegar, honey, oil or brine (Shephard, 2000). Fluid preservation today uses a variety of chemicals including alcohols, aldehydes and glycol (Simmons, 2014). Modern practices generally include a fixation step prior to storage in preservative (Andrei and Genoways, 1999; Simmons and Voss, 2009; Simmons, 2014); typically, museums currently submerge and/or inject

specimens with formalin (an aqueous solution of formaldehyde) followed by transferring them into 70% ethanol for storage (Andrei and Genoways, 1999).

The use of chemicals for fluid fixation has been shown to have variable effects on anatomical specimens that include dehydration, shrinking, and swelling as well as changes in biomass and coloration (Simmons, 2014). Studies that have investigated the effects of fluid preservation on anatomical specimens have been conducted in various taxa including anurans (Lee, 1982; Deichmann et al., 2009), fish (Hoar, 1939; Burgner, 1962; Engel, 1974; Billy, 1982; Al-Hassan et al., 2000; Jawad, 2003), and reptiles (Klauber, 1943; Reed, 2001; Vervust et al., 2009) but have been more focused on the gross morphometrics of whole specimens (finding, for instance, that green iguanas lose 3.19% of their snout-vent length and 13.39% of their whole body mass when stored for 57 days in 70% ethanol; (Vervust et al., 2009) rather than the influence on muscle architecture specifically.

### *Summary*

Ultimately, these four independent studies evaluate some of the most fundamental assumptions made by functional morphologists about muscle fiber architecture ó those pertaining to the consistency of these key variables during ontogeny, across anatomical region and based on their short- and long-term preservation. Unfortunately, these studies elucidate the fundamental errors incorporated into much of the previous work by not accounting for these effects, but, collectively, they provide foundational knowledge that can be used to correct the samples. I predict that these correction factors can be used to explain some of the previously problematic findings and to give more accurate biological conclusions moving forward.

## References:

- Al-Hassan LA, Bujawari J, El-Silini O. 2000. The effect of some preservatives and freezing on certain body dimensions of two species of the family Mullidae collected from Benghazi waters, Libya. *Acta Ichthyol Piscat* 2.
- Anapol F, Barry K. 1996. Fiber architecture of the extensors of the hindlimb in semiterrestrial and arboreal guenons. *Am J Phys Anthropol* 99:429-447.
- Anapol F, Shahnour N, Ross CF. 2008. Scaling of reduced physiologic cross-sectional area in primate muscles of mastication. In: *Primate craniofacial function and biology*: Springer. p 201-216.
- Andrei MA, Genoways HH. 1999. Changes in pH in museum storage fluids. In: *Collection Forum*. p 63-75.
- Añón MC, Calvelo A. 1980. Freezing rate effects on the drip loss of frozen beef. *Meat Sci* 4:1-14.
- Billy AJ. 1982. The effects of formalin and isopropyl alcohol on length and weight measurements of *Sarotherodon mossambicus* Trewavas. *J Fish Bio* 21:107-112.
- Birdseye C. 1929. Some Scientific Aspects of Packaging and Quick-Freezing Perishable Flesh Products Iô More Rapid Freezing Means Better Preservation. *Ind Eng Chem* 21:414-417.
- Bodine SC, Roy R, Meadows D, Zernicke R, Sacks R, Fournier M, Edgerton V. 1982. Architectural, histochemical, and contractile characteristics of a unique biarticular muscle: the cat semitendinosus. *Journal of Neurophysiology* 48:192-201.
- Burgner RL. 1962. *Studies of red salmon smolts from the Wood River Lakes, Alaska*. Seattle, Washington: University of Washington Press.
- Cachel S. 1984. Growth and allometry in primate masticatory muscles. *Arch Oral Biol* 29:287-293.
- Callow E. 1952. Frozen meat. *J Sci Food Agric* 3:145-150.
- Carlson DS. 1983. Growth of the masseter muscle in rhesus monkeys (*Macaca mulatta*). *Am J Phys Anthropol* 60:401-410.
- Carroll R, Cavanaugh J, Rorer F. 1981. Effects of frozen storage on the ultrastructure of bovine muscle. *J Food Sci* 46:1091-1094.
- Close R. 1972. Dynamic properties of mammalian skeletal muscles. *Physiol Rev* 52:129-197.

- Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel J-P, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M. 2010. Sarcopenia: European consensus on definition and diagnosis. *Age Ageing* 39:412-423.
- Curtis AA, Santana SE. 2018. Jaw-dropping: functional variation in the digastric muscle in bats. *Anat Rec* 301:279-290.
- Deichmann JL, Boundy J, Williamson GB. 2009. Anuran artifacts of preservation: 27 years later. *Phyllomedusa: J Herpetol* 8:51-58.
- Deutsch AR, Dickinson E, Leonard KC, Pastor F, Muchlinski MN, Hartstone-Rose A. 2019. Scaling of anatomically derived maximal bite force in primates. *Anatomical record* (Hoboken, NJ : 2007).
- Dick TJM, Clemente CJ. 2016. How to build your dragon: scaling of muscle architecture from the world's smallest to the world's largest monitor lizard. *Frontiers in Zoology* 13:8.
- Dickinson E, Fitton LC, Kupczik K. 2018. Ontogenetic changes to muscle architectural properties within the jaw-adductor musculature of *Macaca fascicularis*. *Am J Phys Anthropol* 167:291-310.
- Dumont ER, Herrel A, Medellin RA, Vargas Contreras JA, Santana SE. 2009. Built to bite: cranial design and function in the wrinkle faced bat. *J Zool* 279:329-337.
- Eltoum I, Fredenburgh J, Myers RB, Grizzle WE. 2001. Introduction to the theory and practice of fixation of tissues. *J Histotechnol* 24:173-190.
- Eng CM, Ward SR, Vinyard CJ, Taylor AB. 2009. The morphology of the masticatory apparatus facilitates muscle force production at wide jaw gapes in tree-gouging common marmosets (*Callithrix jacchus*). *J Exp Biol* 212:4040.
- Engel S. 1974. Effects of formalin and freezing on length, weight and condition factor of cisco and yellow perch. *Trans Am Fish Soc* 103:136-138.
- Fabre PH, Herrel A, Fitriana Y, Meslin L, Hautier L. 2017. Masticatory muscle architecture in a water-rat from Australasia (Murinae, *Hydromys*) and its implication for the evolution of carnivory in rodents. *J Anat* 231:380-397.
- Gans C. 1982. Fiber architecture and muscle function. *Exerc Sport Sci Rev* 10:160-207.
- Gans C, Bock WJ. 1965. The functional significance of muscle architecture--a theoretical analysis. *Ergeb Anat Entwicklungsgesch* 38:115-142.
- Gans C, De Vree F. 1987. Functional bases of fiber length and angulation in muscle. *Journal of morphology* 192:63-85.

- Gonzalez Sanguinetti S, Anon M, Calvelo A. 1985. Effect of thawing rate on the exudate production of frozen beef. *Journal of Food Science* 50:697-700.
- Gruji R, Petrovi L, Pikula B, Amidfi L. 1993. Definition of the optimum freezing rate 1. Investigation of structure and ultrastructure of beef *M. longissimus dorsi* frozen at different freezing rates. *Meat Sci* 33:301-318.
- Hartstone-Rose A, Deutsch AR, Leischner CL, Pastor F. 2018. Dietary correlates of primate masticatory muscle fiber architecture. *Anat Rec* 301:311-324.
- Hartstone-Rose A, Hertzig I, Dickinson E. 2019. Bite Force and Masticatory Muscle Architecture Adaptations in the Dietarily Diverse Musteloidea (Carnivora). *Anatomical record* (Hoboken, NJ : 2007) 302:2287-2299.
- Hartstone-Rose A, Perry JMG, Morrow CJ. 2012. Bite force estimation and the fiber architecture of felid masticatory muscles. *Anat Rec* 295:1336-1351.
- Hoar WS. 1939. The weight-length relationship of the Atlantic salmon. *Journal of the Fisheries Board of Canada* 4:441-460.
- Huq E, Wall CE, Taylor AB. 2015. Epaxial muscle fiber architecture favors enhanced excursion and power in the leaper *Galago senegalensis*. *J Anat* 227:524-540.
- Imamura K, Ashida H, Ishikawa T, Fujii M. 1983. Human major psoas muscle and sacrospinalis muscle in relation to age: A study by computed tomography. *J Gerontol* 38:678-681.
- Janssen I, Heymsfield SB, Wang Z, Ross R. 2000. Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. *Journal of Applied Physiology* 89:81-88.
- Jawad LA. 2003. The effect of formalin, alcohol and freezing on some body proportions of *Alepes djeddaba* (Pisces: Carangidae) collected from the Red Sea coast of Yemen. *Revista de biología marina y oceanografía* 38:77-80.
- Jones D. 1976. Chemistry of fixation and preservation with aldehydes. In: Steedman HF, editor. *Zooplankton fixation and preservation*. Paris, France: UNESCO Press. p 155.
- Kikuchi Y. 2010. Comparative analysis of muscle architecture in primate arm and forearm. *Anat Histol Embryol* 39:93-106.
- Kim H-W, Kim J-H, Seo J-K, Setyabrata D, Kim YHB. 2018. Effects of aging/freezing sequence and freezing rate on meat quality and oxidative stability of pork loins. *Meat Sci* 139:162-170.
- Klauber LM. 1943. Tail-length Differences in Snakes: With Notes on Sexual Dimorphism and the Coefficient of Divergence [and] A Graphic Method of Showing Relationships. San Diego, CA: Zoological Society of San Diego.

- Knuttgen HG. 1976. Development of muscular strength and endurance. In: Neuromuscular mechanisms for therapeutic and conditioning exercise. p 97-118.
- Langenbach GEJ, Weijs WA. 1990. Growth patterns of the rabbit masticatory muscles. *J Dent Res* 69:20-25.
- Lee JC. 1982. Accuracy and precision in anuran morphometrics: artifacts of preservation. *Syst Biol* 31:266-281.
- Leischner CL, Crouch M, Allen KL, Marchi D, Pastor F, Hartstone Rose A. 2018. Scaling of primate forearm muscle architecture as it relates to locomotion and posture. *Anat Rec* 301:484-495.
- Leygonie C, Britz TJ, Hoffman LC. 2012. Impact of freezing and thawing on the quality of meat. *Meat sci* 91:93-98.
- Lieber RL, Fridén J. 2001. Clinical significance of skeletal muscle architecture. *Clinical orthopaedics and related research* 383:140-151.
- Linuma M, Yoshida S, Funakoshi M. 1991. Development of masticatory muscles and oral behavior from suckling to chewing in dogs. *Comparative biochemistry and physiology A, Comparative physiology* 100:789-794.
- Lowie A, Herrel A, Abdala V, Manzano AS, Fabre AC. 2018. Does the morphology of the forelimb flexor muscles differ between lizards using different habitats? *Anatomical record (Hoboken, NJ : 2007)* 301:424-433.
- Marchi D, Leischner CL, Pastor F, Hartstone Rose A. 2018. Leg muscle architecture in primates and its correlation with locomotion patterns. *Anat Rec* 301:515-527.
- Maughan RJ, Watson JS, Weir J. 1983. Strength and cross-sectional area of human skeletal muscle. *J Physiol* 338:37-49.
- Méndez J, Keys A. 1960. Density and composition of mammalian muscle. *Metab: Clin Exp* 9:184-188.
- Michilsens F, Vereecke EE, D'Août K, Aerts P. 2009. Functional anatomy of the gibbon forelimb: adaptations to a brachiating lifestyle. *Journal of Anatomy* 215:335-354.
- Mills A. 1975. Measuring changes that occur during frozen storage of fish: a review. *J Food Technol* 10:483-496.
- Murphy RA, Beardsley AC. 1974. Mechanical properties of the cat soleus muscle in situ. *The American journal of physiology* 227:1008-1013.

- Narici MV, Maganaris CN, Reeves ND, Capodaglio P. 2003. Effect of aging on human muscle architecture. *J Appl Physiol* 95:2229-2234.
- Newton J, Yemm R, Abel R, Menhinick S. 1993. Changes in human jaw muscles with age and dental state. *Gerodontology* 10:16-22.
- Oishi M, Ogihara N, Endo H, Asari M. 2008. Muscle architecture of the upper limb in the orangutan. *Primates; Journal of Primatology* 49:204-209.
- Perry JMG, Hartstone-Rose A. 2010. Maximum ingested food size in captive strepsirrhine primates: Scaling and the effects of diet. *Am J Phys Anthropol* 142:625-635.
- Perry JMG, Hartstone-Rose A, Wall CE. 2011. The jaw adductors of strepsirrhines in relation to body size, diet, and ingested food size. *Anat Rec* 294:712-728.
- Perry JMG, Macneill KE, Heckler AL, Rakotoarisoa G, Hartstone Rose A. 2014. Anatomy and adaptations of the chewing muscles in *Daubentonia* (Lemuriformes). *Anat Rec* 297:308-316.
- Perry JMG, Wall CE. 2008. Scaling of the chewing muscles in prosimians. In: *Primate craniofacial function and biology*. Boston, MA: Springer. p 217-240.
- Pfaller JB, Gignac PM, Erickson GM. 2011. Ontogenetic changes in jaw-muscle architecture facilitate durophagy in the turtle *Sternotherus minor*. *J Exp Biol* 214:1655-1667.
- Pfaller JB, Herrera ND, Gignac PM, Erickson GM. 2009. Ontogenetic scaling of cranial morphology and bite force generation in the loggerhead musk turtle. *J Zool* 280:280-289.
- Prufrock KA, Perry JMG. 2018. Strepsirrhine diets and the pattern of masticatory muscle development. *FASEB J* 32:780-785.
- Qi J, Li C, Chen Y, Gao F, Xu X, Zhou G. 2012. Changes in meat quality of ovine longissimus dorsi muscle in response to repeated freeze and thaw. *Meat Sci* 92:619-626.
- Ramsbottom J, Koonz C. 1939. Freezing temperature as related to drip of frozen defrosted beef. *Journal of Food Science* 4:425-431.
- Reed RN. 2001. Effects of museum preservation techniques on length and mass of snakes. *Amphibia Reptilia* 22:488-491.
- Rosenberg IH. 1997. Sarcopenia: origins and clinical relevance. *J Nutr* 127:990S-991S.
- Sacks RD, Roy RR. 1982. Architecture of the hind limb muscles of cats: functional significance. *Journal of morphology* 173:185-195.

- Santana SE, Cheung E. 2016. Go big or go fish: morphological specializations in carnivorous bats. *Proc R Soc B* 283:20160615.
- Schumacher G-H. 1961. *Funktionelle morphologie der kaumuskulatur*. Jena: G. Fischer.
- Scott NJ, Aquino-Shuster AL. 1989. The effects of freezing on formalin preservation of specimens of frogs and snakes. In: *Collection Forum*. p 41-16.
- Sen A, Sharma N. 2004. Effect of freezing and thawing on the histology and ultrastructure of buffalo muscle. *Asian-australasian Journal of Animal Sciences* 17:1291-1295.
- Shephard S. 2000. *Pickled, potted and canned: How the art and science of food preserving changed the world*. New York: Simon and Schuster.
- Simmons JE. 2014. *Fluid preservation: a comprehensive reference*. Lanham, MD: Rowman & Littlefield.
- Simmons NB, Voss RS. 2009. *Collection, preparation, and fixation of specimens and tissues*, 2 ed. Baltimore, MD: Johns Hopkins University Press.
- Strange ED. 1987. Quantitation and Characterization of Drip from Frozen-Thawed and Refrigerated Pork Liver. *Journal of Food Science* 52:910-915.
- Taylor AB, Jones KE, Kunwar R, Ravosa MJ. 2006. Dietary consistency and plasticity of masseter fiber architecture in postweaning rabbits. *Anat Rec* 288:1105-1111.
- Taylor AB, Vinyard CJ. 2009. Jaw-muscle fiber architecture in tufted capuchins favors generating relatively large muscle forces without compromising jaw gape. *J Hum Evol* 57:710-720.
- Vervust B, Van Dongen S, Van Damme R. 2009. The effect of preservation on lizard morphometrics: an experimental study. *Amphibia-Reptilia* 30:321-329.
- Williams PE, Goldspink G. 1978. Changes in sarcomere length and physiological properties in immobilized muscle. *Journal of anatomy* 127:459-468.
- Xia X, Kong B, Liu Q, Liu J. 2009. Physicochemical change and protein oxidation in porcine longissimus dorsi as influenced by different freeze-thaw cycles. *Meat Sci* 83:239-245.
- Yang Y, Wang H, Zhang ZH. 2015. Muscle architecture of the forelimb of the Golden Pheasant (*Chrysolophus pictus*) (Aves: Phasianidae) and its implications for functional capacity in flight. *Avian Res* 6:8.

## CHAPTER 2: The ontogeny of masticatory muscle architecture in *Microcebus murinus*

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### Abstract:

The masticatory apparatus has been the focus of many studies in comparative anatomy especially analyses of skulls and teeth, but also of the mandibular adductor muscles which are responsible for the production of bite force and the movements of the mandible during food processing and transport. The fiber architecture of these muscles has been correlated to specific diets (e.g., prey size in felids) and modes of foraging (e.g., tree gouging in marmosets). Despite the well-elucidated functional implications of this architecture, little is known about its ontogeny. To characterize age-related myological changes, I studied the masticatory muscles in a large (n=33) intraspecific sample of a small, Malagasy primate, *Microcebus murinus* including neonatal through geriatric individuals. The mandibular adductors individually and the mass as well as other linear measurements recorded. Each muscle was chemically dissected to study its architecture of fascicle length and physiological cross-sectional area (PCSA) which relate to stretch (gape) and force capabilities respectively. I observed PCSA and muscle mass to increase rapidly and plateau in adulthood through senescence. Fascicle lengths remained relatively

constant once maximal length was reached, which occurred early in life, suggesting that subsequent changes in PCSA are driven by changes in muscle mass. Quadratic curvilinear models of each of the architectural variables of all adductors combined as well as individual muscles regressed against age were all significant.

Keywords: mastication, development, senescence, growth, muscle atrophy

### **Introduction:**

The arrangement of muscle fascicles within the masticatory apparatus has been shown to correlate with dietary adaptations in both primates (Perry and Wall, 2008; Eng et al., 2009; Taylor and Vinyard, 2009; Perry and Hartstone-Rose, 2010; Perry et al., 2011; Perry et al., 2014; Hartstone Rose et al., 2018) and other mammals (Taylor et al., 2006; Herrel et al., 2008; Hartstone-Rose et al., 2012; Santana and Cheung, 2016; Fabre et al., 2017; Curtis and Santana, 2018; Santana, 2018). Despite the understanding of the relationship between fascicular architecture and masticatory function, however, few studies have considered how this architecture changes throughout the lifetime of an animal (Huhov et al., 1988; Langenbach and Weijs, 1990; Pfaller et al., 2009; Pfaller et al., 2011), especially within the primate order (though see Dickinson et al., 2018). Importantly, characterizing the architectural properties of muscles at various stages of life could provide valuable insights into dynamic functional demands throughout ontogeny and its impact upon the masticatory apparatus.

The functionality of a particular muscle is directly related to its architectural properties. A muscle of a given volume can be highly pennate and therefore optimized for maximal force production. Alternatively, muscles may have no pennation and long fibers and therefore, are optimized for maximal stretch and speed (Gans and Bock, 1965; Gans, 1982; Otten, 1988; Gans and Gaunt, 1991; Anapol and Barry, 1996; Lieber and Friden, 2000). Some muscles may also

fall somewhere along this continuum between highly pennate and not pennate at all (i.e., intermediary amounts of pennation (i.e., intermediary amounts of pennation; see Anapol and Barry, 1996; Lieber and Friden, 2000). This architecture is studied by analyzing the configuration of the muscle fascicles, which are bundles of individual muscle fibers (e.g., Gans and Bock, 1965). For a given muscle fiber type, total force producing capacity is directly correlated with a muscle's cross-sectional area (i.e., thicker muscles comprised of more fiber bundles in parallel are stronger). Muscle fibers themselves are comprised of serially arranged sarcomeres, which shorten during contraction. As longer muscle fibers are comprised of more sarcomeres, they are capable of both greater contractile velocity and greater total excursion. These same principals are generalizable to the bundles of fibers— fascicles.

The reason that muscles of a given volume can be optimized for either maximal force production or maximal stretch and speed relates to the distribution of fascicles therein: to maximize fascicle length, muscle fibers should be arranged in parallel, spanning the entire length of the muscle along the muscle's line of action from origin to insertion such that each muscle fascicle is roughly equivalent to the length of the whole muscle (Gans and Bock, 1965). By arranging fascicles in a pennate configuration, by contrast, more fascicles can be accommodated within a muscle's volume; however, packing more fibers into the same volume means that each is relatively shorter, typically spanning from the muscle's medial or lateral border to a central tendon (Gans, 1982; Anapol and Barry, 1996).

Previously, it has been determined that cross-sectional area is directly proportional to maximal force production capabilities (Knuttggen, 1976; Maughan et al., 1983). However, this is not the case in instances of pennate muscles— muscles with fascicles with an angular arrangement (Leischner et al., 2018). To accurately account for fascicular orientation, a cross-

sectional area is taken that is perpendicular to all muscle fascicles – a variable known as physiological cross-sectional area (PCSA). Physiological cross-sectional area is a function of mass, fascicle length and the specific density of muscle (Schumacher, 1961). Often PCSA is “reduced” (RPCSA) to include only the force of pull along the muscle’s line of action (i.e., the perpendicular vector of these angular pennate fibers is removed) – especially for limb muscles that are long, straight and move along one clear line of action (Anapol and Barry, 1996). However, the masticatory muscles are rotational (i.e., they move the mandible along an arc, not in a straight line) and each individually have angularity in multiple planes in ever changing axes (Hartstone-Rose et al., 2018).

Muscles of mastication and their fascicular architecture have been well studied and while the abducting digastric muscles and the anterior translator, the lateral pterygoid are important for chewing, it is the mandibular adductors that have been the central focus (though see Curtis and Santana, 2018). The mandibular adductors include the masseter, temporalis, and medial pterygoid and have been of particular interest within anatomical research because their architecture correlates with bite force and diet (Taylor et al., 2006; Anapol et al., 2008; Perry and Wall, 2008; Perry et al., 2011; Hartstone-Rose et al., 2012; Perry et al., 2014; Fabre et al., 2017; Hartstone Rose et al., 2018; Santana, 2018).

For instance, Hartstone-Rose and colleagues (2012) observed that muscle fascicle lengths in felids are correlated with relative prey size. That is, cats that exploit relatively large prey (e.g., herbivores that are larger than the predators themselves) have relatively longer masticatory muscle fascicles whereas cat species that specialize in relatively small prey (e.g., small rodents) have relatively short muscle fascicles. Other similar findings have related the architectural properties of the masticatory muscles to carnivory in bats (Santana and Cheung, 2016), food size

and diet in bats (Dumont et al., 2009; Curtis and Santana, 2018; Santana, 2018), dietary consistency in rabbits (Taylor et al., 2006) and carnivory in water-rats (Fabre et al., 2017).

The muscle architecture of the masticatory apparatus has also been the focus of considerable research within the primate order (Antón, 1999; Antón, 2000; Anapol et al., 2008; Perry and Wall, 2008; Eng et al., 2009; Taylor and Vinyard, 2009; Perry and Hartstone-Rose, 2010; Perry et al., 2011; Perry et al., 2014; Hartstone Rose et al., 2018). From these inquiries researchers have a better understanding of 1. the uniformity within different portions of the masticatory muscles (Antón, 1999), 2. the way that masticatory muscles are adapted to overcome mechanically disadvantaged leverages (e.g., prognathic faces; Antón, 1999; Antón, 2000), 3. the allometric scaling of the architectural properties of masticatory muscles (Anapol et al., 2008; Perry and Wall, 2008; Perry et al., 2011; Hartstone Rose et al., 2018), and 4. the correlations between masticatory muscle architecture and dietary processing and acquisition (Eng et al., 2009; Taylor and Vinyard, 2009; Perry et al., 2014; Hartstone Rose et al., 2018). However, only a few studies of primates have sought to clarify how these architectural properties might develop and change throughout the life of the animal (Carlson, 1983; Cachel, 1984; Dickinson et al., 2018; Prufrock and Perry, 2018).

Through the use of radiopaque markers embedded into portions of the developing masseter within female immature rhesus macaques (*Macaca mulatta*), it was observed that the masseter underwent elongation throughout ontogeny (Carlson, 1983). This phenomenon was attributed to the addition of sarcomeres during development (Carlson, 1983). A more comprehensive interspecific study conducted by Cachel (1984) investigated growth and allometry in primate masticatory muscles on the basis of dry muscle weights, determining that the mass of the masticatory muscles scaled isometrically with body mass during development.

However, architectural properties of these muscles were not considered. More recently Dickinson and colleagues (2018) investigated ontogenetic changes in muscle architecture across the adductor musculature of the crab-eating macaque (*M. fascicularis*). They observed that muscle mass, PCSA and fascicle lengths scaled with positive allometry relative to both jaw length and condyle-molar length across the life span of their focal species (Dickinson et al., 2018).

#### *Ontogeny within Microcebus murinus*

The grey mouse lemur, *Microcebus murinus*, is a small nocturnal primate native to Western Madagascar (Mittermeier et al., 2010). Due to its small size and manageability ó especially for a primate ó it exists as a model organism in numerous colonies across Europe and the United States (Martin, 1971; Rassoul et al., 2010; Ezran et al., 2017) in larger numbers than most other primate species. The life span for these animals in the field has been reported to be between three and four years (Bons et al., 2006); however, in captive environments they tend to live to be around five years of age with a maximal lifespan of up to eleven years. (Perret, 1997; Castanet et al., 2004). They are weaned around two months of age, reach their adult mass by six months (Castanet et al., 2004) and are sexually mature by around nine months (Lutermann et al., 2006). Individuals older than five years of age are considered to be senescent within this species (Bons et al., 2006).

In addition to their manageability and relatively short lifespans, another characteristic of *M. Murinus* making them an ideal model system is their rather generalized diet. Mouse lemurs in the consume a wide array of foods consisting of fruit, insects, gum, and some small vertebrates (Dammhahn and Kappeler, 2008). This variation in diet is accompanied by variation in dietary size and ultimately the manipulation strategies utilized. For instance, when consuming relatively

small foods, *Microcebus murinus* utilizes mouth grasping more so than when consuming larger foods (Reghem et al., 2011).

A recent study into the ontogeny of bite force in this species identified a strong positive correlation between *in vivo* bite force and age within *M. murinus* (Chazeau et al., 2012). The authors report that older individuals were capable of generating greater bite forces than their younger counterparts. However, their oldest age group (5.5 years), demonstrated a decline in force production relative to prime-age adults (Chazeau et al., 2012), suggesting that bite force may decline in senescent individuals.

### *Aims and Predictions*

Following previous studies conducted on the ontogeny of primate mastication, this study aims to quantify ontogenetic changes to the size and architecture of the jaw-adductor musculature in *M. murinus* using the largest intraspecific study in primates to date. While an *in vivo* study pertaining to the ontogeny of bite force in *M. murinus* has been previously conducted by Chazeau and colleagues (2012), this study specifically focuses on the age-associated anatomical changes of the mandibular adductors and includes individuals more advanced in age, which may further elucidate the functional changes they observed. On the basis of this previous work, in addition to recent studies into the life history and bite force potential of this species, I hypothesized the following:

**H1: PCSA increases throughout ontogeny, driven by an increase in muscle mass, as the functional demands on the masticatory musculature change from suckling to chewing.**

As an organism makes the transition from suckling to chewing solid foods, additional adductive force is needed. This requires greater PCSA or potentially more orthognathic faces (i.e., greater mechanical advantage). Increasing PCSA requires either an increase in muscle mass

or a reduction in fascicle length (because, for the same volume, a muscle with shorter fascicles has *more* fascicles and therefore a greater PCSA). As a reduction in fascicle length would reduce the maximal possible gape distance (unlikely, given that masticating animals would be required to attain larger gapes than suckling infants), this increase in force capacity is likely to be driven by an increase in overall muscle mass and volume.

### **H2: Muscle mass and PCSA decline at onset of senescence at 5.5 years of age.**

A recent study conducted by Chazeau and colleagues (2012) reports that whereas bite force is strongly correlated with age with in *M. murinus*, the oldest age group within their study, which was 5.5 years of age, experienced a decline in force production relative to younger adults (Chazeau et al., 2012). They suggested that the decline observed may be due to age-associated muscle atrophy. Previous studies have similarly suggested that, after the onset of senescence, sarcopenia results in a loss in overall muscle mass (Rosenberg, 1997; Cruz-Jentoft et al., 2010) which may decrease force production capacity. The oldest individuals studied by Chazeau and colleagues (2012) were just above the threshold of senescence; as this study incorporates individuals of more advanced ages, I expect to be able to more fully demonstrate the effects of senescence on the PCSA of the jaw adductors (which are responsible for the production of bite force) and anticipate that it will decline in animals over the age of 5.5 years old.

**H3: Muscle fascicle length increases throughout postnatal development until adult size is reached and remains relatively constant throughout adulthood, then decreases after the onset of senescence.**

Muscle fascicles increase in length throughout postnatal development (Goldspink, 1980). This increase in length is the result of an increase in the number of sarcomeres present in each fiber and not due to an increase in the length of the sarcomeres already present (Elliott and

Crawford, 1965; Close, 1972; Goldspink, 1980). Based on this expectation, I hypothesized that fascicle length increases with body size from birth until the cessation of growth which occurs around the age of 6 months (Castanet et al., 2004). After this period, it is expected that the length of the muscle fascicles to remain relatively constant until senescence. A study conducted by Narici and colleagues (2003) evaluated the effects of aging on human gastrocnemius medialis muscle and observed reductions in PCSA, fascicle length, and pennation angles in elderly men (Narici et al., 2003). This suggests that sarcopenia is not only characterized by a loss of muscle mass but also a loss of sarcomeres which results in a decrease in fascicle length (Narici et al., 2003).

## **Materials and Methods:**

### *Sample*

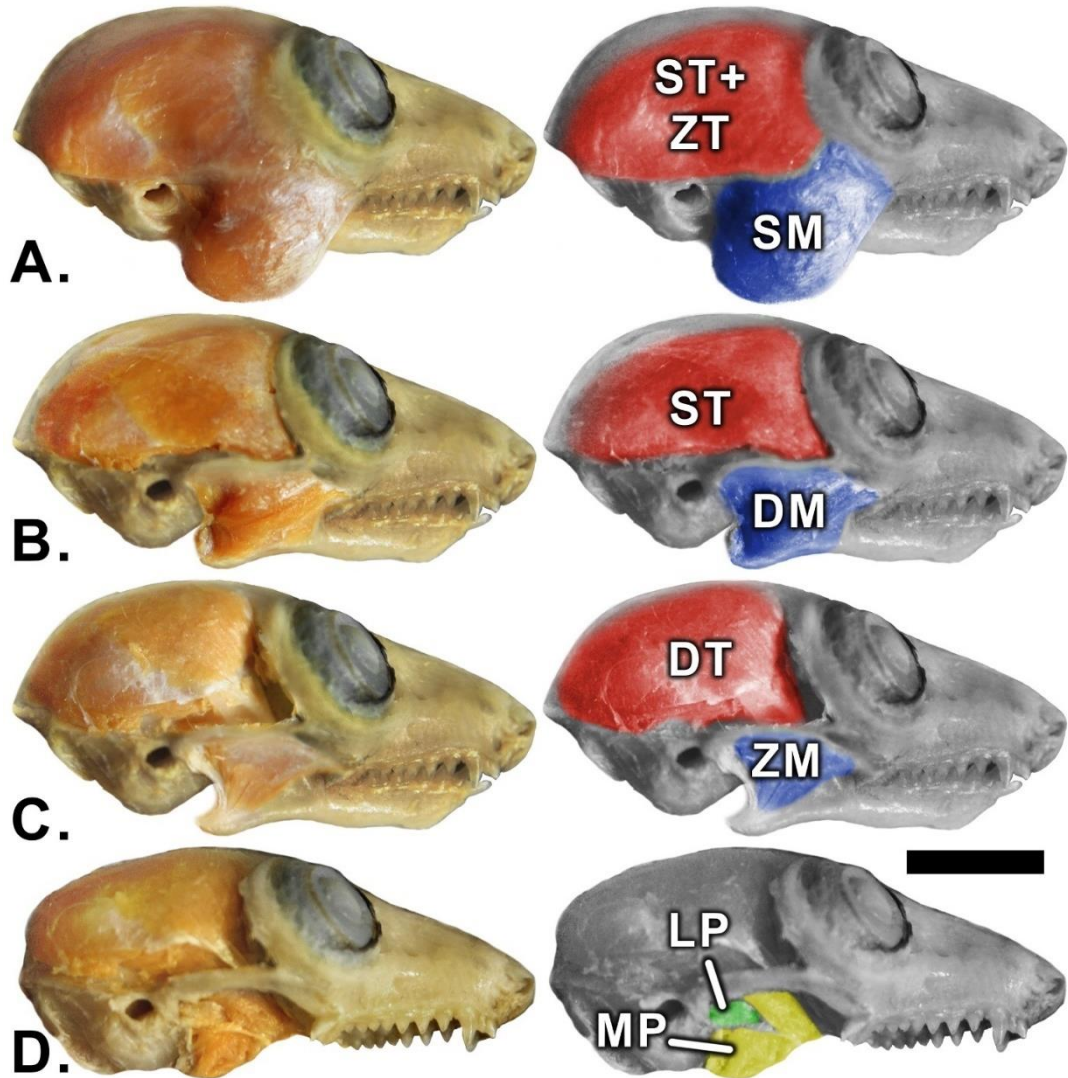
To test these hypotheses, thirty-three (23 males and 10 females) *M. murinus* ranging in age from six days to eight years of age were dissected (Table S1). The animals were born and raised in captivity in the laboratory breeding colony located in Brunoy, France (license number # F91-114-1). None of these animals were euthanized for the purpose of this study. The specimens had been previously fixed in 10% formalin and stored in 70% ethanol. This is the largest intraspecific sample of any primate to have been evaluated for ontogenetic muscle fiber architecture changes.

The sample was divided into four age-cohorts, infant, juvenile, adult, and senescent, based on the life history of captive *Microcebus murinus*. The infant cohort consisted of individuals less than two months of age (pre-weaning) (Castanet et al., 2004). Individuals over the age of two months old but prior to sexual maturity which occurs around nine months of age

(Lutermann et al., 2006) were classified as juveniles. Individuals over the age of nine months but younger than 5.5 years were classified as adults and individuals over 5.5 years of age were classified as senescent (Chazeau et al., 2012).

### *Gross Dissection*

Following careful skinning such that no muscle fibers were removed or damaged, all three of the mandibular adductors were excised (the masseter, temporalis, and medial pterygoid) (Figure 2.1). When feasible, each constituent muscle portion (e.g., superficial, deep and zygomatic (i.e., zygomaticomandibularis) portions of masseter, and temporalis) were excised individually; however, in instances in which the muscle bellies had become fused, muscle portions were removed as one. The mass of each muscle portion was recorded immediately following excision.



**Figure 2.1.** Muscles of mastication of *Microcebus murinus* shown in situ. ST, superficial temporalis; ZT, zygomatic temporalis; DT, deep temporalis; SM, superficial masseter; ZM, zygomaticomandibularis; DM, deep masseter; LP, lateral pterygoid; MP, medial pterygoid.

### *chemical Dissection*

A protocol modified from Rayne and Crawford (1972) was utilized to chemically dissect the muscles. The excised muscles were placed individually into a 35% aqueous nitric acid solution to dissolve the connective tissue binding their fascicles, until fascicles could be separated without damage. This process took 12-24 hours depending on the size and amount of connective tissue in and surrounding the muscle. Once fascicles could be teased apart, muscles were transferred into 50% aqueous glycerol to neutralize the reaction and cease further chemical digestion. The separated muscle fascicles were arranged such they were lying flat and photographed, alongside a scale bar, using a Nikon D3000 camera. Fascicle lengths were subsequently measured using the software package ImageJ (IJ1.46r). When available, a minimum of 40 muscle fascicles per muscle were measured in order to calculate a representative fascicle length; though some smaller muscles had fewer measurable fascicles.

### *Data Analysis*

Using the muscle mass and average fascicle length for each muscle, the physiological cross-sectional area (PCSA) was calculated— an estimator of force production using the following formula modified from (Schumacher, 1961):

$$PCSA = \frac{\text{muscle mass(g)}}{\text{avg. fascicle length [cm]} * \text{specific density of muscle (g/cm}^3\text{)}}$$

The specific density of mammalian skeletal muscle used was a constant of 1.0564 g/cm<sup>3</sup>, following Murphy and Beardsley (1974). To evaluate the total adductors as a single functional unit, three aggregate measures were calculated: total muscle mass, total PCSA and weighted average fascicle length. Total muscle mass and total PCSA were calculated by summing the individual components. An average weighted fascicle length was calculated by using the following formula adapted from (Hartstone-Rose et al., 2012):

$$FL_{\bar{x}} = \frac{\sum FL_{SM} m_{SM} + \sum FL_{DM} m_{DM} + \sum FL_{ZM} m_{ZM} + \sum FL_{ST} m_{ST} + \sum FL_{DT} m_{DT} + \sum FL_{ZT} m_{ZT} + \sum FL_{MP} m_{MP}}{(m_{SM} + m_{DM} + m_{ZM} + m_{ST} + m_{DT} + m_{ZT} + m_{MP})}$$

where  $FL_X$  is the weighted fascicle length and  $FL_{SM}$ ,  $FL_{DM}$ ,  $FL_{ZM}$ ,  $FL_{ST}$ ,  $FL_{DT}$ ,  $FL_{ZT}$ ,  $FL_{MP}$ , are the average fascicle length and  $m_{SM}$ ,  $m_{DM}$ ,  $m_{ZM}$ ,  $m_{ST}$ ,  $m_{DT}$ ,  $m_{ZT}$ ,  $m_{MP}$  are the muscle masses for the superficial masseter, deep masseter, zygomaticomandibularis, superficial temporalis, deep temporalis, zygomatic temporalis, and medial pterygoid respectively.

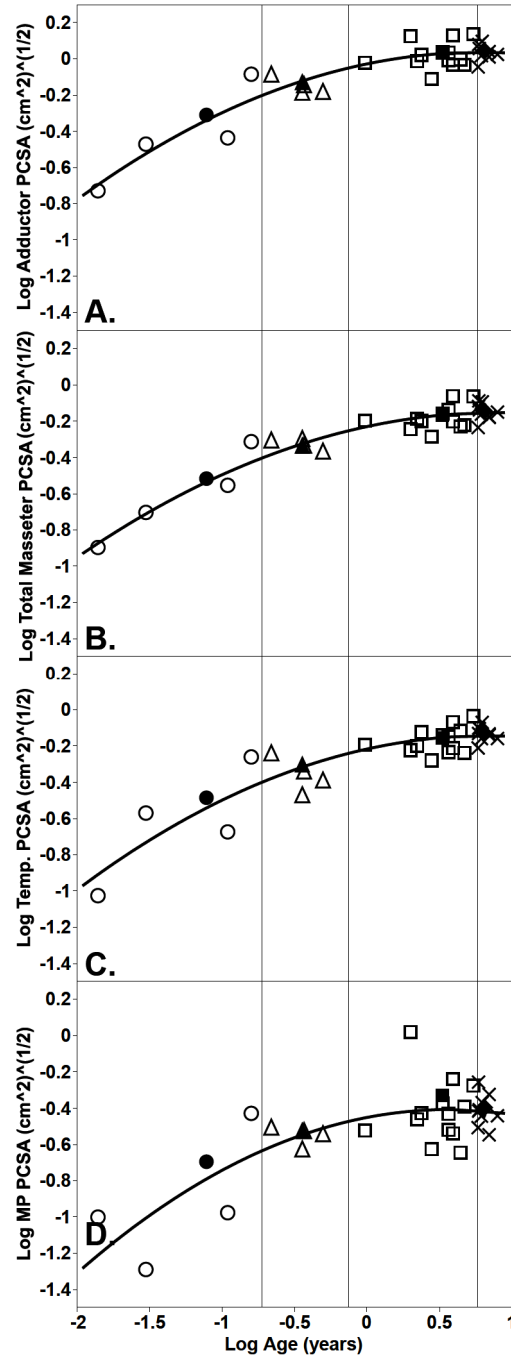
Statistical analyses of all variables were conducted using JMP Pro 13 (SAS). All variables were first linearized to a consistent power (i.e., the cubic- and square-roots of the volumetric variables of mass and square variables of areas were taken respectively) and logged. Log-transformations were performed to prevent overemphasizing any age group. Curvilinear regressions (polynomial fit degree = 2) were conducted for each architectural variable against both age and body mass. Curvilinear regressions were selected based on the hypothesized trends of a increase in each architectural variable followed by a plateau and subsequent decline in senescence. To further clarify the ontogenetic trends observed, I calculated the mean for each architectural variable for each age-cohort and then determined the slopes between each mean point to determine the magnitude of change between infancy and juvenility, juvenility and adulthood and adulthood and senescence.

## Results:

### *Physiological Cross-Sectional Area*

When the PCSA of each mandibular adductor was regressed against age, quadratic curvilinear relationships yielded significant  $p$ -values  $< 0.05$  (Figure 2.2). Equations for each of the fitted curves shown in Figures 2.1-2.5 are provided in supplementary materials (Table S2). This suggests that the PCSA of the adductors increases and peaks in mid-to-late adulthood

(supporting H1) before plateauing during senescence (refuting H2). This trend is further indicated by analyzing the slope of total adductor PCSA between the mean points for each group (Table 1.1): while the slopes from infancy to juvenility and juvenility to adulthood are both clearly positive (0.272 and 0.169, respectively), the slope from adulthood to senescence is essentially nil (0.035). This trend is consistent within both the temporalis and the masseter; however, a negative senescent slope (-0.233) is observed for the medial pterygoid, suggesting a decline in the force production of this muscle within older individuals. However, the decline observed in this muscle may be exaggerated by a single adult specimen with an exceptionally high medial pterygoid PCSA, which inflates the adult mean (slope with individual excluded= 0.081).



**Figure 2.2** Bivariate plots of the log PCSA (cm<sup>2</sup>)<sup>(1/2)</sup> of total sum and each separate mandibular adductor regressed against log age. Open circles = infants, open triangles = juveniles, open squares = adults, x = senescent, closed shapes = mean for their respective age cohorts (closed

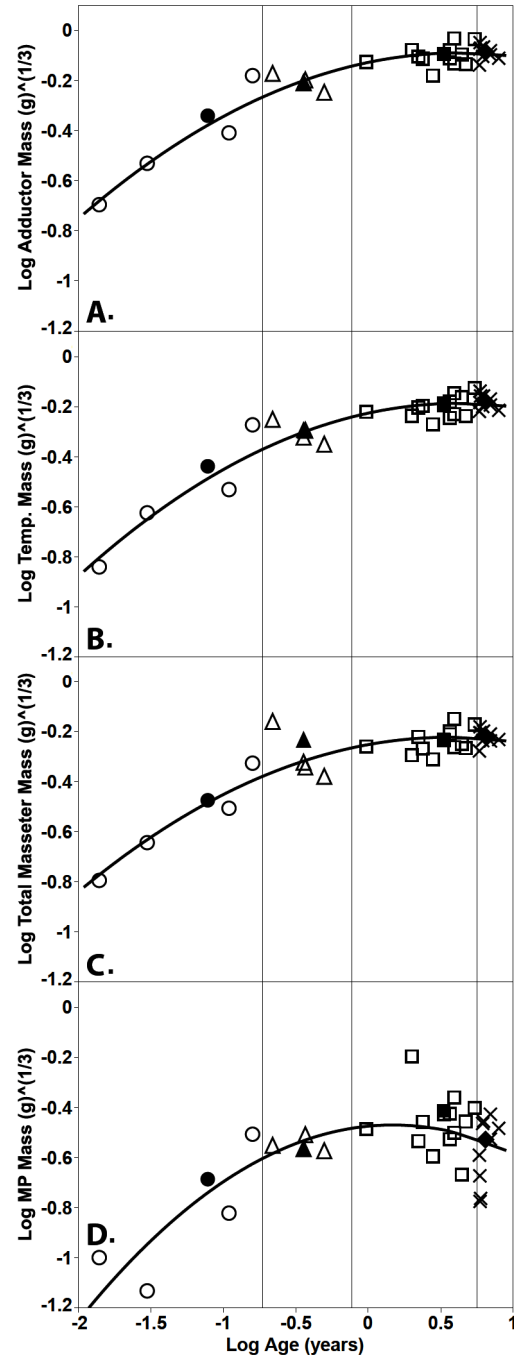
diamonds = mean for senescent individuals). The vertical lines indicate the boundaries between the following life history stages: infantójuvenile, juvenileóadult, and adultósenescent.

**Table 2.1.** Slopes calculated between the mean of each age cohort infancy to juvenility, juvenility to adult and adult to senescence for each architectural variable of each mandibular adductor. Positive slopes indicate that the architectural variable is increasing between the two age-cohorts, while negative values indicate a decrease.

<b>Muscle(s)</b>	<b>Age Cohort Range</b>	<b>PCSA Slope</b>	<b>MM Slope</b>	<b>FL Slope</b>
Total Adductors	Infant Juvenile	0.272	0.202	0.172
	Juvenile Adult	0.169	0.116	0.012
	Adult Senescent	0.035	0.031	-0.002
Total Masseter	Infant Juvenile	0.275	0.364	0.031
	Juvenile Adult	0.174	0.004	0.087
	Adult Senescent	0.079	0.042	-0.028
Temporalis	Infant Juvenile	0.276	0.217	0.184
	Juvenile Adult	0.151	0.101	0.007
	Adult Senescent	0.091	0.059	-0.019
Medial Pterygoid	Infant Juvenile	0.257	0.185	0.105
	Juvenile Adult	0.202	0.155	0.097
	Adult Senescent	-0.233	-0.397	-0.168

### *Muscle Mass*

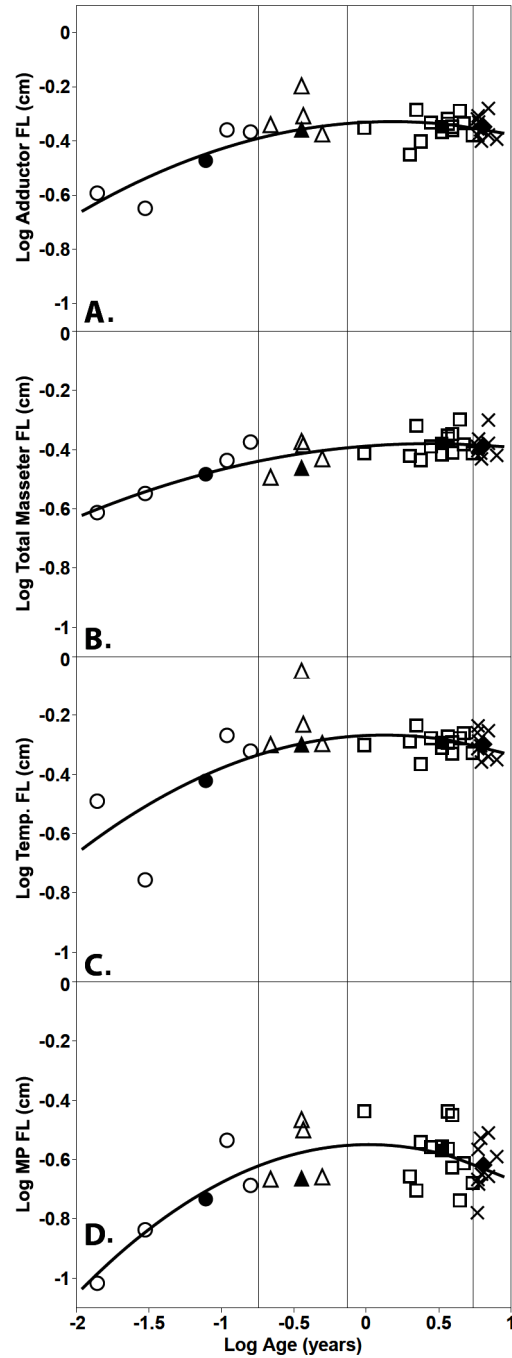
When regressing adductor mass against age, a quadratic curvilinear relationship was observed to be significant in each case with p-values  $<0.01$  (Figure 2.3). Muscle mass of the mandibular adductors increases and peaks during early to mid-adulthood (supporting H1) and plateaus into senescence (refuting H2). This trend is also observed when evaluating the slopes between each age cohort within the total adductor and temporalis mass. Masseter displays a similar trend, though with an earlier plateau occurring between juvenility and adulthood (Table 2.1). However, as for its PCSA, the medial pterygoid exhibits a distinctive trend, with a steep decline (slope =  $-0.397$ ) in muscle mass from adulthood to senescence (Figure.2.3d, Table 2.1). This decline, again, may have been exaggerated by the inclusion of one adult individual with an exceptionally massive medial pterygoid (slope with individual excluded =  $-0.224$ ).



**Figure 2.3.** Bivariate plots of the log MM (g)<sup>(1/3)</sup> of total sum and each separate mandibular adductor regressed against log age. Legend: same as Figure 2.

### *Fascicle Length*

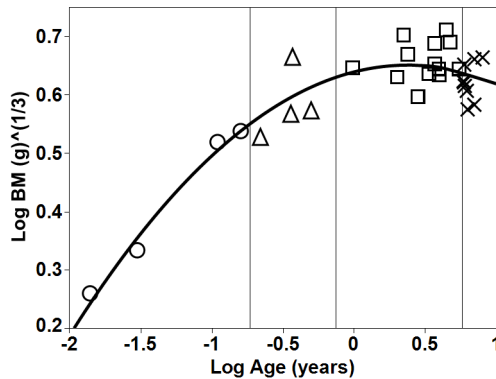
When the logged weighted average fascicle length and the average fascicle length from each individual adductor are regressed with log age, quadratic curvilinear relationships are significant, with  $p < 0.01$  (Figure 2.4). For weighted average fascicle lengths, an increase is observed from infants to juveniles (partially supporting H3), but this trend plateaus through all further ages (partially refuting H3) (Table 2.1). The same trend is observed for the temporalis alone. In the case of the masseter and the medial pterygoid, however, average fascicle lengths show a slight increase through to adulthood, before decreasing into senescence (Table 2.1). This decline is particularly pronounced within the medial pterygoid (slope = -0.168; Table 2.1).



**Figure 2.4.** Bivariate plots of the log FL (cm) weighted average of combined adductors and of each mandibular adductor regressed against log age. Legend same as in Figure 2.2.

### Body Mass

A significant curvilinear relationship is observed when body mass is regressed against age, with  $p < 0.0001$  (Figure 2.5). This trend reflects a rapid increase in body mass occurring throughout infancy and juvenility, which peaks in the early stages of adulthood. Following this, a decline in body mass is associated with the onset of senescence.



**Figure 2.5.** Bivariate plot of  $\log \text{BM (g)}^{(1/3)}$  regressed against  $\log \text{age}$ . Legend same as in Figure 2.2.

### Discussion:

Masticatory muscle fiber architecture has been a growing focus of anatomical research over the last several decades. This study is one of the first to comprehensively evaluate the fiber architecture within all of the mandibular adductors in a large intraspecific sample with known ages; it is the largest intraspecific primate sample for which the ontogeny of muscle fiber architecture has been evaluated. This allowed thorough investigation into the ontogenetic changes that occur within the fiber architecture of the mandibular adductors within *M. murinus*.

Based on the need for increased force production capabilities as the feeding mechanism transitions from suckling to chewing, I hypothesized that PCSA would increase throughout early ontogeny in *M. murinus*, driven largely by an increase in muscle mass. These findings support

this hypothesis (H1). Physiological cross-sectional area increased throughout infancy and juvenility then plateaued during adulthood; a trend which closely mirrored the changes in muscle mass. However, a decline in PCSA associated with the onset of senescence was predicted, following the functional analysis of the ontogeny of bite force in *M. murinus* by Chazeau and colleagues (2012). The findings of this study did not support this hypothesis (H2): rather, PCSA remains relatively consistent between adult and senescent groups. When looking at the bivariate plots of the log PCSA for each mandibular adductor against log age, the plateau during adulthood is relatively apparent (Figure 2.2); however, the anticipated point of inflection associated with senescence is not. The exception to this trend was the medial pterygoid, which displays a notable shift in both mass and PCSA during senescence, with both variables showing a marked decline.

These findings appear curious in light of the findings of Chazeau and colleagues (2012) that bite force was reduced within senescent individuals of *M. murinus*. Despite this functional separation, there is no clear physiological decline in the force production capacity of the adductor muscles between prime-age and senescent individuals. Consequently, other factors may therefore be limiting bite force production within senescent individuals, such as a decline in motor coordination or perhaps even a behaviorally reduced willingness to bite with relative maximal force. The maxillae and mandibles of older individuals do, based on qualitative observation, seem to be more lightly built (e.g., osteoporotic). It is therefore possible that older individuals are less capable of safely transmitting the forces associated with maximal biting through this region of the skull, and thus adjust their behavior to prevent micro- or macro-damage to the facial skeleton. Future work could explore this phenomenon in greater depth

through the use of CT scanning to examine bone density and produce finite-element models to evaluate jaw resistance to deformation at differing bone densities (e.g., Röhrle and Pullan, 2007).

In terms of fascicle lengths, it was observed that adult lengths were attained early during development, and remained relatively constant throughout the animal's life span, across the adductor musculature. This trend partially supports the hypothesis that fascicle lengths increased until adult size was reached, which occurs around six months of age (H3). However, this observation also refutes the hypothesis (H3) that fascicle lengths would experience a decrease in length at the onset of senescence contradicting a study conducted by Narici and colleagues (2003) which characterized sarcopenia as not just a loss in muscle mass but also a subsequent loss in sarcomeres. If sarcomeres were lost in the sample, it appears that the loss was entirely in parallel fibers (i.e., cross-section/mass) and not in serial (i.e., no shortening of fascicles).

Overall, it was anticipated that quadratic models to be significant because for each architectural property, because a rapid increase through infancy and juvenility, a plateau occurring in adulthood, and a decline with senescence was expected. Although the clear points of inflection were expected in late adulthood to early senescence were not obvious in most cases, quadratic curvilinear relationships were observed to be significant when each architectural variable was regressed with age.

From these results, representing the most comprehensive ontogenetic and intraspecific samples of primate masticatory muscle architecture, which characterized myological changes across the lifespan of *M. murinus*. These findings suggest that the changes in PCSA are driven mostly by changes in muscle mass including growth through early adulthood, and a plateau into senescence, whereas masticatory fascicle lengths remain surprisingly constant throughout life.

Future studies are needed to determine whether or not these trends are consistent throughout other muscular regions and if these findings are species specific.

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## References:

- Anapol F, Barry K. 1996. Fiber architecture of the extensors of the hindlimb in semiterrestrial and arboreal guenons. *Am J Phys Anthropol* 99:429-447.
- Anapol F, Shahnoor N, Ross CF. 2008. Scaling of reduced physiologic cross-sectional area in primate muscles of mastication. In: *Primate craniofacial function and biology*: Springer. p 201-216.
- Antón SC. 1999. Macaque masseter muscle: internal architecture, fiber length and cross-sectional area. *Int J Primatol* 20:441-462.
- Antón SC. 2000. Macaque pterygoid muscles: internal architecture, fiber length, and cross-sectional area. *Int J Primatol* 21:131-156.
- Bons N, Rieger F, Prudhomme D, Fisher A, Krause KH. 2006. *Microcebus murinus*: a useful primate model for human cerebral aging and Alzheimer's disease? *Genes Brain Behav* 5:120-130.
- Cachel S. 1984. Growth and allometry in primate masticatory muscles. *Arch Oral Biol* 29:287-293.
- Carlson DS. 1983. Growth of the masseter muscle in rhesus monkeys (*Macaca mulatta*). *Am J Phys Anthropol* 60:401-410.
- Castanet J, Croci S, Aujard F, Perret M, Cubo J, De Margerie E. 2004. Lines of arrested growth in bone and age estimation in a small primate: *Microcebus murinus*. *J Zool* 263:31-39.
- Chazeau C, Marchal J, Hackert R, Perret M, Herrel A. 2012. Proximate determinants of bite force capacity in the mouse lemur. *J Zool* 290:42-48.
- Close R. 1972. Dynamic properties of mammalian skeletal muscles. *Physiol Rev* 52:129-197.
- Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel J-P, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M. 2010. Sarcopenia: European consensus on definition and diagnosis. *Age Ageing* 39:412-423.
- Curtis AA, Santana SE. 2018. Jaw-dropping: functional variation in the digastric muscle in bats. *Anat Rec* 301:279-290.
- Dammhahn M, Kappeler PM. 2008. Comparative feeding ecology of sympatric *Microcebus berthae* and *M. murinus*. *Int J Primatol* 29:1567.
- Dickinson E, Fitton LC, Kupczik K. 2018. Ontogenetic changes to muscle architectural properties within the jaw-adductor musculature of *Macaca fascicularis*. *Am J Phys Anthropol* 167:291-310.

- Dumont ER, Herrel A, Medellin RA, Vargas Contreras JA, Santana SE. 2009. Built to bite: cranial design and function in the wrinkle faced bat. *J Zool* 279:329-337.
- Elliott D, Crawford G. 1965. The thickness and collagen content of tendon relative to the cross-sectional area of muscle during growth. *Proc R Soc Lond B* 162:198-202.
- Eng CM, Ward SR, Vinyard CJ, Taylor AB. 2009. The morphology of the masticatory apparatus facilitates muscle force production at wide jaw gapes in tree-gouging common marmosets (*Callithrix jacchus*). *J Exp Biol* 212:4040.
- Ezran C, Karanewsky CJ, Pendleton JL, Sholtz A, Krasnow MR, Willick J, Razafindrakoto A, Zohdy S, Albertelli MA, Krasnow MA. 2017. The mouse lemur, a genetic model organism for primate biology, behavior, and health. *Genetics* 206:651.
- Fabre PH, Herrel A, Fitriana Y, Meslin L, Hautier L. 2017. Masticatory muscle architecture in a water-rat from Australasia (Murinae, *Hydromys*) and its implication for the evolution of carnivory in rodents. *J Anat* 231:380-397.
- Gans C. 1982. Fiber architecture and muscle function. *Exerc Sport Sci Rev* 10:160-207.
- Gans C, Bock WJ. 1965. The functional significance of muscle architecture--a theoretical analysis. *Ergeb Anat Entwicklungsgesch* 38:115-142.
- Gans C, Gaunt AS. 1991. Muscle architecture in relation to function. *J Biomech* 24:53-65.
- Goldspink G. 1980. Growth of muscle. Development and specialization of skeletal muscle:19-35.
- Hartstone-Rose A, Perry JMG, Morrow CJ. 2012. Bite force estimation and the fiber architecture of felid masticatory muscles. *Anat Rec* 295:1336-1351.
- Hartstone Rose A, Deutsch AR, Leischner CL, Pastor F. 2018. Dietary correlates of primate masticatory muscle fiber architecture. *Anat Rec* 301:311-324.
- Herrel A, De Smet A, Aguirre LF, Aerts P. 2008. Morphological and mechanical determinants of bite force in bats: do muscles matter? *J Exp Biol* 211:86-91.
- Huhov J, Henry Ward W, Phillips L, German R. 1988. Growth allometry of craniomandibular muscles, tendons, and bones in the laboratory rat (*Rattus norvegicus*): Relationships to oromotor maturation and biomechanics of feeding. *Am J Anat* 182:381-394.
- Knuttgen HG. 1976. Development of muscular strength and endurance. In: *Neuromuscular mechanisms for therapeutic and conditioning exercise*. p 97-118.
- Langenbach GEJ, Weijs WA. 1990. Growth patterns of the rabbit masticatory muscles. *J Dent Res* 69:20-25.

- Leischner CL, Crouch M, Allen KL, Marchi D, Pastor F, Hartstone Rose A. 2018. Scaling of primate forearm muscle architecture as it relates to locomotion and posture. *Anat Rec* 301:484-495.
- Lieber RL, Friden J. 2000. Functional and clinical significance of skeletal muscle architecture. *Muscle Nerve* 23:1647-1666.
- Lutermann H, Schmelting B, Radespiel U, Ehresmann P, Zimmermann E. 2006. The role of survival for the evolution of female philopatry in a solitary forager, the grey mouse lemur (*Microcebus murinus*). *Proc R Soc B* 273:2527-2533.
- Martin R. 1971. A laboratory breeding colony of the lesser mouse lemur. In: *Breeding primates*: Karger Publishers. p 161-171.
- Maughan RJ, Watson JS, Weir J. 1983. Strength and cross-sectional area of human skeletal muscle. *J Physiol* 338:37-49.
- Mittermeier RA, Louis EE, Richardson M, Schwitzer C, Langrand O, Rylands AB, Hawkins F, Rajaobelina S, Rasimbazafy J, Rasoloarison RM, Roos C, Kappeler PM, Mackinnon J. 2010. *Lemurs of Madagascar*, Third ed. Arlington, Virginia: Conservation International.
- Murphy RA, Beardsley AC. 1974. Mechanical properties of the cat soleus muscle in situ. *The American journal of physiology* 227:1008-1013.
- Narici MV, Maganaris CN, Reeves ND, Capodaglio P. 2003. Effect of aging on human muscle architecture. *J Appl Physiol* 95:2229-2234.
- Otten E. 1988. Concepts and models of functional architecture in skeletal muscle. *Exerc Sport Sci Rev* 16:89-138.
- Perret M. 1997. Change in photoperiodic cycle affects life span in a prosimian primate (*Microcebus murinus*). *J Biol Rhythms* 12:136-145.
- Perry JMG, Hartstone-Rose A. 2010. Maximum ingested food size in captive strepsirrhine primates: Scaling and the effects of diet. *Am J Phys Anthropol* 142:625-635.
- Perry JMG, Hartstone-Rose A, Wall CE. 2011. The jaw adductors of strepsirrhines in relation to body size, diet, and ingested food size. *Anat Rec* 294:712-728.
- Perry JMG, Macneill KE, Heckler AL, Rakotoarisoa G, Hartstone Rose A. 2014. Anatomy and adaptations of the chewing muscles in *Daubentonia* (Lemuriformes). *Anat Rec* 297:308-316.
- Perry JMG, Wall CE. 2008. Scaling of the chewing muscles in prosimians. In: *Primate craniofacial function and biology*. Boston, MA: Springer. p 217-240.

- Pfaller JB, Gignac PM, Erickson GM. 2011. Ontogenetic changes in jaw-muscle architecture facilitate durophagy in the turtle *Sternotherus minor*. *J Exp Biol* 214:1655-1667.
- Pfaller JB, Herrera ND, Gignac PM, Erickson GM. 2009. Ontogenetic scaling of cranial morphology and bite force generation in the loggerhead musk turtle. *J Zool* 280:280-289.
- Prufrock KA, Perry JMG. 2018. Strepsirrhine diets and the pattern of masticatory muscle development. *FASEB J* 32:780-785.
- Rassoul RA, Alves S, Pantesco V, De Vos J, Michel B, Perret M, Mestre-Frances N, Verdier J-M, Devau G. 2010. Distinct transcriptome expression of the temporal cortex of the primate *Microcebus murinus* during brain aging versus Alzheimer's disease-like pathology. *PLoS One* 5:e12770.
- Rayne J, Crawford GNC. 1972. The relationship between fibre length, muscle excursion and jaw movements in the rat. *Arch Oral Biol* 17:859-IN811.
- Reghem E, Tia B, Bels V, Pouydebat E. 2011. Food prehension and manipulation in *Microcebus murinus* (Prosimii, Cheirogaleidae). *Folia Primatol* 82:177-188.
- Röhrle O, Pullan AJ. 2007. Three-dimensional finite element modelling of muscle forces during mastication. *J Biomech* 40:3363-3372.
- Rosenberg IH. 1997. Sarcopenia: origins and clinical relevance. *J Nutr* 127:990S-991S.
- Santana SE. 2018. Comparative anatomy of bat jaw musculature via diffusible iodine-based contrast-enhanced computed tomography. *Anat Rec* 301:267-278.
- Santana SE, Cheung E. 2016. Go big or go fish: morphological specializations in carnivorous bats. *Proc R Soc B* 283:20160615.
- Schumacher G-H. 1961. Funktionelle morphologie der kaumusculatur. Jena: G. Fischer.
- Taylor AB, Jones KE, Kunwar R, Ravosa MJ. 2006. Dietary consistency and plasticity of masseter fiber architecture in postweaning rabbits. *Anat Rec* 288:1105-1111.
- Taylor AB, Vinyard CJ. 2009. Jaw-muscle fiber architecture in tufted capuchins favors generating relatively large muscle forces without compromising jaw gape. *J Hum Evol* 57:710-720.

### CHAPTER 3: Anatomical and Ontogenetic Influences on Muscle Density

#### Abstract:

Physiological cross-sectional area (PCSA), an important biomechanical variable, is an estimate of a muscle's contractile force potential and is derived from dividing muscle mass by the product of a muscle's average fascicle length and a theoretical constant representing the density of mammalian skeletal muscle. This density constant is usually taken from experimental studies of several model taxa using tissue samples collected predominantly from the lower limbs of adult animals. However, the generalized application of this constant to broader analyses of mammalian myology assumes that muscle density 1. is consistent across anatomical regions and 2. is unaffected by the aging process. To investigate the validity of these assumptions, excised several whole muscles were excised from numerous anatomical regions from three different age cohorts of New Zealand white rabbits (*Oryctolagus cuniculus*). Differences in muscle density as a consequence of age and anatomical region were evaluated using Tukey HSD tests. Further these muscles were histologically evaluated by taking cross-sections perpendicular to the muscle fibers, and preparing slides using a hematoxylin and eosin protocol. Overall, it was observed that older individuals tend to have denser muscles than younger individuals. These findings also demonstrated significant differences in muscle density between anatomic regions within the 8 month and 2-year age cohorts. However, no regional differences in muscle density were observed in the 3-week-old cohort. These differences can be explained in part by the histology of the muscles themselves: based on gross measurements of muscle fiber areas relative to other connective tissues, approximately 50% of the variation in muscle density can be explained by the average muscle fiber area and the average percent fiber area. That is, muscles with larger average fiber areas and a higher proportion of fiber area tend to be denser. These data suggest that using

the age and region dependent measurements of muscle density presented here may increase the validity of PCSA estimations. Although statistically significant differences were observed related to ontogeny and anatomical region, the magnitude of these differences was small and, if density cannot be measured directly, then a value of 1.0558 and 1.0502 g/cm<sup>3</sup> would reasonable constants across all adult and juvenile muscles respectively.

Keywords: muscle region, PCSA, development, aging

### **Introduction:**

Physiological cross-sectional area (PCSA) is an architectural property of muscle that directly relates to force production capabilities ó muscles with higher PCSAs can produce proportionally more force than muscles with lower PCSAs. As such, PCSA has been frequently used to contextualize dietary (Perry and Wall, 2008; Eng et al., 2009; Taylor and Vinyard, 2009; Perry and Hartstone-Rose, 2010; Perry et al., 2011; Hartstone-Rose et al., 2012; Perry et al., 2014; Fabre et al., 2017; Curtis and Santana, 2018; Hartstone-Rose et al., 2018; Prufrock and Perry, 2018) and locomotor adaptations (Crook et al., 2008; Oishi et al., 2008; Kikuchi, 2010; Leischner et al., 2018; Marchi et al., 2018) across taxa. Unlike muscle mass and fascicle length, which are measured directly, PCSA is derived: calculated as a function of muscle mass, average fascicle length and muscle density (Schumacher, 1961). The constants used for muscle density (~1.06 g/cm<sup>3</sup>) are commonly taken from several different studies (Gersh et al., 1944; Méndez and Keys, 1960; Murphy and Beardsley, 1974). These sources share some commonalities: their model specimens were all adult individuals and their samples were taken from similar anatomical regions (i.e., the lower limb). Therefore, the use of these constants makes the

assumptions that muscle density is static regardless of age and anatomical region—assumptions that this study aims to address.

Gersh and colleagues (1944) used mature guinea pigs as their model system and were analyzing the specific gravity or relative density of skeletal muscle due to changes in pressure; however, did not specify the anatomical region from which they took their tissue sample, but found no significant differences in the specific density of the muscle after decompression. Mendez and Keys (1960) used muscles of the lower limb and “sometimes” include the psoas muscle of mature rabbits and dogs to evaluate muscle density. Though they specify the muscles they utilized, which include the quadriceps cruralis, gastrocnemius, and tibialis, they did not specify what muscles and how many of each were included in each of the 13 muscle samples for rabbits and 12 muscle samples for dogs (Méndez and Keys, 1960). Lastly, Murphy and Beardsley (1974) evaluated the mechanical properties of the soleus muscle of adult cats ( $n=6$ ) and as an aside measured density because it was necessary to calculate PCSA. The conclusions of these studies (Gersh et al., 1944; Méndez and Keys, 1960; Murphy and Beardsley, 1974) all converged upon very similar densities (1.065, 1.0597, and 1.0564 g/cm<sup>3</sup>, respectively). This convergence may reflect similarities in study design between these experiments, which were all comprised exclusively of adult individuals, and limited variation among anatomical regions. Therefore, given these similarities, using a constant for muscle density makes some inherent assumptions.

### *Assumption 1*

The first assumption made seemingly universally by functional morphologists that is important to evaluate is that muscle density remains relatively constant throughout an individual’s life. For this assumption to be true, muscle mass and muscle volume would have to

change proportionally because density is defined as the mass per unit of volume. However, given that other architectural properties of muscle have been demonstrated to be dynamic and change throughout the lifespan of animals (see, for example, Dickinson et al., 2018; Boettcher et al., 2019; Leonard et al., 2019), this is also likely to be the case for muscle density. For example, muscle mass has been shown to decline with increasing age—a condition that has been termed as sarcopenia (Rosenberg, 1997; Cruz-Jentoft et al., 2010). Other previous studies conducted provide more direct evidence that muscle density is variable with age (Bulcke et al., 1979; Imamura et al., 1983; Newton et al., 1993). For instance, Imamura and colleagues (1983) investigated the size and density of human sacrospinalis and psoas major muscles with respect to age using computed tomography and observed an increase in density until middle-age with a subsequent decline. While they noted that the differences they observed were significant, they did not specify what these differences were. Additional evidence suggesting muscle density changes with age is provided by a study conducted by Newton and colleagues (1993) who observed a decline in the density of the masseter and medial pterygoid muscles with advancing age. Overarching trends within this literature suggest that muscle density will increase until approximately middle-age and decline throughout senescence.

In addition, to these studies that suggest muscles will vary based on their gross characteristics (e.g., mass), it is also likely that they will change microscopically and in composition especially during growth—resulting in changes in muscle density. Muscles grow three different ways which include increasing the number of muscle fibers, increasing the size of the muscle fibers, and lastly, increasing the length of the muscle fibers (Pearson, 1990). Skeletal muscle is predominantly comprised of muscle fibers with connective tissue such as collagen and fat dispersed throughout (Listrat et al., 2016). Therefore, as muscle grows it is likely that the

proportions of the microscopic components will change resulting in differences in muscle density.

### *Assumption 2*

It is assumed in most functional myology studies that muscle density is not influenced by the anatomical region from which the samples are taken. The study conducted by Méndez and Keys (1960) used lower limb muscles including the quadriceps cruralis, gastrocnemius, tibialis, and occasionally included the hip flexor psoas, while the other most commonly cited reference for muscle density, the study by Murphy and Beardsley (1974), only evaluated the soleus. This is potentially problematic as the density constants determined in these studies are used to make determinations about muscles from all anatomical regions even though it has been clearly demonstrated that muscles are variable in composition. For instance, Faucitano and colleagues (2004) found that fat content within individual muscle fascicles can vary throughout the same muscle. Muscles that are higher in fat (i.e., greater degree of marbling as the food industry calls it) should have a lower density relative to leaner muscles because fat has a significantly lower density of 0.936g/ml (Entenman et al., 1958). Additionally, an inverse trend has been demonstrated within muscles between fat content and water content meaning that tissue with higher water content has lower fat content (Ramsbottom and Strandine, 1948; Swift and Berman, 1959; Lawrie et al., 1963). This variability in muscle composition will presumably be reflected in muscle density. Fat content within skeletal muscle has been demonstrated to be correlated with its microscopic organization. For instance, Kauffman and Safanie noted that organized, but widely dispersed fasciculi correlated with high lipid content (Kauffman and Safanie, 1967). The present study plans to address this by sampling a certain area of a cross-section of each muscle to

determine the percent of this area comprised of muscle fibers. A higher percentage of muscle fibers in theory should be correlated to less fat content and ultimately greater density.

In addition to variable fat content, it has been reported that collagen is the most substantial constituent element of connective tissue within skeletal muscle and can comprise between 3 and 30% of a muscle's total protein (Walls, 1960). Therefore, muscles with a lot of connective tissue will likely be denser. A good example of this would be the masseter because it is a complex muscle consisting of several fascial layers with connective tissue throughout.

### *Predictions*

In effort to address these assumptions and based on previous literature, I predict the following:

1. Based on previous studies (Bulcke et al., 1979; Imamura et al., 1983), I predict that muscle density will vary based on age within my sample—increasing until prime adulthood.
2. Furthermore, I anticipate that this increase in density will be histologically correlated with an increase in the size of the muscle fibers. I expect this to influence density because larger fibers will inherently contain more proteins which are denser than water resulting in a slightly higher density.

### **Materials and Methods:**

To test these hypotheses a sample (n = 66) of New Zealand white *Oryctolagus cuniculus* rabbit cadavers was obtained from a commercial meat farm, Brittany Ridge Farms. All animals were euthanized according to USDA standards prior to obtaining them and were therefore deemed exempt by NC State IACUC. The specimens were subdivided into three age-cohorts

that consisted of individuals approximately 3 weeks (n=18), 8 months (n=30) and 2 years of age (n=18; Table 3.1). These age-cohorts were selected based on the life history of this breed of rabbit and availability. (As commercial breeders of rabbits for the food industry do not keep animals beyond prime breeding age, the effects of senescence in this species, which lives on average to be up to 7 years old, were not studied; Reddan et al., 1982; see limitations below.) Three-week old rabbits were chosen to represent the juvenile cohort as they do not wean until approximately 30 days of age (Hudson et al., 1996; von Holst et al., 2002). Sexual maturity is achieved by 6 months of age (Macari and Machado, 1978), therefore the 8-month old rabbits represent sexually mature individuals. By the age of 2 years (the oldest age-cohort that was obtained from the commercial farm) rabbits have reached full adult size.

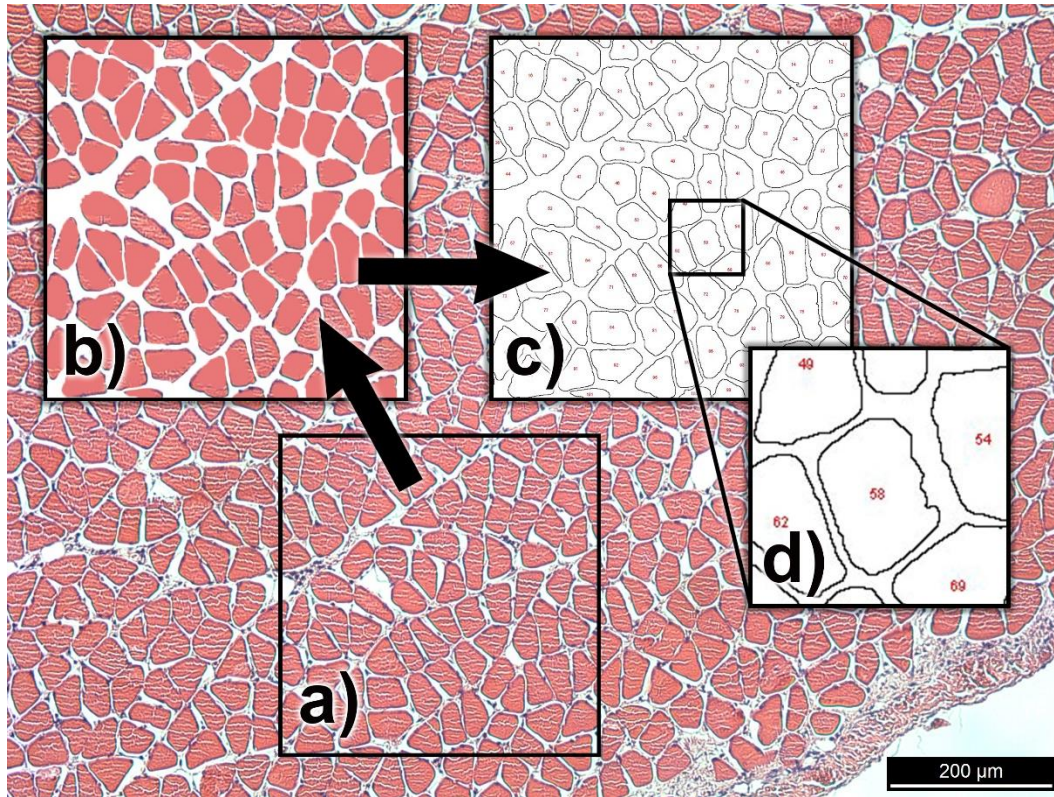
**Table 3.1.** *Oryctolagus cuniculus* sample demographics

Cohort	Age	Males	Females	Total
C1	3-weeks old	7	11	18
C2	8-months old	11	19	30
C3	2-years old	10	8	18

In order to evaluate the effects of anatomical region on muscle density, muscles from four different regions were selected, including the head, forelimb, hindlimb and the trunk. Individual muscles were chosen as representative of these regions and also relatively diverse in subjective myological properties (e.g., muscles with a lot of connective tissue, ðleanö muscles, and states in between), and included the masseter, digastric, quad labii, extensor carpi radialis longus (ECRL), pronator teres (PT), gastrocnemius, soleus, plantaris and psoas minor. After excision, muscles were weighed to the nearest 0.0001g using a Mettler Toledo New Classic (MS-

105) analytical balance and the density of each muscle at 20.0°C was determined using a Mettler Toledo density kit (MS-DNY-54), which automatically calculates density using Archimedes principle. The kit requires first weighing the sample in air and then in an auxiliary liquid, for which deionized water was used.

In an effort to better account for differences in muscle composition, a subsample of muscles was histologically evaluated (Figure 3.1). To do this, all sampled muscles from 6 rabbits from each cohort were cross-sectioned perpendicular to their fibers. For muscles that have multiple components in which the fascicular orientation varies throughout (e.g., masseter), the section was taken perpendicular to the most superficial fascicles. These samples were then embedded in paraffin, sectioned at 10-12  $\mu\text{m}$  and then stained using hematoxylin and eosin. The slides were photographed using a Leica (CTR5500) microscope and three 400  $\mu\text{m}$  by 400  $\mu\text{m}$  sections were sampled (chosen to minimize major vessels, nerves and tendons) using Photoshop (CC2019). These sections were then processed further using ImageJ (IJ1.46r) (Figure 3.1). After the scale was set appropriately, the images were binarized, and then any holes within the individual fibers were filled using the "fill holes" tool. The "Analyze particles" function was then used to collect information about the area of each muscle fiber and the percent of each sampled section that consisted of fiber area. To determine the average fiber area, any partial fibers that may have been captured within the sampled section were excluded (e.g., on the perimeter of the frame). The percent fiber area was calculated by summing all of the fiber areas for each of the three sampled sections and dividing by the total sampled area.



**Figure 3.1.** Stages of the histological image processing: a) raw image obtained of an 8-month old rabbit soleus (Specimen ID: A54\_Soleus) muscle, b) 400 x 400 μm section preprocessed in Photoshop to simplify for measurement c) outline schematic produced by using the 'analyze particles' function in ImageJ d) partial fibers are excluded by number.

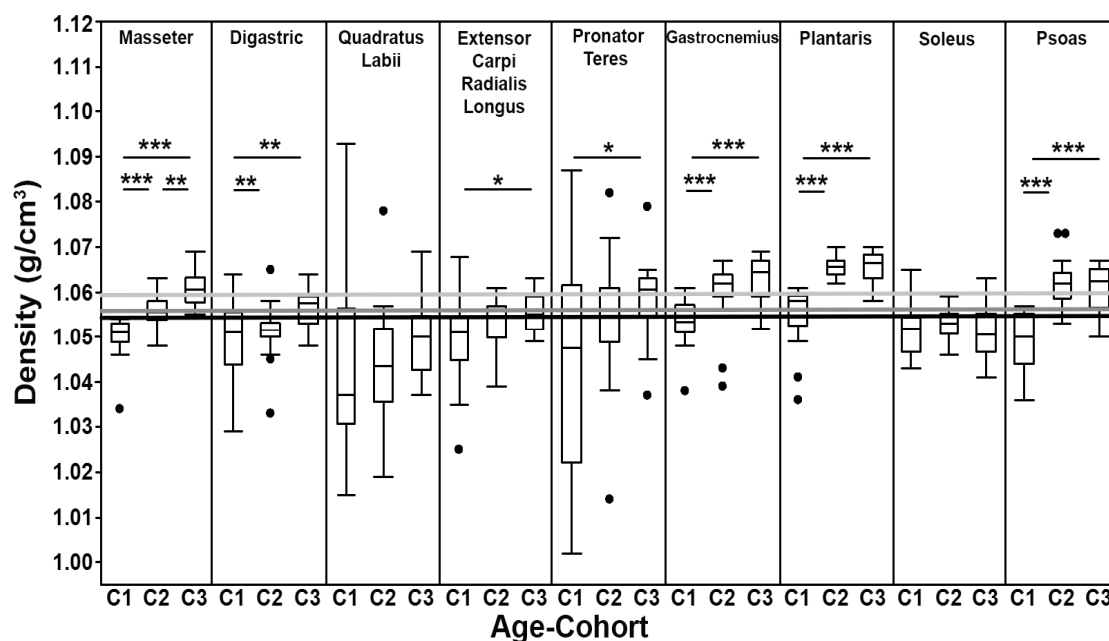
All statistical analyses were conducted using JMP Pro14 (SAS). Tukey-Kramer HSD tests (essentially an all-pairs t-tests;  $\alpha=0.05$ ) were conducted to compare the mean densities between age-cohorts and anatomical regions. Tukey Kramer-HSD tests were conducted to identify not only the presence of significant differences in mean density but to determine where the significances lie. This test was used to compare density across the anatomical regions of each age-cohort as well as across the age-cohorts for each muscle. Further Tukey-Kramer HSD tests were conducted to evaluate to determine if average fiber area and percent of the sampled area

that was comprised of muscle fibers differed significantly between age-cohorts. To evaluate the scaling relationship between muscle density and average fiber area and percent fiber area reduced major axis (RMA) linear regressions were conducted. This type of analysis accounts for error in both the x and y axes (Sokal and Rohlf 1995) and is commonly employed when evaluating scaling relationships between variables.

### **Results:**

The distributions of muscle density for the three age-cohorts and each muscle indicates variation based on both age and anatomical region (Figure 3.2). The quadratus labii was consistently the least dense while the plantaris was the densest (Table 3.2). All muscles except the soleus became denser with increasing age (Table 3.2; Figure 3.2).

The average density of the masseter increased with increasing age and the masseter is the only muscle that differed significantly between each age-cohort (Table 3.2). A similar trend was observed when all muscles for each age-cohort were analyzed collectively (C1 n = 162; C2 n= 270; C3 n= 162) (Table 3.2). Contrastingly the quadratus labii and the soleus were the only two muscles that did not show any significant differences in muscle density between the three age-cohorts (Table 3.2). The digastric, gastrocnemius, plantaris and psoas followed similar trends to one another. The youngest individuals had muscle densities that were significantly different than the 8-month olds and 2-year olds, but the average densities between the 8-month olds and 2-year olds were not significantly different from one another (Table 3.2). The extensor carpi radialis longus (ECRL) and pronator teres both exhibited the same pattern. The average muscle density within the 3-week age-group was not significantly different than the 8-month olds but was significantly different than the 2-year olds. The 8-month olds and 2-year olds were also not significantly than one another (Table 3.2).



**Figure 3.2.** The distributions for the density of each muscle per each of the three cohorts: C1=3 weeks; C2= 8 months; C3=2 years old. The lines above the box plots indicate significant differences between connected columns (\*=  $p < 0.05$ , \*\*=  $p < 0.01$ , \*\*\*=  $p < 0.0001$ ) for each muscle. The light gray and medium gray lines depict the constants taken from Mendez and Keys (1.0597 g/ml; 1960) and Murphy and Beardsley (1.0564 g/ml; 1974) respectively. The black line represents the grand mean of all the muscles measured in this study (1.0546 g/ml;  $n=594$  muscles). One data outlier (C1 of the quadratus labii; density of 1.16 g/cm<sup>3</sup>) omitted for graphical clarity.

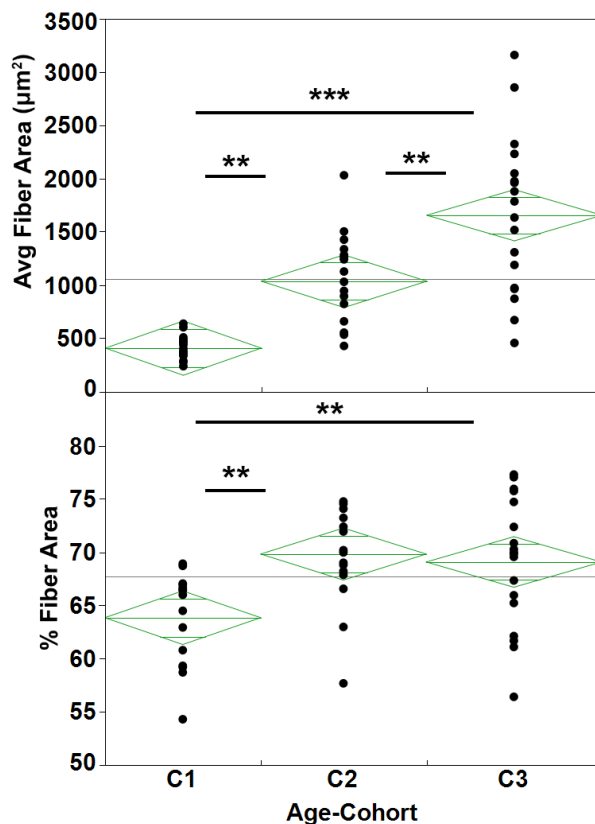
**Table 3.2.** The mean muscle density, mean standard error and results within each age cohort (C1=3 weeks old; C2= 8 months old; C3= 2 years old).

<b>Muscle</b>	<b>Age-Cohort</b>	<b>Mean Muscle Density (g/cm<sup>3</sup>)</b>	<b>Mean Standard Error</b>
Masseter	C1	1.0501	0.0011
	C2	1.0558	0.0007
	C3	1.0606	0.0009
Digastric	C1	1.0497	0.0020
	C2	1.0513	0.0009
	C3	1.0565	0.0010
Quadratus Labii	C1	1.0482	0.0079
	C2	1.0432	0.0022
	C3	1.0499	0.0019
Extensor Carpi Radialis Longus	C1	1.0494	0.0025
	C2	1.0529	0.0011
	C3	1.0556	0.0009
Pronator Teres	C1	1.0453	0.0060
	C2	1.0542	0.0022
	C3	1.0589	0.0021
Gastrocnemius	C1	1.0534	0.0013
	C2	1.0606	0.0011
	C3	1.0630	0.0011
Plantaris	C1	1.0551	0.0016
	C2	1.0653	0.0003
	C3	1.0656	0.0008
Soleus	C1	1.0514	0.0014
	C2	1.0529	0.0007
	C3	1.0508	0.0013
Psoas	C1	1.0493	0.0015
	C2	1.0616	0.0009
	C3	1.0611	0.0013
All Regions	C1	1.0502	0.0012
	C2	1.0553	0.0006
	C3	1.0580	0.0006



When comparing the distributions of the histologically measured average fiber areas between each age cohort, (Figure 3.3; Table 3.4), average fiber area was significantly higher in the 8-month old cohort than the 3-week old cohort and also significantly higher in the 2-year old cohort than the 8-month old cohort. The percent of the sampled histological area that was occupied by muscle fibers was also compared (Figure 3.3; Table 3.5) and it was observed significantly lower percent area was occupied by muscle fibers in the 3-week cohort than either of the older cohorts. However, the average percent fiber area of the sample area was not significantly different between the 8-month and 2-year old cohort.

To evaluate the relationship between muscle density and average fiber area and average percent fiber area reduced major axis linear regressions (Figure 3.5) were conducted. Approximately 49% of the variation observed in muscle density is explained by the average fiber area and average percent fiber area with individuals with denser muscles having larger fiber areas (i.e., bigger cross-sectional areas of the fibers) and the sample regions consisting of a greater proportion of muscle fibers within the sampled regions (i.e., higher percent of the sampled region was made up of muscle fibers; Figure 3.5).



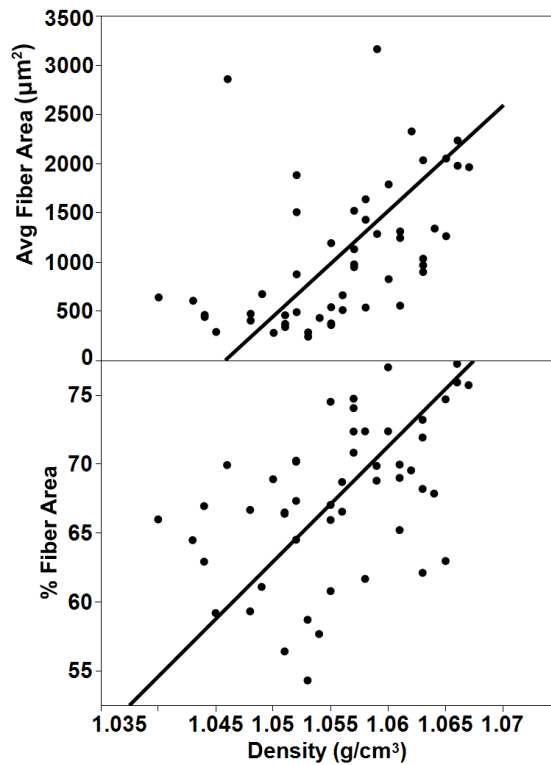
**Figure 3.3.** Distributions and mean diamonds for the histologically measured average fiber area and percent fiber area for each of the muscles ( $n=18$  muscles from 2 individuals) measured in each of the three cohorts (C1=3 weeks old; C2= 8 months old; C3= 2 years old). The lines above indicate significant differences between connected columns (\*=  $p<0.01$ , \*\*=  $p<0.001$ , \*\*\*=  $p<0.0001$ ).

**Table 3.4.** Average fiber area, standard deviation and standard error for each muscle of a subset of each age cohort (C1=3 weeks old; C2= 8 months old; C3= 2 years old) and the connecting letter report from a Tukey-Kramer HSD test comparing the three groups.

Age-Cohort	Average Fiber Area	Std. Dev.	Std. Error Mean
C1	393.8651	129.4763	31.4026
C2	1037.6525	427.6838	103.7286
C3	1657.0180	736.3184	173.5519

**Table 3.5.** Average percent fiber area, standard deviation and standard error for each muscle for a subset of each age cohort (C1=3 weeks old; C2= 8 months old; C3= 2 years old) and the connecting letter report from a Tukey-Kramer HSD test comparing the three groups.

Age-Cohort	Average % Fiber Area	Std. Dev.	Std. Error Mean
C1	62.4018	7.2156	1.7500
C2	69.8209	4.4263	1.0735
C3	69.0772	6.0642	1.4293



**Figure 3.4.** Reduced major axis regressions of average fiber area (top;  $r^2 = 0.4901$ ) and % fiber area against (bottom;  $r^2 = 0.4862$ ) density.

**Discussion:**

Accurate representation of muscle density is crucial when calculating PCSA, an important biomechanical variable that is a direct correlate of muscle force production potential. However, the current practice in the field of substituting a value for muscle density as universally applicable from previous studies that included very conscribed samples (Gersh et al., 1944; Méndez and Keys, 1960; Murphy and Beardsley, 1974), obscures the variability in this value that relates to ontogeny and anatomical region. To address this, this study comprehensively evaluated variation in muscle density in different age groups and parts of the body and sought to explain these differences histologically.

As predicted based on the findings of Imamura and colleagues (1983) consisting of increasing muscle density within the sacrospinalis and the psoas major muscles until middle-age in humans, a general trend was observed that for each muscle evaluated, the average density was successively higher within the latter age-cohorts (Table 3.2). However, these differences were not always significant between the age-cohorts for each muscle suggesting differential trends in development based on the functional demands throughout the animal's life history. Histologically, some of the differences were explained based on the observation that with increasing age, the average fiber area also increased. Additionally, the percent of the sampled area that consisted of muscle fibers increased significantly between the 3-week old cohort and the 8-month cohort and the 3-week old and 2-year old cohorts but did not differ significantly between the 8-month old and 2-year old cohort.

Overall, these findings demonstrate significant differences in muscle density occurring both across ontogeny and anatomical region. Although no statistically significant regional differences were observed in muscle density within 3-week old rabbits, significant trends were

observed within the two older cohorts. The lack of significant differences observed in youngest cohort could have two contrasting explanations. The first is that, the musculature has not developed enough to reflect the diversity of their inherent anatomical properties. The second plausible explanation is that muscles of the juveniles even within the same region could vary too widely ó perhaps because of important differences in maturation at this age ó such that the Tukey-Kramer test would not be able to detect more subtle differences.

While the findings of this study demonstrate statistically significant differences in muscle density based on ontogeny and anatomical region, the practical significance of this may be relatively small: Inter-muscular differences appear rather subtleó meaning that if it is *not feasible* to directly measure muscle density, the use of a õconstantö is reasonable. In other words, although there are significant differences, the magnitude of those differences do not warrant extreme consideration if density cannot be measured on a muscle by muscle basis. However, the use of the previously determined constants that were derived from a small subset of muscles from adult individuals may not be the best practice. This study presents a variety of values for muscle density (Table 3.6) that could be applied to more specific analyses based on age and structural characteristics of the muscle. For example, if a researcher was studying the temporalis muscleó a complex muscle consisting of several constituent layers and substantial fascial sheets, substituting a value for density that was taken from the masseter would at least provide some structural similarity. Another example, for a specimen of an unknown age, a density value of  $1.0546 \text{ g/cm}^3$  (Table 3.1) may be more appropriate as it was derived from a large sample ( $n=594$  muscles) of muscles from individuals of various ages, spanning greater anatomical breadth than the previously used constants. Researchers could also select other tailored constants if the muscle of a similarly aged-cohort has been incorporated here. For instance, densities of  $1.0558 \text{ g/cm}^3$

and 1.0502 g/cm<sup>3</sup> would be appropriate for general adult and juvenile muscles respectively; and 1.05494 g/cm<sup>3</sup> and 1.0474 g/cm<sup>3</sup> for adult and juvenile for *forelimb* mm; 1.0597 g/cm<sup>3</sup> and 1.0533 g/cm<sup>3</sup> for adult and juvenile *leg* mm.; and 1.0518 g/cm<sup>3</sup> and 1.0493 g/cm<sup>3</sup> for adult and for juvenile *head* mm.

**Table 3.6.** Average muscle density by structural characteristics and age.

Muscle	Structural Description	Age-cohort	Average Muscle Density (g/cm <sup>3</sup> )
Masseter	Robust and complex containing multiple fascial layers	C1	1.0501
		C2	1.0558
		C3	1.0606
Digastric	Cylindrical with a distinct distal tendon	C1	1.0497
		C2	1.0513
		C3	1.0565
Quadratus Labii	Thin and highly associated with the skin and connective tissue	C1	1.0482
		C2	1.0432
		C3	1.0499
Extensor Carpi Radialis Longus	Strap-like muscle with a distinct distal tendon	C1	1.0494
		C2	1.0529
		C3	1.0556
Pronator Teres	Strap-like muscle with a highly integrated and inseparable tendon	C1	1.0453
		C2	1.0542
		C3	1.0589
Gastrocnemius	Consists of a lateral and medial head that converge at a central tendinous sheet	C1	1.0534
		C2	1.0606
		C3	1.0630
Plantaris	Fusiform shaped with a substantial distal tendon	C1	1.0551
		C2	1.0653
		C3	1.0656
Soleus	Cylindrical with a distinct distal tendon and	C1	1.0514
		C2	1.0529
		C3	1.0508
Psoas minor	Thin and fragile--lacking substantial sheets of connective tissue	C1	1.0493
		C2	1.0616
		C3	1.0611

### *Limitations and Future Directions*

This study produced significant findings; however, it is not without limitations. For example, because histological samples are, by their nature generally relatively small, I was not able to evaluate what are likely more subtle significant differences in average fiber area and percent fiber area between the older two cohorts.

While this study found significant differences histologically in the average fiber area and percent fiber area between age-cohorts I was still not able to explain other important elements of the tissue in a comprehensively quantitative manner. For instance I was able to make some inferences about how an increase in fiber area may be driving an increase in muscle density with age (i.e., larger muscle fiber areas thereby an increase in the amount of protein present), but was not able to quantify the proportion of other types of tissues present that may also be contributing to these differences (i.e., fat and collagen etc.). Future studies could incorporate a trichrome stain into the methods allowing for the differentiation of these tissues which could then be quantified using the methods developed within this study.

Another histological element that is not incorporated here that may prove useful to incorporate into future studies is immunohistochemical fiber typing. For example, fiber type composition has been demonstrated to differ between the gastrocnemius and soleus muscles with the soleus muscle containing a greater proportion of Type I or slow twitch fibers (Gollnick et al., 1974). Fiber-type analyses may help to explain why within the latter two age-cohorts the plantaris and gastrocnemius were consistently denser than the soleus muscle. This might also help to elucidate the similarities in density like, for example, was observed between the ECRL and the PT despite functional and structural differences.

Another limitation to this study is that the sample did not include an age-cohort that was a true representation of senescence. I procured a highly controlled sample from a commercial meat farm where they do not have animals that reach senescence because they would no longer be reproductively efficient. In a future extension of this work, it would be valuable to find and incorporate some data from senescent individuals perhaps from show rabbit breeders or to replicate this density research on a taxon for which a broader age range is available (e.g., the mouse lemurs used in Boettcher et al., 2019; Leonard et al., 2019, though a larger taxon would probably yield more accurately measured densities). Additionally, further studies are needed to determine the species-specificity of these values of muscle density presented here.

### *Conclusions*

This study is the first study to explicitly evaluate the density of muscles across broad anatomical regions and ages while the highly cited standard values for muscle density were collected on limited samples as part of studies that were not explicitly trying to establish a constant that has become so broadly used. Ultimately this study has demonstrated the variability in muscle density associated with ontogeny and anatomy. I have also provided a variety of values for specific muscles if researchers happen to be studying the same or similar muscles, and more general values that can be used more generically (namely a value of  $1.0558 \text{ g/cm}^3$  for adult muscles,  $1.0502 \text{ g/cm}^3$  for juveniles,  $1.0597 \text{ g/cm}^3$  for adult legs and  $1.0518 \text{ g/cm}^3$  for adult head muscles) if muscle density cannot be directly measured. However, more work needs to be done to elucidate how factors such as specimen handling and fluid preservation (the subjects of other chapters herein) and future studies on the effects of how senescence and muscle fiber type may also influence muscle density.

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## References:

- Boettcher ML, Leonard KC, Dickinson E, Aujard F, Herrel A, Hartstone Rose A. 2019. The forearm musculature of the gray mouse lemur (*Microcebus murinus*): An ontogenetic study. *Anat Rec*.
- Bulcke J, Termote J-L, Palmers Y, Crolla D. 1979. Computed tomography of the human skeletal muscular system. *Neuroradiology* 17:127-136.
- Crook TC, Cruickshank SE, McGowan CM, Stubbs N, Wakeling JM, Wilson AM, Payne RC. 2008. Comparative anatomy and muscle architecture of selected hind limb muscles in the Quarter Horse and Arab. *J Anat* 212:144-152.
- Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel J-P, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M. 2010. Sarcopenia: European consensus on definition and diagnosis. *Age Ageing* 39:412-423.
- Curtis AA, Santana SE. 2018. Jaw-dropping: functional variation in the digastric muscle in bats. *Anat Rec* 301:279-290.
- Dickinson E, Fitton LC, Kupeczik K. 2018. Ontogenetic changes to muscle architectural properties within the jaw-adductor musculature of *Macaca fascicularis*. *Am J Phys Anthropol* 167:291-310.
- Eng CM, Ward SR, Vinyard CJ, Taylor AB. 2009. The morphology of the masticatory apparatus facilitates muscle force production at wide jaw gapes in tree-gouging common marmosets (*Callithrix jacchus*). *J Exp Biol* 212:4040.
- Entenman C, Goldwater WH, Ayres NS, Behnke Jr AR. 1958. Analysis of adipose tissue in relation to body weight loss in man. *J Appl Physiol* 13:129-134.
- Fabre PH, Herrel A, Fitriana Y, Meslin L, Hautier L. 2017. Masticatory muscle architecture in a water-rat from Australasia (Murinae, *Hydromys*) and its implication for the evolution of carnivory in rodents. *J Anat* 231:380-397.
- Faucitano L, Rivest J, Daigle J, Lévesque J, Gariépy C. 2004. Distribution of intramuscular fat content and marbling within the longissimus muscle of pigs. *Can J Anim Sci* 84:57-61.
- Gersh I, Hawkinson GE, Rathbun EN. 1944. Tissue and vascular bubbles after decompression from high pressure atmospheres ó correlation of specific gravity with morphological changes. *J Cell Physiol* 24:35-70.
- Gollnick PD, Sjödin B, Karlsson J, Jansson E, Saltin B. 1974. Human soleus muscle: A comparison of fiber composition and enzyme activities with other leg muscles. *Pflügers Archiv* 348:247-255.

- Hartstone-Rose A, Deutsch AR, Leischner CL, Pastor F. 2018. Dietary correlates of primate masticatory muscle fiber architecture. *Anat Rec* 301:311-324.
- Hartstone-Rose A, Perry JMG, Morrow CJ. 2012. Bite force estimation and the fiber architecture of felid masticatory muscles. *Anat Rec* 295:1336-1351.
- Hudson R, Bilko A, Altbäcker V. 1996. Nursing, weaning and the development of independent feeding in the rabbit (*Oryctolagus cuniculus*). *Zeitschrift für Säugetierkunde* 61:39-48.
- Imamura K, Ashida H, Ishikawa T, Fujii M. 1983. Human major psoas muscle and sacrospinalis muscle in relation to age: A study by computed tomography. *J Gerontol* 38:678-681.
- Kauffman R, Safanie A. 1967. Influence of porcine muscle structure on its lipid accumulation during growth. *Journal of Food Science* 32:283-286.
- Kikuchi Y. 2010. Comparative analysis of muscle architecture in primate arm and forearm. *Anat Histol Embryol* 39:93-106.
- Lawrie R, Pomeroy R, Cuthbertson A. 1963. Studies on the muscles of meat animals III. Comparative composition of various muscles in pigs of three weight groups. *J Agric Sci* 60:195-209.
- Leischner CL, Crouch M, Allen KL, Marchi D, Pastor F, Hartstone Rose A. 2018. Scaling of primate forearm muscle architecture as it relates to locomotion and posture. *Anat Rec* 301:484-495.
- Leonard KC, Boettcher ML, Dickinson E, Malhotra N, Aujard F, Herrel A, Hartstone Rose A. 2019. The ontogeny of masticatory muscle architecture in *Microcebus murinus*. *Anat Rec*.
- Listrat A, Leuret B, Louveau I, Astruc T, Bonnet M, Lefaucheur L, Picard B, Bugeon J. 2016. How muscle structure and composition influence meat and flesh quality. *The Scientific World Journal* 2016.
- Macari M, Machado CR. 1978. Sexual maturity in rabbits defined by the physical and chemical characteristics of the semen. *Lab Anim-UK* 12:37-39.
- Marchi D, Leischner CL, Pastor F, Hartstone Rose A. 2018. Leg muscle architecture in primates and its correlation with locomotion patterns. *Anat Rec* 301:515-527.
- Méndez J, Keys A. 1960. Density and composition of mammalian muscle. *Metab: Clin Exp* 9:184-188.
- Murphy RA, Beardsley AC. 1974. Mechanical properties of the cat soleus muscle in situ. *The American journal of physiology* 227:1008-1013.

- Newton J, Yemm R, Abel R, Menhinick S. 1993. Changes in human jaw muscles with age and dental state. *Gerodontology* 10:16-22.
- Oishi M, Ogihara N, Endo H, Asari M. 2008. Muscle architecture of the upper limb in the orangutan. *Primates; Journal of Primatology* 49:204-209.
- Pearson AM. 1990. Muscle growth and exercise. *Critical reviews in food science and nutrition* 29:167-196.
- Perry JMG, Hartstone-Rose A. 2010. Maximum ingested food size in captive strepsirrhine primates: Scaling and the effects of diet. *Am J Phys Anthropol* 142:625-635.
- Perry JMG, Hartstone-Rose A, Wall CE. 2011. The jaw adductors of strepsirrhines in relation to body size, diet, and ingested food size. *Anat Rec* 294:712-728.
- Perry JMG, Macneill KE, Heckler AL, Rakotoarisoa G, Hartstone Rose A. 2014. Anatomy and adaptations of the chewing muscles in *Daubentonia* (Lemuriformes). *Anat Rec* 297:308-316.
- Perry JMG, Wall CE. 2008. Scaling of the chewing muscles in prosimians. In: *Primate craniofacial function and biology*. Boston, MA: Springer. p 217-240.
- Prufrock KA, Perry JMG. 2018. Strepsirrhine diets and the pattern of masticatory muscle development. *FASEB J* 32:780-785.
- Ramsbottom J, Strandine E. 1948. Comparative tenderness and identification of muscles in wholesale beef cuts. *J Food Sci* 13:315-330.
- Reddan JR, Dziedzic DC, Mostafapour MK, McGee SJ, Schwartz CA. 1982. Establishment and characterization of a lens epithelial cell line from an eight year old rabbit. *Curr Eye Res* 2:633-640.
- Rosenberg IH. 1997. Sarcopenia: origins and clinical relevance. *J Nutr* 127:990S-991S.
- Schumacher G-H. 1961. *Funktionelle morphologie der kaumusculatur*. Jena: G. Fischer.
- Sokal, R., & Rohlf, F. (1995). *Biometry*. New York: WH Freeman.
- Swift C, Berman M. 1959. Factors affecting the water retention of beef. 1. Variations in composition and properties among 8 muscles. *Food Technol* 13:365-370.
- Taylor AB, Vinyard CJ. 2009. Jaw-muscle fiber architecture in tufted capuchins favors generating relatively large muscle forces without compromising jaw gape. *J Hum Evol* 57:710-720.

von Holst D, Hutzelmeyer H, Kaetzke P, Khaschei M, Rödel HG, Schrutka H. 2002. Social rank, fecundity and lifetime reproductive success in wild European rabbits (*Oryctolagus cuniculus*). Behav Ecol Sociobiol 51:245-254.

Walls E. 1960. The microanatomy of muscle. New York: Academic Press.

## Chapter 4: Effects of Freezing and Short-term Fluid Preservation on the Properties of Skeletal Muscle

### Abstract:

Preventing postmortem deterioration is an important component of anatomical research in order to preserve the integrity of soft-tissue structural characteristics. Two common methods used in the field include freezing and fluid preservation, both of which have proven to have adverse effects on soft tissues including shrinking and reductions in mass. This study evaluates the acute effects of freezing, formalin fixation and ethanol storage on mammalian muscles using a sample (n =46) of New Zealand White rabbits (*Oryctolagus cuniculus*). Bilateral dissections were performed on each specimen, with contralateral muscles divided between different preparations (fresh, formalin only, formalin followed by varying lengths of ethanol storage, frozen once and frozen twice). Muscles from different anatomical regions (head/face: masseter, digastric, quadratus labii; forelimb: pronator teres and extensor carpi radialis longus; leg: gastrocnemius, plantaris and soleus; trunk: psoas) with varying structural characteristics were individually excised and measured for mass, volume and density. The findings indicate that freezing/thawing the specimens at -20°C once or twice had consistently benign effects on the gross properties of the musculature being examined; specimens declined on average 3% and 9% for both mass and volume when frozen/thawed once or twice respectively with no change in density, and the mass and volume changes were not statistically significant. Formalin fixation also had significant effects on muscle mass, volume and density, with both mass and volume being approximately 24% reduced, with a 1% reduction in density, compared to fresh muscle tissue. Formalin fixation followed by various durations of ethanol storage were also found to have significant effects on the mass, volume and density of the muscles. On average, muscles lost ~3% density within the first several months of ethanol storage, resulting from a 41%, 43%,

and 39% loss of mass and a 39%, 41% and 36% loss of volume over the first 1, 3 and 6 months, respectively. Although these data cannot be extrapolated to infer changes in muscles that have been preserved for years (the subject of another chapter in this dissertation), two overarching observations can be determined. Firstly, muscles preserved through freezing and formalin fixation differ little from fresh muscles. Additionally, muscles lose ~40% of their mass and volume within the first month of ethanol storage and continue to decrease in these metrics slightly over subsequent months. Based these findings, the following correction factors (multipliers) were calculated for each preparation: *Mass*: ethanol 1.69; 10% formalin 1.32; frozen once 1.03; frozen twice 1.09; *Volume*: ethanol 1.64; 10% formalin 1.32 ; frozen once 1.03 ; frozen twice 1.10 ; *Density*: ethanol 1.03; 10% formalin 1.00; frozen once 1.00; frozen twice 1.00.

Keywords: preparation, correction, ethanol, formalin, mass, volume, density

### **Introduction:**

Fixation and preservation practices are essential in preventing the postmortem deterioration of soft tissue and maintaining representative morphological characteristics of anatomical soft tissues (Eltoum et al., 2001; Simmons, 2014). Unmitigated deterioration is due to a combination of microbial activity, cell lysis and osmotic damage (Jones, 1976). Preserving the soft tissues and preventing such degradation of specimens is particularly important in anatomical research because characteristics of these tissues are used to inform functionality. Two common methods of preserving anatomical specimens include freezing and formalin fixation followed by ethanol storage. This study aims to evaluate the acute effects these methods of preservation have on muscle mass, volume and density, and to provide corrections that can be used for scientists

wishing to use a sample containing specimens for which different preservation techniques were utilized.

### *Freezing*

Anatomical specimens are commonly frozen as a method of preservation. Using freezing as a method of preservation can influence the data collection on biological tissues by introducing instrument error or generating alterations in the tissue in general. For instance, Hale and Ross (2017) evaluated the effects of freezing on bone mineral density (BMD) and found that freezing overtime led to significantly different BMD measurements than what was obtained for fresh tissue. However, they also observed that when the tissue was thawed and then scanned the BMD measurements did not differ significantly from that of fresh tissue (Hale and Ross, 2017). The effects of freezing on muscle tissue specifically have been relatively well investigated within the meat industry as it pertains to meat quality (Callow, 1952; Añón and Calvelo, 1980; Carroll et al., 1981; Grujić et al., 1993; Sen and Sharma, 2004; Xia et al., 2009; Leygonie et al., 2012; Qi et al., 2012; Kim et al., 2018). One particular effect that has been extensively evaluated is how freezing impacts the microscopic or ultrastructure of muscle tissue (Cook et al., 1926; Luyet, 1964; Carroll et al., 1981; Rahelić et al., 1985; Grujić et al., 1993; Ngapo et al., 1999), with the formation of ice crystals disrupting the microscopic structure and arrangement of the muscle fibers (Birdseye, 1929; Mills, 1975; Scott and Aquino-Shuster, 1989). Indeed, as early as 1929, Birdseye evaluated the effects of freezing on the microscopic structure of haddock and noted that the muscle fibers appeared swollen and closely packed but unevenly distributed throughout the tissue. The author suggested that the spaces between the clusters of closely packed muscle fibers were the result of the disintegration of the connective tissue between them (Birdseye, 1929). On a macroscopic level, Birdseye (1929) also observed that when cutting the subsequently thawed

fish, it seemed to fall apart and lacked structural integrity. Similarly, Scott and Aquino-Shuster (1989) noted that in frozen garter snakes and leopard frogs this disruption took the form of irregularly shaped spaces around and within the muscle fibers, as well as distortion of the membranes.

Not only do the ice crystals formed during high-temperature (e.g.,  $-20^{\circ}\text{C}$  as opposed to  $-80^{\circ}\text{C}$ ) freezing disrupt the structure of the muscle, they also result in a loss of fluid as an exudate. This fluid is known in the meat sciences as "drip" and consists of water and sarcoplasmic proteins that leach from inside the cells to the extracellular space (Ramsbottom and Koonz, 1939; Añón and Calvelo, 1980; Gonzalez Sanguinetti et al., 1985; Strange, 1987). Ramsbottom and Koonz (1939) evaluated how freezing temperatures of beef ribs and steaks influence the amount of exudate produced upon thawing and found that smaller steaks frozen at lower temperatures lose a greater amount of fluid. While this loss of fluid is of particular interest in the meat industry as it adversely affects the quality and monetary value of meat (Ngapo et al., 1999), this principle is also important for consideration with anatomical specimens because a substantial loss of intramuscular fluid will result in a loss of mass and volume and thus distorting the measure of myological structures.

Anatomical specimens are often frozen as an initial means of preservation, but also frequently thawed and refrozen for various reasons such as freezer failure, transport between institutions and examination of specific structures then refreezing the remainder of the specimen. Although the existing literature contains conflicting findings (Carroll et al., 1981; Sen and Sharma, 2004), some studies have shown that repetitive freezing and thawing is detrimental to tissues (Benjakul and Bauer, 2000; Qi et al., 2012). The current study seeks to more thoroughly elucidate the gross effects of freeze-thaw cycles on muscles.

### *Fluid Preservation*

Fluid preservation involves submerging specimens in, or injecting specimens with liquid chemicals to prevent deterioration (Simmons, 2014). The chemicals used in the process vary but include alcohols, aldehydes or glycol (Simmons, 2014). The use of such chemicals has been demonstrated to have variable effects on specimens which include shrinking, dehydration, swelling, and rehydration, as well as alterations in mass, coloration, texture, and chemical composition (Simmons, 2014).

The fluid-preserved collections of museums are rich sources of anatomical specimens and the protocol for fluid preservation at these museums commonly consists of submerging and/or injecting specimens with formalin (10% formaldehyde) followed by a rinse and then long-term storage in 70% ethanol (Andrei and Genoways, 1999). Generally, specimens fixed in formalin will swell and increase in mass but when transferred to alcohols (e.g., ethanol), will shrink and exhibit a decrease in mass (Simmons, 2014). Studies investigating the effects of these practices have focused more on the gross morphometrics of specimens rather than the effects on muscle tissue specifically. This has been particularly well studied in anurans (Lee, 1982; Deichmann et al., 2009), fish (Hoar, 1939; Burgner, 1962; Engel, 1974; Billy, 1982; Al-Hassan et al., 2000; Jawad, 2003) and reptiles (Klauber, 1943; Reed, 2001). In one study by Lee (1982) the snout-vent length of Cane toads (*Bufo marinus*) was observed to reduce by 6.19% after 14 months in ethanol. Similarly, Klauber (1943) reported a range of 2.09 and 3.15% of a reduction in the body length of snakes.

While the focus of studies investigating the effects of fluid fixation and preservation has been predominantly on the gross morphometrics on whole specimens, a pair of studies have placed more of an emphasis on muscle tissue (Cutts, 1988; Ward and Lieber, 2005). Cutts (1988)

investigated the effects of fluid preservation on human musculature and evaluated the differential effect of fixation when tissue was fixed intact on the skeleton as well as isolated. When fixed intact, there was no significant loss of length observed in the muscle; however, when removed from the skeleton prior to fixation, there was a small but significant amount of shrinkage (Cutts, 1988). More recently, Ward and Lieber (2005) evaluated how the concentration of formalin influences preserved muscle density. They observed that muscle tissue preserved by immersion in a 4% formaldehyde solution was less dense than tissue preserved using perfusion of a 37% solution—a finding the authors acknowledge could be due to the differences in application (i.e., immersion versus perfusion). In addition to the inconsistency in the application of the fixative, they utilized tissue samples from different regions of the body (upper limb vs. lower limb) which could have also influenced the differences observed (Ward and Lieber, 2005).

The present study seeks to use controlled methods to elucidate the acute effects on muscles of the commonly used preservational methods. Specifically, I aim to quantify the effects of freeze-thaw cycling and formalin fixation accompanied by ethanol storage on muscle mass, muscle volume and muscle density, and to ultimately provide correction factors for these effects with which data can be standardized. These variables are of particular interest as they are essential in informing what is known about muscle function. The most widespread application of muscle mass and density data is in the calculation of physiological cross-sectional area (PCSA) — an anatomically derived correlate of a muscle's contractile force potential calculated by dividing muscle mass by the product of average fascicle length and muscle density (Schmacher, 1961; Gans and Bock, 1965; Close, 1972; Gans, 1982; Otten, 1988; Gans and Gaunt, 1991; Anapol and Barry, 1996; Lieber and Friden, 2000). The value used for muscle density is commonly taken as a previously determined constant (Méndez and Keys, 1960; Murphy and Beardsley, 1974).

Méndez and Keys (1960) as well as Murphy and Beardsley (1974) both used fresh tissue in their studies and therefore, any potential effects of freezing or formalin fixation and ethanol storage are not accounted for in these commonly used density constants. Thus, reevaluating the impact of preservation upon these muscular properties will also test the overall applicability of the use of a generalized constant representing muscle density.

### *Predictions*

Based on this previous work, I predict the following:

1. As freezing has been shown to damage cells ó producing ödripö exudate ó it is anticipated that muscle mass and volume will decline after each successive freezing due to water loss.
2. Since formalin is denser than water, it is anticipated that specimens fixed in formalin (but not stored in ethanol) will be more dense than unfixed specimens due to the replacement of water with formalin within the tissues.
3. Ethanol has been demonstrated to induce dehydration due to its ability to interfere with cellular membranes (Klemm, 1990). Because of this, as well as the findings of previous studies, it is predicted that muscles stored in ethanol will exhibit a reduction in mass and volume. Furthermore, as ethanol is less dense than water and as the water is replaced with ethanol within the tissue, I anticipate density to decrease as well.
4. It is anticipated that this loss of mass and density will be greater in specimens stored for successively longer periods of time.

## **Materials and Methods:**

To elucidate the acute effects of these commonly employed preservational practices when working with anatomical specimens, a sample of fresh 8-month-old New Zealand White rabbit (*Oryctolagus cuniculus*) cadavers (n=46) was obtained from a commercial meat farm (Brittany Ridge Farms, NC). All animals were euthanized by farm staff prior to us obtaining them and in compliance with USDA regulations. Cadaveric specimens are exempt from IACUC review at NC State University. Bilateral dissections were performed on each specimen and all rabbits were assigned to multiple treatment groups.

### *Freeze-Thaw Cycling*

The effects of freeze-thaw cycling by freezing 6 fully intact rabbits in a standard -20°C freezer was evaluated. The rabbits remained frozen for one month, after which they were removed and thawed fully. The masseter, digastric, quadratus labii, psoas, pronator teres, extensor carpi radialis longus, plantaris, soleus, and gastrocnemius muscles were dissected from one side. Once removed, each muscle was weighed to the nearest 0.0001g using a Mettler Toledo New Classic (MS 105) analytical balance. After obtaining dry weights, density determinations were made using this balance and an associated Mettler Toledo density kit (MS-DNY-54). All weight and density measurements were recorded. The specimens were then refrozen in the same freezer and repeated the process on the contralateral side once an additional month had elapsed.

### *Formalin Fixation and Ethanol Storage*

For the portion of the study addressing the short-term effects of formalin fixation and ethanol storage a subsample of forty individuals was used. Ten of these individuals were randomly assigned to each of the four treatment groups: 2-weeks of 10% formalin fixation only, 2-weeks of formalin fixation followed by 1,3, and 6 months of storage in 70% ethanol. Muscles

were preserved on their associated skeletal supports with skin intact (e.g., whole heads and limbs, not simply excised muscles).

At each designated time point, treatment group specimens were extracted, patted dry and the muscles of interest were excised individually. The focal muscles for the fixation analyses were the same as those used in the freeze/thaw study with the exception of the psoas ó a trunk muscle that was not retained for the fixation study when the specimens were broken down into subunits for more efficient fluid fixation and storage. Upon removal, the muscles were measured as described in the freeze-thaw protocol above.

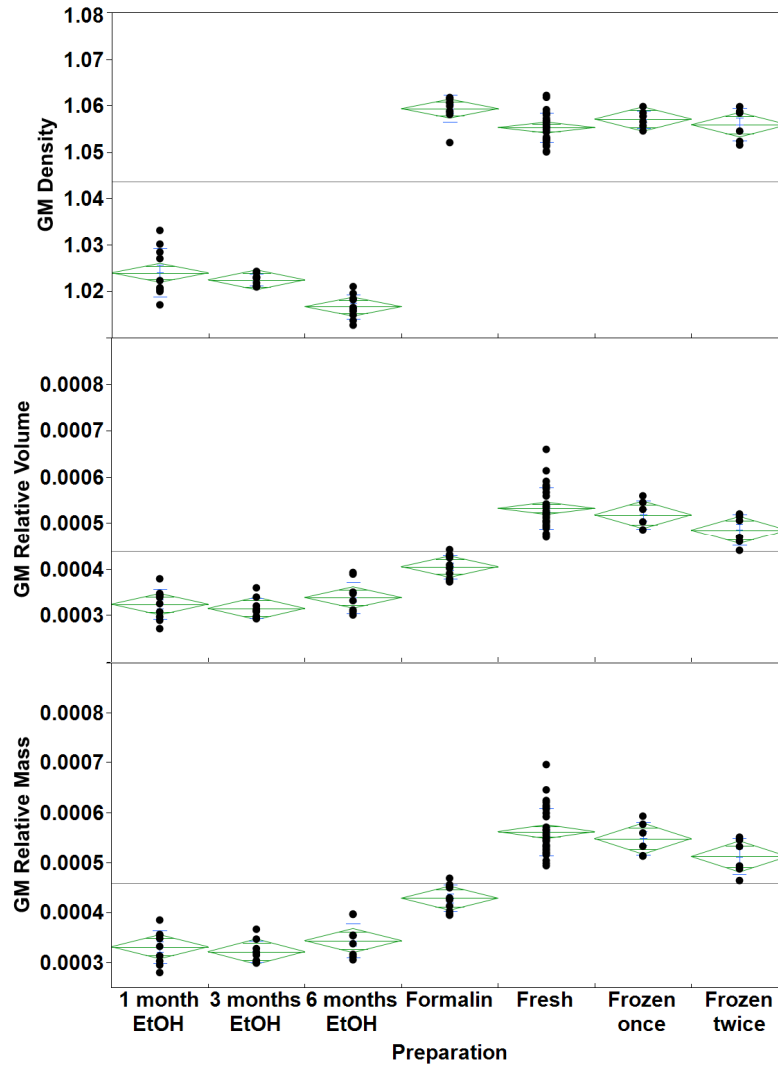
### *Statistical Analyses*

Geometric means (GM) were calculated for the muscle mass and volume for all the muscles of each individual in each treatment group, which provided a representative muscle density per individual per preparation. Geometric mean is calculated by multiplying each measurement for an individual together and taking the to the nth root where n is the number of observations being multiplied (8 for the formalin and EtOH treatment groups and 9 for the fresh). The GM for each individual was divided by body mass to produce a GM that was relative to body size.

All statistical analyses were conducted using JMP Pro 14 (SAS) and statistically significant differences were evaluated between the geometric means (GM) of each preparation relative to body mass using a Tukey-Kramer HSD test which allows for the determination of how preparation groups differ from one another. Exponential 3P models were fit using days in ethanol as the independent regressor and GM relative mass, GM relative volume, and GM density as the dependent variables. A chi-square goodness of fit test was also conducted to determine how well the model fit the data.

**Results:***Freezing*

The average mass and volumes observed did not differ between the fresh tissue and tissue that had been frozen once ( $p = 0.9825$ ;  $p = 0.9763$  respectively), between the fresh tissue and tissue frozen twice ( $p = 0.0680$ ;  $p = 0.069$ ), or between tissue that had been frozen once versus twice ( $p = 0.6690$ ;  $p = 0.6964$ ; Table 4.1). Muscle density also did not differ between either the fresh and frozen groups (once  $p = 0.8689$ ; twice  $p = 0.9996$ ) or between the frozen treatment groups ( $p = 0.9946$ ; Table 4.1). This indicates that freeze/thaw cycling has little impact on the muscle mass, volume and density. Although these differences were not significant, absolute mass and volume decreased by approximately 3% and 9% when frozen once and twice, respectively, compared to the fresh tissue. Subsequently, the prediction that freezing would result in a decrease in mass and volume is not statistically significantly supported. Density also remained virtually unchanged with less than a 1% change when compared to the fresh tissue (Table 4.1).



**Figure 4.1.** Distributions of relative muscle mass (bottom) and volume (middle) as well as density (top) grouped by preparation group. The grand mean is represented by the horizontal line shown on each graph.

**Table 4.1.** The mean, percent change from the mean of the fresh tissue, mean standard error and the connecting letters report (CLR) from the Tukey-Kramer tests ( $p < \alpha = 0.05$ ) for the relative mass and volumes as well as density. Full statistical results for each pairwise comparison are provided in Tables 4.S1-S3.

Variable	Preparation	Mean	% Change from Fresh	Mean Std. Error	Tukey-Kramer HSD CLR			
					A	B	C	D
GM Relative Mass	1 month EtOH	0.00033	-40.961	1.04E-05				
	3 months EtOH	0.00032	-42.687	7.46E-06				
	6 months EtOH	0.00034	-38.772	1.06E-05				
	Fresh	0.00056		8.70E-06				
	Formalin	0.00043	-23.665	8.45E-06				
	Frozen Once	0.00055	-2.491	1.37E-05				
	Frozen Twice	0.00051	-8.861	1.45E-05				
GM Relative Volume	1 month EtOH	0.00032	-39.136	1.02E-05				
	3 months EtOH	0.00032	-40.845	7.32E-06				
	6 months EtOH	0.00034	-36.432	1.04E-05				
	Fresh	0.00053		8.26E-06				
	Formalin	0.00040	-23.962	8.02E-06				
	Frozen Once	0.00052	-2.648	1.27E-05				
	Frozen Twice	0.00049	-8.901	1.35E-05				
GM Density	1 month EtOH	1.02398	-2.965	0.0016753				
	3 months EtOH	1.02247	-3.108	0.0004255				
	6 months EtOH	1.01670	-3.655	0.0008262				
	Fresh	1.05527		0.0005758				
	Formalin	1.05933	0.384	0.0009085				
	Frozen Once	1.05709	0.172	0.0007803				
	Frozen Twice	1.05587	0.056	0.0014567				

### *Formalin*

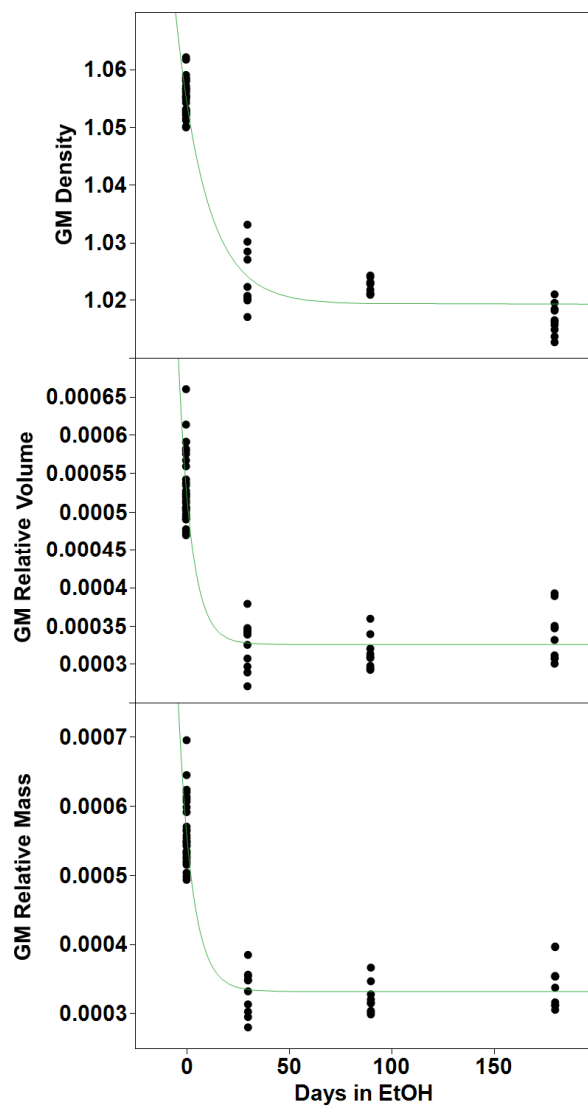
Relative to fresh muscle tissue, formalin-treated samples decreased in both mass and volume by approximately 24% and exhibited a minor increase in density (<1%) (Table 4.1). All three variables were significantly different from those of fresh tissue with  $p$ -values of  $< 0.0001$ ,

$<0.0001$ ,  $= 0.0160$ , respectively (Tables 4.S1-3). These findings supported the prediction that formalin fixation will lead to an increase in muscle density compared to that of the fresh muscle treatment group.

### *Ethanol Storage*

Following 1, 3 and 6 months of ethanol storage, muscle mass decreased by approximately 41%, 43% and 39% compared to the fresh tissue respectively. The average muscle mass for each one of these treatment groups was significantly smaller than the average of the fresh muscle group (p-values  $<0.0001$ , Table S1; Table 1). Masses did not differ significantly between the 1, 3 and 6 month ethanol groups. Similarly, when an exponential model is fit regressing muscle mass against time in ethanol, the percent difference from the average of the fresh tissue to the asymptote is also approximately 41% (Figure 4.2; Table 4.2). Thus, the changes in muscle due to time in ethanol appears to plateau sometime between 0 and 30 days.

The trends observed in muscle volume associated with ethanol storage mirrored closely the trends in muscle mass decreasing by 39%, 41% and 36% compared to the fresh tissue after 1, 3 and 6 months in ethanol respectively. The average muscle volume for each one of the ethanol treatment groups was found to be significantly less than the average muscle volume of the fresh treatment group (p-values  $<0.0001$ , Table 4.S2; Table 4.1), but as was the case for mass, volume did not differ significantly between the ethanol treatment groups. The exponential model was also found to be relatively consistent with this in that the percent drop from the average muscle volume of the fresh treatment group to the asymptote was approximately 39% and also appeared to plateau between 0 and 30 days in ethanol (Figure 4.2; Table 4.2). The findings related to muscle mass, volume and density support the predictions that each of these variables would decrease with ethanol storage.



**Figure 4.2.** Exponential (3P) models for mass, volume and density versus days in ethanol displayed graphically.

**Table 4.2.** Exponential (3P) models for mass, volume and density versus days in ethanol.

<b>Exponential Decay Models (3P)</b>							
<b>Variable</b>	<b>Parameter</b>	<b>Estimate</b>	<b>Std. Error</b>	<b>Wald Chi Square</b>	<b>Prob &gt; Chi Square</b>	<b>% Change from Average Fresh to Asymptote</b>	<b>R<sup>2</sup></b>
GM Relative Mass	Asymptote	0.00033	9.30E-06	1273.87	<.0001*	-40.91	0.89
	Scale	0.00023	0.0000119	373.63	<.0001*		
	Growth Rate	-0.14750	0.1911315	0.60	0.4403		
GM Relative Volume	Asymptote	0.00033	8.95E-06	1323.30	<.0001*	-38.85	0.88
	Scale	0.00021	1.14E-05	327.04	<.0001*		
	Growth Rate	-0.15957	0.2937462	0.30	0.587		
GM Density	Asymptote	1.01933	0.0008705	1371237.90	<.0001*	-3.41	0.96
	Scale	0.03594	0.0011015	1064.78	<.0001*		
	Growth Rate	-0.06707	0.0098306	46.55	<.0001*		

**Discussion:**

Understanding the acute effects of common preservation techniques on muscle tissue is important because these practices are likely to influence muscular properties frequently used as anatomical correlates of functional performance. This study evaluates the two most common preservation practices applied to anatomical specimens - freezing and fluid preservation. These findings add to and complement the previous body of literature documenting the larger scale implications (i.e., fluid loss and deviations in the gross morphometrics of specimens) of these methods.

The findings indicate that freeze-thaw cycling at -20°C had little impact on muscle mass, volume or density, suggesting that short-term freezing is not significantly detrimental to gross muscle characteristics. This was different than what was expected based on the substantial

evidence that freezing disrupts the microscopic structure of the tissue (Cook et al., 1926; Luyet, 1964; Raheli et al., 1985; Gruji et al., 1993; Ngapo et al., 1999) and leads to a loss of fluid (Ramsbottom and Koonz, 1939; Añón and Calvelo, 1980; Gonzalez Sanguinetti et al., 1985; Strange, 1987) alterations expected to be reflected in the gross characteristics of the tissue (i.e., a decrease in mass; decrease in volume; increase in density). While freezing did not significantly impact these characteristics, it was observed that, on average, freezing once decreases muscle mass by 2.49% and muscle volume by 3.0%. Differences in density, meanwhile, were more negligible still (averaging 0.17%). While the density difference is still very small after freezing/thawing twice, the changes in mass and volume are increased (8.86% and 8.90% respectively), but remain statistically insignificant relative to fresh muscle tissues. While future studies may wish to correct for specimens which have undergone additional freeze-thaw cycles and for the long-term effects of freezing due to freezer burn, overall, these findings suggest that short-term freezing and thawing has a relatively small, and statistically insignificant, effect on the quality of musculature at the macroscopic level of anatomical specimens.

The short-term formalin fixation of specimens (2 weeks in a 10 percent buffered formalin solution) had a statistically significant effect on the muscle tissue. Relative to fresh tissues, muscle mass and volume decreased by 23.67% and 23.96% and muscle density increased by approximately 0.38%. This effect is likely a response to the increased density of formalin. Although 10 percent buffered formalin is a modern standard for preservation (Simmons and Voss, 2009), it should be noted that fixation in a higher formalin concentration may lead to significant differences in muscle anatomy relative to fresh specimens (see Ward and Lieber, 2005).

Formalin fixation followed by ethanol storage was observed to most significantly impact muscular properties. Following 1 month, 3 months, and 6 months storage in ethanol, muscle mass decreased by about 40.96%, 42.69% and 38.77% respectively while muscle volume decreased by 39.14%, 40.85% and 36.43%. Density was also observed to decrease by 2.97%, 3.11%. and 3.70% after 1,3, and 6 months respectively. These findings mirrored the *a priori* predictions due to the anticipated impact of ethanol, the dehydrating effects of which appear to produce a reduction in mass and volume (Klemm, 1990). Additionally, the replacement of water within the tissue with ethanol, which is less dense than water, will decrease overall tissue density (Simmons, 2014). Though a slight (but statistically insignificant) increase was observed in the mass and volume of tissues stored in ethanol for 6 months relative to 3 months, I hypothesized that fluid exchange between external ethanol and water from the tissue may have diluted the concentration of the solution over time (Simmons, 2014), resulting in an eventual equilibrium.

Ethanol had relatively substantial effects on the muscle tissue (~40% loss of muscle mass and volume) it may be useful to evaluate other combinations and concentrations of fixatives, in this case 10% buffered formalin was used, followed by 70% ethanol to see if any of these effects could be mitigated.

### *Limitations*

While short-term freezing and thawing had little effect on muscle tissues at the macroscopic level, this may not be the case if histological data are needed. This study evaluated freeze-thaw cycling at -20°C which induces a slower rate of freezing than does a -80°C freezer. The rate of freezing has been correlated to the location and size of ice crystals that form within muscle tissue (Cook et al., 1926; Luyet, 1964; Raheli et al., 1985; Gruji et al., 1993; Petrovi et al., 1993). More specifically, slower rates of freezing have been shown to lead to the formation

of larger ice crystals between muscle fibers, which leads to water loss from the fibers, while faster rates of freezing result in smaller ice crystals that form within the fibers themselves (Cook et al., 1926; Luyet, 1964; Raheli et al., 1985; Petrovi et al., 1993). Therefore, it is important to consider freezing rate when pursuing histological data collection.

Another important consideration is that this study only addresses two cycles of short-term freezing and thawing. That is to say that while short duration freezing (1 month) between freezing and thawing is relatively harmless to the gross properties of the muscle, effects of long duration freezing and repetitive freeze thaw cycling need to be further elucidated in order to determine their effects. Furthermore, many specimens that undergo multiple freeze-thaw cycles do so because freezers fail, potentially resulting in tissue decay of the extent of which is affected by multiple variables (time, insulation, ambient temperature etc.) that are potentially difficult to control. Thus, these findings are applicable only to specimens that have undergone controlled freeze-thaw processes.

### *Conclusions*

Overall, this study shows that while short-term freezing and thawing have little effect on muscle morphology, even short-term formalin fixation and ethanol storage significantly reduces muscle mass and volume. Although freezing at -20C in the short-term may have less of an altering effect on the gross morphometric characteristics of the musculature compared to ethanol storage, slow freezing likely is detrimental to the microanatomy of muscle cells and therefore a faster freezing rate may be preferable. Ultimately, this study fills a gap in the literature by providing the first systematic, controlled examination of the effects of acute preservation on the mass, volume and density of muscles of data that will be of value to studies which integrate such variables into functional analyses of skeletal muscle. Based on these results the following

correction factors for each preparation (multiplication factors) were derived: Mass: ethanol 1.69; 10% formalin 1.32; frozen once 1.03; frozen twice 1.09; Volume: ethanol 1.64; 10% formalin 1.32 ; frozen once 1.03 ; frozen twice 1.10 ; Density: ethanol 1.03; 10% formalin 1.00; frozen once 1.00; frozen twice 1.00.

**List of Abbreviations:**

PCSAô physiological cross-sectional area

EtOHô ethanol

GMô geometric mean

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**References:**

- Al-Hassan LA, Bujawari J, El-Silini O. 2000. The effect of some preservatives and freezing on certain body dimensions of two species of the family Mullidae collected from Benghazi waters, Libya. *Acta Ichthyol Piscat* 2.
- Andrei MA, Genoways HH. 1999. Changes in pH in museum storage fluids. In: *Collection Forum*. p 63-75.
- Añón MC, Calvelo A. 1980. Freezing rate effects on the drip loss of frozen beef. *Meat Sci* 4:1-14.
- Benjakul S, Bauer F. 2000. Physicochemical and enzymatic changes of cod muscle proteins subjected to different freeze-thaw cycles. *Journal of the Science of Food and Agriculture* 80:1143-1150.
- Billy AJ. 1982. The effects of formalin and isopropyl alcohol on length and weight measurements of *Sarotherodon mossambicus* Trewavas. *J Fish Bio* 21:107-112.
- Birdseye C. 1929. Some Scientific Aspects of Packaging and Quick-Freezing Perishable Flesh Products Iô More Rapid Freezing Means Better Preservation. *Ind Eng Chem* 21:414-417.
- Burgner RL. 1962. *Studies of red salmon smolts from the Wood River Lakes, Alaska*. Seattle, Washington: University of Washington Press.
- Callow E. 1952. Frozen meat. *J Sci Food Agric* 3:145-150.
- Carroll R, Cavanaugh J, Rorer F. 1981. Effects of frozen storage on the ultrastructure of bovine muscle. *J Food Sci* 46:1091-1094.
- Cook G, Love E, Vickery J, Young WJ. 1926. Studies on the refrigeration of meat. I. Investigations into the refrigeration of beef. *Australian Journal of Experimental Biology and Medical Science* 3:15-31.
- Cutts A. 1988. Shrinkage of muscle fibres during the fixation of cadaveric tissue. *J Anat* 160:75.
- Deichmann JL, Boundy J, Williamson GB. 2009. Anuran artifacts of preservation: 27 years later. *Phyllomedusa: J Herpetol* 8:51-58.
- Eltoum I, Fredenburgh J, Myers RB, Grizzle WE. 2001. Introduction to the theory and practice of fixation of tissues. *J Histotechnol* 24:173-190.
- Engel S. 1974. Effects of formalin and freezing on length, weight and condition factor of cisco and yellow perch. *Trans Am Fish Soc* 103:136-138.

- Gonzalez Sanguinetti S, Anon M, Calvelo A. 1985. Effect of thawing rate on the exudate production of frozen beef. *J Food Sci* 50:697-700.
- Gruji R, Petrovi L, Pikula B, Amidfi L. 1993. Definition of the optimum freezing rate. 1. Investigation of structure and ultrastructure of beef *M. longissimus dorsi* frozen at different freezing rates. *Meat Sci* 33:301-318.
- Hale AR, Ross AH. 2017. The impact of freezing on bone mineral density: implications for forensic research. *J Forensic Sci* 62:399-404.
- Hoar WS. 1939. The weight-length relationship of the Atlantic salmon. *Journal of the Fisheries Board of Canada* 4:441-460.
- Jawad LA. 2003. The effect of formalin, alcohol and freezing on some body proportions of *Alepes djeddaba* (Pisces: Carangidae) collected from the Red Sea coast of Yemen. *Revista de biología marina y oceanografía* 38:77-80.
- Jones D. 1976. Chemistry of fixation and preservation with aldehydes. In: Steedman HF, editor. *Zooplankton fixation and preservation*. Paris, France: UNESCO Press. p 155.
- Kim H-W, Kim J-H, Seo J-K, Setyabrata D, Kim YHB. 2018. Effects of aging/freezing sequence and freezing rate on meat quality and oxidative stability of pork loins. *Meat Sci* 139:162-170.
- Klauber LM. 1943. Tail-length Differences in Snakes: With Notes on Sexual Dimorphism and the Coefficient of Divergence [and] A Graphic Method of Showing Relationships. San Diego, CA: Zoological Society of San Diego.
- Klemm W. 1990. Dehydration: A new alcohol theory. *Alcohol* 7:49-59.
- Lee JC. 1982. Accuracy and precision in anuran morphometrics: artifacts of preservation. *Syst Biol* 31:266-281.
- Leygonie C, Britz TJ, Hoffman LC. 2012. Impact of freezing and thawing on the quality of meat. *Meat sci* 91:93-98.
- Luyet BJ. 1964. Effects of Freezing on Muscle Tissue. In: *Proc. 17th Reciprocal Meat Conf.*
- Méndez J, Keys A. 1960. Density and composition of mammalian muscle. *Metab: Clin Exp* 9:184-188.
- Mills A. 1975. Measuring changes that occur during frozen storage of fish: a review. *J Food Technol* 10:483-496.
- Murphy RA, Beardsley AC. 1974. Mechanical properties of the cat soleus muscle in situ. *Am J Physiol* 227:1008-1013.

- Ngapo T, Babare I, Reynolds J, Mawson R. 1999. Freezing rate and frozen storage effects on the ultrastructure of samples of pork. *Meat Sci* 53:159-168.
- Petrovi L, Gruji R, Petrovi M. 1993. Definition of the optimal freezing rate. 2. Investigation of the physico-chemical properties of beef *M. longissimus dorsi* frozen at different freezing rates. *Meat Sci* 33:319-331.
- Qi J, Li C, Chen Y, Gao F, Xu X, Zhou G. 2012. Changes in meat quality of ovine longissimus dorsi muscle in response to repeated freeze and thaw. *Meat Sci* 92:619-626.
- Raheli S, Pua S, Gawwad AH. 1985. Structure of beef Longissimus dorsi muscle frozen at various temperatures: Part 1. histological changes in muscle frozen at 10, 22, 33, 78, 115 and 196°C. *Meat Sci* 14:63-72.
- Ramsbottom J, Koonz C. 1939. Freezing temperature as related to drip of frozen defrosted beef. *J Food Sci* 4:425-431.
- Reed RN. 2001. Effects of museum preservation techniques on length and mass of snakes. *Amphibia Reptilia* 22:488-491.
- Scott NJ, Aquino-Shuster AL. 1989. The effects of freezing on formalin preservation of specimens of frogs and snakes. In: *Collection Forum*. p 41-16.
- Sen A, Sharma N. 2004. Effect of freezing and thawing on the histology and ultrastructure of buffalo muscle. *Asian-australasian J Anim Sci* 17:1291-1295.
- Simmons JE. 2014. *Fluid preservation: a comprehensive reference*. Lanham, MD: Rowman & Littlefield.
- Simmons NB, Voss RS. 2009. *Collection, preparation, and fixation of specimens and tissues*, 2 ed. Baltimore, MD: Johns Hopkins University Press.
- Strange ED. 1987. Quantitation and Characterization of Drip from Frozen-Thawed and Refrigerated Pork Liver. *J Food Sci* 52:910-915.
- Ward SR, Lieber RL. 2005. Density and hydration of fresh and fixed human skeletal muscle. *J Biomech* 38:2317-2320.
- Xia X, Kong B, Liu Q, Liu J. 2009. Physicochemical change and protein oxidation in porcine longissimus dorsi as influenced by different freeze-thaw cycles. *Meat Sci* 83:239-245.

## CHAPTER 5: Effects of Long-term Ethanol Preservation on Muscle Architecture

### Abstract:

Studies of muscle gross anatomy and architecture rely on accurate measurements of, for instance, muscle dimensions, mass, volume, fascicle length and density to estimate measures of functional performance such as excursion and force potential. Although many studies collect these data on fresh specimens, others rely on specimens that have been preserved in ethanol, and some studies even mix these types of samples. However, fluid preservation has been demonstrated to alter the gross morphology of anatomical specimens. This study aims to elucidate in greater detail the effects of long-term ethanol storage on these gross and architectural properties of muscle by evaluating a sample of *Mus musculus* specimens ranging from fresh to having been stored in ethanol for 16-130 years. Three sample sites (biceps femoris, quadriceps group and triceps surae) were excised *en bloc* from each individual and the masses, volumes and densities measured. Additionally, for a subset of individuals, histological cross-sections stained using a hematoxylin and eosin protocol were used to evaluate the microscale effects of long-term fluid preservation. For the remainder of the sample, the muscles were chemically dissected, and fascicle lengths were obtained to evaluate the fixation effects on muscle architecture linear dimensions. The geometric means of relative muscle mass, volume, fascicle length, average fiber area (femoral length as the body size proxy for each), density, and percent fiber area were regressed against years stored in ethanol. It was observed that muscle mass and volume dropped steeply between the fresh and stored samples, but then plateaued at less than half (38% and 40%, respectively) their original mass and volume. These losses in mass and volume correlate with histologically measured shrinking of average muscle fiber area. The statistically significant drop in density followed a similar pattern though plateaued only 5% below the fresh density. As

expected, although muscles lost size (mass and volume) during long-term ethanol storage, because they were preserved on the bone (attached on either end), fascicle lengths did not shorten significantly. This study demonstrates that mixing mass, volumetric and density samples of fresh and uncorrected fixed specimens is problematic and provides a means to correct for these *long-term* effects of fluid preservation, namely the mass, volume and density of decades-old ethanol stored specimens should be corrected by factors of 2.64, 2.49 1.054 respectively. (Another study, see chapter 4, evaluates the *acute* effects of preservation.)

Keywords: PCSA, mass, volume, density, correction

### **Introduction:**

The functional capabilities of skeletal muscle (e.g., contractile force and excursion potential) are conferred by a muscle's gross morphology and architectural structure (Gans and Bock, 1965; Gans, 1982; Otten, 1988; Gans and Gaunt, 1991; Anapol and Barry, 1996; Lieber and Fridén, 2001), and correlations have been found between these properties and, for instance, dietary (Taylor et al., 2006; Herrel et al., 2008; Perry and Wall, 2008; Eng et al., 2009; Taylor and Vinyard, 2009; Perry and Hartstone-Rose, 2010; Perry et al., 2011; Hartstone-Rose et al., 2012; Perry et al., 2014; Santana and Cheung, 2016; Fabre et al., 2017; Curtis and Santana, 2018; Hartstone-Rose et al., 2018; Santana, 2018; Deutsch et al., 2019; Hartstone Rose et al., 2019) and locomotor adaptations (Oishi et al., 2009; Berbel-Filho et al., 2013; Ogihara et al., 2017; Marchi and Hartstone Rose, 2018). Studies investigating these architectural properties of musculature, even from the same lab, do so using fresh (e.g., Hartstone-Rose et al., 2012; Hartstone Rose et al., 2018; Leischner et al., 2018; Deutsch et al., 2019), preserved (e.g., Boettcher et al., 2019; Leonard et al., 2019) specimens, or a mix of the two (e.g., Hartstone

Rose et al., 2019) as a key source of rare comparative specimens can be obtained from the fluid collections of museums. Museums commonly use formalin fixation followed by ethanol storage as a means to preserve the structural integrity of the portion of their collections devoted to whole-organism preservation (Simmons, 2014). Studies have shown that this ethanol storage leads to alterations in the gross morphology of whole specimens (Lee, 1982; Guillette et al., 1988; Bininda-Emonds and Russell, 1994; Cunningham et al., 2000; Voight, 2001; Moku et al., 2004; Buchheister and Wilson, 2005; Deichmann et al., 2009; Vervust et al., 2009; Berbel-Filho et al., 2013; Gaston et al., 2013; Larochelle et al., 2016; Hetherington et al., 2019; Sotola et al., 2019; Thacker and Gkenas, 2019; Leonard, 2020). Therefore, it is likely that finer-scale alterations are taking place within the musculature as well. However, little has been done to precisely quantify and correct for these effects despite the fact that mixed samples are at times unavoidable, for example, in comparative studies when trying to accrue adequate taxonomic breadth or when rare specimens are available only through a museum (e.g., Hartstone Rose et al., 2019). This study aims to evaluate the effects of fixation on muscle architecture to address these potential discrepancies.

### *The Functional Morphology of Muscle Fiber Architecture*

Muscles are comprised of bundles of muscle fibers known as fascicles, the length of which has been shown to correlate with stretch and speed capabilities (Bodine et al., 1982; Sacks and Roy, 1982). Sarcomeres, the contractile subunit of muscle, are arranged in series within muscle fibers, with each sarcomere capable of producing force when stretched a certain percentage beyond its resting potential. Therefore, longer muscle fascicles, containing more sarcomeres arranged serially, are able to stretch farther than are shorter muscle fascicles. Sarcomeres shorten simultaneously when muscles contract; therefore, longer muscle fascicles

comprised of more sarcomeres in serial are also able to contract with greater speed because they close a greater amount of distance per unit of time. This has functional implications: for instance longer masticatory muscle fascicle lengths have been demonstrated to correlate with larger relative prey size in felids (Hartstone-Rose et al., 2012) and fruit eating in lemurs (Perry et al., 2011) and monkeys (Hartstone-Rose et al., 2018).

Physiological cross-sectional area (PCSA) correlates with force production capabilities and is a function of muscle mass, average fascicle length and muscle density. Physiological cross-sectional area is calculated using the following formula modified from Schumacher (1961):

$$q = m/lp$$

where  $q$  is PCSA ( $\text{cm}^2$ ),  $m$  is muscle mass (g),  $l$  is average fascicle length (FL) (cm), and  $p$  is the specific density of the muscle. Most myological studies use constant muscle densities of  $1.0564 \text{ g/cm}^3$  or  $1.0597 \text{ g/cm}^3$  as described by Murphy and Beardsley (1974) and Méndez and Keys (1960) respectively. (Chapter 3 of this dissertation gives a more comprehensive analysis of density constants that relate to specific anatomical regions and ontogenetic stages, while Chapter 4 gives density corrections that pertain to frozen and thawed specimens and the effects of acute fixation on specimens. The current chapter will add to the literature density corrections for long term fixation. All of these should be preferentially used over the less thoroughly evaluated but widely cited studies when specific specimen density cannot be measured directly during anatomical analysis.)

*What is already known about ethanol preservation of specimens*

Myological studies examining muscle architecture require the use of cadaveric materials, and fresh specimens ó especially of rare and/or exotic animals ó are not always readily available. Therefore, fixed specimens are often included in these types of studies and are frequently obtained from museums. The purpose of fixation and preservation are to halt changes that occur after death and to preserve their structure by preventing microbial activity and cell lysis (Carleton et al., 1967; Simmons, 2014). Preservation protocols vary (Carter, 2003; Simmons, 2014). Although, formalin-fixation and ethanol preservation has been the most common whole-organism preservation practice by museums for the past century, and despite being used for over 350 years, the effects of ethanol as a fluid preservative is not fully understood and even modern practices remain imperfect in specimen maintenance (Simmons, 2014). Ethanol has a very strong effect on the structural characteristics of cell membranes (Patra et al., 2006), and the effects are evident throughout the entire membrane. Given certain properties of alcohol (i.e., possessing hydrophilic properties and fluidity potential in water), the hydrogen bonding capability of ethanol can dehydrate several regions of cells and their structures (Klemm, 1990, 1998). This dehydration causes morphological variation between ethanol stored and fresh specimens (Lee, 1982; Guillette et al., 1988; Bininda-Emonds and Russell, 1994; Cunningham et al., 2000; Voight, 2001; Moku et al., 2004; Buchheister and Wilson, 2005; Deichmann et al., 2009; Vervust et al., 2009; Berbel-Filho et al., 2013; Gaston et al., 2013; Laroche et al., 2016; Hetherington et al., 2019; Sotola et al., 2019; Thacker and Gkenas, 2019). For instance, Vervust and colleagues (2009) observed that after sixty-eight days of being preserved in ethanol, the average snout-vent length (SVL) of a slaughter of 65 green iguanas (*Iguana iguana*) was significantly reduced. They noted that this reduction in SVL occurred relatively quickly after

being placed in ethanol with a plateau occurring after only fifteen days. Similarly, other studies found that tissue size underwent an initial shrinkage associated with alcohol preservation, and this altered size remained constant over time (Voight, 2001; Moku et al., 2004; Von Schiller and Solimini, 2005; Gaston et al., 2013; Larochelle et al., 2016). More systematically, chapter 4 of this dissertation also found significant effects of formalin fixation and ethanol preservation in a sample of 46 rabbits that took place most dramatically in the first 30 days, plateauing at a loss of 40% of myological mass and volume. Thus, there is clear reason to worry about the comparability of ethanol preserved and fresh myological specimens ó especially given that most preserved specimens in museums are decades old.

Inaccurate representation of a specimen is especially detrimental to studies aiming to analyze anatomical variation within species. Subsequently, given that ethanol preservation has shown effects on gross morphometrics, it is likely that muscles and their architecture will be affected as well. Though not done using ethanol, Cutts (1988) observed a slight, but insignificant decrease, in the fascicle lengths of muscle fibers when preserved in embalming fluid while still attached to the skeleton--further reinforcing the need for ethanol-specific investigations.

To evaluate the long-term effects of ethanol preservation, this study uses *Mus musculus* an abundantly represented taxon within museum collections that allows us to examine the potential effects of fixation and preservation over a vast temporal timescale ó in this case, spanning more than 100 years of preservation.

### *Predictions*

Based on previous work and the known dehydrating effects of ethanol, I predict the following trends within each variable of interest:

1. Since skeletal muscles are composed of more than 70% water (Hargens et al., 1983; Reinoso et al., 1997; Ruotsalainen et al., 1997) and due to the dehydrating effects of ethanol, it is anticipated that muscle mass, muscle volume, and fiber cross-sectional area will decline (i.e., a result of fluid loss) and then plateau with the achievement of fluid stasis.
2. With ethanol being less dense than water, it is anticipated that a reduction in overall muscle density will occur because of the cellular fluid exchange between water and ethanol in each muscle tissue (Klemm, 1990, 1998).
3. Alternatively, it is plausible that because ethanol removes water from the muscle tissue this will concentrate the muscle proteins which are denser than water resulting in a net increase in muscle density.
4. Due to alterations in the gross morphology of whole specimens, it is anticipated that there will be a small but significant decrease in the average fascicle length similar to the overall body length reduction seen in the iguana study (Simmons, 2014). Because the specimens in this study were preserved intact (i.e., tissues attached to the skeleton), I anticipate that this decrease will be minimal (Cutts, 1988)

### **Materials and Methods:**

To evaluate these predictions and the overarching effects of ethanol storage on muscle architecture, a sample of fresh cadaveric control *Mus musculus* from North Carolina State University (NCSU) was obtained as well as fixed specimens from the American Museum Natural History (AMNH) and the North Carolina Museum of Natural Sciences (NCMNS) that had been stored in ethanol for varying 16-130 years (Table 5.1).

Table 5.1. Sample.

Specimen ID	Species	Collection	Years in EtOH	Femur Length (mm)
KCL-MA	<i>Mus musculus</i>	NCSU	0	18.07
KCL-MB	<i>Mus musculus</i>	NCSU	0	19.45
KCL-MC	<i>Mus musculus</i>	NCSU	0	19.18
KCL-MD	<i>Mus musculus</i>	NCSU	0	18.50
KCL-ME	<i>Mus musculus</i>	NCSU	0	19.30
KCL-MF	<i>Mus musculus</i>	NCSU	0	18.80
275208	<i>Mus musculus</i>	AMNH	16	14.31
264459	<i>Mus musculus</i>	AMNH	28	16.22
5579	<i>Mus musculus</i>	AMNH	32	16.18
5582	<i>Mus musculus</i>	AMNH	32	17.02
262266	<i>Mus musculus</i>	AMNH	34	17.14
256401	<i>Mus musculus castaneus</i>	AMNH	35	23.76
255991	<i>Mus musculus</i>	AMNH	36	16.67
9496	<i>Mus musculus</i>	AMNH	39	17.58
239270	<i>Mus musculus</i>	AMNH	42	15.27
229862	<i>Mus musculus</i>	AMNH	44	13.34
229870	<i>Mus musculus</i>	AMNH	44	17.27
242313	<i>Mus musculus</i>	AMNH	47	16.70
9495	<i>Mus musculus</i>	NCMNS	48	15.97
255691	<i>Mus musculus</i>	AMNH	50	17.13
212448	<i>Mus musculus</i>	AMNH	54	16.06
197443	<i>Mus musculus</i>	AMNH	56	16.01
197446	<i>Mus musculus</i>	AMNH	56	15.87
197452	<i>Mus musculus</i>	AMNH	56	13.61
234805	<i>Mus musculus</i>	AMNH	62	15.96
234807	<i>Mus musculus</i>	AMNH	62	17.74
175396	<i>Mus musculus brevisrostris</i>	AMNH	63	17.48
166959	<i>Mus musculus</i>	AMNH	65	15.37
170071	<i>Mus musculus</i>	AMNH	66	15.02
153471	<i>Mus musculus</i>	AMNH	71	16.65
150190	<i>Mus musculus</i>	AMNH	72	20.90
143678	<i>Mus musculus</i>	AMNH	75	22.54
129348	<i>Mus musculus</i>	AMNH	81	13.57
88946	<i>Mus musculus</i>	AMNH	81	17.33
90185	<i>Mus musculus</i>	AMNH	87	17.24
85391	<i>Mus musculus</i>	AMNH	90	14.97
80025	<i>Mus musculus</i>	AMNH	93	15.45
86967	<i>Mus musculus</i>	AMNH	94	18.49

**Table 5.1.** (Continued).

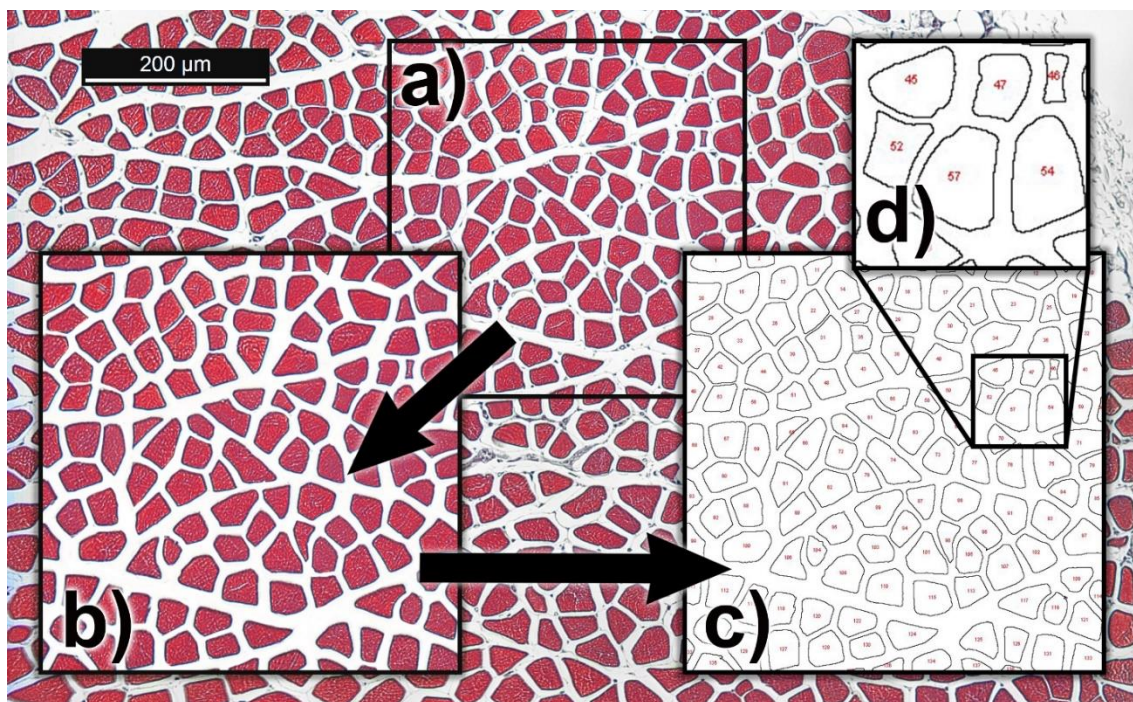
60642	<i>Mus musculus</i>	AMNH	98	16.57
7329	<i>Mus musculus</i>	AMNH	128	22.17
7335	<i>Mus musculus</i>	AMNH	128	21.87
1903	<i>Mus musculus</i>	AMNH	130	14.28
1906	<i>Mus musculus</i>	AMNH	130	15.14

From each specimen, the quadriceps group and the triceps surae *en bloc* and the biceps femoris was excised after reflecting just enough skin to access the muscles. After excision each removed group was weighed to the nearest 0.0001g using a Mettler Toledo New Classic (MS-105) analytical balance. Muscle density was then measured using a Mettler and Toledo density kit (MS-DNY-54). Using the values obtained for mass and density, density was calculated.

For a subset of the sample (n=75 muscles from 25 specimens), muscles were chemically digested to break them down into individual fascicles (following Boettcher et al., 2019; Leonard et al., 2019) to evaluate the effects of long-term ethanol storage on average fascicle length. To do this, once the mass and densities had been obtained for each of these muscles, they were submerged into 35% nitric acid until the muscle fascicles could be separated without tearing them. The nitric acid was then replaced with 50% glycerol to stop further digestion and manually separated fascicles using forceps. Each set of fascicles was photographed using a Canon EOS 5Ds with an identification tag and scale bar. A representative sample of fascicles (~40) were measured for each of the compartments using ImageJ (IJ1.46r) in order to calculate average fascicle length.

For another portion of the sample (n=54 muscles from 18 specimens), the muscles were evaluated histologically. For the fixed specimens, sections perpendicular to the direction of the fibers were taken and processed using standard paraffin embedding and a hematoxylin and eosin

staining protocol. To evaluate fresh muscle tissue, the muscles were frozen whole in isopentane using liquid nitrogen, and the same cross-sections of these muscles taken using a cryostat. Following the same methods discussed in chapter 3, the slides were photographed using a Leica CTR 5500 light microscope and three 400 by 400  $\mu\text{m}$  sections taken per muscle. These sections were then cleaned digitally in Photoshop to isolate the fibers and the measured percent fiber area and the average fiber area measured using the "analyze particles" function in ImageJ (IJ1.46r).



**Figure 5.1.** Stages of the histological image processing of the triceps surae of AMNH *Mus musculus* 143678\_posterior leg a) a 400 by 400  $\mu\text{m}$  section is cut from the original photograph slide b) extraneous noise was removed by cleaning in photoshop and c) the cells were analyzed in ImageJ d) partial fibers were excluded by number.

In order to discuss these variables on a specimen by specimen basis rather than for each muscle group, the geometric means (GM) were calculated for the muscle mass, volume, density, average fiber area, and the percent fiber area for each individual. Geometric means were calculated by multiplying each observation for each variable together and then taking the  $n$ th root where  $n$  is number of observations being multiplied. In order to adjust for the differences in size between specimens geometric means of muscle mass, muscle volume, and average fiber area were divided by femoral length as a body size proxy. Although total body mass is a more common body size proxy, femoral length was used for two reasons 1. overall body mass is likely influenced by all of the same effects of fluid preservation that are being evaluated and 2. many of the specimens obtained from the museums were not fully intact ó i.e., some were previously sectioned, decapitated or eviscerated.

All statistical analyses were conducted in JMP Pro 14 (SAS). Exponential decay 3P models, which are fit based on an estimated asymptote, were fit with years in EtOH on the x-axis and the geometric means of mass, volume, density, average on fiber area, percent fiber area and average fascicle length on the y-axes. A chi-square goodness of fit was also conducted to determine how well the model fit the data. To determine how these properties changed after the steep initial drop, the fresh specimens were excluded and an ordinary least squares regression was conducted with years in EtOH on the x-axis and the geometric means of mass, volume, density, average fiber area, percent fiber area and average fascicle length on the y-axes. Ordinary least squares regression was selected because it allows for the prediction of how y-changes in response to changes in  $x$  which assumes that changes in y are dependent on independent changes in x. To get a better idea of how the muscles were changing relative to their histology, I fit reduced major axis (RMA) lines of average fiber length and percent fiber area against mass,

volume and density. Reduced major axis linear regression was selected because it assumes error in both the x and y axes (Sokal and Rohlf, 1995) and is commonly used in evaluating scaling relationships.

### **Results:**

In order to study the effects of preservation over time on these variables, each was regressed relative to years in ethanol and then fit with a 3P exponential decay model - a line that best demonstrates the properties of a decaying sample as it reaches an asymptote. (Figure 5.2; Table 5.2).

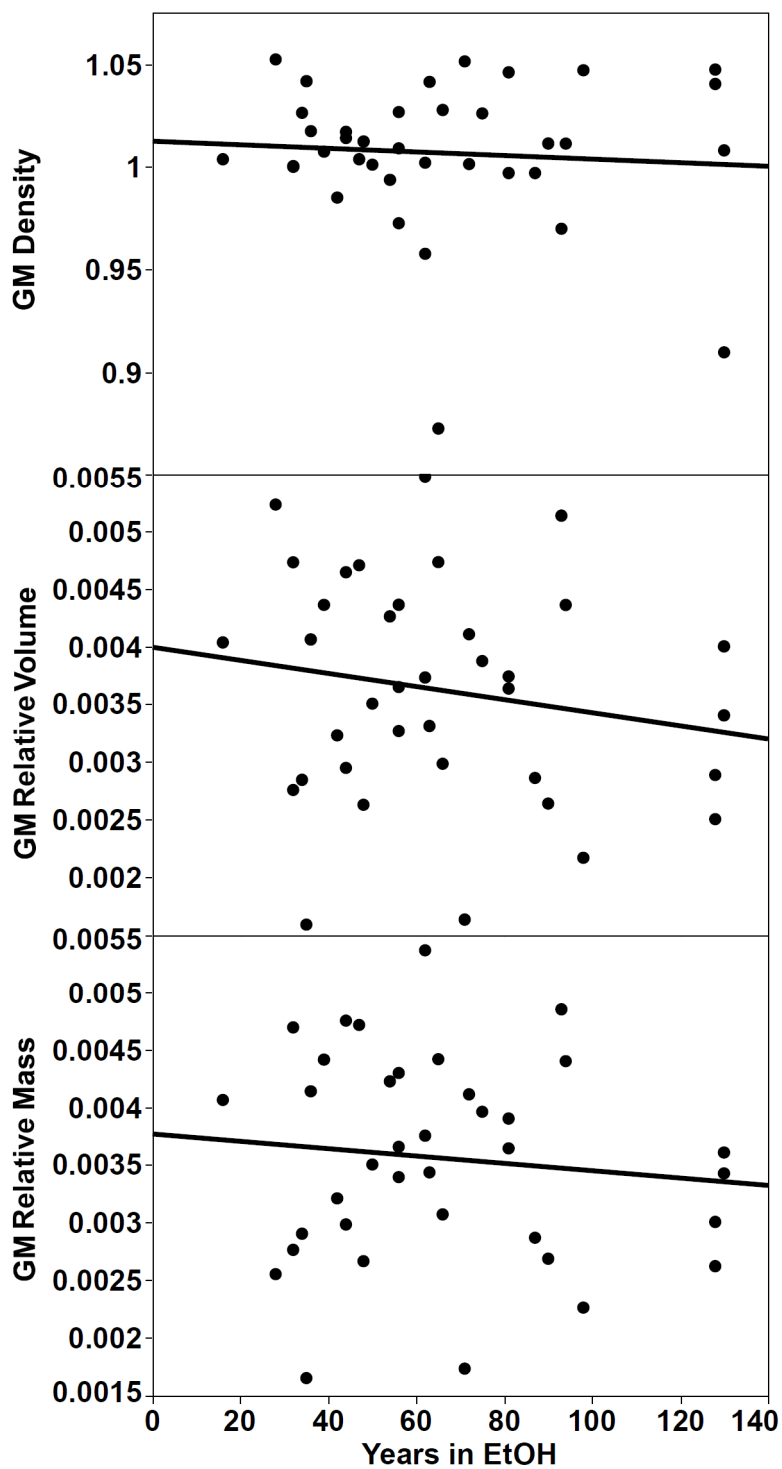
The mass and volume of all of the ethanol preserved specimens was substantially lower than those of the fresh specimens; relative muscle mass decreased by approximately 62% and relative muscle volume decreased by about 60% (Table 5.2). Density also exhibited a decrease compared to the fresh tissue, but this decline was more subtle at approximately 5% (Table 5.2). However, for each of these variables, the decline occurred relatively early, seemingly before the preservational age of the youngest specimens in my sample (in ethanol for ~16 years; Figure 5.2).



**Table 5.2.** Exponential model parameters for the geometric means of the relative mass, relative volume, density, relative average fiber area, average % fiber area and the % change from the average of the fresh tissue to the asymptote.

Variable	Parameter	Estimate	Std. Error	Wald Chi Square	Prob > Chi Square	% Change from Average Fresh to Asymptote	R <sup>2</sup>
GM Relative Mass	Asymptote	0.0035	0.0002	511.8611	<.0001*	-62.1719	0.8377
	Scale	0.0058	0.0004	205.0488	<.0001*		
	Growth	-0.1917	0.2129	0.8105	0.368		
GM Relative Volume	Asymptote	0.0036	0.0002	402.0453	<.0001*	-59.7936	0.7901
	Scale	0.0053	0.0004	148.3245	<.0001*		
	Growth	-0.1267	0.0799	2.5167	0.1126		
GM Density	Asymptote	1.0033	0.0091	12025.7170	<.0001*	-5.1141	0.2248
	Scale	0.0538	0.0163	10.8399	0.0010*		
	Growth	-0.0558	0.0523	1.1358	0.2865		
GM Relative Average Fiber Area	Asymptote	38.4113	2.7321	197.6671	<.0001*	-64.4704	0.9281
	Scale	69.7093	5.4222	165.2861	<.0001*		
	Growth	-0.0799	0.0259	9.5458	0.0020*		
GM % Fiber Area	Asymptote	62.9965	1.9403	1054.1101	<.0001*	-20.8111	0.6897
	Scale	16.2835	3.1243	27.1632	<.0001*		
	Growth	-0.0485	0.0300	2.6206	0.1055		
GM Relative Average Fascicle Length	Asymptote	0.0512	0.0024	445.3258	<.0001*	12.2466	0.0337
	Scale	-0.0056	0.0066	0.7229	0.3952		
	Growth	-2.8451	1.52E+38	0.0000	1.0000		

Because the exponential models indicated that this plateau occurs relatively quickly ó clearly before the youngest specimen in the sample ó I also fit as ordinary least squares (OLS) line excluding the fresh specimens (Figure 5.3; Table 5.3) ó essentially to study the decrease in variables over time from 16-130 years of ethanol preservation. The linear models reinforced what was observed with the asymptotes of the exponential decay models for muscle mass, volume and density; the slopes are approximately zero in the linear models.

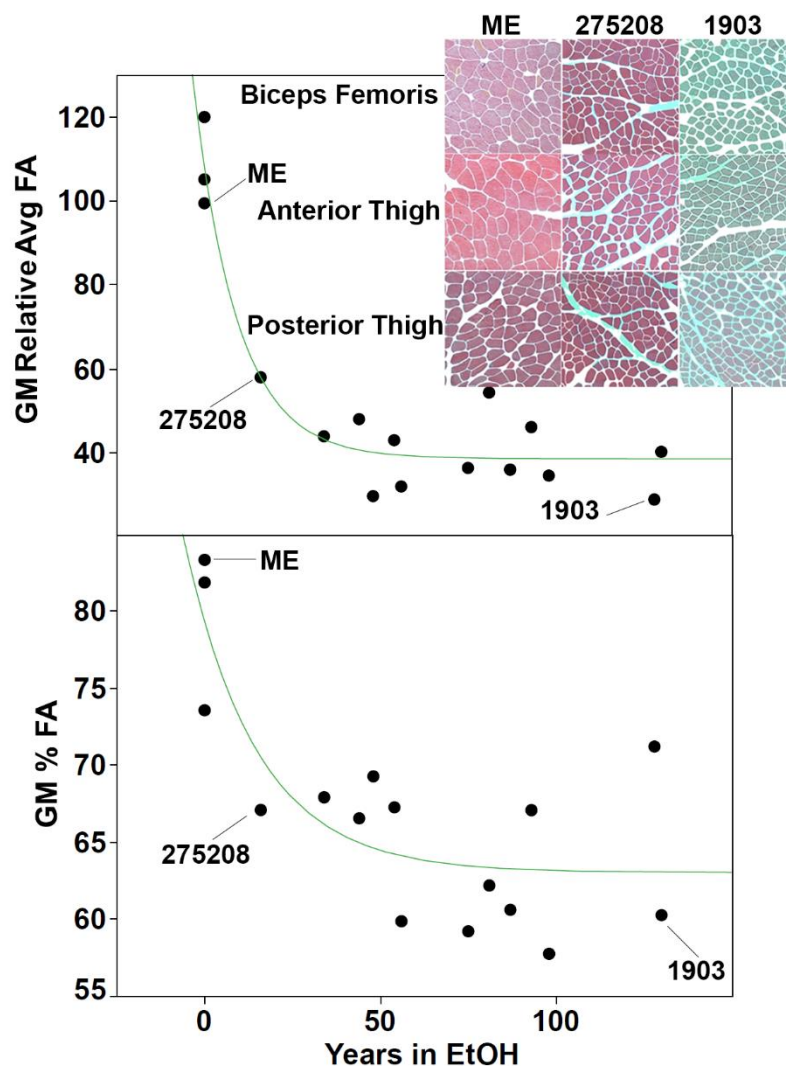


**Figure 5.3.** Ordinary least squares regressions of the geometric means of the relative mass (top), volume (middle) and density (bottom) of the preserved specimens (excluding the fresh specimens) regressed against years in ethanol.

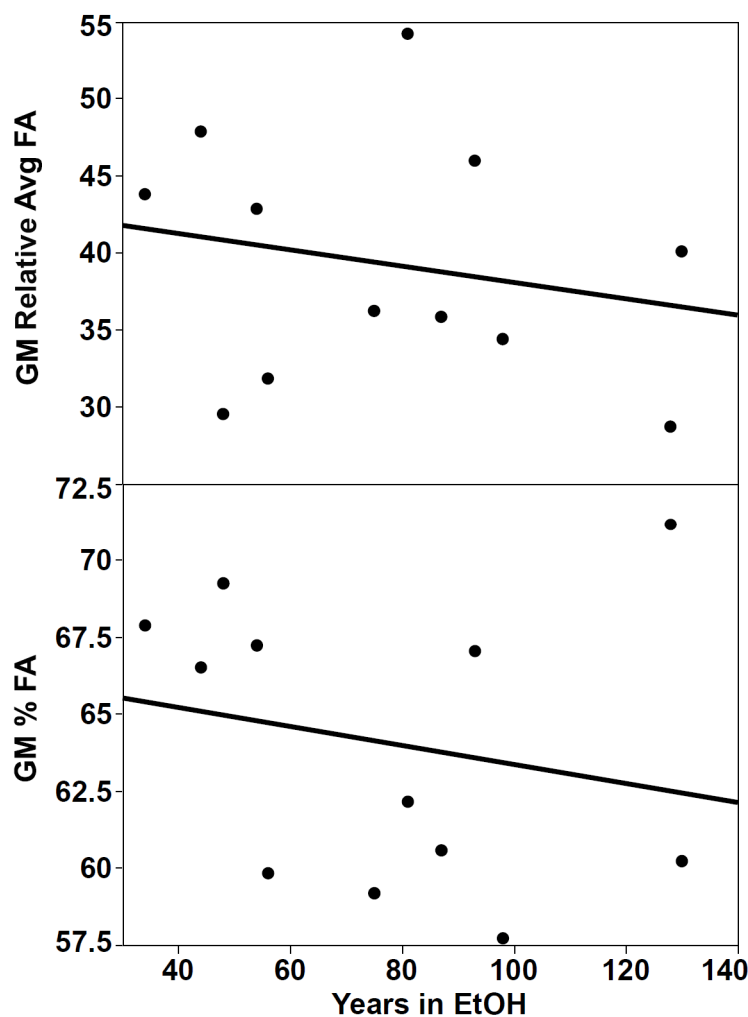
**Table 5.3.** Ordinary least squares term estimates for the geometric means of the mass, volume, density, average fiber area, average percent fiber area and fascicle length.

Variable	Term	Estimate	Std. Error	t Ratio	prob> t	R <sup>2</sup>
GM Relative Mass	Intercept	0.0038	0.0004	10.65	<.0001*	1.18E-02
	Slope	0.0000	0.0000	-0.65	0.5224	
GM Relative Volume	Intercept	0.0040	0.0004	10.52	<.0001*	0.0319
	Slope	0.0000	0.0000	-1.07	0.29	
GM Density	Intercept	1.0124	0.0146	69.17	<.0001*	0.0052
	Slope	-0.0001	0.0002	-0.43	0.6705	
GM Relative Average Fiber Area	Intercept	43.3703	6.4015	6.78	<.0001*	0.0451
	Slope	-0.0530	0.0771	-0.69	0.5078	
GM Percent Fiber Area	Intercept	66.4516	3.7087	17.92	<.0001*	0.0454
	Slope	-0.0308	0.0447	-0.69	0.5059	
GM Relative Fascicle Length	Intercept	0.0522	0.0042	12.4	<.0001*	0.0098
	Slope	0.0000	0.0001	-0.47	0.6447	

I also evaluated trends within the histology of a subset of these anatomical specimens (Figure 4). The average fiber area decreased by approximately 64 % from the fresh tissue to the asymptote observed (Table 5.2). The OLS trend for the relative average fiber area versus time in ethanol when excluding the fresh specimens indicated a very slight downward trend with a slope of approximately  $-0.05 \text{ m}^2$  per year (Figure 5.5; Table 5.3). The average % fiber area followed more subtle trends. The asymptote for the exponential decay model of the geometric mean of the average % fiber area versus time in ethanol indicated a decline of about 21% from the average of the fresh tissue to the asymptote (Figure 5.4; Table 5.2). The OLS trend excluding the fresh specimens had a slope of approximately zero suggesting no further measurable decay in the ethanol preserved sample (Table 5.2; Table 5.3).



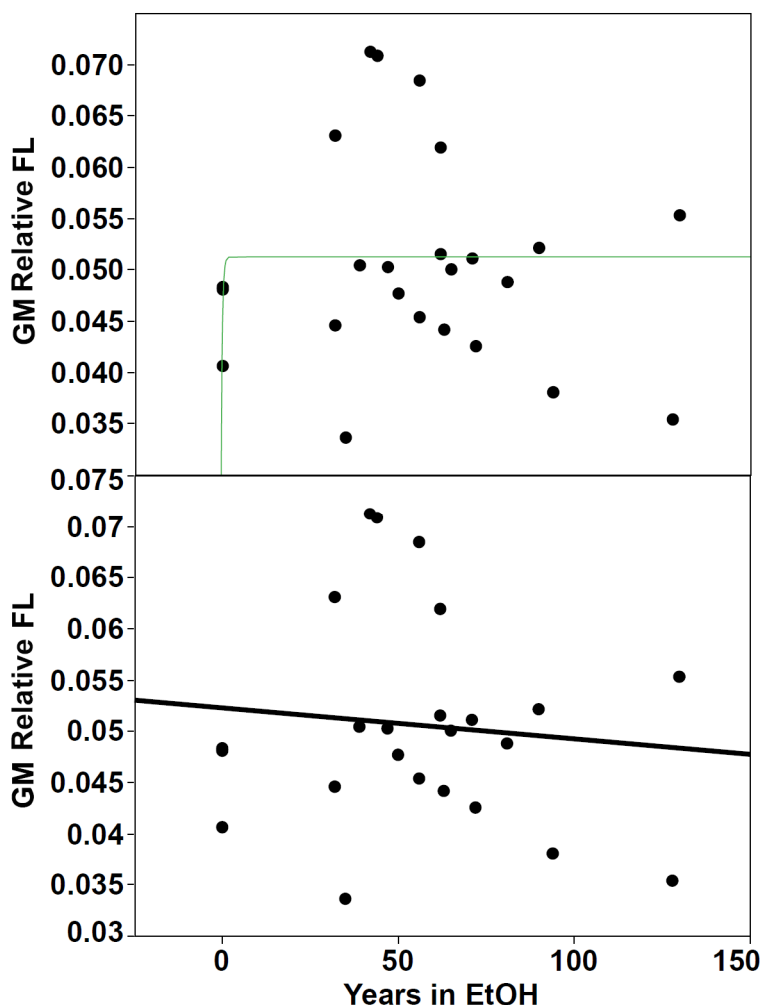
**Figure 5.4.** Exponential decay (3P) models for the geometric means of the relative average percent fiber area (top) and the average percent fiber area (bottom) regressed against years in ethanol. The inset (top) shows representative 400 by 400  $\mu\text{m}$  sections of each of the three muscle compartments (biceps femoris, anterior thigh, and posterior thigh) sampled from three individuals (Specimen IDs: ME, 275208 and 1903 which had been in ethanol for 0, 16 and 130 years respectively. Note that there are no real qualitative differences in the muscles within individuals but there is a clear (and typical) difference in fiber area and percent area particularly noticeable between the fresh and fixed specimens.



**Figure 5.5.** Ordinary Least Squares regressions of the geometric means of the relative average fiber area  $m^2$  (top) and the average percent fiber area (bottom) of the ethanol preserved specimens (excluding the fresh specimens) regressed against years in ethanol.

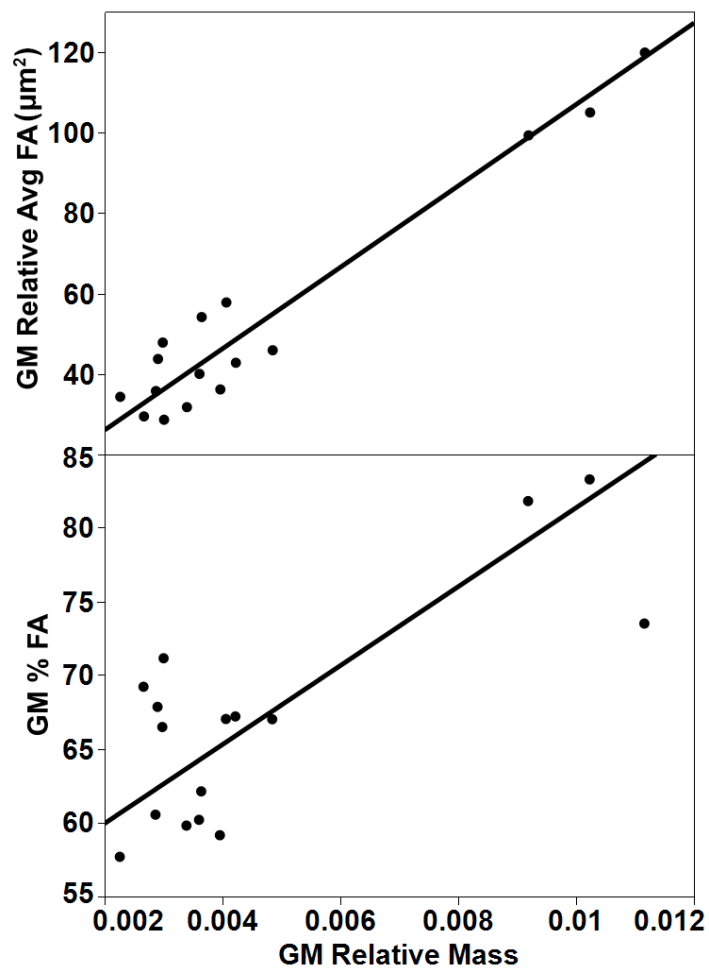
The exponential trend observed within the geometric means of average fascicle lengths was dramatically different than what was observed for the other architectural variables ó actually showing an apparent inversion (i.e., an increase in fascicle lengths then a plateau; Figure 5.6; Table 5.2). However, this is clearly an artifact of the fact that fascicle length really does not change relative to time in ethanol ó affirmed by the very low  $r^2$  value (0.0337) for both the fit in

the exponential model and the linear model ( $r^2=0.0098$ ). Thus, as predicted, fascicle lengths of muscles preserved on the bone (and therefore constrained from shrinking) do not change length.

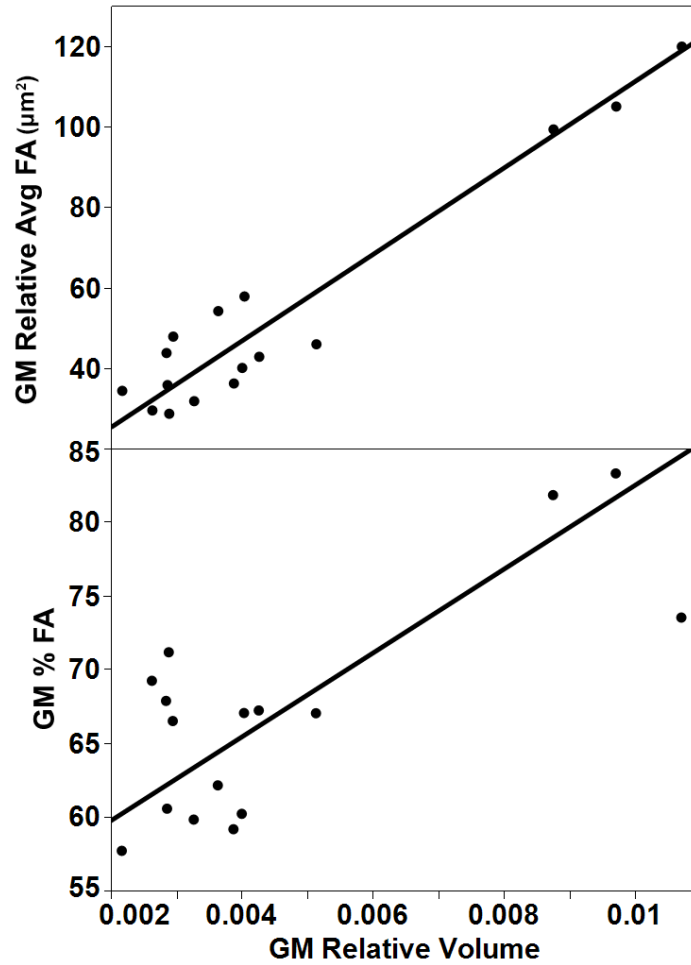


**Figure 5.6.** Exponential (3P) model for the geometric means of average fiber length (top) and OLS regression of geometric means of average fiber length (bottom) regressed against years in ethanol. Note, the inversion of the exponential line and the extreme scatter of the points in both lines ( $r^2 = 0.0337$  and  $0.0098$  respectively) demonstrate that there is no significant loss of fascicle length over time in ethanol ó as expected for muscles preserved on the skeleton, and therefore, unable to shrink in length.

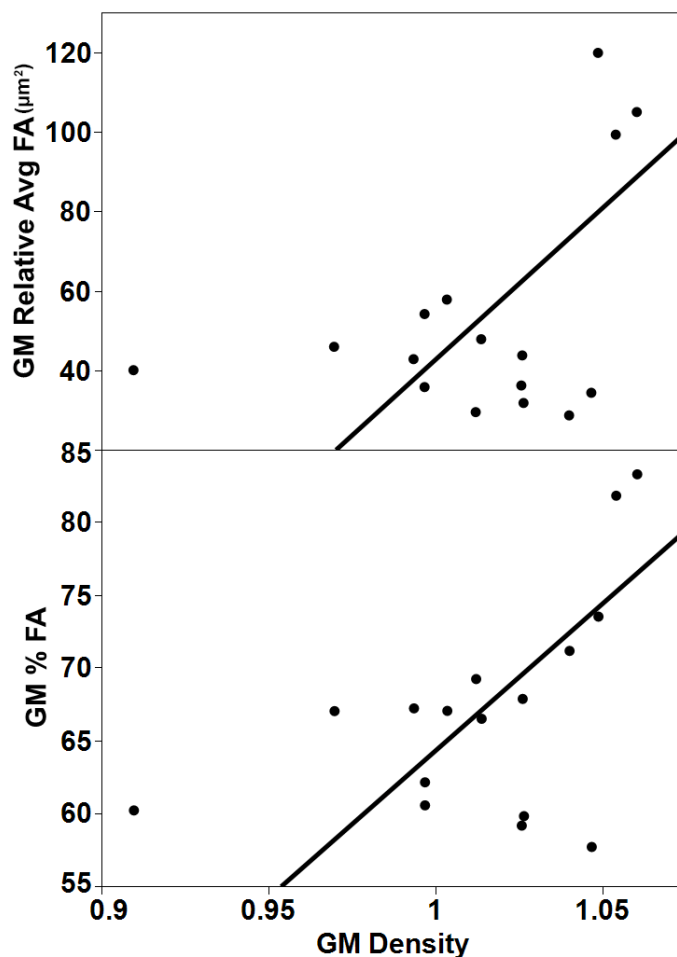
To determine the relationship between mass, volume, density and the relative average fiber area (  $\mu\text{m}^2$ ) and the relative percent fiber area, I fit RMA lines (Figures 5.7-9). There were very strong positive correlations between the average fiber area with mass and volume ( $r^2 = 0.9648$  and  $0.9597$ , respectively). There were also strong positive correlations between percent fiber area and muscle mass and volume ( $r^2 = 0.7725$  and  $0.7595$  respectively). The correlations between average fiber area and percent fiber and density area were not as strong ( $r^2 = 0.4289$  and  $0.4925$  respectively). This may be explained by the small variation in muscle density.



**Figure 5.7.** Reduced major axis regressions (RMA) of (top) relative average fiber area ( $r^2 = 0.9648$ ) and (bottom) percent fiber area ( $r^2 = 0.7725$ ) regressed against relative muscle mass.



**Figure 5.8.** Reduced major axis (RMA) regressions of (top) relative average fiber area ( $r^2 = 0.9597$ ) and (bottom) percent fiber area ( $r^2 = 0.7595$ ) regressed against relative muscle volume.



**Figure 5.9.** Reduced major axis (RMA) regressions of (top) relative average fiber area ( $r^2 = 0.4289$ ) and (bottom) percent fiber area ( $r^2 = 0.4925$ ) regressed against relative muscle density.

### Discussion:

Studies pertaining to muscle architecture provide important insight into the functionality of muscles and evolutionary adaptations thereof. While it would be ideal if all studies of muscle architecture could incorporate only fresh tissue (the types of tissues on which most physiological parameters have been established), this is not always feasible. The fluid collections of museums are a rich source of anatomical specimens. However, these collections have been fixed and stored for various durations of time—some being stored in ethanol for over 100 years. While fluid

preservation has been demonstrated to have some morphological effects on specimens (Lee, 1982; Guillette et al., 1988; Bininda-Emonds and Russell, 1994; Cunningham et al., 2000; Voight, 2001; Moku et al., 2004; Buchheister and Wilson, 2005; Deichmann et al., 2009; Vervust et al., 2009; Berbel-Filho et al., 2013; Gaston et al., 2013; Laroche et al., 2016; Hetherington et al., 2019; Sotola et al., 2019; Thacker and Gkenas, 2019) these effects have not been systematically quantified in typical museum specimens, until now.

Because skeletal muscle is comprised of approximately 70% water (Hargens et al., 1983; Reinoso et al., 1997; Ruotsalainen et al., 1997) and due to the dehydrating effects of ethanol (Klemm, 1990, 1998), I anticipated a decline in relative muscle mass, volume and density with increasing time in ethanol. These findings supported this prediction as muscle mass and volume decreased by 62% and 60% respectively. The decrease observed in density (~5% over time) supported the prediction that muscle density decreases in time in ethanol as the ethanol is less dense than water and replaces the water within the tissue. The decline in these variables occurred relatively early as indicated by the asymptotes as well as the slopes of the linear trends excluding the fresh specimens being nearly zero. The steep decline in mass, volume, and density that occurs before the youngest specimens (~16 years in ethanol) highlights the need to correct for the effects of fluid preservation in decades old museum specimens; not doing so leads to vast underestimations of, for instance, calculated physiological cross-sectional area (PCSA) and therefore, the force production capabilities inferred for the muscles of these specimens.

As predicted, this drop in mass and volume is correlated histologically both with reductions in relative average muscle fiber areas and percent fiber areas. As was shown that muscle fibers do not shorten when stored in ethanol (since they are fixed on the skeleton and can therefore not change in length while attached to their origins and insertions), all of the reductions

in volume are to their cross-sections. The shrinkage of the individual muscle fibers suggests that the loss of both mass and volume is likely due to the loss of fluid from the muscle cells. This is due to the alcoholic properties of ethanol which allow it to disrupt the lipid bilayer of cells (Klemm, 1990) and because of its hydrogen bonding capabilities it displaces water from the cells (Klemm, 1998).

Fascicle lengths, as predicted, remained relatively static—this was indicated by the lack of correlation between relative fascicle length and time in ethanol. This supports the findings by Cutts (1988) that when muscles are fixed intact that any reduction in fascicle length will be insignificant. However, while a loss in fascicle length was not observed, a reduction in fiber area was observed. This was demonstrated by the strong correlations between fiber area and muscle mass and volume which suggests that while the fascicles were not shrinking in length they were shrinking in cross-section.

Fluid preserved specimens are important resources in anatomical research, and the present study not only demonstrates that mixing samples of fresh and fluid preserved specimens is problematic without corrections, but also quantifies the effects of long-term ethanol storage such as to provide correction factors for muscle mass, muscle volume and density; namely masses, volumes and densities of muscles measured from decades-old ethanol stored specimens should be corrected by factors of 2.64, 2.49 1.054 respectively.

#### *Limitations and Future Directions*

One limitation of this study is that there is a large gap in the sample between 0 years (fresh) and the first fluid preserved specimen having been stored in ethanol for ~16 years. Therefore, the correction factors presented in this study would not be appropriate to correct for more acute effects of ethanol preservation.

A study by Leonard and colleagues (Chapter 4 and In Review) has elucidated the acute effects of fluid preservation by comparing fresh muscle tissue and tissue after 6 months of ethanol storage in a sample of New Zealand White rabbits, *Oryctolagus cuniculus*. Based on exponential decay models and the asymptotes observed, this study reported an approximate 40% reduction in mass and volume and a 3% decrease in density compared to what was observed for the fresh tissue. This is substantially different than the trends presented in the current study describing the long-term effects of ethanol storage. In short, the current study found reductions in mass and volume in decades-long preserved specimens that are almost three times as substantial as those found in specimens preserved for up to 6 months. There are several potential explanations for these differences.

The study conducted by Leonard et. al. (Chapter 4 and In Review) was much more controlled in that all of the specimens were obtained fresh and were fixed in 10% formalin prior to storage in 70% ethanol. The study presented here was not subject to the same degree of experimental control because the specimens were initially preserved by potentially dozens of curators over a vast time scale and preservation practices have evolved over time (Simmons, 2014). For instance, modern preservation practices involve fixation followed by storage in a preservative (Andrei and Genoways, 1999; Simmons and Voss, 2009; Simmons, 2014). However, this has not always been the case and preserving specimens directly in alcohol has been demonstrated to excessively dehydrate the tissue which therefore, would likely lead to greater reductions in mass, volume and density (Simmons, 2014). Even in instances where a fixative is used prior to alcohol storage, the concentrations of these chemicals have varied as well. Although modern museum collections tend to use a standard fixation method (days or weeks in 10% formalin) and an even more consistent storage method (almost uniformly using

70% ethanol), it is not possible to determine exactly when these standards were implemented in many collections, and certainly not possible to ensure that they have been consistently applied to specimens collected 130 years ago.

To address this inconsistency, future studies should strive to collect data from early collections with documented preservation techniques.

Also, in order to better understand the difference between my acute and long-term preservational effects studies, it would be valuable to find similarly preserved collections containing specimens preserved over the last couple of decades. It is unfortunate that the two major collections targeted for sampling did not happen to have specimens that span this time.

Beyond accounting for potential inconsistencies in methodology brought about by utilizing a sample accrued over such a vast decadal time scale, another factor that may help to explain the difference between the effects observed by Leonard and colleagues (Chapter 4 and In Review) and the study presented here could be a result of species-specific influences with the previous study focusing on rabbits and the current one focusing on mice. A future study should be conducted with another common taxon that could be studied from samples spanning long periods of time for instance fluid collections often have relatively large samples of other species of rodents (e.g., *Rattus* and *Peromyscus*) and bats (e.g., *Myotis*).

As there may be a scaling relationship driving the extent of mass and volume loss, another future study could examine fresh and fixed pairs or small samples of specimens spanning broad size ranges ó for instance a sample of carnivorans spanning orders of magnitude that would somewhat control for phylogeny by looking within a single order or constrained families.

### *Conclusions*

This study has demonstrated the significant effects of long-term ethanol storage on the gross and histological characteristics of skeletal muscle. More specifically I observed that the masses, volumes and densities of muscles measured from decades-old ethanol stored specimens should be corrected by factors of 2.64, 2.49 1.054 (62%, 60%, 5% respectively). It was also noted that there was no correlation between fiber length and time in ethanol suggesting that muscle fibers do not shrink lengthwise. However, the reduction in mass and volume correlates with vast decreases in the average fiber area as well as the percent fiber area suggesting that while the fascicles do not change in length they do shrink in cross-section.

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## References:

- Anapol F, Barry K. 1996. Fiber architecture of the extensors of the hindlimb in semiterrestrial and arboreal guenons. *Am J Phys Anthropol* 99:429-447.
- Andrei MA, Genoways HH. 1999. Changes in pH in museum storage fluids. In: *Collection Forum*. p 63-75.
- Berbel-Filho W, Pereira U, Martinez PA. 2013. Preservation effects in geometric morphometric approaches: Freezing and alcohol in a freshwater fish. *Ichthyological Research* 60.
- Bininda-Emonds ORP, Russell AP. 1994. Flight style in bats as predicted from wing morphometry: the effects of specimen preservation. *J Zool* 234:275-287.
- Bodine SC, Roy R, Meadows D, Zernicke R, Sacks R, Fournier M, Edgerton V. 1982. Architectural, histochemical, and contractile characteristics of a unique biarticular muscle: the cat semitendinosus. *Journal of Neurophysiology* 48:192-201.
- Boettcher ML, Leonard KC, Dickinson E, Aujard F, Herrel A, Hartstone Rose A. 2019. The forearm musculature of the gray mouse lemur (*Microcebus murinus*): An ontogenetic study. *Anat Rec*.
- Buchheister A, Wilson MT. 2005. Shrinkage correction and length conversion equations for *Theragra chalcogramma*, *Mallotus villosus* and *Thaleichthys pacificus*. *Journal of Fish Biology* 67:541-548.
- Carleton HM, Drury RAB, Wallington EA. 1967. Carleton's Histological technique. 1-520.
- Carter J. 2003. The Effects of Preservation and Conservation Treatments on the DNA of Museum Invertebrate Fluid Preserved Collections. In.
- Cunningham M, Jr W, Pope K. 2000. Shrinkage of Inland Silverside Larvae Preserved in Ethanol and Formalin. *North American Journal of Fisheries Management* 20:816-818.
- Curtis AA, Santana SE. 2018. Jaw dropping: Functional variation in the digastric muscle in bats. *Anat Rec* 301:279-290.
- Cutts A. 1988. Shrinkage of muscle fibres during the fixation of cadaveric tissue. *J Anat* 160:75.
- Deichmann J, Boundy J, Williamson G. 2009. Anuran artifacts of preservation: 27 years later. *Phyllomedusa : Journal of Herpetology* 8.
- Deutsch AR, Dickinson E, Leonard KC, Pastor F, Muchlinski MN, Hartstone-Rose A. 2019. Scaling of anatomically derived maximal bite force in primates. *Anatomical record* (Hoboken, NJ : 2007).

- Eng C, Ward S, Vinyard C, Taylor A. 2009. The morphology of the masticatory apparatus facilitates muscle force production at wide jaw gapes in tree-gouging common marmosets (*Callithrix jacchus*). *Journal of Experimental Biology* 212:4040-4055.
- Fabre PH, Herrel A, Fitriana Y, Meslin L, Hautier L. 2017. Masticatory muscle architecture in a water rat from Australasia (Murinae, *Hydromys*) and its implication for the evolution of carnivory in rodents. *J Anat* 231:380-397.
- Gans C. 1982. Fiber architecture and muscle function. *Exerc Sport Sci Rev* 10:160-207.
- Gans C, Bock WJ. 1965. The functional significance of muscle architecture--a theoretical analysis. *Ergeb Anat Entwicklungsgesch* 38:115-142.
- Gans C, Gaunt AS. 1991. Muscle architecture in relation to function. *J Biomech* 24:53-65.
- Gaston K, Jacquemin S, Lauer T. 2013. The Influence of Preservation on Fish Morphology in Museum Collections Based on Two Species of the Genus *Lepomis* (*Actinopterygii*: *Perciformes*: *Centrarchidae*). *Acta Ichthyologica Et Piscatoria* 43:219-227.
- Guillette LJ, Rand MS, Demarco V, Etheridge K. 1988. Fixative and Alcohol-induced Weight Change in Eggs from the Lizard, *Sceloporus undulatus* *J Herpetol* 22:115-118.
- Hargens AR, Tipton CM, Gollnick PD, Mubarak SJ, Tucker BJ, Akeson WH. 1983. Fluid shifts and muscle function in humans during acute simulated weightlessness. *J Appl Physiol* 54:1003-1009.
- Hartstone-Rose A, Deutsch AR, Leischner CL, Pastor F. 2018. Dietary correlates of primate masticatory muscle fiber architecture. *Anat Rec* 301:311-324.
- Hartstone-Rose A, Perry JMG, Morrow CJ. 2012. Bite force estimation and the fiber architecture of felid masticatory muscles. *Anat Rec* 295:1336-1351.
- Hartstone Rose A, Hertzog I, Dickinson E. 2019. Bite force and masticatory muscle architecture adaptations in the dietarily diverse Musteloidea (Carnivora). *Anat Rec* 302:2287-2299.
- Herrel A, De Smet A, Aguirre LF, Aerts P. 2008. Morphological and mechanical determinants of bite force in bats: do muscles matter? *J Exp Biol* 211:86-91.
- Hetherington ED, Kurle CM, Ohman MD, Popp BN. 2019. Effects of chemical preservation on bulk and amino acid isotope ratios of zooplankton, fish, and squid tissues. *Rapid Commun Mass Spectrom* 33:935-945.
- Klemm WR. 1990. Dehydration: A new alcohol theory. *Alcohol* 7:49-59.
- Klemm WR. 1998. Biological Water and Its Role in the Effects of Alcohol. *Alcohol* 15:249-267.

- Larochelle C, Tinoco Pickens F, Burns M, Sidlauskas B. 2016. Long-term Isopropanol Storage Does Not Alter Fish Morphometrics. *Copeia* 104:411-420.
- Lee JC. 1982. Accuracy and Precision in Anuran Morphometrics: Artifacts of Preservation *Systematic Zoology* 31:266-281.
- Leischner CL, Crouch M, Allen KL, Marchi D, Pastor F, Hartstone Rose A. 2018. Scaling of primate forearm muscle architecture as it relates to locomotion and posture. *Anat Rec* 301:484-495.
- Leonard KC. 2020. Leonard Chapter 3. Raleigh NC: Department of Biological Sciences.
- Leonard KC, Boettcher ML, Dickinson E, Malhotra N, Aujard F, Herrel A, Hartstone Rose A. 2019. The ontogeny of masticatory muscle architecture in *Microcebus murinus*. *Anat Rec*.
- Lieber RL, Fridén J. 2001. Clinical significance of skeletal muscle architecture. *Clinical orthopaedics and related research* 383:140-151.
- Marchi D, Hartstone Rose A. 2018. Functional Morphology and Behavioral Correlates to Postcranial Musculature. *AnatRec* 301:419-423.
- Méndez J, Keys A. 1960. Density and composition of mammalian muscle. *Metab: Clin Exp* 9:184-188.
- Moku M, Mori K, Watanabe Y. 2004. Shrinkage in the body length of myctophid fish (*Diaphus* slender-type spp.) larvae with various preservatives. *Copeia*:647-651.
- Murphy RA, Beardsley AC. 1974. Mechanical-properties of cat soleus muscle insitu. *Am J Physiol* 227:1008-1013.
- Ogihara N, Oishi M, Kanai R, Shimada H, Kondo T, Yoshino-Saito K, Ushiba J, Okano H. 2017. Muscle architectural properties in the common marmoset (*Callithrix jacchus*). *Primates* 58:461-472.
- Oishi M, Ogihara N, Endo H, Ichihara N, Asari M. 2009. Dimensions of forelimb muscles in orangutans and chimpanzees. *J Anat* 215:373-382.
- Otten E. 1988. Concepts and models of functional architecture in skeletal muscle. *Exerc Sport Sci Rev* 16:89-138.
- Patra M, Salonen E, Terama E, Vattulainen I, Faller R, Lee BW, Holopainen J, Karttunen M. 2006. Under the influence of alcohol: the effect of ethanol and methanol on lipid bilayers. *Biophys J* 90:1121-1135.

- Perry JMG, Macneill KE, Heckler AL, Rakotoarisoa G, Hartstone Rose A. 2014. Anatomy and adaptations of the chewing muscles in *Daubentonia* (Lemuriformes). *Anat Rec* 297:308-316.
- Perry JMG, Wall CE. 2008. Scaling of the chewing muscles in prosimians. In: *Primate craniofacial function and biology*: Springer. p 217-240.
- Perry JMG, Hartstone-Rose A. 2010. Maximum ingested food size in captive strepsirrhine primates: Scaling and the effects of diet. *Am J Phys Anthropol* 142:625-635.
- Perry JMG, Hartstone-Rose A, Wall CE. 2011. The jaw adductors of strepsirrhines in relation to body size, diet, and ingested food size. *Anat Rec* 294:712-728.
- Reinoso RF, Telfer BA, Rowland M. 1997. Tissue water content in rats measured by desiccation. *Journal of Pharmacological and Toxicological Methods* 38:87-92.
- Ruotsalainen U, Raitakari M, Nuutila P, Oikonen V, Sipila H, Teras M, Knuuti MJ, Bloomfield PM, Iida H. 1997. Quantitative blood flow measurement of skeletal muscle using oxygen-15-water and PET. *J Nucl Med* 38:314-319.
- Sacks RD, Roy RR. 1982. Architecture of the hind limb muscles of cats: functional significance. *J Morphol* 173:185-195.
- Santana SE. 2018. Comparative anatomy of bat jaw musculature via diffusible iodine based contrast enhanced computed tomography. *Anat Rec* 301:267-278.
- Santana SE, Cheung E. 2016. Go big or go fish: morphological specializations in carnivorous bats. *Proceedings of the Royal Society B: Biological Sciences* 283:20160615.
- Schumacher G-H. 1961. *Funktionelle morphologie der kaumusculatur*. Jena: G. Fischer.
- Simmons JE. 2014. *Fluid preservation: a comprehensive reference*. Lanham, MD: Rowman & Littlefield.
- Simmons NB, Voss RS. 2009. *Collection, preparation, and fixation of specimens and tissues*, 2 ed. Baltimore, MD: Johns Hopkins University Press.
- Sokal, R., & Rohlf, F. (1995). *Biometry*. New York: WH Freeman.
- Sotola VA, Craig C, Pfaff P, Maikoetter J, Martin N, Bonner T. 2019. Effect of preservation on fish morphology over time: Implications for morphological studies. *PLoS ONE* 14:1-16.
- Taylor AB, Jones KE, Kunwar R, Ravosa MJ. 2006. Dietary consistency and plasticity of masseter fiber architecture in postweaning rabbits. *Anat Rec Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology: An Official Publication of the American Association of Anatomists* 288:1105-1111.

- Taylor AB, Vinyard CJ. 2009. Jaw-muscle fiber architecture in tufted capuchins favors generating relatively large muscle forces without compromising jaw gape. *J Hum Evol* 57:710-720.
- Thacker CE, Gkenas C. 2019. Morphometric convergence among European sand gobies in freshwater (*Gobiiformes: Gobionellidae*). *Ecology and Evolution* 9:8087-8103.
- Vervust B, Van Dongen S, Van Damme R. 2009. The effect of preservation on lizard morphometrics - An experimental study. *Amphibia-Reptilia* 30:321-329.
- Voight JR. 2001. Morphological deformation in preserved specimens of the deep-sea octopus *Graneledone*. *J Molluscan Stud* 67:95-102.
- Von Schiller D, Solimini A. 2005. Differential effects of preservation on the estimation of biomass of two common mayfly species. *Archiv fur Hydrobiologie* 164:325-334.

## Chapter 6: The Importance of Correcting for Physiological and Preservational Effects in Muscle Fiber Architecture

Studies of muscle architecture provide substantial insight into the natural world and have been used to evaluate adaptations for what organisms eat, how they eat what they eat, how they move, which substrates they live on and, more broadly, how they interact with their environments. Because of these many functional implications of muscle architecture, it is imperative that the physiological and preservational influences on muscle architecture be understood. This body of work has demonstrated not only that these influences exist but has calculated several values that can be used to correct for them— especially those that pertain to the enormous changes related to preservation.

Chapter 2 illustrates that muscle architecture is dynamic throughout ontogeny within the small, Malagasy primate, *Microcebus murinus*. More specifically, it was observed that muscle mass and physiological cross-sectional area (PCSA) increased from infancy to juvenility and leveled-off into adulthood. The increase in muscle mass and PCSA of the mandibular adductors from infancy to juvenility was anticipated due to the transition in feeding mechanism from suckling to consuming solid food. A decline in muscle mass and PCSA at the onset of senescence, which occurs around 5 years of age for this species (Bons et al., 2006) was also anticipated due to the effects of sarcopenia— age-associated atrophy (Rosenberg, 1997; Cruz-Jentoft et al., 2010). Further support for this prediction was found within a study conducted by Chazeau and colleagues (2012) within the same animals pertaining to age and biteforce indicating a functional decline in maximal bite force after 5.5 years of age. However, this decline in either muscle mass or PCSA that would indicate a decline in functionality was not observed—

indicating that from an anatomical perspective, the senescent individuals should have been capable of producing biteforce comparable to that of the more prime-aged individuals. The fact that the age-related behavioral changes were not reflected in anatomical changes seems to indicate that there are other explanations for the deterioration in bite force—such as an unwillingness to bite maximally, which could perhaps be a behavioral protective measure to prevent damage to the jaw and dentition due to the evident osteoporosis and alveolar resorption and or possibly evidence of a loss in motor coordination. The trends observed in fascicle length within the mandibular adductors of *Microcebus murinus* were different in that they increased rapidly and remained relatively constant once maximal length was attained. This could explain why Perry and colleagues (Perry et al., 2011) observed relatively longer fascicles in juvenile and subadult lemurs—it is not that the fascicles are absolutely long, but that lemurs attain adult-length fascicles before attaining adult muscle or overall body size. Overall, quadratic curvilinear models were significant when each architectural variable was regressed relative to age: each variable grows parabolically from birth, then levels off into adulthood and some shrink again slightly into senescence.

Although this particular study is the largest intraspecific sample to comprehensively evaluate the ontogeny of the muscle architecture of the mandibular adductors within a primate species, it is not without limitations. For instance, from this study alone it could not be concluded whether or not the trends observed are consistent within other species (the subject of chapter 3) or between other anatomical regions. However, the latter limitation was addressed by conducting a more recent study (Boettcher et al., 2019) evaluating the ontogenetic trends within the forearm musculature for the same animals included in the study of the mandibular adductors. For the most part similar trends to the trends within the architecture of the mandibular adductors

were observed within the forearm  $\hat{\delta}$  indicating that the ontogenetic trends are relatively similar between anatomical regions and provides further support for the possibility of global control mechanisms for development.

The findings in chapter 2 highlight the need for researchers to be cognizant of age-related trends within the architectural properties of muscle. This is particularly important in sample composition and making sure that comparative samples are equitable in life-stages to ensure that differences observed are species-specific or related to function rather than development to avoid misinterpreting trends.

Chapter 3 addressed both ontogenetic and regional differences in muscle density using a sample of New Zealand white rabbits (*Oryctolagus cuniculus*). These findings demonstrated that muscle density varies according to both age and anatomical region; however, these differences are quite small and therefore, the practical significance is relatively limited. Nonetheless, this study does provide evidence that the use of the previously determined constants (Méndez and Keys, 1960; Murphy and Beardsley, 1974) within the field may not be the best practice. Henceforth, when feasible, muscle density should be determined on a case by case basis.

However, recognizing that density is relatively complex without the specialized kit used in this research (determining muscle volume is difficult due to the irregularity in muscle shape), it is not likely to always be feasible or realistic to determine muscle density for individual muscles. This study is important because it not only demonstrated the difficulty of employing density  $\hat{\delta}$ universal constants $\hat{\delta}$  that have been utilized for years, but provides an array of different values for different age groups as well as anatomical regions that can be selected and more appropriately applied. To this end a density of 1.0558 would be appropriate to use for general

muscles from an adult, 1.0502 for juveniles, and 1.0597 for adult leg muscles and 1.0518 for adult head muscles. (Other specific values can be found in chapter 3.)

Beyond documenting that there are differences in muscle architecture in different ages and anatomical regions, the histological correlates thereof were also demonstrated; for 2 individuals from each age-cohort, each muscle was evaluated histologically and found that with increasing age, the average area of each individual muscle fiber also increased. The percent of the sampled area that consisted of muscle fibers was also observed to be significantly higher between the youngest age-cohort and each of the other two age cohorts.

The other extremely important though strangely overlooked elements evaluated within this body of work were the effects of preservation, both acute and long-term. Chapter 4 addresses the effects of repetitive freeze-thaw cycling and recent fluid preservation on muscle mass, volume and density on mammalian skeletal muscles within the model system, New Zealand White rabbits, *Oryctolagus cuniculus*.

The findings indicate that freezing once or twice led to a 3% and 9% decrease in mass and volume respectively, and these differences were found to be insignificant compared to the fresh tissue. It was also noted that in both of the freezing treatment groups, muscle density was virtually unchanged relative to that of the fresh tissue. When the tissue was fixed in formalin for two weeks, there was a 24% reduction in muscle mass and volume with less than a 1percent reduction in density. Much more extreme differences in the ethanol treatment groups were observed. The findings indicate that on average, muscles lose 41%, 43% and 39% mass after 1, 3, and 6 months of ethanol storage. Slightly smaller changes in muscle volume were observed with an 39%, 41% and 36% decline in volume after 1,3 and 6 months in ethanol respectively.

Changes in muscle density were relatively conserved with approximately a 3% decline after the first few months in ethanol.

In short, muscles that were preserved by freezing did not change significantly relative to the fresh muscles, while muscles fixed in formalin lost approximately 24 % of their mass and volume and muscles stored in ethanol exhibited an approximate 40% loss of mass and volume after the first month and slight subsequent decreases in the months to follow. The findings of this study are particularly important because they provide insight into which methods for short-term preservation are least detrimental to the variables of interest in studies of muscle architecture. Given that these results indicate freezing affects the mass and volume the least (3% for one freeze-thaw cycle and 9% for two) and this can be relatively easily corrected for, freezing may be the preferable short-term preservation method. However, if histological analyses are desired as well then freezing rate/temperature should be considered. Freezing at a slower rate leads to the formation of large ice crystals between muscle fibers, while faster rates of freezing leads to the formation of smaller ice crystals within the muscle fibers themselves (Cook et al., 1926; Luyet, 1964; Raheli et al., 1985; Gruji et al., 1993; Petrovi et al., 1993). Therefore, if the histological target is the muscle fibers themselves, then a faster rate of freezing (e.g., -80°C) may be preferable to prevent the formation of large ice crystals. However, for studies focused on connective tissues surrounding muscle fibers, a faster rate (e.g., -80°C or snap freezing with liquid nitrogen) of freezing may reduce the freezing artifact in the region of interest.

The findings pertaining to the effects of formalin fixation and short-term ethanol storage are also important in that they provide a way to correct for the loss of muscle mass, volume and density associated with the short-term effects of ethanol storage. This can be particularly useful because freezing specimens may not always be a feasible option or specimens may be obtained

from collaborators or museums that used methods for fluid preservation. Therefore, based on the effects of short-term preservation observed, the following correction factors (multipliers) were calculated: *Mass*: ethanol 1.69; 10% formalin 1.32; frozen once 1.03; frozen twice 1.09; *Volume*: ethanol 1.64; 10% formalin 1.32 ; frozen once 1.03 ; frozen twice 1.10 ; *Density*: ethanol 1.03; 10% formalin 1.00; frozen once 1.00; frozen twice 1.00.

However, most of the ethanol stored specimens available for anatomical study are museum specimens that have been preserved for many years ó the subject of Chapter 5, that evaluated the long-term effects of ethanol storage on muscle architecture including muscle mass, volume, density, and fascicle length. To accomplish this, each variable was evaluated over a decadal time scale spanning 130 years in a sample of fresh as well as museum preserved, house mice, *Mus musculus*. Average relative muscle mass, relative volume and density plateaued after decreasing by 62%, 60% and 5% respectively. In addition, no correlation was found between fascicle length and time in ethanol storage suggesting that, when specimens are fixed intact, fascicle length remains fairly constant. This finding mirrors previous reports by Cutts (1988). Additionally, through histological evaluation of these muscles and based on the very strong positive correlation between volume and fiber area it was determined, that, while the muscle fascicles did not shrink lengthwise, they did shrink in cross-section.

These findings are interesting in that the percent differences observed for the long-term effects of ethanol storage are substantially greater than those observed for the acute effects of ethanol storage. There are several possible explanations for these differences. One potential explanation for these differences is that the study addressing the acute effects of ethanol storage (Chapter 4) was much more controlled than the study addressing the long-term effects of ethanol storage (Chapter 5). This is because the entire sample for Chapter 4 was obtained fresh and all

specimens were fixed using identical methods. This is not an assertion that can be made based on the sample for Chapter 5 because this fixed portion of the sample was obtained from museums. Furthermore, because the sample spans such a vast time range (dating back to 1889), the chemicals and methods utilized for fluid preservation have likely evolved overtime. While it is known that each specimen was being stored in 70% ethanol when they were obtained, it is uncertain if this was always the case or if they were fixed prior to being placed in the ethanol. Both of these factors would influence the percent decrease in each of the variables of interest. For instance, many of the oldest fluid preserved specimens were preserved directly in alcohol thus skipping the fixation step altogether which has been demonstrated to rapidly dehydrate the tissue (Simmons, 2014).

Another factor that could help to explain these differences is that some specimens may not have been fixed immediately after obtaining them, and therefore, it is possible that the specimens may have been frozen prior or that the rotting process may have started (more likely within the oldest portion of the sample). Contrastingly, it is known that this was not the case in the acute study as each specimen was fixed quickly after obtaining them.

The manner that these specimens were stored could also help to explain the differences observed. Climate control of fluid collections is critical in that it helps to maintain effective seals on the containers in which the specimens are stored (Horie, 1994; van Dam, 2000). While maintaining a relatively constant ambient temperature is not necessarily a problem today, it would have been during the time some of the oldest specimens in the sample would have been placed in preservative. Fluctuations in temperature resulting in ineffective container seals may have allowed for the evaporation and therefore, concentration of the chemicals and further dehydration.

### *Limitations and Future Directions*

As with most scientific research, the studies contained in this dissertation each had important limitations and opened up new and interesting avenues of questions as well. One of these extensions has already been published: the ontogenetic scaling of the masticatory muscles in Chapter 2 prompted the question about whether the same scaling patterns were held identical in the forearm muscles of the same individual mouse lemurs. The follow-up study (Boettcher et al., 2019) did indeed show similar patterns though key inflections happened at slightly different life history stages. Even though these mouse lemur studies included the largest intraspecific, known-age ontogenetic sample of any primate muscle research ever published, adding more older animals might yield more insight into the effects of senescence on muscle architecture. This was a limitation of Chapter 3 as well, which was constrained to only infants and young and prime aged adults. A study targeting particularly old animals would certainly be warranted.

Another limitation of this dissertation is that it really only evaluated gross structures and their simple histological areas. To this end, only the size of muscle fibers themselves was evaluated and the proportion of the sampled area they occupied. However, the proportion of other types of tissues present such as fat and collagen was not taken into consideration. Future studies could incorporate more complex staining methods, like a trichrome stain for instance, that would differentiate these tissues so that regional and ontogenetic trends of muscle composition could be more holistically evaluated quantitatively. Muscle fiber type composition has been shown to differ based on anatomical region (Gollnick et al., 1974), and different types of fibers have slightly different contractile properties. Given this, it would be valuable to replicate the ontogenetic and regional examination (e.g., the sample evaluated in Chapter 3) with a deeper analysis of the variation in muscle fiber type using, for instance, histochemical

approaches. Understanding the distribution of fiber types and their effects of the muscle contractile properties could help further refine the type of corrections at the heart of this dissertation.

Further, there are other aspects of specimen preservation that would be valuable to examine. For instance, although Chapter 4 evaluated the two most common preservational practices (i.e., freezing at  $-20^{\circ}\text{C}$  and standard formalin fixation followed by ethanol storage), only the effects of a couple cycles of freezing with relatively short periods between them (i.e., 1 month) were evaluated. Therefore, the correction factors might not be appropriate to apply to muscle tissue that has been frozen for extended periods of time. For instance, specimens that are exposed to the air in  $-20\text{C}$  freezers for long periods of time get "freezer burn" a type of preservation that dehydrates the surfaces of the tissues (Lavety, 1991). Furthermore, the freezing and thawing was relatively controlled, a condition which is not always possible, for instance in circumstances like freezer failure. When freezers fail, the contents therein begin to thaw and this introduces a whole other set of confounding variables. For instance, the preservational state of a specimen would be influenced by the degree of thawing that occurred, which itself would be influenced by factors that include freezer insulation and specimen specific properties such as whether it was intact or had been previously skinned. Another potential issue with freezer failure is that depending on the time between the freezer failure and discovery, the specimens could undergo degrees of rotting i.e., some degree of cell lysis which would likely lead to substantial reductions in muscle mass and volume. Therefore, these correction factors should only be applied to specimens that have been frozen and thawed in a controlled manner, and a follow-up study examining the effects of freezer failure might be valuable to colleagues trying to salvage rare specimens that might have been otherwise lost in this manner.

Somewhat related, although this research shows that freezing at -20C has a minimal detrimental effect on muscle architecture, there are potentially even better ways to freeze specimens more rapidly if the highest level of preservation is desired. For instance, many labs have the ability to freeze specimens in -80C freezers or liquid nitrogen. Although this is seldom done in anatomical work beyond that focusing on certain histological aspects that are damaged during slow-freezing, it would be valuable to evaluate the relative value of preserving larger specimens in this manner.

Arguably the biggest limitation of this dissertation is that, like all of the other previous studies that evaluated muscle density and most of the other studies that evaluated muscle ontogeny, it focused on a limited taxonomic sample. It was important for the specific questions being evaluated in these chapters to control for the kinds of variables that taxonomic breadth would have introduced, but this control introduced its own confounding variables. For instance it could be that the differences seen in the preservation chapters (4 & 5) were influenced not only by the time scopes that they covered (acute and long-term respectively), but also by the sizes of the specimens that they focused on. Ethanol does not penetrate tissue very quickly (Simmons, 2014) and therefore, it is possible that the rabbit muscles would not have been as drastically dehydrated due to being larger in size and therefore, less susceptible to severe dehydration than were the mice. More generally, it would be valuable to study different preservational effects in a broad sample of species. For instance, instead of trying to capture as much time depth or intraspecific variation as were the goals in the chapters in this dissertation, it would be valuable to fresh and preserved pairs or small samples of as many species as possible, preferably from a constrained target lineage that spans as much size breadth as possible. This may be possible with lineages like bats (common in museum fluid collections and usually available fresh as well) or

common taxa like raccoons, foxes and other furbearers or perhaps even larger animals like bears and wolves. With samples like these, interspecific differences in the variables and how preservation may affect them could be more comprehensively evaluated.

### *Conclusions*

Overall, this body of work has demonstrated the physiological influences of age and anatomical region as well as the effects of the preservation process on muscle architecture variables. Understanding how all of these factors influence muscle architecture is particularly important because of the vast functional inferences that can be made using these variables. Therefore, it is not only necessary to understand what these influences are, but ultimately it is imperative to correct for them. For the first time with muscle architecture, the studies in this dissertation do just that. Following these results researchers will now be able to use a density measure that was evaluated more systematically than any of those that are widely used, and they can choose more specific values based on a focal region or ontogenetic stage. Further, this research provides the first comprehensive corrections for the kinds of preservational states that most anatomists encounter regularly or states that have dramatic effects on the functional interpretation of the specimens, but have never been accounted for. Therefore, these corrections may revolutionize the way functional morphologists are able to combine complex samples and even potentially go back to previously published studies and correct anomalous findings.

## References:

- Boettcher ML, Leonard KC, Dickinson E, Aujard F, Herrel A, Hartstone Rose A. 2019. The forearm musculature of the gray mouse lemur (*Microcebus murinus*): An ontogenetic study. *Anat Rec*.
- Bons N, Rieger F, Prudhomme D, Fisher A, Krause KH. 2006. *Microcebus murinus*: a useful primate model for human cerebral aging and Alzheimer's disease? *Genes Brain Behav* 5:120-130.
- Chazeau C, Marchal J, Hackert R, Perret M, Herrel A. 2012. Proximate determinants of bite force capacity in the mouse lemur. *J Zool* 290:42-48.
- Cook G, Love E, Vickery J, Young WJ. 1926. Studies on the Refrigeration of Meat. I. Investigations into the Refrigeration of Beef. *Australian Journal of Experimental Biology and Medical Science* 3:15-31.
- Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel J-P, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M. 2010. Sarcopenia: European consensus on definition and diagnosis. *Age Ageing* 39:412-423.
- Cutts A. 1988. Shrinkage of muscle fibres during the fixation of cadaveric tissue. *J Anat* 160:75.
- Gollnick PD, Sjödin B, Karlsson J, Jansson E, Saltin B. 1974. Human soleus muscle: A comparison of fiber composition and enzyme activities with other leg muscles. *Pflügers Archiv* 348:247-255.
- Gruji R, Petrovi L, Pikula B, Amidfi L. 1993. Definition of the optimum freezing rate. 1. Investigation of structure and ultrastructure of beef *M. longissimus dorsi* frozen at different freezing rates. *Meat Sci* 33:301-318.
- Horie V. 1994. Environmental control for spirit specimens. *Newsletter (Biology Curators' Group)* 6:43-44.
- Lavety J. 1991. Physio-chemical problems associated with fish freezing. In: *Food Freezing: Springer*. p 123-131.
- Luyet BJ. 1964. Effects of freezing on muscle tissue. In: *Proc 17th Reciprocal Meat Conf. Madison, Wisconsin: American Foundation for Biological Research*.
- Méndez J, Keys A. 1960. Density and composition of mammalian muscle. *Metab: Clin Exp* 9:184-188.
- Murphy RA, Beardsley AC. 1974. Mechanical properties of the cat soleus muscle in situ. *The American journal of physiology* 227:1008-1013.

- Perry JMG, Hartstone-Rose A, Wall CE. 2011. The jaw adductors of strepsirrhines in relation to body size, diet, and ingested food size. *Anat Rec* 294:712-728.
- Petrovi L, Gruji R, Petrovi M. 1993. Definition of the optimal freezing rate. 2. Investigation of the physico-chemical properties of beef *M. longissimus dorsi* frozen at different freezing rates. *Meat Science* 33:319-331.
- Raheli S, Pua S, Gawwad A. 1985. Structure of beef longissimus dorsi muscle frozen at various temperatures: Part 1. histological changes in muscle frozen at 10, 22, 33, 78, 115 and 196 C. *Meat Sci* 14:63-72.
- Rosenberg IH. 1997. Sarcopenia: origins and clinical relevance. *J Nutr* 127:990S-991S.
- Simmons JE. 2014. *Fluid preservation: a comprehensive reference*. Lanham, MD: Rowman & Littlefield.
- van Dam AJ. 2000. The interactions of preservative fluid, specimen container, and sealant in a fluid collection. *Collection Forum* 14:78-92.

**APPENDICES**

**Appendix A: Chapter 2 Supplemental Materials**

**Table 2.S1.** Ontogenetic sample obtained from the Muséum National d'Histoire Naturelle in Paris, France by age.

	<b>Specimen ID</b>	<b>Sex</b>	<b>Age (years)</b>	<b>Body Mass (g)</b>
<b>Infant</b> (N=4; F=1; M=3)	Infant from 119EB	F	0.014	6
	Infant from 162H	M	0.03	10
	Infant from 172IB	M	0.11	36
	Infant from 276B	M	0.16	41
<b>Juvenile</b> (N=4; F=3; M=1)	Infant from 943GF	F	0.22	38
	102CA	F	0.36	50
	147EE	M	0.37	98
	276AC	F	0.5	52
<b>Adult</b> (N=13; F=5; M=8)	139BB	M	0.97	87
	278ACC	F	2	78
	911FBJ	F	2.22	128
	219E	M	2.38	102
	241AA	M	2.79	62
	189GAA	M	3.33	81
	119CBB	M	3.66	91
	893AAJ	M	3.66	116
	153F	F	3.92	86

Table 2.S1 (Continued).

	206DBA	M	3.93	80
	921BAC	F	4.42	136
	989EB	F	4.7	118
	225A	M	5.42	86
Senescent (N=9; F=1; M=8)	143CAD	M	5.88	74
	143CAC	M	5.92	73
	163DE	M	5.95	90
	100DBA	M	6	70
	223A	M	6.2	66
	113B	M	6.28	53
	245BB	F	7	56
	143CAA	M	7.01	96
	883DEM	M	8	98
Unknown (N=3; F=0; M=3)	No number 1	M	unknown	78
	No number 2	M	unknown	82
	Z93265	M	unknown	68

**Table 2.S2.** Equations for each of the fitted curves from each of the bivariate plots.

Regression	Equation for Fitted Curve
Log Adductor PCSA vs Log Age	$\text{Log Adductor PCSA (cm}^2)^{(1/2)} = -0.027397 + 0.1318052 * \text{Log Age (years)} - 0.1051152 * (\text{Log Age (years)} - 0.15951)^2$
Log Total Masseter PCSA vs Log Age	$\text{Log Total Masseter PCSA (cm}^2)^{(1/2)} = -0.22908 + 0.1416485 * \text{Log Age (years)} - 0.0949447 * (\text{Log Age (years)} - 0.15951)^2$
Log Temp. PCSA vs Log Age	$\text{Log Temp. PCSA (cm}^2)^{(1/2)} = -0.21628 + 0.1432414 * \text{Log Age (years)} - 0.1060308 * (\text{Log Age (years)} - 0.15951)^2$
Log MP PCSA vs Log Age	$\text{Log MP PCSA (cm}^2)^{(1/2)} = -0.449909 + 0.1112891 * \text{Log Age (years)} - 0.1375233 * (\text{Log Age (years)} - 0.15951)^2$
Log Adductor MM vs Log Age	$\text{Log Adductor Mass (g)}^{(1/3)} = -0.124065 + 0.0738649 * \text{Log Age (years)} - 0.0956959 * (\text{Log Age (years)} - 0.24489)^2$
Log Total Masseter MM vs Log Age	$\text{Log Total Masseter Mass (g)}^{(1/3)} = -0.248976 + 0.0593702 * \text{Log Age (years)} - 0.0941387 * (\text{Log Age (years)} - 0.24489)^2$
Log Temp. MM vs Log Age	$\text{Log Temp. Mass (g)}^{(1/3)} = -0.221721 + 0.0763819 * \text{Log Age (years)} - 0.1002467 * (\text{Log Age (years)} - 0.24489)^2$
Log MP MM vs Log Age	$\text{Log MP Mass (g)}^{(1/3)} = -0.466223 - 0.0245 * \text{Log Age (years)} - 0.1658636 * (\text{Log Age (years)} - 0.24489)^2$
Log Adductor FL vs Log Age	$\text{Log Adductor FL (cm)} = -0.328099 - 0.0089892 * \text{Log Age (years)} - 0.0720451 * (\text{Log Age (years)} - 0.24489)^2$
Log Total Masseter FL vs Log Age	$\text{Log Total Masseter FL (cm)} = -0.38599 + 0.0174315 * \text{Log Age (years)} - 0.0416161 * (\text{Log Age (years)} - 0.24489)^2$
Log Temp. FL vs Log Age	$\text{Log Temp. FL (cm)} = -0.263754 - 0.0211211 * \text{Log Age (years)} - 0.0886747 * (\text{Log Age (years)} - 0.24489)^2$
Log MP FL vs Log Age	$\text{Log MP FL (cm)} = -0.542119 - 0.0563075 * \text{Log Age (years)} - 0.1244758 * (\text{Log Age (years)} - 0.24489)^2$
Log BM vs Log Age	$\text{Log BM (g)}^{(1/3)} = 0.6474901 + 0.0173246 * \text{Log Age (years)} - 0.0850562 * (\text{Log Age (years)} - 0.24489)^2$

### Appendix B: Chapter 4 Supplemental Materials

**Table 4.S1.** Pairwise comparisons of GM mass between treatment groups using Tukey-Kramer HSD

Comparisons for all pairs using Tukey-Kramer HSD (GM Mass)						
Level	Level	Difference	Std. Error Difference	Lower CL	Upper CL	p-value
Fresh	3 months EtOH	0.000218	1.39E-05	0.000175	0.000260	<.0001*
Fresh	1 month EtOH	0.000209	1.33E-05	0.000168	0.000249	<.0001*
Frozen once	3 months EtOH	0.000203	1.92E-05	0.000145	0.000262	<.0001*
Frozen once	1 month EtOH	0.000194	1.89E-05	0.000137	0.000251	<.0001*
Fresh	6 months EtOH	0.000194	1.33E-05	0.000154	0.000235	<.0001*
Frozen once	6 months EtOH	0.000180	1.89E-05	0.000123	0.000237	<.0001*
Frozen twice	3 months EtOH	0.000170	1.92E-05	0.000112	0.000228	<.0001*
Frozen twice	1 month EtOH	0.000161	1.89E-05	0.000104	0.000218	<.0001*
Frozen twice	6 months EtOH	0.000147	1.89E-05	0.000090	0.000204	<.0001*
Fresh	Formalin	0.000128	1.33E-05	0.000087	0.000168	<.0001*
Frozen once	Formalin	0.000114	1.89E-05	0.000056	0.000171	<.0001*
Formalin	3 months EtOH	0.000090	1.68E-05	0.000039	0.000141	<.0001*
Formalin	1 month EtOH	0.000081	1.63E-05	0.000031	0.000130	<.0001*
Frozen twice	Formalin	0.000080	1.89E-05	0.000023	0.000137	0.0011*
Formalin	6 months EtOH	0.000066	1.63E-05	0.000017	0.000116	0.0022*
Fresh	Frozen twice	0.000047	1.63E-05	-0.000002	0.000097	0.0691
Frozen once	Frozen twice	0.000033	2.11E-05	-0.000031	0.000097	0.6964
6 months EtOH	3 months EtOH	0.000023	1.68E-05	-0.000027	0.000074	0.8014
6 months EtOH	1 month EtOH	0.000014	1.63E-05	-0.000035	0.000064	0.9743
Fresh	Frozen once	0.000014	1.63E-05	-0.000035	0.000064	0.9763
1 month EtOH	3 months EtOH	0.000009	1.68E-05	-0.000042	0.000060	0.9981

**Table 4.S2.** Pairwise comparisons of GM volume between treatment groups using Tukey-Kramer HSD

<b>Comparisons for all pairs using Tukey-Kramer HSD (GM Volume)</b>						
<b>Level</b>	<b>Level</b>	<b>Difference</b>	<b>Std. Error Difference</b>	<b>Lower CL</b>	<b>Upper CL</b>	<b>p-value</b>
Fresh	3 months EtOH	0.000218	1.39E-05	0.000175	0.000260	<.0001*
Fresh	1 month EtOH	0.000209	1.33E-05	0.000168	0.000249	<.0001*
Frozen once	3 months EtOH	0.000203	1.92E-05	0.000145	0.000262	<.0001*
Frozen once	1 month EtOH	0.000194	1.89E-05	0.000137	0.000251	<.0001*
Fresh	6 months EtOH	0.000194	1.33E-05	0.000154	0.000235	<.0001*
Frozen once	6 months EtOH	0.000180	1.89E-05	0.000123	0.000237	<.0001*
Frozen twice	3 months EtOH	0.000170	1.92E-05	0.000112	0.000228	<.0001*
Frozen twice	1 month EtOH	0.000161	1.89E-05	0.000104	0.000218	<.0001*
Frozen twice	6 months EtOH	0.000147	1.89E-05	0.000090	0.000204	<.0001*
Fresh	Formalin	0.000128	1.33E-05	0.000087	0.000168	<.0001*
Frozen once	Formalin	0.000114	1.89E-05	0.000056	0.000171	<.0001*
Formalin	3 months EtOH	0.000090	1.68E-05	0.000039	0.000141	<.0001*
Formalin	1 month EtOH	0.000081	1.63E-05	0.000031	0.000130	<.0001*
Frozen twice	Formalin	0.000080	1.89E-05	0.000023	0.000137	0.0011*
Formalin	6 months EtOH	0.000066	1.63E-05	0.000017	0.000116	0.0022*
Fresh	Frozen twice	0.000047	1.63E-05	-0.000002	0.000097	0.0691
Frozen once	Frozen twice	0.000033	2.11E-05	-0.000031	0.000097	0.6964
6 months EtOH	3 months EtOH	0.000023	1.68E-05	-0.000027	0.000074	0.8014
6 months EtOH	1 month EtOH	0.000014	1.63E-05	-0.000035	0.000064	0.9743
Fresh	Frozen once	0.000014	1.63E-05	-0.000035	0.000064	0.9763
1 month EtOH	3 months EtOH	0.000009	1.68E-05	-0.000042	0.000060	0.9981

**Table 4.S3.** Pairwise comparisons of GM density between treatment groups using Tukey-Kramer HSD

<b>Comparisons for all pairs using Tukey-Kramer HSD (GM Density)</b>						
<b>Level</b>	<b>Level</b>	<b>Difference</b>	<b>Std. Error Difference</b>	<b>Lower CL</b>	<b>Upper CL</b>	<b>p-value</b>
Formalin	6 months EtOH	0.0426	1.44E-03	0.0382	0.0470	<.0001*
Frozen once	6 months EtOH	0.0404	1.67E-03	0.0353	0.0454	<.0001*
Frozen twice	6 months EtOH	0.0392	1.67E-03	0.0341	0.0442	<.0001*
Fresh	6 months EtOH	0.0386	1.18E-03	0.0350	0.0421	<.0001*
Formalin	3 months EtOH	0.0369	1.48E-03	0.0324	0.0414	<.0001*
Formalin	1 month EtOH	0.0353	1.44E-03	0.0310	0.0397	<.0001*
Frozen once	3 months EtOH	0.0346	1.70E-03	0.0295	0.0398	<.0001*
Frozen twice	3 months EtOH	0.0334	1.70E-03	0.0282	0.0386	<.0001*
Frozen once	1 month EtOH	0.0331	1.67E-03	0.0280	0.0382	<.0001*
Fresh	3 months EtOH	0.0328	1.23E-03	0.0291	0.0365	<.0001*
Frozen twice	1 month EtOH	0.0319	1.67E-03	0.0268	0.0369	<.0001*
Fresh	1 month EtOH	0.0313	1.18E-03	0.0277	0.0349	<.0001*
1 month EtOH	6 months EtOH	0.0073	1.44E-03	0.0029	0.0117	<.0001*
3 months EtOH	6 months EtOH	0.0058	1.48E-03	0.0013	0.0103	0.0040*
Formalin	Fresh	0.0041	1.18E-03	0.0005	0.0076	0.0160*
Formalin	Frozen twice	0.0035	1.67E-03	-0.0016	0.0085	0.378
Formalin	Frozen once	0.0022	1.67E-03	-0.0028	0.0073	0.829
Frozen once	Fresh	0.0018	1.44E-03	-0.0026	0.0062	0.8689
1 month EtOH	3 months EtOH	0.0015	1.48E-03	-0.0030	0.0060	0.9481
Frozen once	Frozen twice	0.0012	1.86E-03	-0.0044	0.0069	0.9946
Frozen twice	Fresh	0.0006	1.44E-03	-0.0038	0.0050	0.9996