



Microanatomical correlates of muscle attachment and their implications for muscular reconstruction
by Tobin Lee Hieronymus

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Sciences in
Biological Sciences

Montana State University

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Abstract:

Reconstructions of the musculature of extinct taxa are often used as a basis for paleobiological inference. Most reconstructions employ a combination of phylogenetic and osteological criteria for the acceptance of specific morphologies. Phylogenetically based reconstructions are often equivocal, given a high morphological diversity in extant representatives. In these cases, osteological criteria must carry the reconstruction.

Actualistic research on osteological correlates of muscle attachment has shown that most features interpreted as muscle attachments have little predictive reliability (e.g. 30% for correlates of direct attachment). Microanatomical features that are directly involved in muscle attachment such as extrinsic fibers (Sharpey's fibers) present better candidates for reliable correlates of muscle attachment.

Seven individuals representing six species were examined. Areas and types of muscle attachment were determined by dissection. Extrinsic fibers at areas of attachment were characterized using scanning electron microscopy. Twenty-nine plots were sampled, representing tendinous, aponeurotic, and direct attachments, as well as areas without muscle attachment. The microscopic relationships of the tissues present at the muscle/bone interface were examined in histological sections from these same specimens.

Density of extrinsic fibers per unit area of attachment is significantly greater for areas of tendinous and aponeurotic attachment than for the other types of attachment studied. Ligamentous attachments also show high densities of extrinsic fibers, indicating that any attachment of dense regular connective tissue to bone is accomplished by extrinsic fibers. The density of extrinsic fibers at tendinous and aponeurotic attachments shows a significant correlation with an index of stress exerted at the attachment, raising the possibility of using extrinsic fiber density as an indicator of force instead of an indicator of muscle attachment. A more accurate biomechanical model is needed to verify these results.

Examinations of high-fidelity casting procedures indicate that accurate casts can be made to examine fossil bone surfaces for microscopic structures, but casts of fossilized bone expected to show extrinsic fibers instead showed ambiguous morphology that could not be assigned to extrinsic fibers with confidence. At the current time, histology remains the most reliable method for determining the existence and density of extrinsic fibers in fossilized bone.

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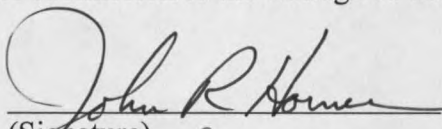
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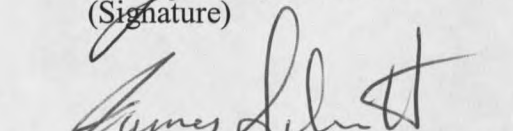
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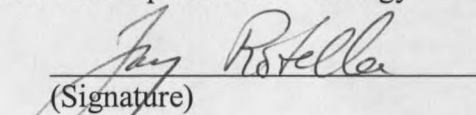
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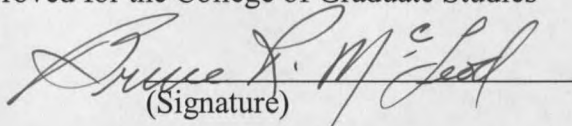
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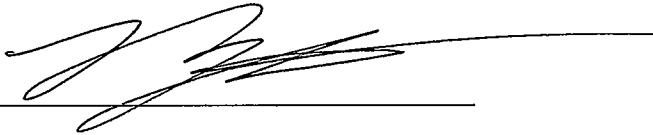
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ABSTRACT

Reconstructions of the musculature of extinct taxa are often used as a basis for paleobiological inference. Most reconstructions employ a combination of phylogenetic and osteological criteria for the acceptance of specific morphologies. Phylogenetically based reconstructions are often equivocal, given a high morphological diversity in extant representatives. In these cases, osteological criteria must carry the reconstruction.

Actualistic research on osteological correlates of muscle attachment has shown that most features interpreted as muscle attachments have little predictive reliability (e.g. 30% for correlates of direct attachment). Microanatomical features that are directly involved in muscle attachment such as extrinsic fibers (Sharpey's fibers) present better candidates for reliable correlates of muscle attachment.

Seven individuals representing six species were examined. Areas and types of muscle attachment were determined by dissection. Extrinsic fibers at areas of attachment were characterized using scanning electron microscopy. Twenty-nine plots were sampled, representing tendinous, aponeurotic, and direct attachments, as well as areas without muscle attachment. The microscopic relationships of the tissues present at the muscle/bone interface were examined in histological sections from these same specimens.

Density of extrinsic fibers per unit area of attachment is significantly greater for areas of tendinous and aponeurotic attachment than for the other types of attachment studied. Ligamentous attachments also show high densities of extrinsic fibers, indicating that any attachment of dense regular connective tissue to bone is accomplished by extrinsic fibers. The density of extrinsic fibers at tendinous and aponeurotic attachments shows a significant correlation with an index of stress exerted at the attachment, raising the possibility of using extrinsic fiber density as an indicator of force instead of an indicator of muscle attachment. A more accurate biomechanical model is needed to verify these results.

Examinations of high-fidelity casting procedures indicate that accurate casts can be made to examine fossil bone surfaces for microscopic structures, but casts of fossilized bone expected to show extrinsic fibers instead showed ambiguous morphology that could not be assigned to extrinsic fibers with confidence. At the current time, histology remains the most reliable method for determining the existence and density of extrinsic fibers in fossilized bone.

INTRODUCTION

Any investigation into the biology of an extinct organism is faced with limited data. While finds from Konservat-Lagerstätten are preserved in very fine detail (e.g. outlines of soft tissues in the Posidonienschiefer and the Grube Messel Lagerstätte; preserved muscle tissue in the Solnhofen and the Green River Lagerstätte) (Martin 1999, pp. 235-267), most fossils are a fragmentary representation of the organism in question. The bony skeleton is the most commonly preserved element of vertebrates. Although information can be taken from fossilized bones, such as biomechanical attributes of the skeletal system, or details of the animal's growth and physiology (Padian *et al.* 1995, de Ricqlès *et al.* 2000), vast amounts of information are lost with the destruction of nonmineralized tissues prior to fossilization.

While nonmineralized tissues are rarely directly preserved, bone and other mineralized tissues form within a soft tissue matrix (the 'functional matrix' of Moss 1971). This functional matrix, specifically the attachment of other connective tissues such as cartilage, ligaments, and tendons, has the potential to modify the morphology and texture of periosteal bone (Moss 1971). Bone is also remodeled in response to purely mechanical stresses (Francillon-Vieillot *et al.* 1990).

Muscle forms part of the functional matrix, and is of considerable importance in defining the potential performance of extinct animals (Parrish 1997). Skeletal muscle is responsible for behaviors as diverse as feeding, locomotion, predation ecology, social behavior and sexual behavior. Any hypothesis of functional morphology using skeletal elements requires that the musculature be considered for complete context.

Attachments of muscle to bone can be grouped into three categories: direct, aponeurotic, and tendinous. Direct attachments form by way of very short ($\sim 20\mu\text{m}$) lengths of reticular connective tissue, arising from the terminal ends of the sarcolemma of individual muscle fibers, and attaching to the periosteal surface of bone (Goss 1944). Aponeurotic and tendinous muscle attachments have similar origins in the sarcolemma of muscle fibers, but they coalesce into longer (up to meters in length) sections of connective tissue (Elliot 1965). The latter two attachment types represent the end members of a continuum; the dense regular connective tissue of an aponeurosis forms a sheet, whereas that of a tendon forms a roughly cylindrical cord. Intermediate morphologies exist between these extremes (Kardong 1997, p. 357).

On a gross level, bone responds to the attachment of muscles in characteristic patterns. Tendinous and aponeurotic muscle attachments are often associated with rugose periosteal bone surfaces, and may be associated with bony processes, such as trochanters or tubercles. Direct muscle attachments are often associated with shallow fossae and smooth areas of periosteal bone, sometimes with thin rugose bands delineating the extent of the muscle attachment (Bryant and Seymour 1990). The bone morphologies associated with muscle attachment are referred to as osteological correlates. They allow for a limited interpretation of the morphology of muscle tissue, and have been used as a basis for reconstructions of musculature in extinct animals.

Muscular Reconstructions: Review

Reconstruction of musculature based on osteological evidence has been regarded as a useful interpretive tool for nearly a century (Parrish 1997). The prevailing use has been for inference of life habits and behaviors of extinct animals. Interpretations of the biology of individual animals are often used as a basis for further paleobiological and paleoecological inference, ranging from associated behaviors to paleocommunity structure and evolution (Witmer 1997).

There are three major approaches to muscular reconstruction, each based on comparative attributes of organismal myology. The first of these approaches is functional reconstruction, in which an osteological setting and function are assumed, and the myology is then reconstructed to the dimensions required to perform that function (Bryant and Russell 1992). In cases of readily defined morphological constraint, such as body shape in large swimmers (e.g. sharks, dolphins, tuna, and ichthyosaurs), this kind of extrapolatory reconstruction is well-founded (Webb 1984, Massare 1988). However, in the case of skeletal musculature, morphological constraints and causal relationships are not well documented (Gans and de Vree 1987). In addition, muscles show great variety in attachment surfaces; many muscles attach to nonmineralized features, such as joint capsules, ligaments, and other muscles (Elzanowski 1993, Gao and Messner 1996, Kim *et al.* 1997). Given these uncertainties, functional reconstruction is best employed as an adjunct to other methods of muscular reconstruction (Hutchinson and Garcia, 2002).

A phylogenetic approach can also be employed for muscular reconstruction. In this approach, the myologies of extant taxa are examined, as well as the morphology of

osteological correlates of related extinct taxa. Muscles are then reconstructed following the morphologies present in extant taxa. In the most strict sense of the phylogenetic approach, hypotheses of homology are clearly stated, and greater confidence is assigned to morphologies that are homologous between the extant taxa studied. Romer (1923a, 1923b, 1927) was the first to employ this more explicit homology-based phylogenetic approach to reconstruction. Many workers before and after Romer used comparisons with related groups without stating phylogenetic criteria for accepting or rejecting a particular reconstruction (Russell 1935, Haas 1969, Newman 1970, Russell 1972, Coombs 1978, 1979).

With the application of cladistics to taxonomy, hypotheses of homology are more rigorously testable. As a consequence, support for phylogeny-based reconstructions can now be discussed in more definite terms. Two examples of this shift are Bryant and Russell's (1992) research program for the inference of attributes of fossils and Witmer's (1995) extant phylogenetic bracket approach. In both, closely related extant taxa from above and below the node of the extinct taxon of interest are used as bracket taxa. Reconstructions of any feature of an extinct taxon are then ranked by their degree of agreement with the states of that feature present in the extant bracket taxa. In the former, a wider set of outgroup taxa are examined in addition to the bracket taxa, and functional extrapolation is used to corroborate phylogenetic inferences.

The third approach, reconstruction from osteological features, relies on comparisons derived from the study of extant animals without necessarily specifying a phylogenetic context. Studies of the gross morphology of the muscle-bone interface in

extant taxa are used to define osteological correlates of muscle attachment, which are then applied to the osteology of extinct taxa. The presence of rugosities and fossae are often cited as osteological correlates of muscle attachment (Osborn 1916, Romer 1923b, Coombs 1978). This is the most frequently employed approach to muscular reconstruction in the dinosaur literature (Table 1). The popularity of this approach is no doubt related to the relative ease and robustness of the method. If existing criteria are used to define osteological correlates, an osteologically based reconstruction requires only the gross observation of fossil specimens. It can be used to reconstruct musculature in groups with morphologies that are widely divergent from the morphologies of their extant relatives, and in groups with unresolved phylogenetic relationships.

Table 1.: A survey of muscular reconstructions of dinosaurian taxa in the literature, including the type of inferences supported by those reconstructions, and the approaches used to create the reconstructions.

Publication	Taxa	Paleobiological Inferences	Methods Used
Osborn 1916	<i>Struthiomimus altus</i> (Saurischia:Ornithomimidae)	Feeding, locomotion	Osteological inference
Gregory and Camp 1918	<i>Ornitholestes</i> (Saurischia:Ornitholestidae)	Stance and locomotion	Osteological and phylogenetic inference
Romer 1923	Saurischia	Stance and locomotion	Osteological and phylogenetic inference
Romer 1927	<i>Thescelosaurus neglectus</i> (Ornithischia: Hypsilophodontidae)	Stance and locomotion	Osteological and phylogenetic inference

Table 1, cont.

Publication	Taxa	Paleobiological Inferences	Methods Used
Russell 1935	Neoceratopsia	Locomotion, feeding, agonistic behavior	Osteological inference, some phylogenetically based comparison
Haas 1955	Neoceratopsia	Diet/Feeding	Osteological and phylogenetic inference
Ostrom 1961	Hadrosauridae	Feeding behavior	-
Galton 1969	<i>Hypsilophodon foxii</i> (Ornithischia: Hypsilophodontidae)	Stance and locomotion	Osteological inference
Ostrom 1969	<i>Deinonychus antirrhopus</i> (Saurischia: Dromaeosauridae)	Stance and locomotion	-
Haas 1969	Ankylosauria	Feeding behavior	Osteological inference, some phylogenetically based comparison
Newman 1970	<i>Tyrannosaurus rex</i> (Saurischia: Tyrannosauridae)	Stance and locomotion	Osteological inference, some phylogenetically based comparison
Russell 1972	<i>Dromiceiomimus</i> (Saurischia:Ornithomimidae)	Locomotion and ecology	Osteological inference, some phylogenetically based comparison
Coombs 1975	Sauropoda	Feeding behavior and ecology	Osteological and phylogenetic inference

Table 1, cont.

Publication	Taxa	Paleobiological Inferences	Methods Used
Coombs 1978, 1979	Ankylosauria	Stance and locomotion, agonistic behavior	Osteological inference with some phylogenetically based comparison
Galton 1983	<i>Dryosaurus</i> (Ornithischia: Hypsilophodontidae)	Feeding behavior	Osteological inference
Tarsitano 1983	<i>T. rex</i>	Stance and locomotion	Osteological and phylogenetic inference
Galton 1985	<i>Sellosaurus gracilis</i> (Saurischia: Plateosauridae)	Feeding behavior	Osteological inference
Norman and Weishampel 1985	Ornithopoda	Feeding behavior	Osteological inference
Molnar and Farlow 1990	"Carnosauria"	Feeding behavior	Osteological inference
Molnar 1993	<i>T. rex</i>	Feeding behavior	Osteological inference
Johnson and Ostrom 1995	<i>Torosaurus latus</i> (Ornithischia: Ceratopsidae)	Stance and locomotion	Osteological inference
Dilkes 2000	<i>Maiasaura peeblesorum</i> (Ornithischia: Hadrosauridae)	Stance and locomotion	Phylogenetic and osteological inference
Dilkes 2001	<i>M. peeblesorum</i>	Stance and locomotion	Osteological inference

The methods of many muscular reconstructions include a combination of phylogenetic and osteological approaches (Table 1). In these approaches, phylogeny is most often used to determine muscle architecture, and osteology is used to assign the size and exact location of attachments. However, in spite of advances in the use of phylogenetic hypotheses, and the combination of osteological and phylogenetic lines of evidence, the explicitly stated approach to most muscular reconstructions remains largely unchanged, with osteological indicators being the primary guides to the assignment of muscular attachments and muscle size (Johnson and Ostrom 1995, Dilkes 2001).

The Importance of Muscular Reconstruction in Paleobiology

The reconstruction of musculature is most often undertaken as a means to infer behaviors that play an important role in the paleobiology and paleoecology of the taxon in question, such as behaviors of locomotion and feeding. As Witmer (1995) has indicated, these initial inferences regarding individual facets of an organism's paleobiology are often the foundation for a series of further inferences. An example of this chain of inference can be found based on the reconstruction of the cranial and pelvic musculature of *Dromiceiomimus* by Russell (1972). From the strength and morphology of the reconstructed musculature, Russell suggests feeding habits and locomotion, extending the latter to paleoecology in the form of potential predation avoidance strategies. Although these suggestions (and most others made from muscular reconstructions) are clearly set forth as speculations, they do influence understanding of the paleobiology of

these animals, and in doing so they have an effect on how additional paleobiological questions are framed.

Problems Facing Phylogenetic and Osteological Muscular Reconstruction

Both phylogenetic and osteological muscle reconstructions encounter circumstances which lower the confidence of their results. The most challenging problem facing phylogenetic reconstructions is presented by taxa that are widely divergent in morphology from their extant bracket taxa. Hadrosaurine and sauropod dinosaurs are examples of such divergences. Both present body plans with major divergences from those of their bracket taxa, Aves and Crocodilia. It may be possible to establish homology between the myologies of the extant bracket taxa, but the morphology of the extinct taxon may be so divergent as to rule out analogy with either bracket taxon. Because phylogenetic reconstruction proceeds by identifying shared character states, it cannot recover novel adaptive response in extinct taxa (Bryant and Russell 1992).

Hutchinson (2001) has addressed this problem by examining phylogenetically intermediate extinct taxa to track the evolution of derived morphologies. The myologies of phylogenetically and morphologically intermediate extinct taxa are reconstructed, and these reconstructions are used to bracket the divergent extinct taxon. For example, in reconstructing the musculature of a hadrosaurine dinosaur, specimens from Aves and Crocodilia would first be used as extant bracket taxa to reconstruct the musculature of a morphologically conservative basal ornithischian dinosaur such as *Lesothosaurus* (Weishampel and Witmer 1990). The reconstruction of *Lesothosaurus* would then be

used to clarify ambiguous osteological correlates within Ornithischia, and would essentially be applied as an extinct bracket taxon (with Aves) to reconstruct the musculature of the hadrosaurine dinosaur. While this method represents the most thorough application of the phylogenetic approach to reconstruction, and hence the most likely to yield accurate results, it also increases the role that osteological information from extinct taxa plays in creating reconstructions.

Osteological approaches to muscular reconstruction are hampered by the problem of low reliability. Recently there have been quantitative studies of the reliability of these relationships (McGowan 1979, 1982, 1986, Bryant and Seymour 1990). Although osteological correlates do very well in representing the rough position and the presence or absence of muscle attachments, the full extent of a muscle attachment and the force exerted by that muscle are not well recorded by gross osteological features.

Actualistic Studies of Gross Osteological Correlates

The quantitative studies of osteological correlates listed in Table 2 were based on the dissection of extant animals to ascertain the true location and extent of muscle attachment. The cleaned bones were examined, and osteological correlates were identified using criteria that have been previously cited in muscular reconstructions of extinct animals. The reliability of the osteological correlates to indicate the true outlines of muscle attachments is represented in these studies by a percent value:

$$\text{Reliability} = \frac{\text{x muscles with accurately recorded muscle attachments}}{\text{n muscles examined}}$$

Using this measurement of reliability, approximately 30% of the muscles of the shoulder region in *Ursus* and *Canis* (Bryant and Seymour 1990), and less than 30% of the muscles in the limb musculature of the Brown Kiwi *Apteryx* (McGowan 1979, 1982) and the flightless rail *Gallirallus* (McGowan 1986) are represented by osteological features that accurately delimit the extent of their attachment.

Bryant and Seymour (1990) also address the differential preservation of osteological correlates of fleshy, tendinous, and aponeurotic muscle attachments. Aponeurotic muscle attachments are relatively well outlined as rugose scars on bone (67% and 91% of the shoulder muscles in *Canis* and *Ursus*, respectively), tendinous attachments are more poorly preserved (50% and 17%), and direct or fleshy attachments are the least well preserved (32% and 17%). The differential representation of the three types of muscle attachments with macroscopic indicators would confound a straightforward correlation between muscle attachment *sensu lato* and any osteological indicator.

With these results, the reliability of these osteological correlates is called into question. Because muscular reconstructions based on osteological evidence represent a large portion of the published work, and because these reconstructions form the basis of further inferences in paleobiology, it is important to identify a more reliable method of testing hypotheses of myology.

Table 2.: A survey of quantitative studies of relationships between muscle attachment and bone morphology.

Publication	Taxa and Region	Reliability of Reconstruction
McGowan 1979	<i>Apteryx mantelli</i> , leg musculature	23%
McGowan 1982	<i>Apteryx mantelli</i> , wing musculature	29%
McGowan 1986	<i>Gallirallus australis</i> , wing musculature	30%
Bryant and Seymour 1990	<i>Ursus</i> and <i>Canis</i> , forelimb musculature	Aponeurotic attachments: 91% in <i>Ursus</i> , 67% in <i>Canis</i> Tendinous attachments: 17% in <i>Ursus</i> , 50% in <i>Canis</i> Direct attachments: 17% in <i>Ursus</i> , 32% in <i>Canis</i>

Other Potential Correlates of Muscle Attachment

The use of gross osteological correlates can be thought of as an examination of the organ-level organization of bone. Although there is not a reliable relationship between the gross morphology of osteological correlates and muscle attachment, the degree of relationship that does exist most likely occurs as a result of a relationship between bone and attached structures on a tissue level of organization (Francillon-Vieillot *et al.* 1990). Examination at the tissue level using histology and scanning electron microscopy may reveal reliable microanatomical correlates of muscle attachment.

The Microscopic Anatomy of Bone

There are two major mechanisms of bone formation: endochondral bone growth and periosteal bone growth. These mechanisms produce morphologically distinct bone tissue (Reith & Ross 1977). As nearly all muscle attachments are found in areas that have been affected by periosteal bone growth, the latter is the mechanism of most concern in this study.

The surface of periosteal bone is covered by a thin layer of tissue called the periosteum. The periosteum comprises a fibrous outer layer of dense irregular connective tissue and an inner cellular layer containing fibroblast cells. In periosteal bone growth, fibroblasts which are furthest away from the outer surface of the periosteum differentiate into osteoblasts. Osteoblasts begin laying down osteoid, a connective tissue matrix similar to cartilage, which contains regular arrays of intrinsic collagen fibers (Boyde 1972). As the periosteum grows away from the osteoblasts, they begin to change the chemistry of the surrounding osteoid, causing the precipitation of carbonate hydroxyapatite, or bone mineral. Periosteal bone is thus formed by the mineralization of an existing intrinsic collagenous framework, most often in the plane of the bone surface (Reith and Ross 1977).

Quantifiable microscopic features on the surface of periosteal bone include ossified intrinsic collagen fibers, which make up the bulk of the visible surface of periosteal bone, and various pits or projections which can be identified as either osteoblast lacunae, osteoclast lacunae, or variably ossified extrinsic fibers (Boyde 1972, Jones and Boyde 1974). Osteoblasts and osteoclasts are functionally related to the

deposition and remodeling of bone. Extrinsic fibers are implicated in the attachment of various structures to the bone surface, such as scales, muscles, ligaments, and other bones (Zylberberg and Meunier 1981, Francillon-Vieillot *et al.* 1990) and provide a strong candidate for a microanatomical correlate of muscle attachment.

Form and Function of Extrinsic Fibers

Extrinsic fibers were first described from the human mandible, where they secure teeth within the thecae (Sharpey 1849-1856; "Sharpey's fibers" in Kolliker 1889). Publications in the human anatomy literature propose a naming convention for Sharpey's fibers which includes all extrinsic fibers entering periosteal bone (Francois *et al.* 2001). However, no study has shown the structural equivalence of the extrinsic fibers anchoring muscles to bone with the extrinsic fibers anchoring teeth in their sockets. The term extrinsic fibers will be used to describe the features studied in this work.

Extrinsic fibers are bundles of collagen fibers, usually ranging from 2-15 μ m in diameter, which penetrate the surface of periosteal bone (Boyde 1972, Cohn 1972a, 1972b, Shackleford 1973, Jones and Boyde 1974, Hamagami 1980, Anderson *et al.* 1993, Kuroiwa *et al.* 1994). It is possible to contrast extrinsic fibers with the smaller intrinsic fibers (Figure 1) which make up the surface of periosteal bone with scanning electron microscopy (SEM). Extrinsic fibers are often mineralized at a slower rate than intrinsic fibers. As a result, they may appear either as projections above the periosteal surface, usually with incompletely mineralized cores (on resting bone surfaces), or as pits with

small projecting fibers (on bone surfaces actively growing by periosteal deposition) (Figure 2) (Boyde 1972, Shackleford 1973).

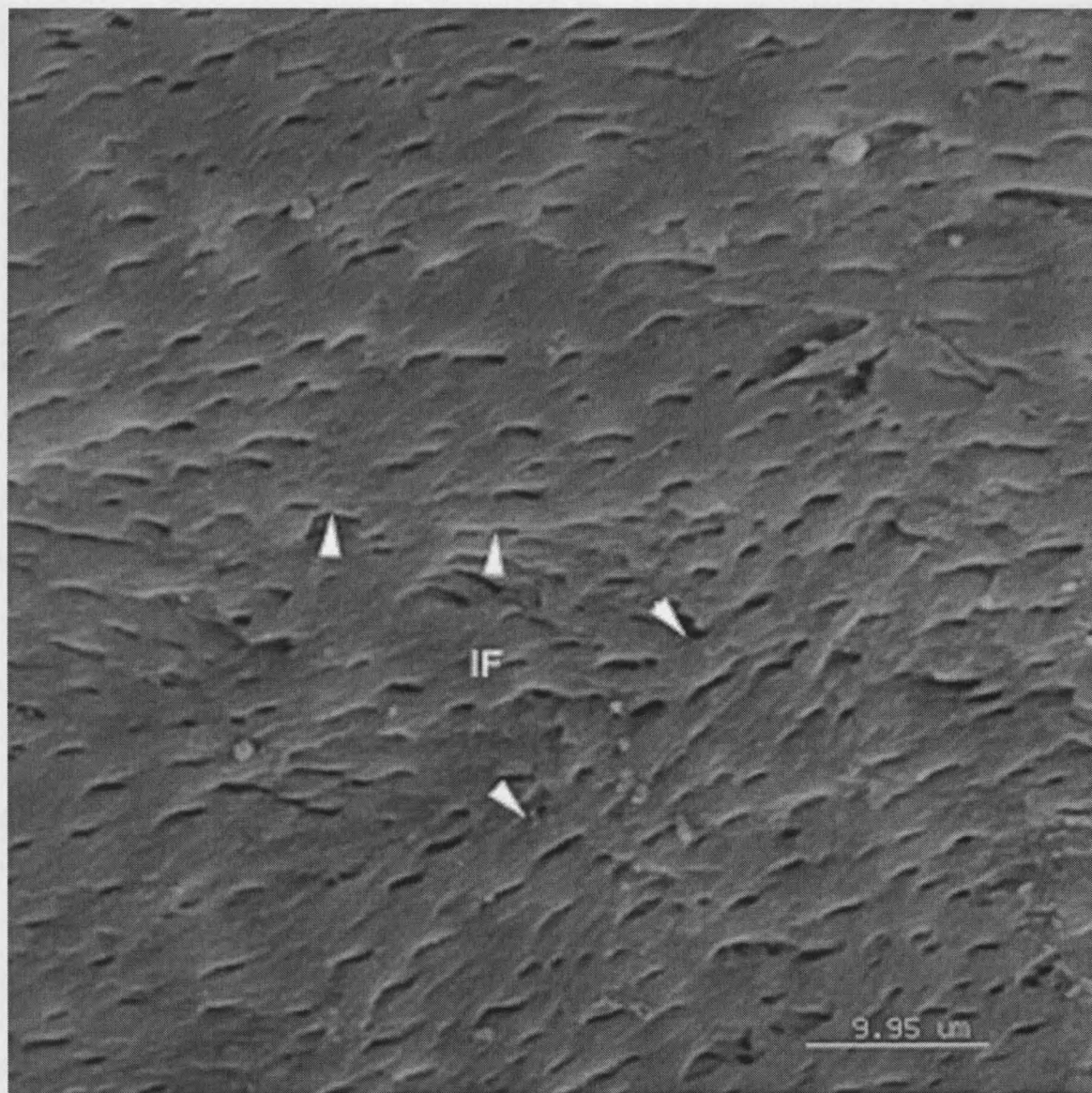


Figure 1. Intrinsic fibers on the periosteal surface of bone. IF: Intrinsic fibers. White arrowheads point to two overlapping tracts of fibers at an oblique angle. *Gekko gecko*, 1500x.

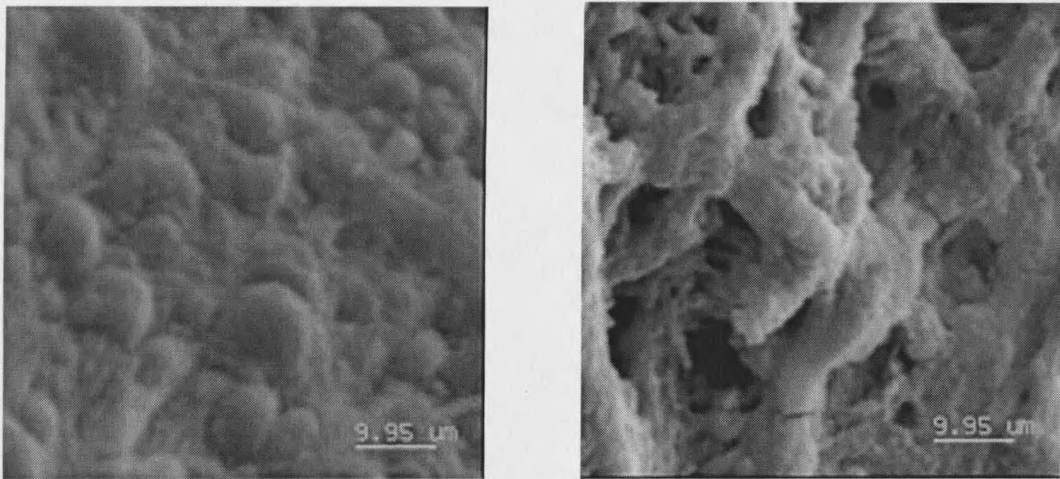


Figure 2. Examples of extrinsic fibers at resting surfaces (left, *Gekko gecko*) and surfaces of active periosteal growth (right, *Dromaius novaehollandiae*). Both at 1500x.

Extrinsic Fibers as Microanatomical Correlates: Review

Several published descriptions indicate the possible use of extrinsic fibers as microanatomical correlates of muscle attachment. Boyde (1972) noted that areas of tendinous and ligamentous attachment have high densities of extrinsic fibers. Jones and Boyde (1974) suggested a clumped arrangement of extrinsic fiber pits as characteristic of areas of muscle attachment. They also found a broad range of densities of extrinsic fibers, from covering ~20% of the periosteal surface at direct muscle attachments to covering ~100% of the periosteal surface at tendinous muscle attachments. Witmer (1997) examined the relationship between muscle attachment *sensu lato* and the pits that extrinsic fibers leave in the periosteal bone surface. Preliminary studies not included in the published work demonstrated approximately a 60% correlation (Witmer, pers. comm.).

Studies by Barton and Keenan (1967) and Anderson *et al.* (1993) have examined the relationships between the diameter or density of extrinsic fibers and stresses exerted on the fibers at their attachments. Both studies describe an increase in diameter and density related to the increased stress placed on functional extrinsic fibers.

Kawamoto (1992) used histological sections to study the relationship between extrinsic fiber diameter and density and the type of attachment present in the origin of the human temporalis muscle. His results indicate an increased density and diameter of extrinsic fibers at the aponeurotic insertion on the ventral aspect of the zygomatic arch, and a relatively lower density and diameter of extrinsic fibers at the direct attachment to the temporal fossa.

One potential confounding factor with using extrinsic fibers as microanatomical correlates of muscle attachment is variation in extrinsic fiber morphology between major phylogenetic groups. Jones and Boyde (1974) reported on a wide range of mean extrinsic fiber diameters for several species within Mammalia. If the morphology of extrinsic fibers varies solely in response to function, their use as a microanatomical correlate of muscle attachment may be straightforward. However, if phylogenetic patterns of variation exist, a documentation of the effects of phylogeny on extrinsic fiber morphology will be necessary before they can be applied as microanatomical correlates.

Purpose and Scope of This Study

This study divides the relationship between muscle attachment and bone into several comparisons, to take into account different attachment types, as well as different

sizes, densities, and distributions of extrinsic fibers. If there are distinct relationships of tissues at different attachment types, their separation will protect any signal that may exist in one relationship from the 'noise' presented by other relationships. This allows a more precise characterization of the attributes of the extrinsic fibers in that relationship, allowing in turn a more precise description of any functional and morphological relationships between bone surface microstructure and muscle attachments. Quantifying the strength of these relationships is the first step in determining whether microscopic bone texture can be used as a reliable tool for inferring myology in extinct taxa.

HYPOTHESES

Relationship Between Extrinsic Fiber Diameter and Attachment Category

The diameter of extrinsic fibers (d , D) has been reported to vary between attachments. To determine whether this variation in diameter represents a trend that can be used to reconstruct attachments from microanatomical evidence, extrinsic fiber diameters from three categories of attachment area (areas of direct attachment [DIR], areas of tendinous and aponeurotic attachment [TEN], and areas of no attachment [NON]) were examined. The general hypothesis regarding the relationship between diameter and attachment category can be stated as follows:

First Hypothesis

There is a significant difference in extrinsic fiber median diameter between attachment types. See appendix A for the statistical treatment of this hypothesis.

Relationship Between Extrinsic Fiber Density and Attachment Category

Extrinsic fiber density (δ , Δ) has been reported to vary between attachments in a manner similar to extrinsic fiber diameter. Values of density from each attachment category were examined for consistent differences using the same tests as those given above for differences in diameter. The general hypothesis regarding the relationship between extrinsic fiber density and attachment type can be stated as:

Second Hypothesis

There is a significant difference in extrinsic fiber median densities between attachment types. See appendix A for statistical treatment.

Relationship Between Extrinsic Fiber

Diameter and Attachment Stress

Extrinsic fiber diameters have been reported to vary with increased stress applied to their attachment site. To determine whether this relationship can be described and predicted, mean extrinsic fiber diameter values (d) were correlated with an index of stress at their attachment (σ) obtained from a biomechanical model . The general hypothesis can be stated as:

Third Hypothesis

Extrinsic fiber diameters show a linear relationship with the stress applied to their attachments. See appendix A for statistical treatment.

Relationship Between Extrinsic Fiber

Density and Attachment Stress

Extrinsic fiber density has been reported to increase with increased stress at an attachment site, in a manner similar to extrinsic fiber diameters. To test this relationship,

mean extrinsic fiber densities (δ) were correlated with an index of stress obtained from a biomechanical model. The general hypothesis can be stated as:

Fourth Hypothesis

Extrinsic fiber densities show a linear relationship with the stress applied at their attachment. See appendix A for statistical treatment.

Relationship Between Spatial Distribution of Extrinsic Fibers and Attachment Type

Extrinsic fiber spatial distribution has been noted to vary between areas of muscle attachment and areas of no attachment. An index of aggregation (b) was examined between different attachment types to determine if this variation can be used to diagnose attachments. This hypothesis can be stated as:

Fifth Hypothesis

The index of aggregation is greater at areas of muscle attachment than at areas of no muscle attachment. See appendix A for statistical treatment.

Influence of Phylogeny on Extrinsic Fiber Morphology

As mean extrinsic fiber diameter has been noted to vary between species, extrinsic fiber morphology was examined over a broad phylogenetic range of specimens, to determine if phylogenetic effects preclude the use of extrinsic fibers as a microanatomical correlate. This qualitatively examined hypothesis can be stated as:

Sixth Hypothesis

Extrinsic fiber diameter and density vary with phylogeny.

Methods to Recover Microanatomical Data from Fossil Specimens

As histology is a destructive process and scanning electron microscopy is potentially so, the effectiveness of a high fidelity casting process was tested to examine its potential for application in recovering microanatomical data from fossilized periosteal bone. This qualitatively examined hypothesis can be stated as:

Seventh Hypothesis

High fidelity epoxy replication allows the identification of extrinsic fibers on fossilized periosteal bone.

METHODS AND MATERIALS

Data used to test hypotheses one through seven have been taken from observations following the methods outlined below. The full set of data includes data on microscopic bone texture, muscle character, and attachment character. Table 3 gives a complete list of variables and the level at which they were measured.

Table 3.: List of variables measured from experimental observations along with the level of measurement.

Datum & Abbreviation	Level of Examination
Attachment Character	
TEN, DIR, NON – Attachment type	Gross observation; confirmed by histological examination
SA – Net area of attachment in mm ²	Gross measurement
Muscle Character	
L – Length of muscle belly in mm	Gross measurement
M – Wet mass of muscle belly in gm	Gross measurement
Microscopic Bone Texture	
d – Diameter of extrinsic fiber pits in μm	Scanning electron microscopy
D – Median diameter of extrinsic fiber pits per plot in μm	
δ – Density of extrinsic fiber pits per quadrat	Scanning electron microscopy,
Δ – Median density of extrinsic fiber pits plot	confirmed by histological observation
Composite Data	
σ – Index of stress	$(M/L) / SA$
b – Index of aggregation	Exponent b of the equation $s^2 = a \cdot \mu^b$, describing the relationship of a set of relative variances, s^2/μ

Specimen Selection and Accession

Attributes of bony tissues visible at the SEM level are known to vary with the age of an individual (Jones and Boyde 1974). Exact chronological ages were not available for most of the specimens used in this study. However, based on size (and secondary sex characteristics of plumage in the birds), all of the specimens were either subadult or adult.

There are also indications that the range of motion of soft-tissue attachments and patterns of use have an effect on tissue-level morphology (Gao and Messner 1996). To limit the possibility of mechanical differences between species introducing bias, this study was constrained to the attachments of mandibular adductors. Differences do exist in the mechanics of the mandible between the study species, but to a lesser degree than is seen in the appendicular skeleton and musculature (George and Berger 1966). The primary foci are the attachments of the *Muscularis adductor mandibulae*, *Muscularis pseudotemporalis*, and *Muscularis pterygoideus* (George and Berger 1966). The contraction of these muscles may vary in speed, period of sustained activity, and net force from group to group, but their action as mandibular adductors remains constant across all the taxa studied (Kesteven 1944, George and Berger 1966, Throckmorton 1978, Busbey 1989, Sato *et al.* 1992).

While variations caused by differences in ontogeny and biomechanics can be explained by established causal relationships, variation related to phylogeny is much more difficult to predict. Six species were selected from a range of diapsids to allow the placement of correlation results in a phylogenetic framework. The relationships between the study species are shown in Figure 3. Attributes of extrinsic fiber morphology were

mapped onto this cladogram in an attempt to identify any phylogenetic trends. Specimens were stored by freezing or fixation in 10% neutral phosphate buffered formalin until they were to be used. Table 4 lists the details of the accession of each specimen.

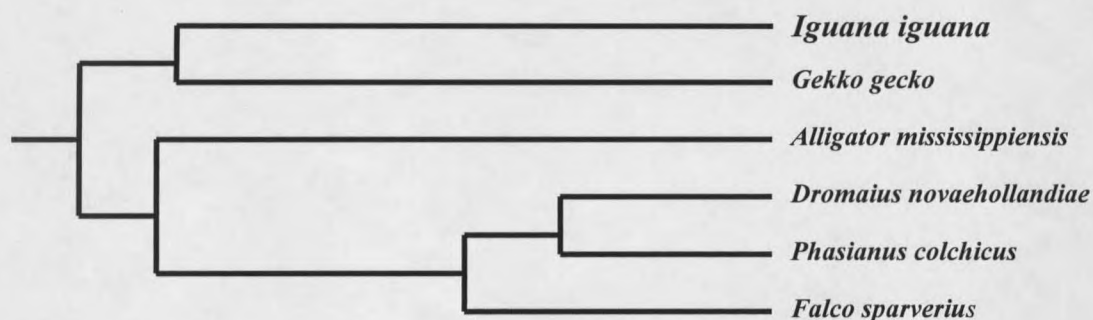


Figure 3. Cladogram of species used in this study. After Estes *et al.* (1988), Sibley and Ahlquist (1990), and Janke and Arnasson (1997)

Table 4. List of specimens used in this study, with accession information.

Species	MOR Histology Project Number	Source
<i>Phasianus colchicus</i> Ring-necked Pheasant	1999-16R-1	MOR collections, Bozeman, MT
<i>Dromaius novaehollandiae</i> Emu	2000-16R-2	Mule Bridge Ratite Farms, Santa Rosa, CA
<i>Gekko gecko</i> Tokay Gecko	2001-04R-1, 2001-04R-2	MOR collections, Bozeman, MT
<i>Iguana iguana</i> Green Iguana	2001-05R-1	MOR collections, Bozeman, MT
<i>Falco sparverius</i> American Kestrel	2001-14R-1	Montana Raptor Conservation Center, Bozeman, MT
<i>Alligator mississippiensis</i> American Alligator	2001-20R-1	Rockefeller NWR Grand Chenier, LA

Specimen heads were cut in half along a midsagittal plane. The right half was prepared for histological sectioning, while the left half was dissected and then prepared

for SEM analysis. This approach allowed comparison of SEM data with histological data from the same specimen.

Dissection and Gross Observation

The left half of each head was first dissected to ascertain the location and extent of muscle attachments. This data was recorded by capturing a digital image of the area with a digital camera. The type of muscle attachment (TEN or DIR) was determined by gross observation. Tendinous and aponeurotic muscle attachments were grouped in the same category, as they are morphologically similar (Kardong 1997, p. 357). Approximately six to twelve lengths of the muscle belly were measured parallel to fiber alignment from the captured image using a scale bar for calibration. These lengths were averaged to describe the mean muscle length (L). The entire muscle belly was then excised. Muscles dissected from fresh specimens were fixed in 10% neutral phosphate buffered formalin, and all muscle bellies were then stored in 70% ethanol.

The number of fascicles contracting in tandem in a muscle shows a linear relationship with the maximum force exerted during contraction (Elliot and Crawford 1965). This number can be roughly estimated from the cross-sectional area of a muscle belly at its widest point. Methods that describe muscle force in terms of muscle morphology often use the term physiological cross-section to describe estimates derived from fascicle morphology.

Elliot and Crawford (1965) describe a method for determining the physiological cross-sectional area of muscles. This method involves measuring the length of several

fascicles of a dissected muscle, and taking the dry mass of the muscle belly. The dry mass is divided by the mean fascicle length to give an estimation of physiological cross-sectional area that is independent of variation in fascicle size and swelling due to different osmolarities and physiological effects of preservative solutions.

I modified this method for use on very small muscles, as the lab equipment that was readily available during dissection was not sensitive enough to take an accurate reading of a mass as small as the dried *M. pseudotemporalis* from an America Kestrel. Instead, the wet mass (M) was taken after the muscle belly had been allowed to equilibrate at least 24 hours in a 0.9% NaCl saline solution.

After dissection, the left half of the head was enzymatically macerated in a solution of porcine pancreatin as per Anderson (1960). The cleaning of skulls by NaOCl or EDTA maceration carries with it the likelihood of introducing preparation artifacts (Anderson 1960, Marshall *et al.* 2001, Witmer, pers. comm.). As this study is intended to have relevance to fossil material, enzymatic maceration is an attempt to more closely model natural muscle decomposition (Martin 1999, pp. 153-155). In addition, this technique provides readily reproducible results in a shorter length of time than processing by bacterial maceration. Following maceration, skulls were cleaned of any remaining organic material by careful manual removal with forceps under a dissecting microscope and rinsing in a sonic washer.

Areas of attachment on cleaned skull elements were digitally photographed at an angle perpendicular to the attachment surface. Areas with topographically complex muscle attachments were divided into component flat areas, and these surfaces were

separately photographed. The outline of the muscle attachment was inscribed on the image of each attachment site, using photographs of the original dissection as a guide. Images were then calibrated, and the area of muscle attachment (SA) was determined using Omnimet® image analysis software (Beuhler 1999).

Scanning Electron Microscopy and Microscale Analysis

Individual cranial elements were mounted to aluminum stubs and gold-coated. Specimens were examined using an RJLee Instruments Ltd. Personal SEM®. Areas of muscle attachment were digitally photographed, as well as areas determined to be free of muscle or ligament attachment, such as the dorsal surface of the frontal bones. Table 5 provides a complete list of the cranial elements examined with SEM.

Approximately sixteen $3,600\mu\text{m}^2$ quadrats in a four by four square plot (Figure 4) were digitally photographed from the surface of each SEM-prepared skull element. Extrinsic fiber pit diameter (d) and density (δ) were measured from digital capture SEM micrographs using previously mentioned image analysis software. Extrinsic fiber pit diameter was measured perpendicular to the apparent axis of the extrinsic fiber (Figure 5), as many fibers meet the bone surface at an oblique angle and present a foreshortened or elongate profile at the surface along their long axis.

Table 5. List of cranial elements examined with SEM.

Taxon	Plot name	Cranial element	Aspect	Number of Quadrats
<i>Phasianus colchicus</i>	PcP1-16	Parietal	Lateral	16
	PcP17-32	Frontal	Lateral	16
<i>Dromaius novaehollandiae</i>	DnA1-16	Angular	Ventral	16
	DnC1-16	Coronoid	Dorsal	16
	DnF1-16	Frontal	Dorsal	16
	DnP1-16	Parietal	Lateral	16
	DnPt1-16	Pterygoid	Ventral	16
	GgD21-36	Coronoid	Mediodorsal	13
<i>Gekko gekko</i>	GgF1-16	Frontal	Dorsal	16
	GgF17-32	Frontal	Dorsal	16
	GgP17-32	Parietal	Ventral	16
	GgPt1-16	Pterygoid	Ventral	16
	GgPt17-32	Pterygoid	Ventral	14
	IiArt1-16	Articular	Ventral	16
<i>Iguana iguana</i>	IiC1-16	Coronoid	Lateral	16
	IiF1-16	Frontal	Dorsal	16
	IiP1-16	Parietal	Dorsal	16
	IiSA1-16	Surangular	Dorsal	16
	FsA1-16	Angular	Lateral	16
<i>Falco sparverius</i>	FsA17-32	Angular	Lateral	16
	FsD1-16	Coronoid	Dorsal	16
	FsP1-16	Frontal	Lateral	4
	FsP17-32	Parietal	Lateral	16
	FsPt1-16	Pterygoid	Ventral	16
	AmArt1-16	Articular	Medial	16
<i>Alligator mississippiensis</i>	AmF1-16	Frontal	Dorsal	16
	AmP1-16	Parietal	Dorsal	16
	AmQ1-16	Quadrato	Ventral	16
	AmSA1-16	Surangular	Dorsal	16

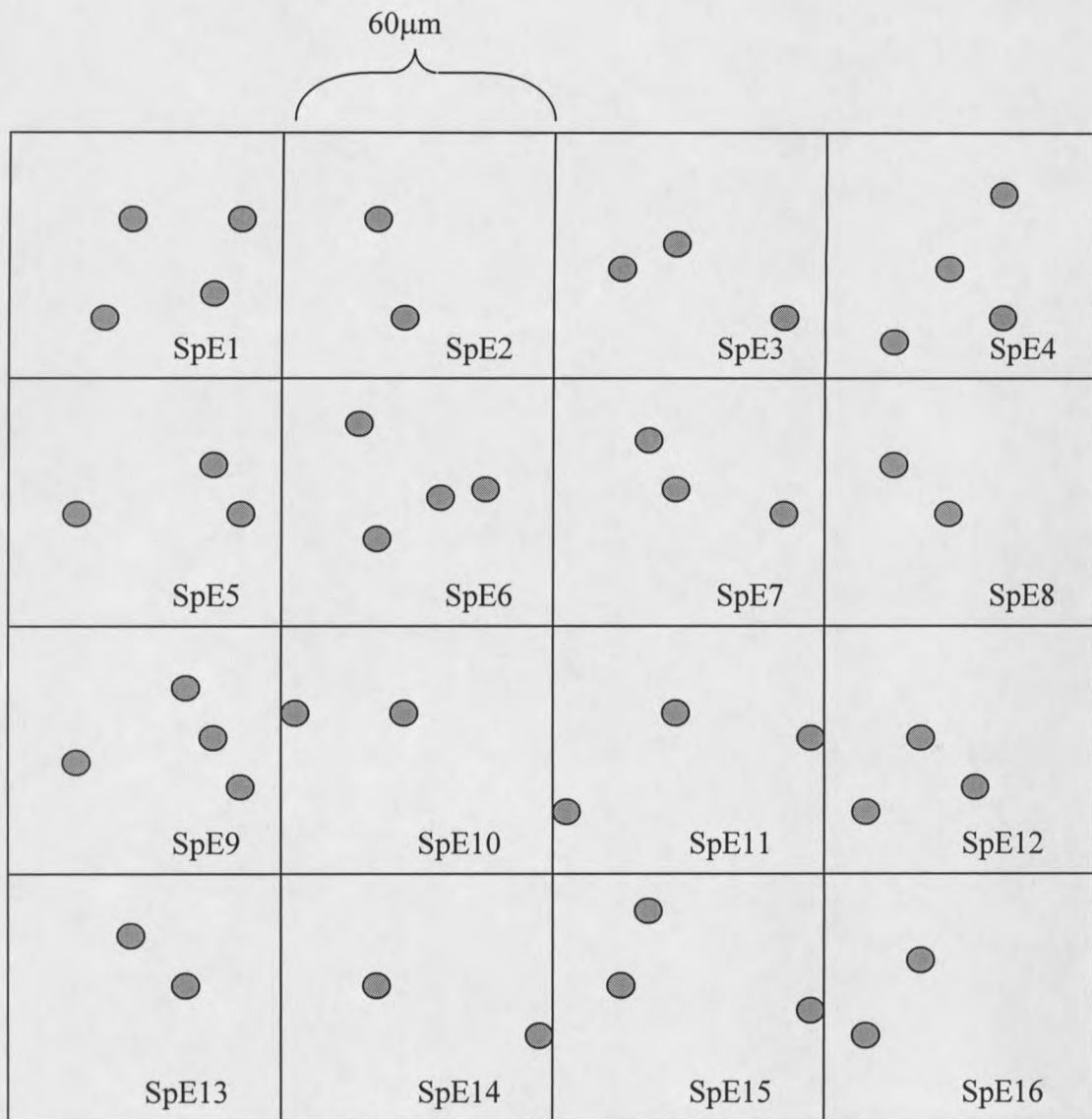


Figure 4. An example of the sampling system used in SEM analysis. Plots are labeled by the first letters of the genus and species epithets and an abbreviation of the skeletal element - plot SpE in this example. Each plot is divided into sixteen $3,600\mu\text{m}^2$ ($60\mu\text{m} \times 60\mu\text{m}$) quadrats, which are numbered in rows from left to right and top to bottom.

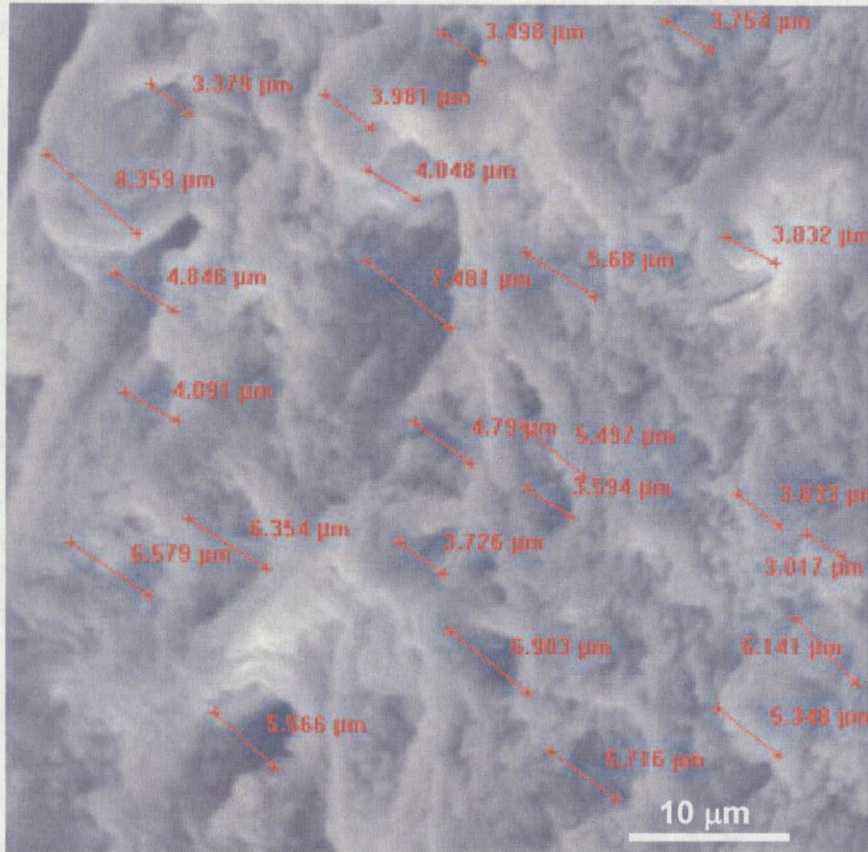


Figure 5. Diameter measurements on individual extrinsic fibers. *Iguana iguana*, 1500x

The index of aggregation (b) used to describe extrinsic fiber pit distribution was determined using a power curve analysis (Hayek and Buzas 1997, pp. 84-112). Each plot of sixteen $3,600\mu\text{m}^2$ quadrats was assigned to one of three groups based upon the type of attachment observed at that plot. The relative variances (variance over mean value, s^2/μ) of extrinsic fiber pit density of the plots within a group were then used to define a power curve following the formula:

$$s^2 = a \cdot \mu^b$$

where a and b are the y-intercept and slope, respectively, of a logarithmic line defining the relationship between the mean density of extrinsic fiber pits (μ) and the variance of extrinsic fiber pit density (s^2). The exponent b can be used to describe spatial distribution. Values of b close to 1.00 describe a random distribution. Values lower than 1.00 describe increasingly ordered and regular distributions (e.g., the distribution of *Tellina*, a regularly spaced benthic invertebrate, at $b = 0.70$), and values above 1.00 describe increasingly aggregated and clumped distributions (e.g., the distribution of haddock, a schooling fish, at $b = 2.35$) (Taylor 1961, Vezina 1988). Figure 6 provides a graphic example of these two extremes in distribution.

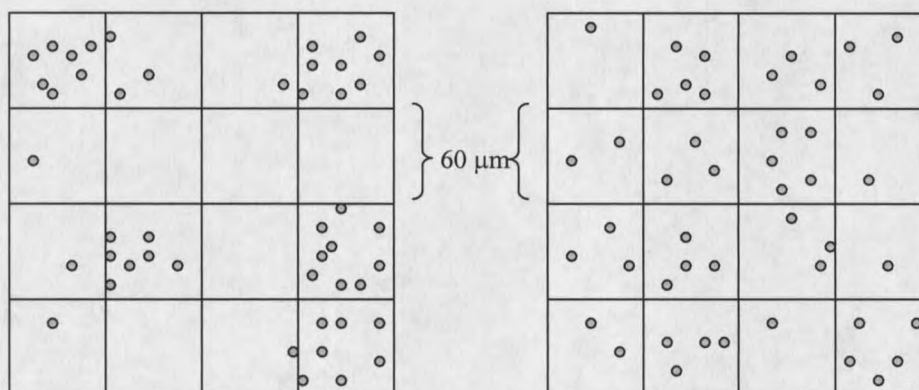


Figure 6. Hypothetical examples of clumped distributions vs. ordered distributions. Both plots have the same mean density of extrinsic fibers per quadrat, but the variance of the mean is much higher for the plot on the left. Given a y-intercept of one, the plot on the left would fit to a power curve describing a clumped distribution ($b \approx 2.35$), while the plot on the right would fit to a power curve describing an ordered distribution ($b \approx 0.70$).

There are other structures on the periosteal surface of bone that bear a superficial resemblance to extrinsic fiber pits. Osteoblast lacunae (Figure 7) appear as large ($\sim 10\mu\text{m}$)

ovoid pits in the bone surface, but are readily distinguished from extrinsic fibers by the presence of canaliculi and a smooth resting surface at the bottom of the pit. Figure 8 shows the two features side by side for comparison. Osteoclast resorption lacunae, or Howship's lacunae (Figure 9), are much larger than extrinsic fiber pits ($\sim 20\mu\text{m}$), and have a smooth profile that lacks the projecting mineralized fibrils of an extrinsic fiber pit. Figure 10 shows an extrinsic fiber pit inside an Howship's lacuna for comparison (Boyde 1972, Jones and Boyde 1974).

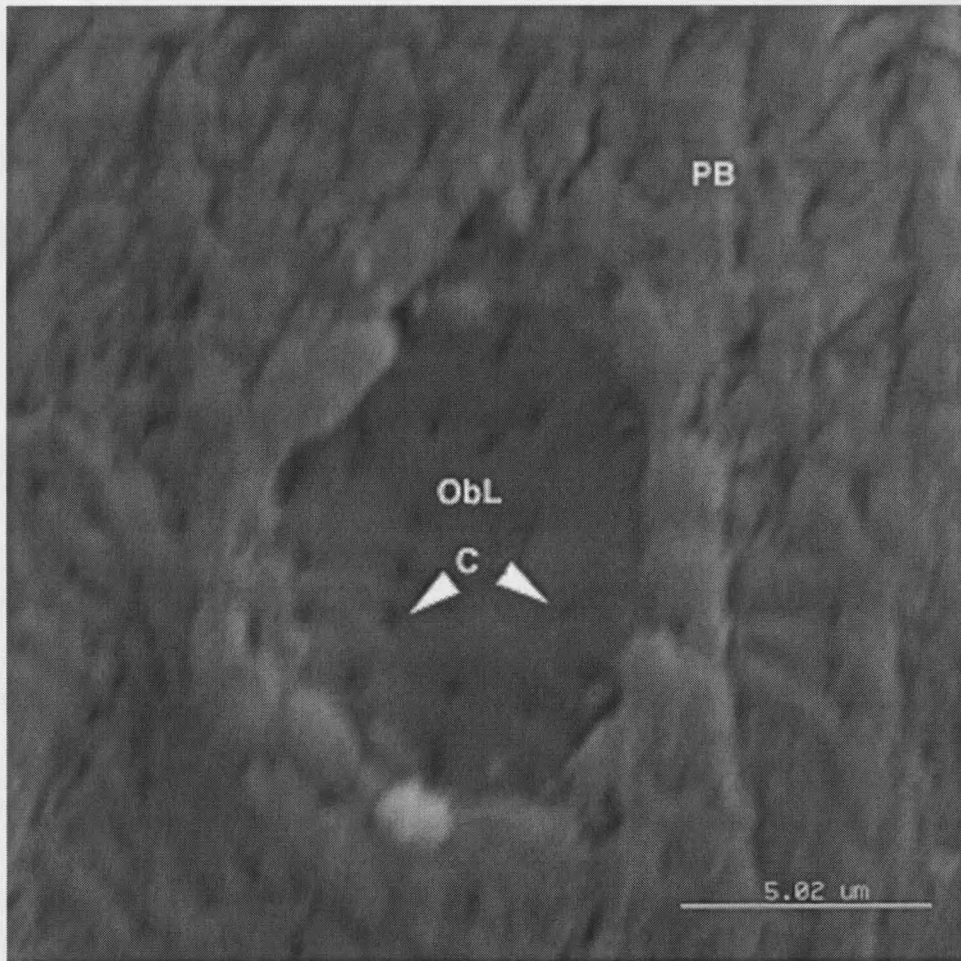


Figure 7. Osteoblast lacuna. Note the presence of canaliculi and the smooth internal surface. ObL: osteoblast lacuna; C: canaliculi; PB: periosteal bone. *Falco sparverius*, 4,000x.

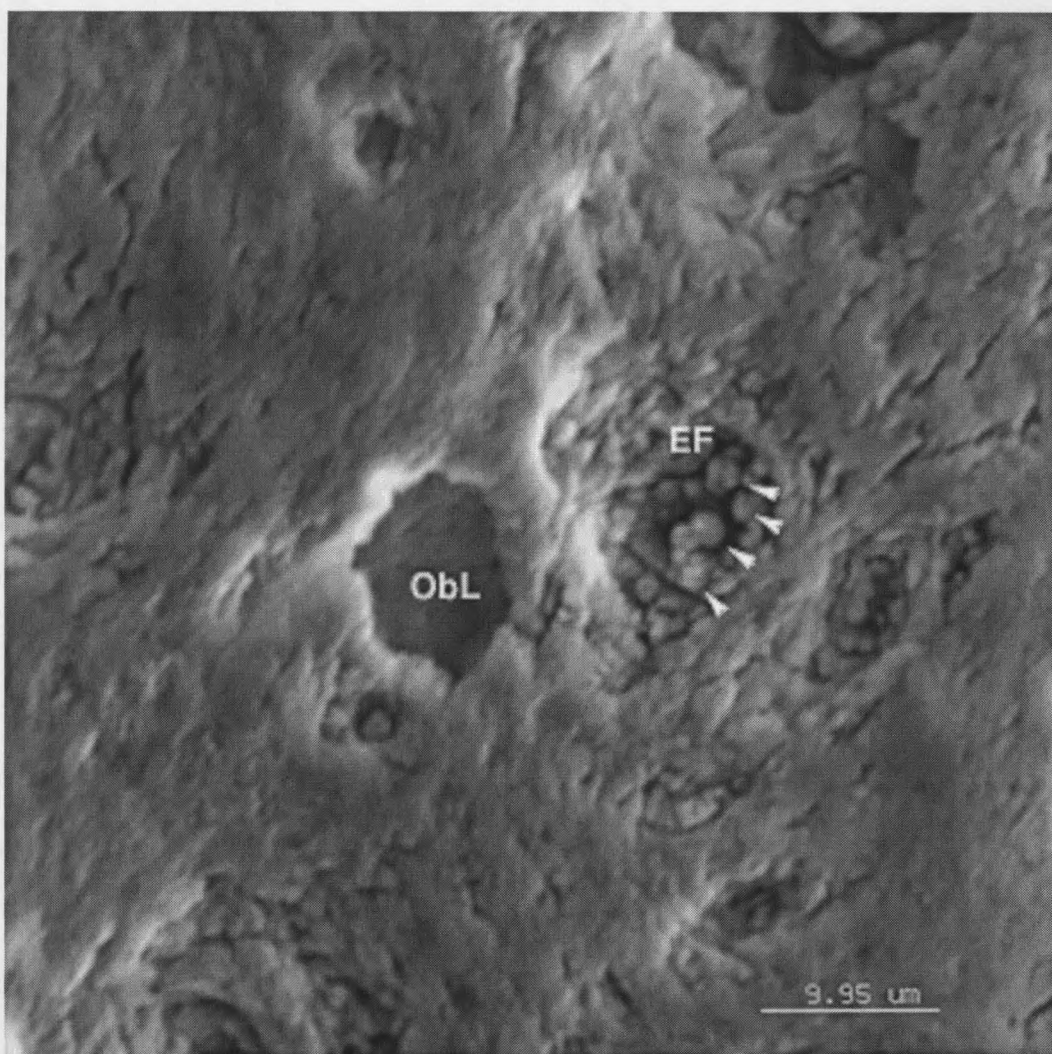


Figure 8. Osteoblast lacuna (ObL) and extrinsic fiber pit EF. White arrowheads point to individual ossified fibrils in the extrinsic fiber. *Falco sparverius*, 1,500x.

Positive evidence of extrinsic fibers was obtained in cases where specimens had been incompletely macerated and retained collagenous fibers visible in SEM analysis (Figure 11). Ambiguous pits that showed a mean diameter well below the expected value for extrinsic fiber pits on the surface of the pterygoid of *Gekko gecko* were determined to be extrinsic fiber pits through such an observation (Figure 12).

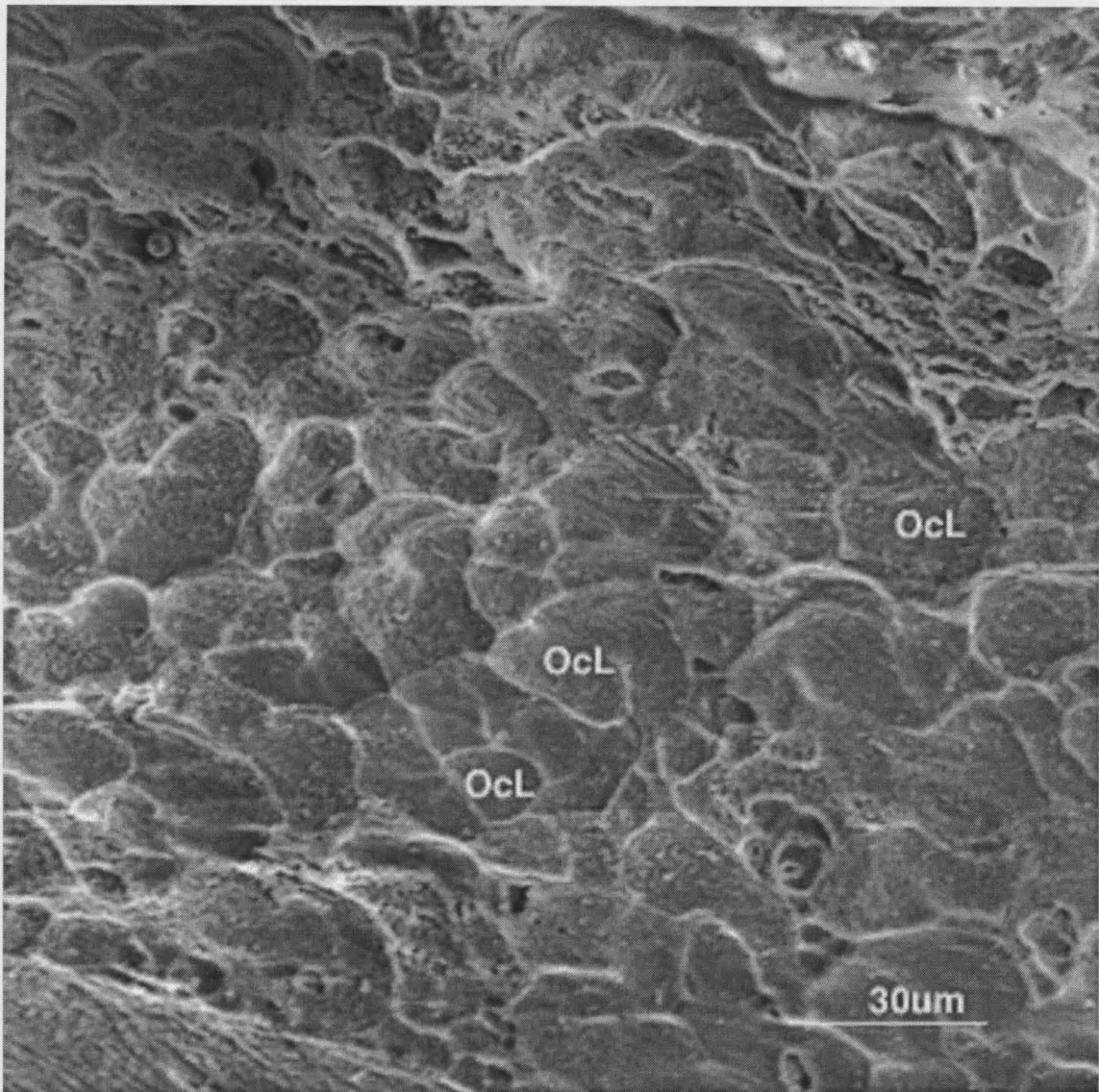


Figure 9. Osteoclast (Howship's) lacunae, indicating a resorptive surface. OcL: osteoclast lacuna. *Gekko gecko*, 340x.

Phylogenetic Analysis

To examine change between groups, median diameters and densities from homologous attachment sites were compared between branches at each node, following Felsenstein's (1985) method for producing phylogenetically independent contrasts (PIC). Although PIC values were originally intended for correlation with ecological traits, they

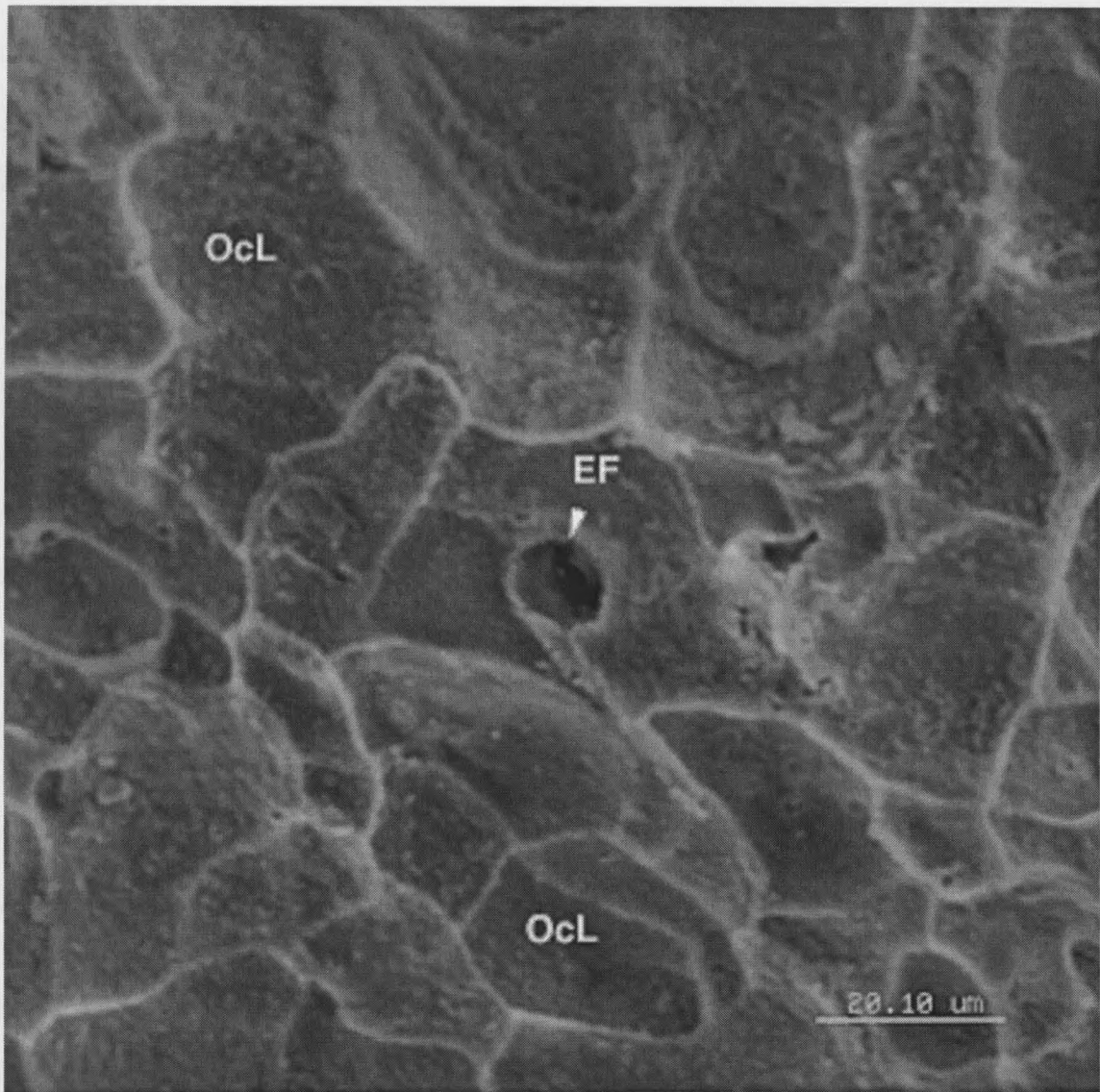


Figure 10. Osteoclast lacuna with truncated extrinsic fiber pit. Note the difference in size and character between the two types of depression. OcL: osteoclast lacuna; EF: extrinsic fiber. *Gekko gecko*, 760x.

can provide a measure of the rate of change of a trait over time. Higher PIC values can be considered indicative of adaptive change. A detailed explanation of the derivation of PIC data from raw data can be found in appendix B.

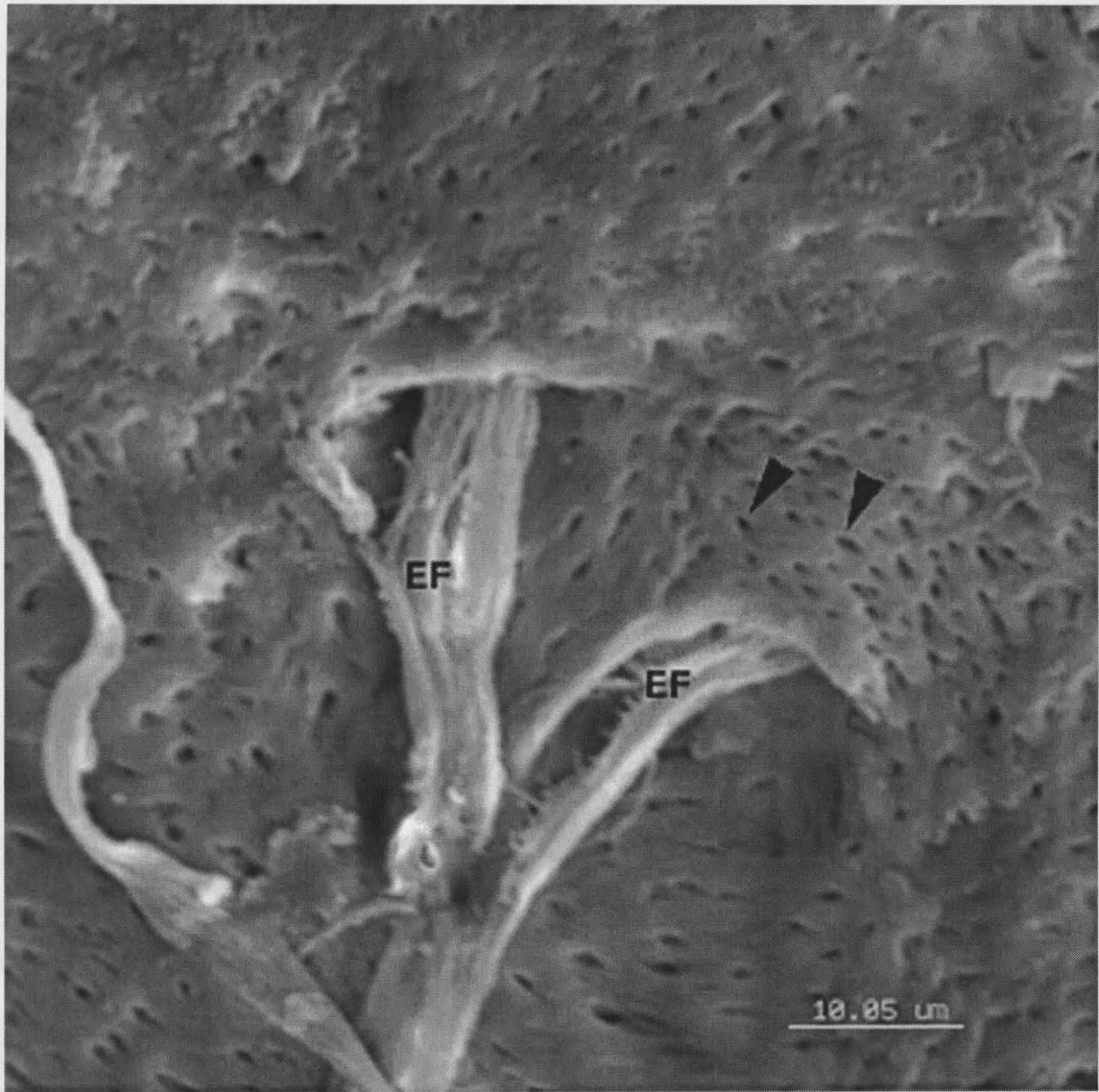


Figure 11. Extrinsic fiber (EF) exiting extrinsic fiber pit on incompletely macerated specimen. Black arrowheads point to canaliculi. *Gekko gecko*, 1400x.

Divergence dates from genetic phylogenies and fossil evidence were used to set the branch lengths of the phylogeny (Carroll 1988 pp. 230-231, Sibley and Ahlquist 1990, Janke and Arnason 1997, Janke *et al.* 2001). A relatively high PIC value between

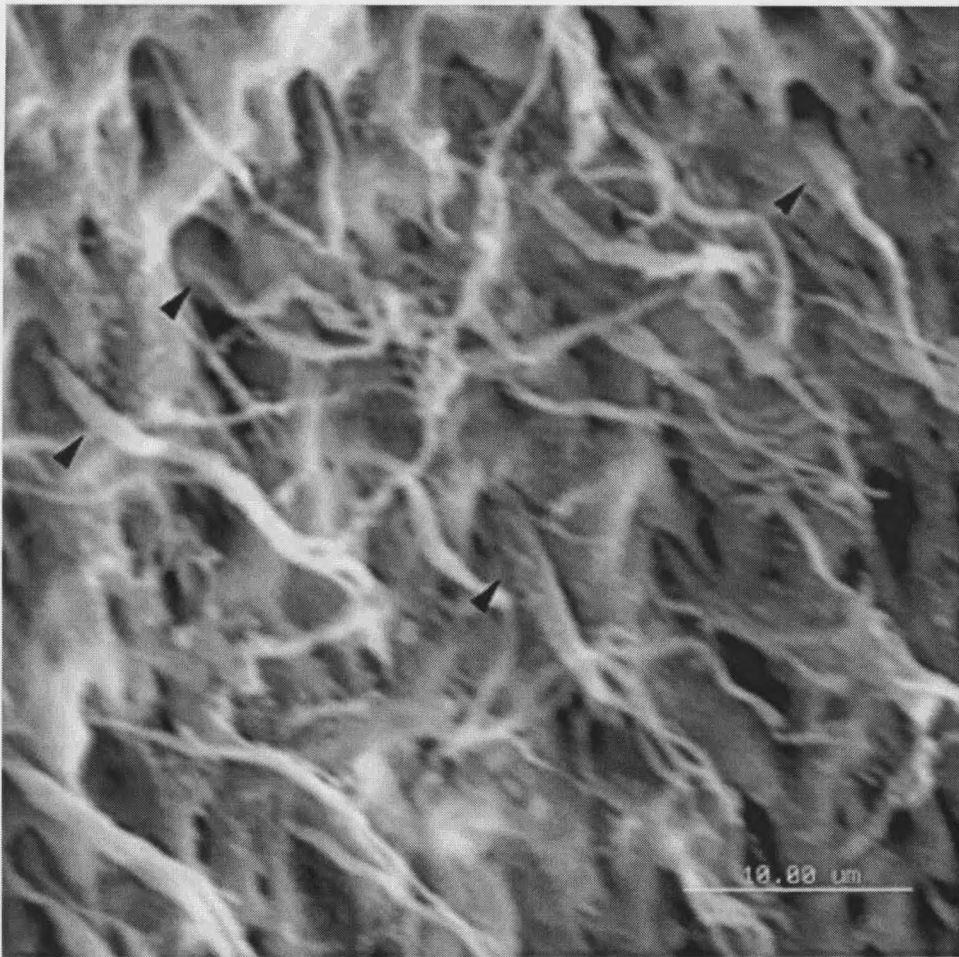


Figure 12. Small extrinsic fibers exiting pits that would warrant an ambiguous interpretation if fully cleaned, due to their unusually small diameter. Black arrowheads indicate examples of individual fibers. *Gekko gekko*, 2100x.

any of the major clades for diameter or density will indicate a departure from a random rate of change, and the possibility of a phylogenetically related difference in extrinsic fiber morphology.

Relationships between extrinsic fiber morphology and attachment type (hypotheses one and two) were also examined within each species. Relationships that held true between D or Δ and attachment type in the pooled plots from all specimens

were tested between d or δ and attachment type in each single specimen. The absence of a relationship previously identified in the pooled plots from any single specimen would indicate a potential phylogenetically related difference.

Histological Analysis

The right half of each head was fixed in formalin for six to eight days. The portion of the head containing the *M. adductor mandibularis* and *M. pseudotemporalis* and a portion free of any muscular attachments were then removed using a handsaw. These portions were put through a dehydration sequence of ethanol, cleared in xylene, and embedded in polymethylmethacrylate (PMMA, commonly referred to as Plexiglas®) for sectioning. Embedding in PMMA allows the examination of calcified bone and attached muscle tissue in the same slide.

All tissue embedded in PMMA was rough-cut with a high-speed tile saw, then cut with a variable-speed diamond wafering blade parallel to the alignment of the *M. adductor mandibulae superficialis* at its point of insertion. Sections were then cut from the face of each tissue portion and mounted onto plastic slides using PS100 cyanoacrylate glue. Mounted sections were ground to a thickness of ~90um and then stained. The choice of stains was constrained by the properties of PMMA to basic and hydrophobic stains (Horobin 1983). Toluidine blue allows a clear differentiation of bone, cartilage, and calcified cartilage (Smith and Bruton 1977, Kiernan 1990 pp. 177-179).

Histological sections were examined under polarized light to identify the orientation of collagen fibers, which aided in the positive identification of extrinsic fibers.

Sections were used to corroborate attachment topographies and types, and to examine extrinsic fiber densities as they passed beneath the periosteal surface of the bone.

Casting of Fossil Material

The production of high-fidelity epoxy casts of fossil material was adapted from a standard SEM technique for silicone molds and epoxy casts (Bozzola and Russell 1991 pp. 53-54). There are two major departures from the published technique. The first is a preliminary cleaning of the surface to be cast with acetone to remove any plastic-based preservative compounds such as Vinac®. The second departure is the omission of a vacuum stage during molding. Although the application of a vacuum during the molding process contributes to the fidelity of the eventual cast, many fossil specimens are highly porous and susceptible to damage caused by outgassing during a sudden drop in pressure.

A small piece of hadrosaur chevron (MOR 1071) was chosen for initial tests of casting fidelity. The epoxy replica was examined alongside the original fossil under SEM, and any differences between the two were noted. Following these tests, a coronoid process of the hadrosaur *Brachylophosaurus canadensis* was molded and cast. The replica was examined for features that could be interpreted as extrinsic fibers.

Statistical Analysis

Extrinsic fiber diameter (d) and density (δ) were recorded as continuous variables. As their distributions did not conform to a normal curve, standard parametric statistics could not be used to test inferences concerning their mean values. Median values were

instead used as the measure of central tendency (Chase and Bown 1997). An α level of 0.05 was used throughout statistical analysis as a threshold for rejection of null hypotheses.

Tests of identity between direct attachment plots from single specimens (Appendix A) showed that neither the diameter nor the density of extrinsic fibers from direct attachments remained the same across different attachment areas. As a result, values of d for individual extrinsic fibers and values of δ for individual quadrats cannot be considered independent between plots. Median values of diameter per plot (D) and median values of density per plot (Δ) were used for comparisons instead.

The first and second hypotheses were tested using the Mann-Whitney U -test, a test specifically designed to recognize a state of identity between the median values of two independent non-normally distributed samples (Chase and Bown 1997). The Mann-Whitney U -test returns two values of the test statistic U for each comparison: one describing the relative value of the median of each of the categories being compared. If either of the values of U are less than or equal to a critical value determined by the sample size of both categories, the median values of those categories can be considered significantly different. Sets of D values were compared pairwise between the three categories of attachment type (TEN, DIR, and NON), for a total of three comparisons (TEN vs. DIR, TEN vs. NON, & DIR vs. NON). Sets of Δ values were compared in the same manner.

The third and fourth hypotheses were tested by correlation. Linear correlation coefficients (r) were tested against one-tailed critical values of r from a normal

distribution. Values of r greater than the critical values can be said to demonstrate significant correlation between the two variables in question (Chase and Bown 1997).

Correlation is sensitive to the set of assumptions made for linear regression. The first of these assumptions is that values of the variable y that correspond to each value of the variable x show a normal distribution. This can be tested by examining the residuals of the correlation for a normal distribution. Quartile plots for the residuals from each regression in this study indicate a degree of normality that is acceptable for use in correlation.

The second assumption of the linear regression model is the assumption of homoscedasticity, meaning that the variance of all possible y values for a given value of x should remain the same across the range of possible x values. Homoscedasticity can be tested for by examining a scatter plot of the residuals of the regression and their corresponding x values. Scatter plots of the data used in regressions in this study did not show departures from homoscedasticity.

The third assumption of the linear regression model is that values of y are independent of each other. Any pattern in the value of residuals over values of x indicates some degree of dependence between values of y . There is no evidence that the variables used in regression in this study are dependent.

The fifth hypothesis was tested by a confidence interval of the slope b . The standard deviation of residual values from the projected slope is used to create a $(1 - \alpha)$ confidence interval, in this case a 95% confidence interval, for the slope of each attachment category. If the slope of one category falls within the 95% confidence interval

of the slope of another category, the slopes of the two categories are considered to be equal. Values of r were also tested against critical values to examine the fit of the power curves describing the relative variance of each category.

RESULTS

Tests of Relationship Between Extrinsic
Fiber Diameter and Type of Attachment

This study found no significant relationship between median extrinsic fiber diameter and type of attachment. Medians and ranges of D values are shown in Table 6; results of Mann-Whitney U tests are shown in Table 7. Median extrinsic fiber diameter was found to be significantly different between areas of tendinous attachment and areas of direct attachment, but neither of these were found to be significantly different from areas of no attachment.

Table 6. Median diameter of extrinsic fibers for each category of insertion.

Type of Attachment	Median Diameter D (μm)	Range	n
TEN	4.29	2.69 - 5.94	6
DIR	3.35	1.98 - 3.85	16
NON	3.7	1.72 - 5.65	7

Table 7. Pairwise Mann-Whitney U results for median extrinsic fiber diameter between categories.

Hypothesis	U_x	U_y	df	c at $\alpha = .05$	Result
H_{1A} : MdD_{TEN} vs. MdD_{DIR}	81	15	(6,16)	21	Reject H_0 ; $MdD_{TEN} > MdD_{DIR}$
H_{1B} : MdD_{TEN} vs. MdD_{NON}	29	13	(6,7)	6	Fail to reject H_0 ; $MdD_{TEN} = MdD_{NON}$
H_{1C} : MdD_{DIR} vs. MdD_{NON}	43	69	(16,7)	26	Fail to reject H_0 ; $MdD_{DIR} = MdD_{NON}$

Tests of Relationship Between Extrinsic
Fiber Density and Type of Attachment

There is a significant difference between median extrinsic fiber density at areas of tendinous attachment and median extrinsic fiber density at other types of attachment. Medians and ranges of Δ values are shown in Table 8; results of Mann-Whitney U tests are shown in Table 9.

Table 8. Median density of extrinsic fibers for each category of insertion.

Type of Attachment	Median Density Δ (EF/mm ²)	Range	<i>n</i>
TEN	5,429	4,000 - 11,286	6
DIR	571	0 - 3,286	16
NON	857	571 - 4,286	7

Table 9. Pairwise Mann-Whitney U results for median extrinsic fiber density between categories.

Hypothesis	U_x	U_y	df	c at $\alpha = .05$	Result
H _{2A} : Md Δ_{TEN} vs. Md Δ_{DIR}	96	0	(6,16)	21	Reject H ₀ ; Md Δ_{TEN} > Md Δ_{DIR}
H _{2B} : Md Δ_{TEN} vs. Md Δ_{NON}	41	1	(6,7)	6	Reject H ₀ ; Md Δ_{TEN} > Md Δ_{NON}
H _{2C} : Md Δ_{DIR} vs. Md Δ_{NON}	39	73	(16,7)	26	Fail to reject H ₀ ; Md Δ_{DIR} = Md Δ_{NON}

Tests of Relationship Between Extrinsic
Fiber Diameter and Attachment Stress

This study showed no relationship between mean extrinsic fiber diameter and stress at attachment sites. Plots of these correlations are shown in Figures 13 and 14. Correlation results are shown in Table 10.

Table 10. Results of correlation between mean diameter and stress

Hypothesis	r	n	c at $\alpha = 0.05$	Result
$H_{3A}: \rho(d, \sigma) \neq 0$	0.065	22	0.423	Fail to reject $H_0: \rho(d, \sigma) = 0$
$H_{3B}: \rho(d_{TEN}, \sigma) \neq 0$	0.332	6	0.811	Fail to reject $H_0: \rho(d_{TEN}, \sigma) = 0$
$H_{3C}: \rho(d_{DIR}, \sigma) \neq 0$	0.102	16	0.497	Fail to reject $H_0: \rho(d_{DIR}, \sigma) = 0$

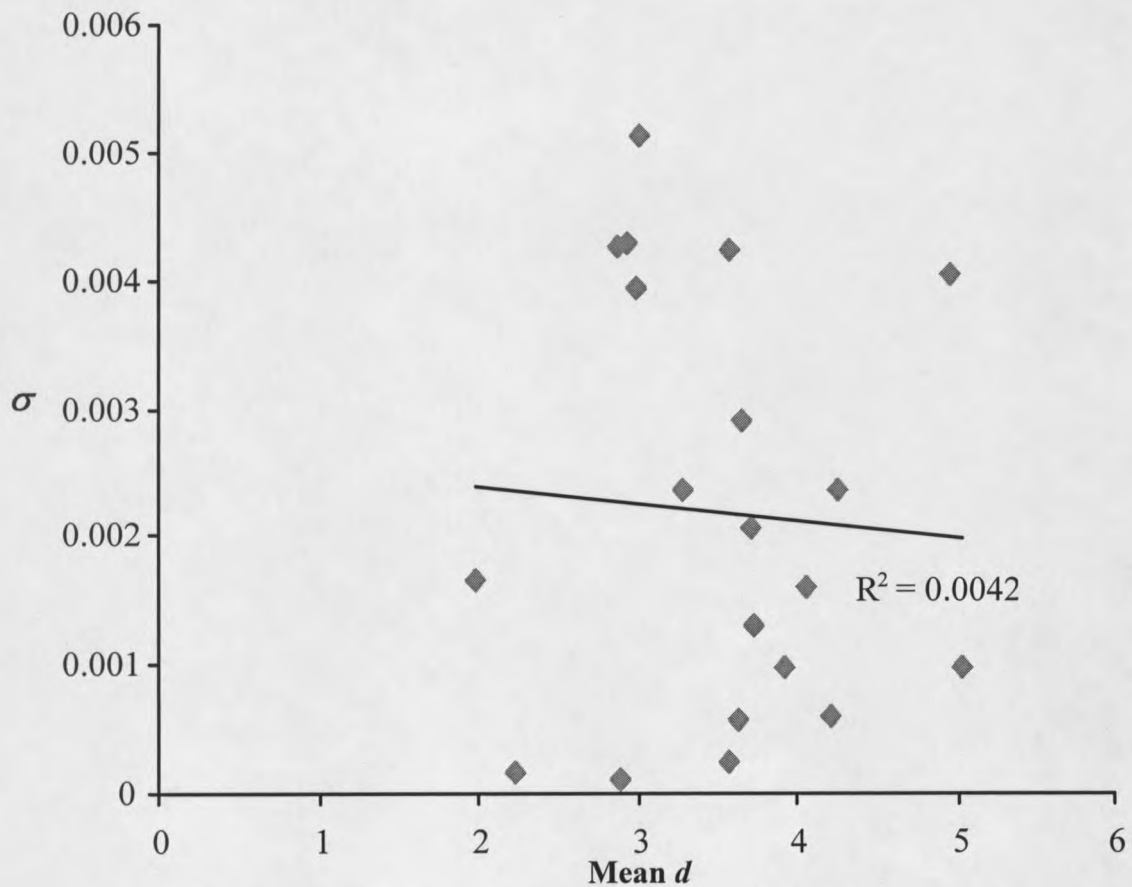


Figure 13. Plot of mean diameter vs. stress

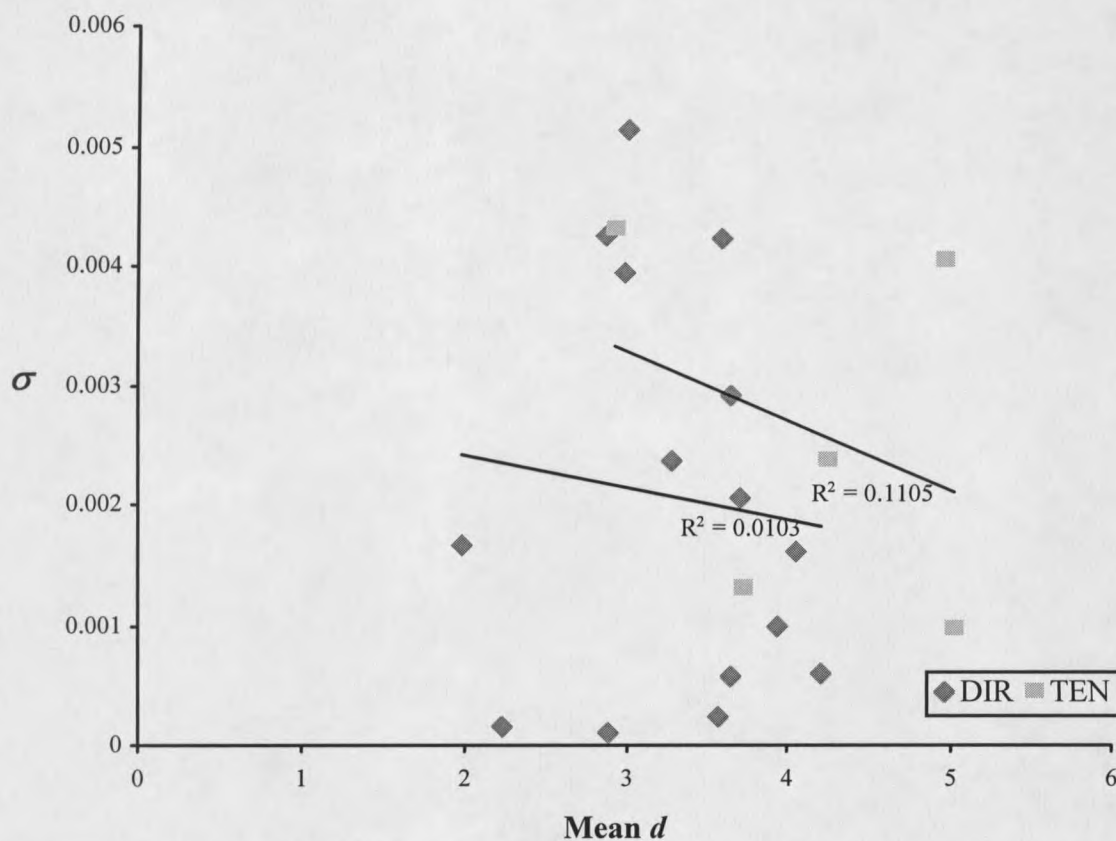


Figure 14. Plot of mean diameter vs. stress by attachment type

Tests of Relationship Between Extrinsic Fiber Density and Attachment Stress

This study showed a significant relationship between extrinsic fiber density and stress at attachment sites. Two of the correlations tested, $\rho(\delta, \sigma)$ and $\rho(\delta_{\text{TEN}}, \sigma)$, had significant values of r . Plots of these correlations are shown in Figures 15 and 16. Correlation results are shown in Table 11.

Table 11. Results of correlation between mean density and stress

Hypothesis	r	n	c at $\alpha = 0.05$	Result
$H_{4A}: \rho(\delta, \sigma) \neq 0$	0.553	22	0.423	Reject $H_0: \rho(\delta, \sigma) > 0$
$H_{4B}: \rho(\delta_{TEN}, \sigma) \neq 0$	0.963	6	0.811	Reject $H_0: \rho(\delta_{TEN}, \sigma) > 0$
$H_{4C}: \rho(\delta_{DIR}, \sigma) \neq 0$	0.442	16	0.497	Fail to reject $H_0: \rho(\delta_{DIR}, \sigma) = 0$

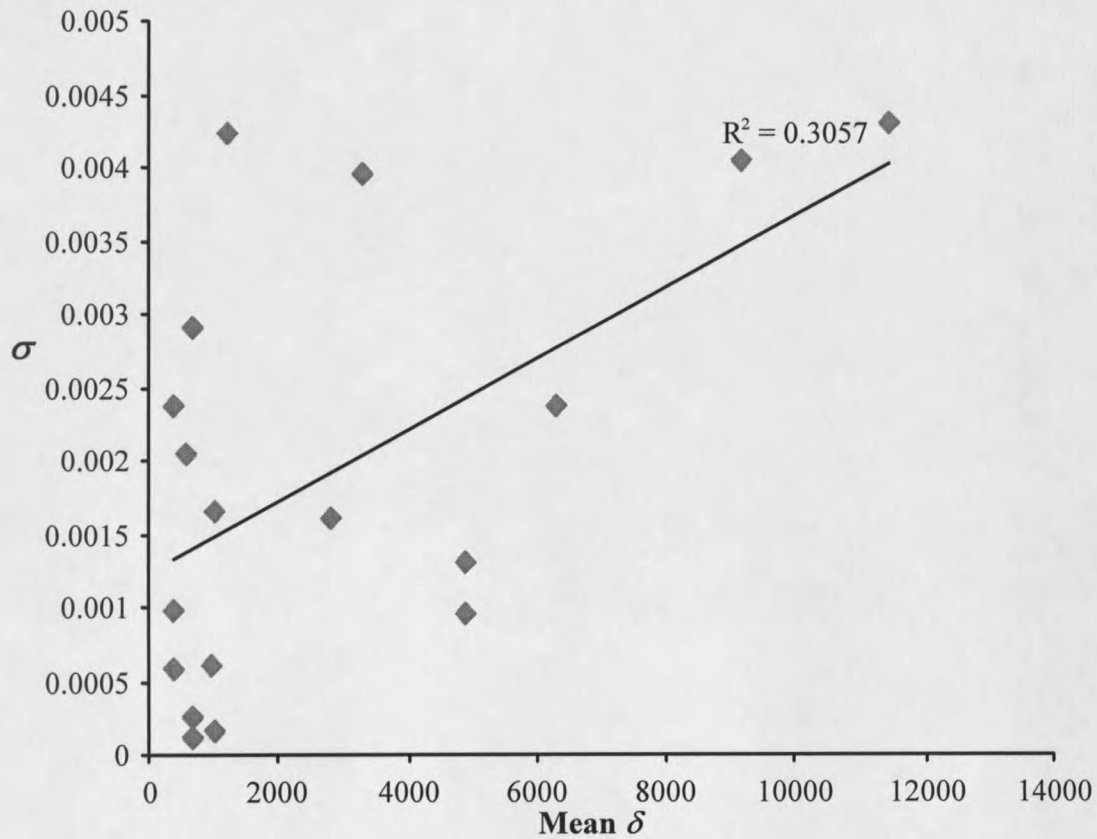


Figure 15. Plot of mean density and stress

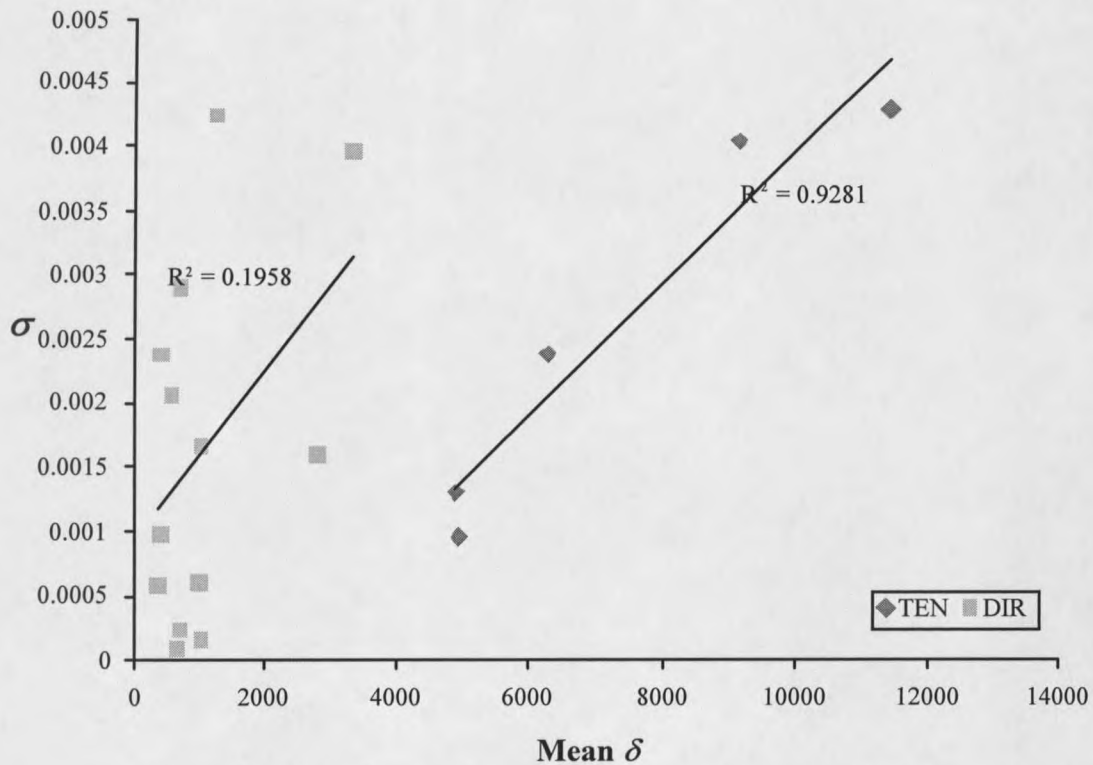


Figure 16. Plot of mean diameter and stress by attachment type.

Test of Relationship Between Extrinsic Fiber

Spatial Distribution and Attachment Type

There is no relationship between extrinsic fiber spatial distribution and attachment type at the scale sampled in this study. Values of the index of aggregation b are given in Table 12. A plot of the power curves describing the relative variances of extrinsic fiber density is shown in Figure 17.

Table 12. Values of b for power curves describing the relative variance of each category.

Type of Attachment	b	r	c at $\alpha = 0.05$	n
TEN	0.87	0.394	0.811	6
DIR	1.06	0.729	0.497	16
NON	0.68	0.653	0.754	7

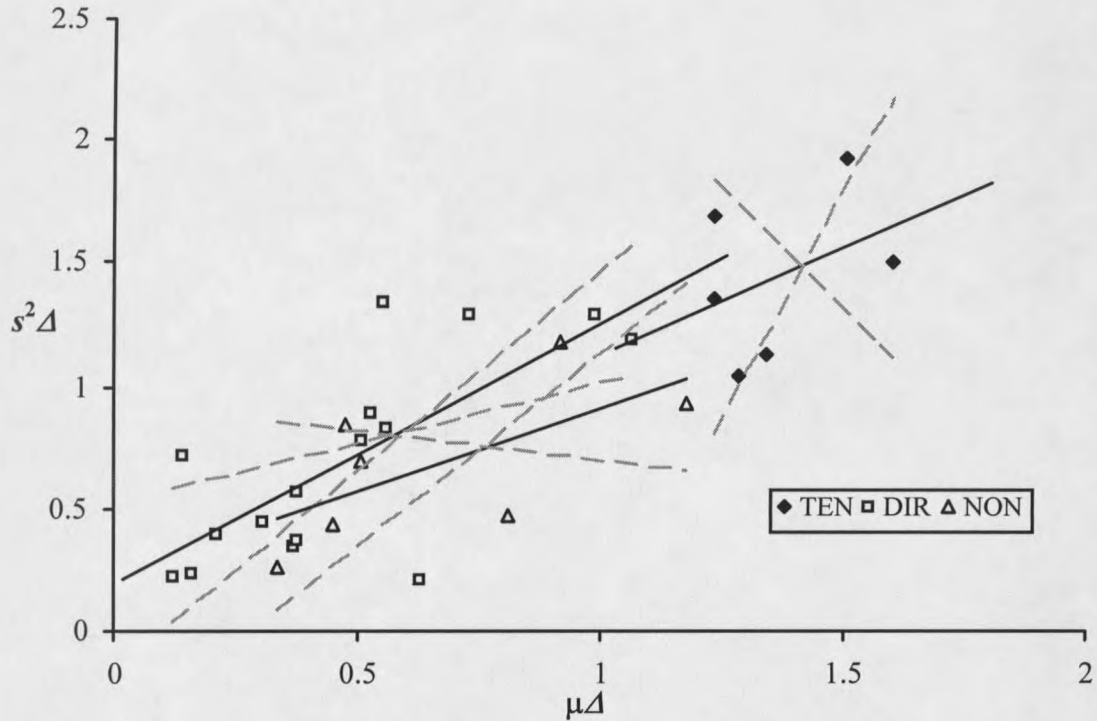


Figure 17. Power curves describing the relative variances of different attachment types, with 95% confidence intervals of slope.

Influence of Phylogeny on Extrinsic Fiber Morphology

Comparison of PICs values for D and Δ does not show any evidence of departures from random change between the major clades. The highest rates of change were seen within each group as opposed to between groups. PICs values are shown in Table 13.

Comparisons of δ between attachment types in individual specimens show that the relationship identified previously ($\Delta_{\text{TEN}} > \Delta_{\text{DIR}}, \Delta_{\text{NON}}$) holds true for each specimen. Comparisons of δ between attachment types are given in Table 14.

Table 13.: PICs values and their interpretations as to rate of change.

Comparison	Rate of Change in D ($\mu\text{m}/\text{my}$)	Rate of Change in Δ (n/my)	Interpretation
<i>Dromaius</i> - <i>Phasianus</i>	2.34×10^{-3}	-	Random change
(<i>Dromaius</i> , <i>Phasianus</i>) - <i>Falco</i>	5.48×10^{-3}	9.11×10^{-2}	Relatively high rate of change in D and Δ
<i>Aves</i> - <i>Alligator</i>	3.06×10^{-3}	4.71×10^{-2}	Random change
<i>Gekko</i> - <i>Iguana</i>	5.87×10^{-3}	8.79×10^{-3}	Relatively high rate of change in D , stasis in Δ
Archosauria - Squamata	9.57×10^{-5}	4.52×10^{-2}	Stasis in D , random change in Δ

Table 14.: Mann-Whitney U -test results of density between attachment types within species.

Taxon	Comparison	U_x	U_y	df	C at $\alpha = 0.05$	Result
<i>Dromaius</i>	δ_{TEN} vs. δ_{DIR}	256	0	(16, 16)	83	$\delta_{\text{TEN}} > \delta_{\text{DIR}}$
	δ_{TEN} vs. δ_{NON}	256	0	(16, 16)	83	$\delta_{\text{TEN}} > \delta_{\text{NON}}$
<i>Falco</i>	δ_{TEN} vs. δ_{DIR}	185	55	(15, 16)	77	$\delta_{\text{TEN}} > \delta_{\text{DIR}}$
	δ_{TEN} vs. δ_{NON}	55	5	(15, 4)	12	$\delta_{\text{TEN}} > \delta_{\text{NON}}$
<i>Alligator</i>	δ_{TEN} vs. δ_{DIR}	256	0	(16, 16)	83	$\delta_{\text{TEN}} > \delta_{\text{DIR}}$
	δ_{TEN} vs. δ_{NON}	256	0	(16, 16)	83	$\delta_{\text{TEN}} > \delta_{\text{NON}}$
<i>Gekko</i>	δ_{TEN} vs. δ_{DIR}	208	0	(13, 16)	65	$\delta_{\text{TEN}} > \delta_{\text{DIR}}$
	δ_{TEN} vs. δ_{NON}	208	0	(13, 16)	65	$\delta_{\text{TEN}} > \delta_{\text{NON}}$
<i>Iguana</i>	δ_{TEN} vs. δ_{DIR}	256	0	(16, 16)	83	$\delta_{\text{TEN}} > \delta_{\text{DIR}}$
	δ_{TEN} vs. δ_{NON}	180	76	(16, 16)	83	$\delta_{\text{TEN}} > \delta_{\text{NON}}$

Recovering Microanatomical Data from Fossil Specimens

Comparisons of a high-fidelity cast and original fossil material show that features as small as $\sim 2\mu\text{m}$ across were faithfully reproduced (Figure 18 and 19). Interpretation of extrinsic fibers from cast fossil material (Figure 20) was less successful. Although some features can be interpreted as extrinsic fibers on this specimen, they are ambiguous. Interpretation hinges upon the expectation of finding extrinsic fibers on the coronoid process, not by any overwhelming evidence presented by their morphology. The casting process, not by any overwhelming evidence presented by their morphology. The casting process of this particular specimen appears to have contributed to the ambiguity; incompletely mixed epoxy resin may have given the surface of this specimen its "half-melted" appearance. However, even at the level of fidelity shown by the initial cast (Figure 19), interpretation of smaller extrinsic fibers ($<3\mu\text{m}$) would be somewhat ambiguous.

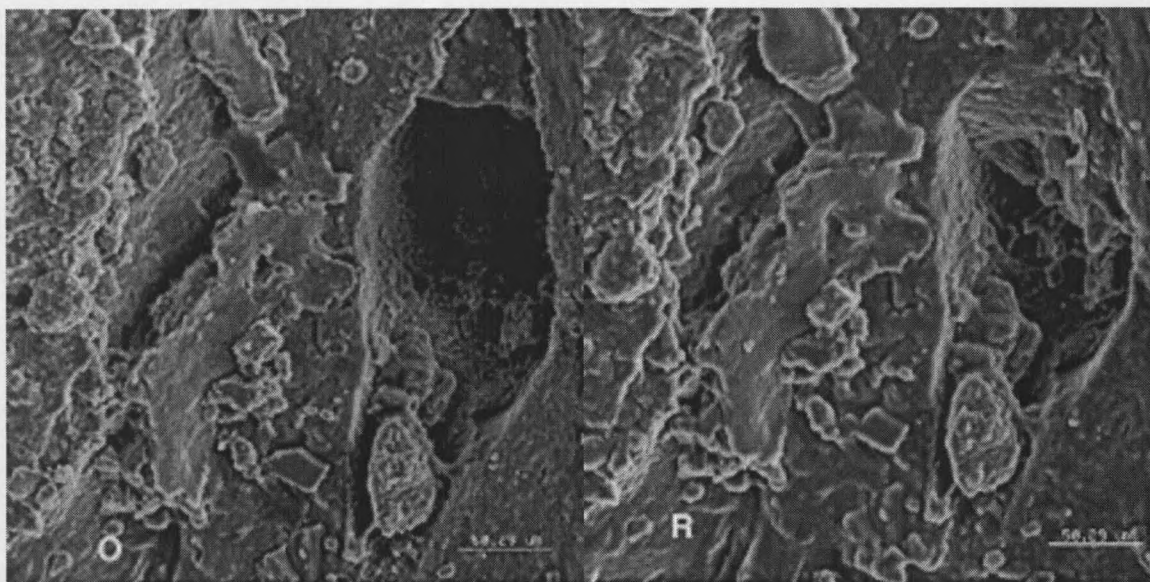


Figure 18. A comparison of original fossil material (O) and a high-fidelity epoxy replica (R). *Brachylophosaurus canadensis*, 300x.

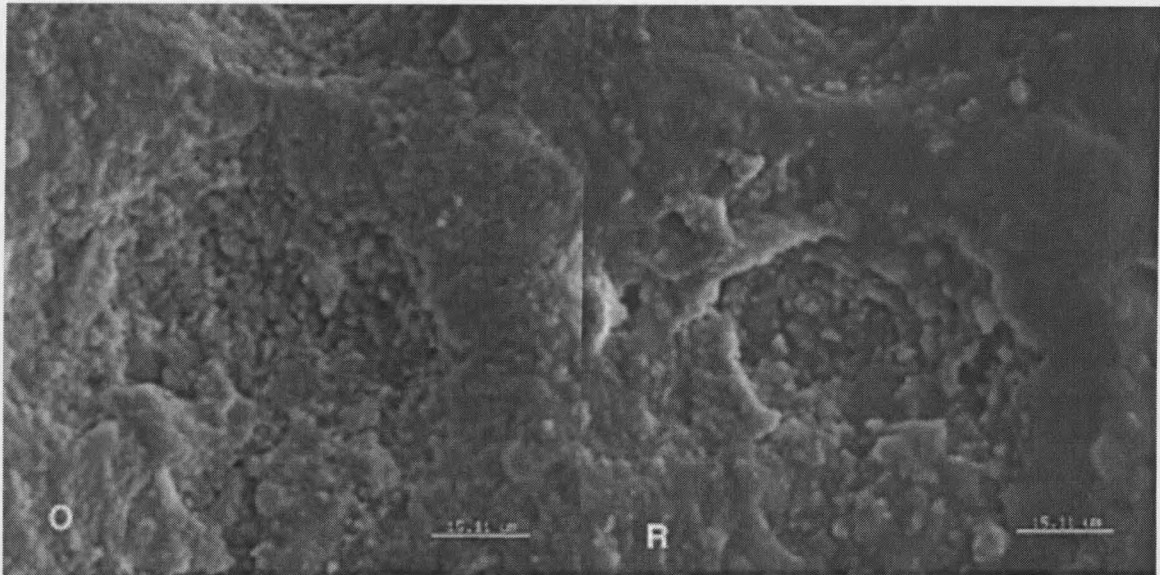


Figure 19. A higher-magnification comparison of original fossil material (O) and a high-fidelity epoxy replica (R). *Brachylophosaurus canadensis*, 1200x.

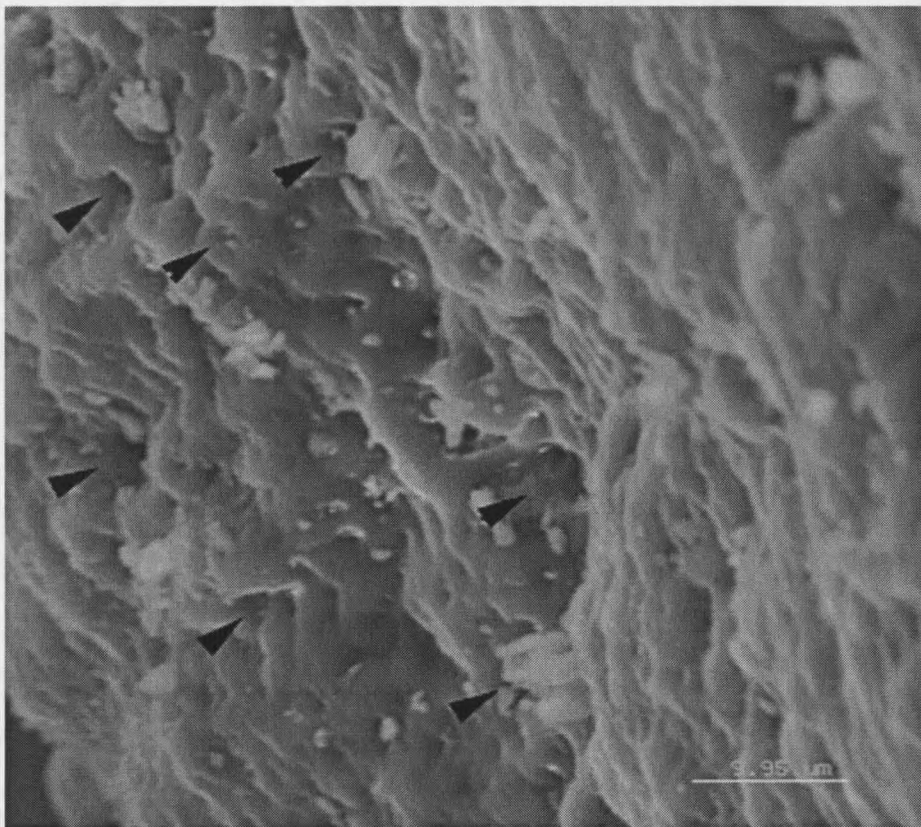


Figure 20. Silicone epoxy cast of the coronoid process of *Brachylophosaurus canadensis*, showing features interpreted as extrinsic fiber pits (black arrowheads). 1500x.

DISCUSSION

Extrinsic Fiber Density at Tendinous & Aponeurotic Attachments

A higher density of extrinsic fibers at areas of tendinous and aponeurotic attachment is qualitatively supported by previous examinations (Boyde 1972, Jones and Boyde 1974, Kawamoto 1992). However, previous examinations of extrinsic fibers at the bone surface also cite ligamentous attachments as areas with a high density of extrinsic fibers. Qualitative examination of ligamentous attachments as part of this study showed similar results. Most ligamentous attachments do have a very high density of extrinsic fibers (Fig. 22). Although tendinous attachments can be differentiated from areas of no attachment or areas of direct attachment, they cannot be differentiated from ligamentous attachments. As a whole, this evidence suggests that there is a characteristic pattern of extrinsic fibers on membrane bone surfaces in areas where dense regular connective tissue attaches.

Extrinsic Fiber Diameter and Stress

Although previous studies have pointed to a change in extrinsic fiber diameter between different levels of stress at the same attachment (Barton and Keenan 1967, Anderson *et al.* 1993), the diameter of extrinsic fibers at different attachment sites does not appear to be dictated by the force exerted at their attachment. Instead it appears that extrinsic fibers at different types of attachment exhibit different diameters. This may be due to developmental effects. Barton and Keenan (1967) have demonstrated that extrinsic

fibers will develop in the absence of stress at their attachment. It is possible that initial diameter is related to the cue for development, and that diameter later varies from its initial state in response to stress.

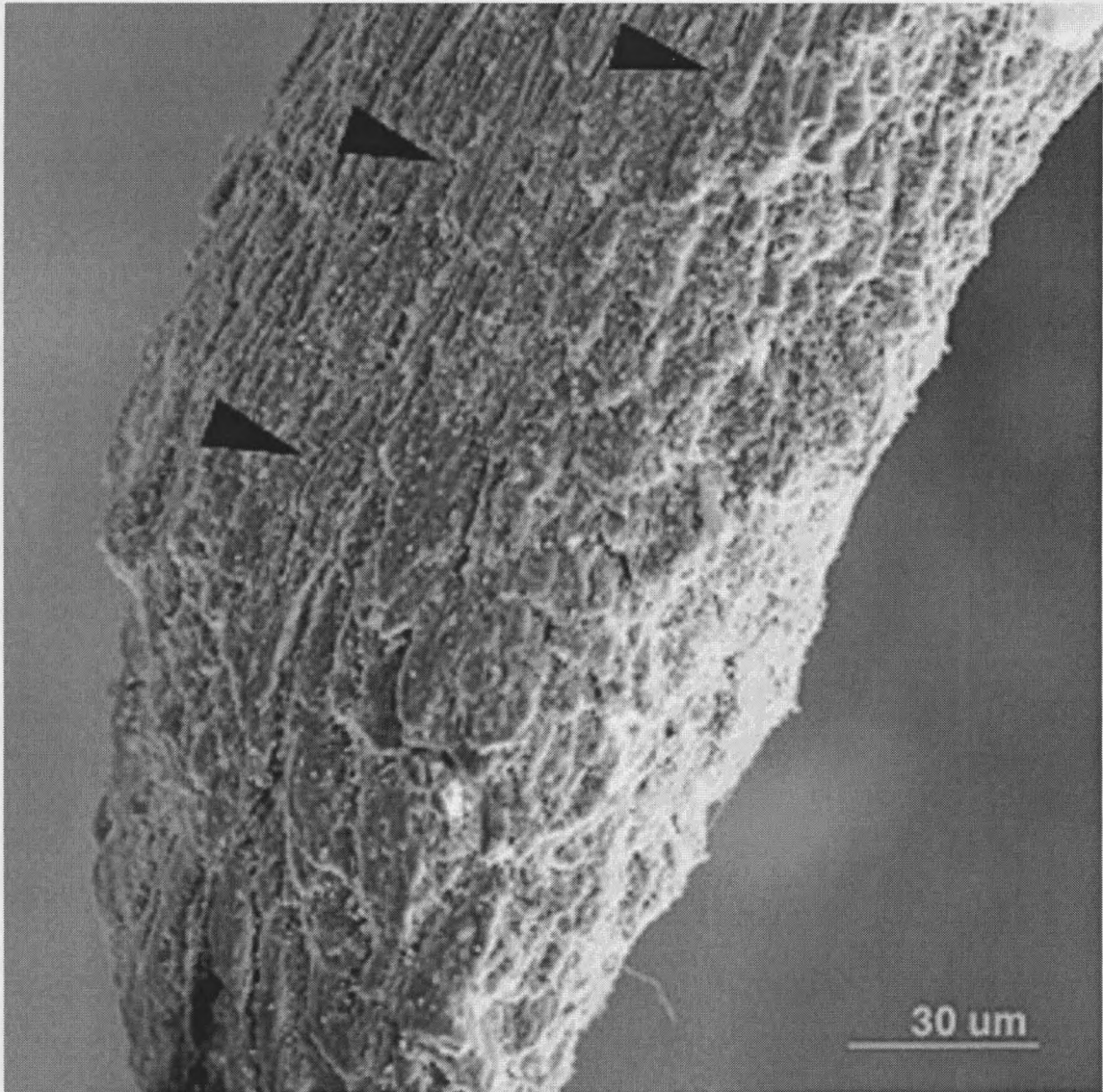


Figure 21. The origin of the postorbital ligament on *Falco sparverius*. Black Arrowheads point to areas where the alignment of fibers is more clear. Almost all of the visible surface is made up of extrinsic fibers. 300x.

Extrinsic Fiber Density and Stress

Extrinsic fiber density and stress at attachment show a significant correlation. Although the small sample size and the abstract nature of the index of stress involved limit the direct utility of this correlation, its significance suggests a relationship that may provide a microanatomical indicator of the actual maximum force applied in life to a dense regular connective tissue attachment. However, the calculation of stress represents a rudimentary biomechanical model of the forces expected at the muscle/bone interface. A more detailed model, taking into account the angle of incidence of the attachment and the physical properties of the extrinsic fiber/bone interface, would be required to properly test this relationship.

Extrinsic Fiber Spatial Distribution

Extrinsic fiber spatial distributions do not vary considerably on the scale analyzed in this study. These results contrast with the description of a clumped distribution of extrinsic fibers at muscle attachments given by Jones and Boyde (1974). Extrinsic fibers at tendinous and aponeurotic attachments in this study did have a tendency to be arranged in contiguous clumps. However, the overall density in these plots was such that individual clumps fit into single quadrats without exerting a strong influence on the relative variance. Sampling these surfaces with a quadrat size smaller than $1,000 \mu\text{m}^2$ may allow a power curve analysis to resolve this small-scale clumping.

Extrinsic Fiber Morphology and Phylogeny

More specimens need to be sampled to provide a definitive answer regarding phylogenetic influence on the morphology of extrinsic fibers. The mean diameters found for extrinsic fibers in this study ($1.98\mu\text{m}$ - $6.75\mu\text{m}$) are smaller than mean diameters reported for mammals ($5.9\mu\text{m}$ - $7.15\mu\text{m}$) (Jones and Boyde 1974), although whether this represents a phylogenetic difference, a body-size related difference, or a difference in the areas sampled is unclear. However, the distribution of extrinsic fiber morphologies in the specimens sampled in this study does not suggest that any major phylogenetically-related trends exist.

Comparisons of Extrinsic Fibers in SEM and in Histological Section

The attachments of tendons, ligaments, and aponeuroses in the samples examined in histological section all show extrinsic fibers continuing beneath the periosteal surface (Figure 22). This corroborates the observation of high extrinsic fiber densities at tendinous, aponeurotic, and ligamentous attachment sites observed in this study and reported elsewhere. Figure 23 shows the insertion of the postorbital ligament in the pheasant both under SEM and in histological section for comparison. In some cases, it was possible to follow extrinsic fibers quite deep into unremodeled periosteal bone, such as at the *M. pseudotemporalis* insertion on the gecko coronoid (Figure 24), suggesting that these areas show high extrinsic fiber densities throughout ontogeny.

In contrast, very few direct muscle attachments had extrinsic fibers in enough density to be visible in a histological section (Figure 25). In sections where muscle fibers

were aligned in the plane of the slide, it was possible to examine the termination of the muscle fiber (Figure 26). In these cases, dense regular connective tissue fibers leading from the spindle-shaped muscle fiber terminus could be seen to join into fibrous periosteum. However, at the edge of direct muscle attachments, both SEM and histological sections show the epimysium of the muscle penetrating the periosteum and continuing into periosteal bone as extrinsic fibers (Figures 27 and 28).

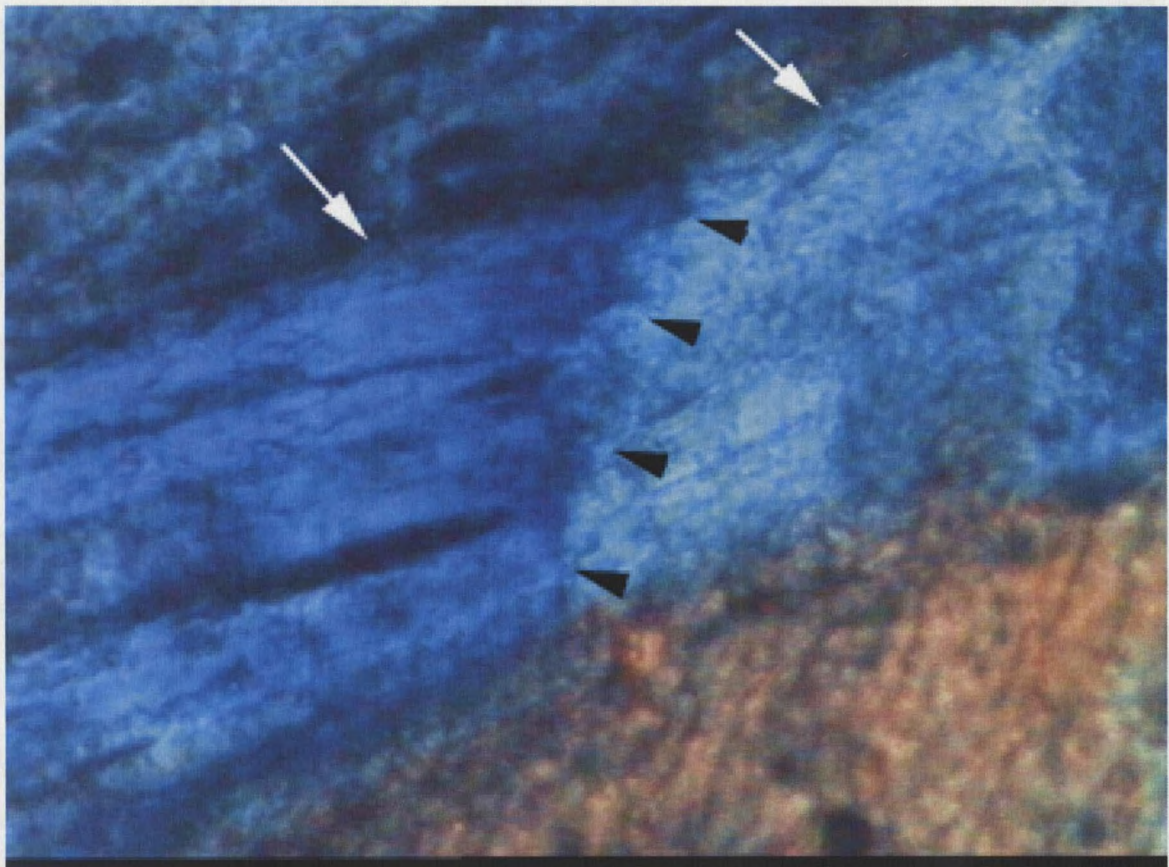


Figure 22. Extrinsic fibers continuing into periosteal bone. White arrows denote the edge of the extrinsic fibers; black arrowheads mark the tendon/bone interface. *Phasianus colchicus*, 400x.

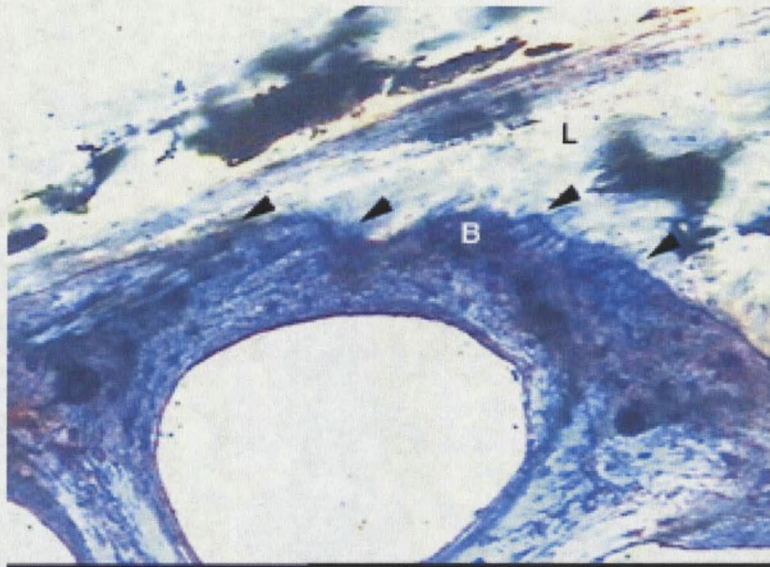


Figure 23. The insertion of the postorbital ligament onto the mandible. L: ligament; B: bone. Black arrowheads mark the ligament/bone interface. *Falco sparverius*, 100x.

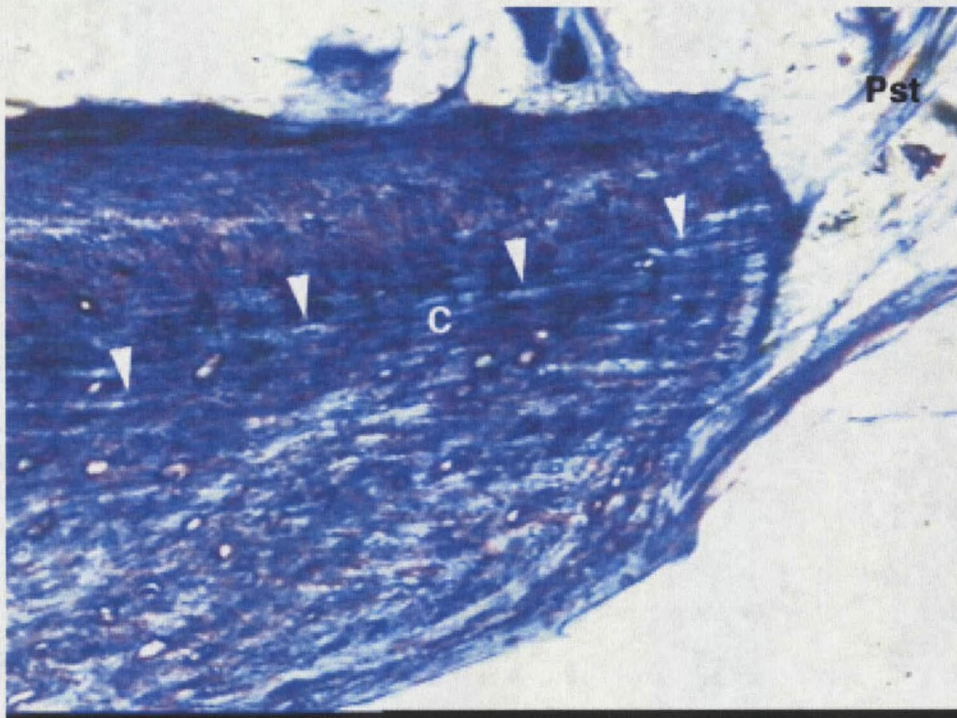


Figure 24. Continuity of extrinsic fiber attachment through ontogeny. The white arrowheads denote extrinsic fibers that have been entrapped in periosteal bone during the growth of the coronoid. C: coronoid; Pst: pseudotemporalis tendon. *Gekko gecko*, 100x.

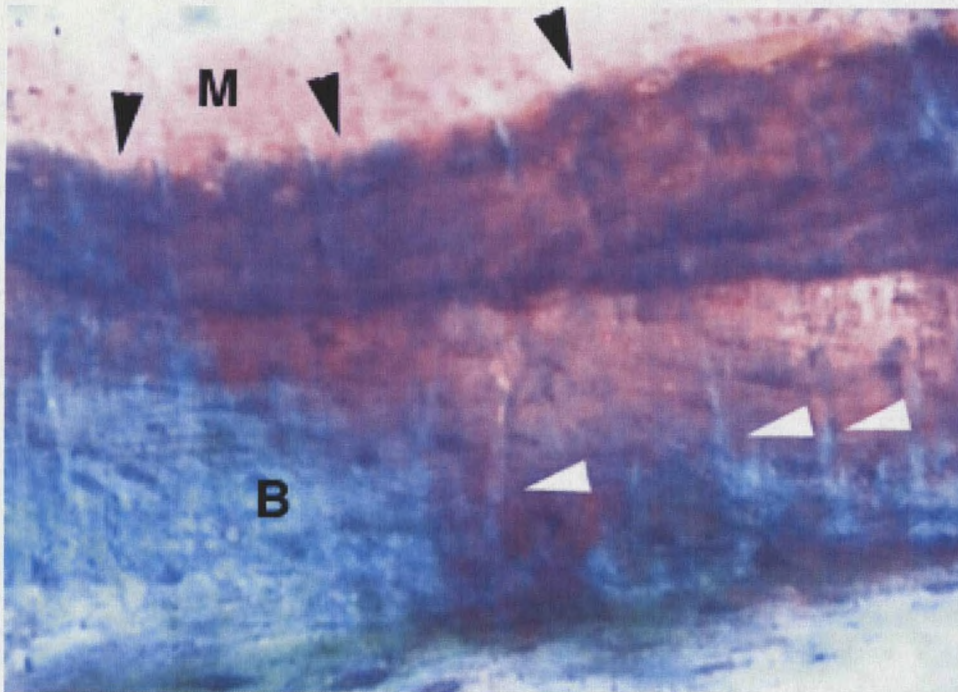


Figure 25. Extrinsic fibers at a direct muscle attachment. B: bone; M: muscle tissue. Black arrowheads: muscle/bone interface; white arrowheads: extrinsic fibers. *Phasianus colchicus*, 100x.

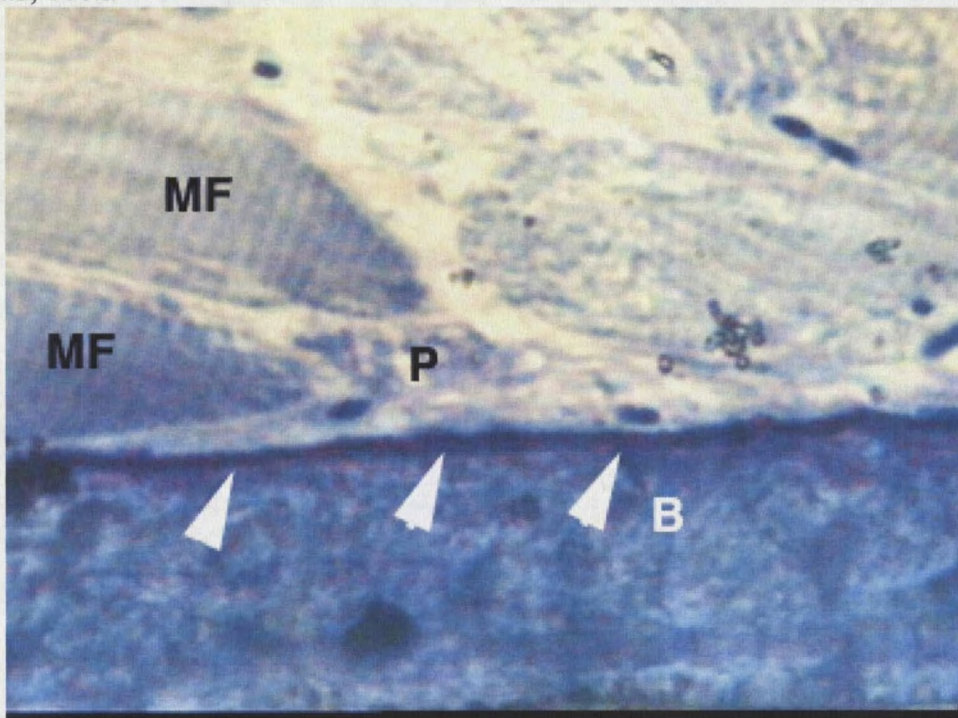


Figure 26. Direct attachment of muscle fibers to the periosteum. MF: muscle fiber; P: periosteum; B: bone. White arrowheads: periosteum/bone interface. *Falco sparverius*, 400x.

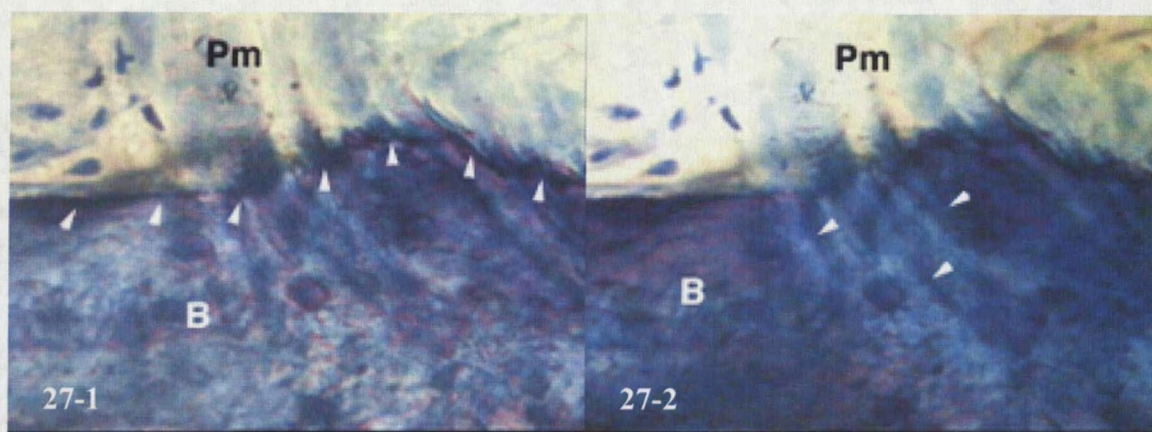


Figure 27. Two views of the origin of the *M. pseudotemporalis* in *Falco sparverius*, showing the fibers of the epimysium continuing into the bone. Pm: Epimysium; B: bone. White arrowheads in 28-1 indicate the muscle/bone interface; white arrowheads in 27-2 indicate extrinsic fibers.

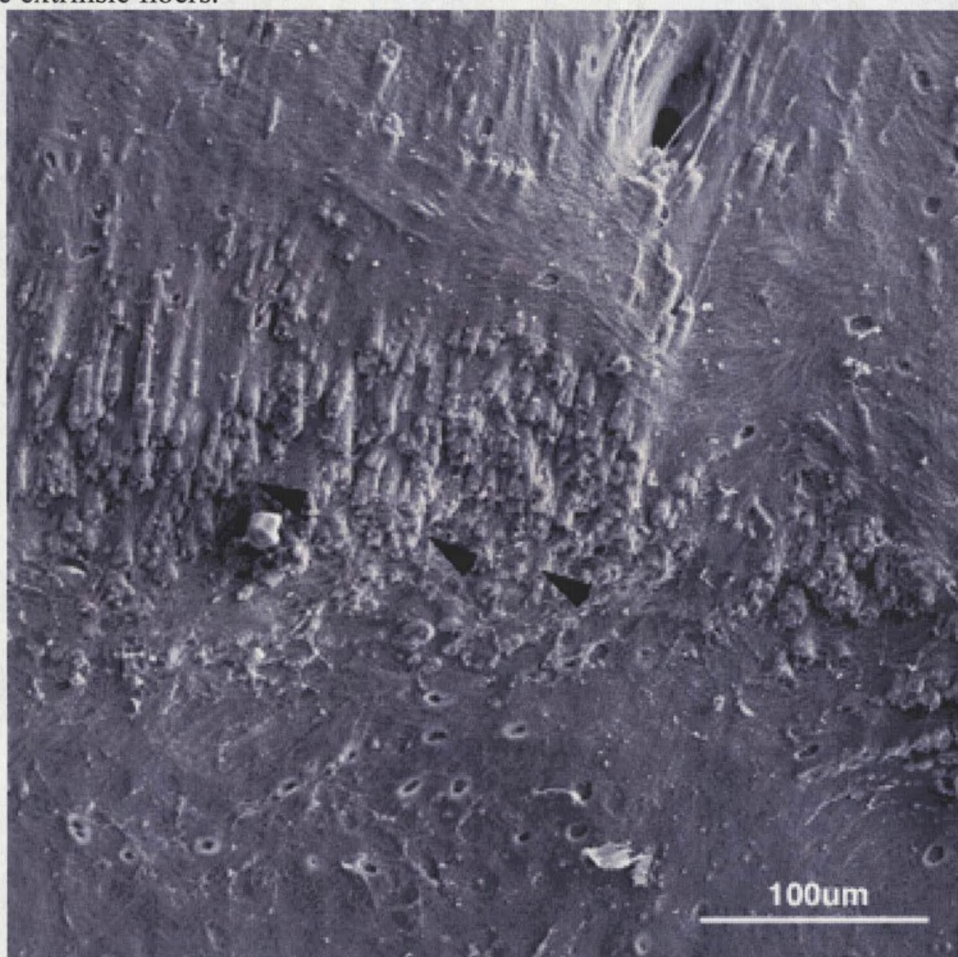


Figure 28. Attachment of the epimysium of the *M. pseudotemporalis* of *Falco sparverius* under SEM. Black arrowheads denote the line of attachment of the epimysium. 200x.

Extrinsic fibers were also seen in high concentrations in histological sections on sutural surfaces (Figure 29), and on the lateral tip of the pterygoid in the two lizards, where they attached a pad of dense irregular connective tissue to the bone surface (Figure 30).

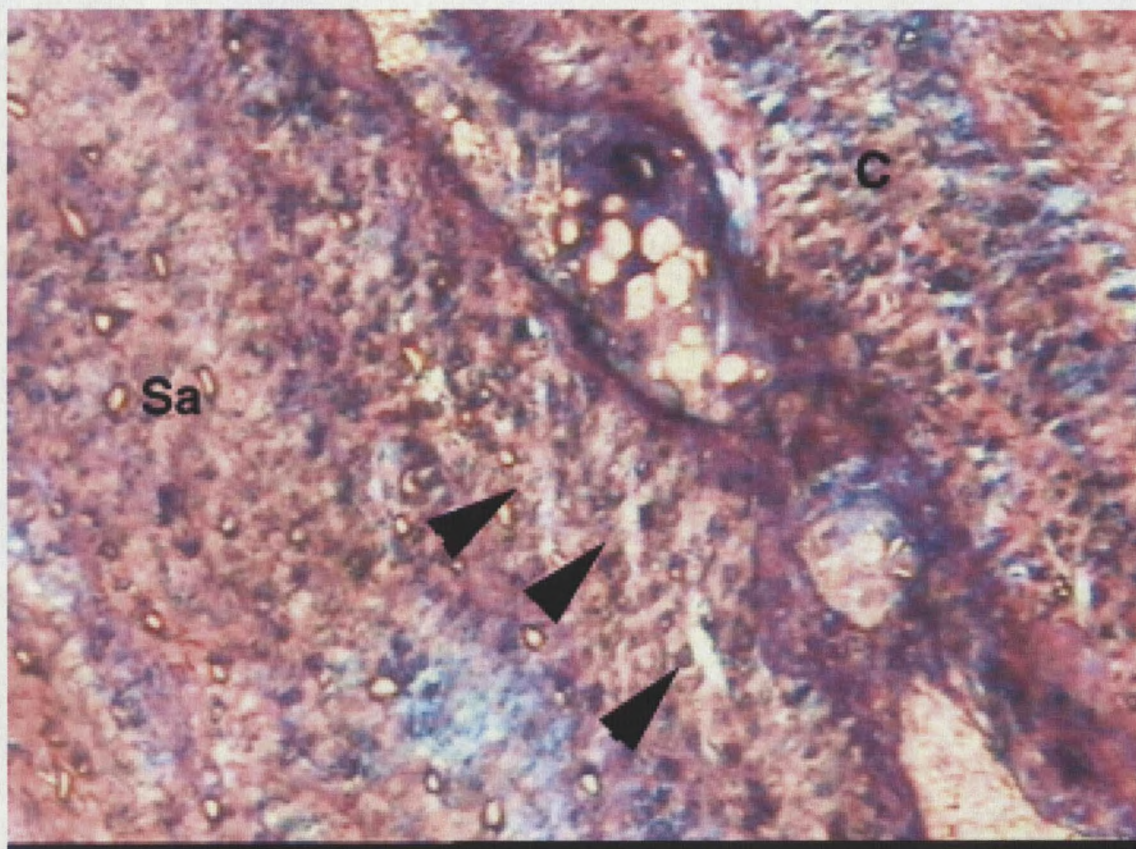


Figure 29. Extrinsic fibers in the coronoid-surangular suture of *Gekko gecko*. Sa: surangular; C: coronoid. Black arrowheads point to individual extrinsic fibers. 100x.

Model of Muscle Attachment at Different Attachment Types

Both SEM and histological evidence indicate that there are different mechanisms of attachment for dense regular connective tissue (tendons, ligaments, aponeuroses, and epimysia) and direct attachments of muscle tissue. Any concentration of dense regular

connective tissue visible at 400x in histological section ($>20\mu\text{m}$ across) exhibits extrinsic fibers continuing into the periosteal bone at its attachment, even when attaching at a low angle of incidence (Figure 28). This type of attachment produces a high density of extrinsic fibers visible on the periosteal surface of bone under SEM.

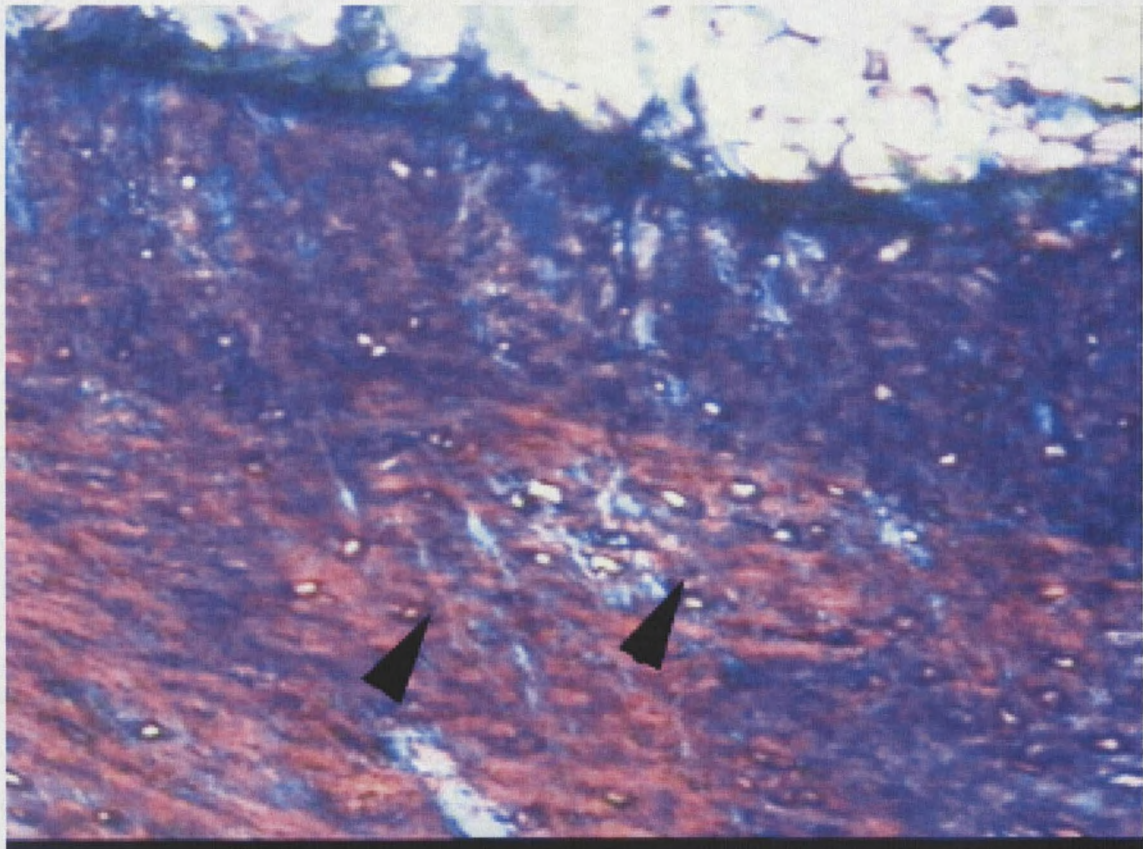


Figure 30. Extrinsic fibers attaching a dense irregular connective tissue pad to the lateral surface of the pterygoid in *Gekko gecko*. 100x.

Direct muscle attachments show extrinsic fibers in histological sections only at high angle of incidence attachments, and their densities of extrinsic fibers cannot be differentiated from that of areas with no muscle attachment. This suggests that extrinsic fibers play no important mechanical role in attaching muscle tissue to periosteal bone.

Effects of Taphonomic Processes on Microanatomy

One concern in the application of these relationships to fossil material is the fidelity and refractory nature of micron-scale features of fossil bone. Micron-scale features are easily destroyed or obscured on fresh collagenous bone with abrasion or pressure. However, the actions of predepositional agents which may destroy microanatomical features are often recognizable either grossly on the surface of a fossil (as with scavenging and weathering) or by microscopic evidence visible under SEM (as with abrasion) (Bromage 1984, Martin 1999 p.p.72-93). Fossils which have obviously been altered on their periosteal surfaces by these processes would be excluded from analysis.

Diagenesis is not necessarily destructive to fine surface features. The predominant replacement reaction in bone tissue is biogenic carbonate hydroxyapatite ($\text{Ca}_5(\text{PO}_4, \text{CO}_3)_3(\text{Cl}, \text{OH}, \text{F})_2$) being replaced with carbonate fluorapatite ($\text{Ca}_5(\text{PO}_4, \text{CO}_3)_3\text{F}_2$) (Zocco and Schwartz 1994, Hubert *et al.* 1996). This replacement should not result in irretrievable distortion of extrinsic fiber pits, as the carbonate fluorapatite crystals follow the original orientation of bone collagen, which diverges around extrinsic fibers (Boyde 1972). Secondary crystallites found in samples of fossilized *Seismosaurus* and theropod bone do not exceed 400nm in length (Zocco and Schwartz 1994), and would not obscure existing extrinsic fiber pits. Although the *Seismosaurus* and theropod samples do not necessarily represent a commonly occurring level or pathway of diagenesis, they do provide evidence that the diagenetic changes present in some fossil specimens do not prevent accurate analysis at the periosteal surface.

Retrieving Microanatomical Information from Fossilized Bone

Although silicone-epoxy casting does produce very high fidelity replicas of fossil material, casts of a fossil surface that was expected to show a very high density of extrinsic fibers only allowed for an ambiguous interpretation of these features. As of yet, it is not clear if extrinsic fibers are preserved on fossil bone of an age comparable to the *Brachylophosaurus* specimen in enough detail to expect accurate estimations of density. Given the descriptions of the effects of diagenesis on bone hydroxyapatite crystallites above, and the fine detail preserved in comparable histological specimens, the preservation of periosteal microanatomy on Cretaceous-age specimens should not be ruled out entirely, but much younger specimens (e.g. Miocene or later) may provide more promising material for examination. As the densities of extrinsic fibers indicative of dense regular connective tissue attachment are visible in histological sections, this process is at present the only reliable way to apply the relationships described in this study for reconstruction of soft tissues.

CONCLUSIONS

Extrinsic fibers cannot be used as direct microanatomical correlates of muscle attachment, but they can be used as indicators of the dense regular connective tissue framework present in soft tissue attaching to membrane bone. There are some indications that the density of extrinsic fibers can provide a record of the maximum expected stress at dense regular connective tissue attachments.

The presence of extrinsic fibers at the epimysium/bone interface of direct muscle attachments does raise the possibility of testing some previous osteologically-based muscular reconstructions. The fact that muscles can also originate and insert on nonmineralized tissue, such as ligaments, tendons, and cartilage, remains a barrier to accurate reconstruction using the morphology of mineralized tissues alone.

The viability of using high-fidelity replication and SEM to identify extrinsic fibers on the surface of fossil bone is unresolved, although studies of diagenesis and histological evidence suggest that there is some basis for continued research in this direction. Although histology is less attractive as an investigative process due to its destructive nature, it remains the only proven method of examining extrinsic fibers in fossil material.

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APPENDICES

APPENDIX A

STATISTICAL TREATMENT OF HYPOTHESES
AND DATA TABLE

STATISTICAL TREATMENT OF HYPOTHESES

First Hypothesis

This hypothesis can be represented mathematically as:

$$H_1: MdD_{TEN} \neq MdD_{DIR} \neq MdD_{NON}$$

However, because the statistical test used to examine this hypothesis (the Mann-Whitney U -test) is limited to two categories, this was tested in a pairwise fashion. As there are three categories, there are three separate pairwise comparisons to be made:

$$H_{1A}: H_0: MdD_{TEN} = MdD_{DIR}$$

$$H_a: MdD_{TEN} \neq MdD_{DIR}$$

$$H_{1B}: H_0: MdD_{TEN} = MdD_{NON}$$

$$H_a: MdD_{TEN} \neq MdD_{NON}$$

$$H_{1C}: H_0: MdD_{DIR} = MdD_{NON}$$

$$H_a: MdD_{DIR} \neq MdD_{NON}$$

Second Hypothesis

This hypothesis can be represented mathematically as:

$$H_2: Md\Delta_{TEN} \neq Md\Delta_{DIR} \neq Md\Delta_{NON}$$

And the three pairwise comparisons can be formulated as:

$$H_{2A}: H_0: Md\Delta_{TEN} = Md\Delta_{DIR}$$

$$H_a: Md\Delta_{TEN} \neq Md\Delta_{DIR}$$

$$H_{2B}: H_0: Md\Delta_{TEN} = Md\Delta_{NON}$$

$$H_a: Md\Delta_{TEN} \neq Md\Delta_{NON}$$

$$H_{2C}: H_0: Md\Delta_{DIR} = Md\Delta_{NON}$$

$$H_a: Md\Delta_{DIR} \neq Md\Delta_{NON}$$

Third Hypothesis

This can be mathematically represented as:

$$H_3: \rho(d, \sigma) \neq 0$$

As different muscle attachment types may display different relationships, this hypothesis will also be tested by attachment type. The complete formulation for statistical testing can be stated as:

$$H_{3A}: H_0: \rho(d, \sigma) = 0$$

$$H_a: \rho(d, \sigma) \neq 0$$

$$H_{3B}: H_0: \rho(d_{TEN}, \sigma) = 0$$

$$H_a: \rho(d_{TEN}, \sigma) \neq 0$$

$$H_{3C}: H_0: \rho(d_{DIR}, \sigma) = 0$$

$$H_a: \rho(d_{DIR}, \sigma) \neq 0$$

Fourth Hypothesis

This can be mathematically stated as:

$$H_4: \rho(\delta, \sigma) \neq 0$$

Taking into account attachment type, there are three correlations which can be formulated for statistical testing as:

$$H_{4A}: H_0: \rho(\delta, \sigma) = 0$$

$$H_a: \rho(\delta, \sigma) \neq 0$$

$$H_{4B}: H_0: \rho(\delta_{TEN}, \sigma) = 0$$

$$H_a: \rho(\delta_{TEN}, \sigma) \neq 0$$

$$H_{4C}: H_0: \rho(\delta_{DIR}, \sigma) = 0$$

$$H_a: \rho(\delta_{DIR}, \sigma) \neq 0$$

Fifth Hypothesis

This can be statistically formulated as:

$$H_5: H_0: b_{TEN, DIR} = b_{NON}$$

$$H_a: b_{TEN, DIR} > b_{NON}$$

Table 15. Attachment type, attachment area, and values of D , Δ , and σ for all plots used in this study.

Plot	Median D	Median Δ	Attachment	Stress σ	Description
PcP1-16	3.27	4	DIR	—	<i>Mpss</i> origin
PcP17-32	4.21	8	NON	0	Frontal bone
DnC1-16	2.69	39.5	TEN	0.0043	<i>Mamer</i> insertion
DnPt1-16	3.63	10.5	DIR	0.00161	<i>Mptv</i> origin
DnP1-16	2.76	2	DIR	0.000107	<i>Mpss</i> origin
DnA1-16	3.575	2	DIR	0.00205	<i>Mptv</i> insertion
DnF1-16	2.365	2.5	NON	0	Frontal bone
GgD21-36	4.7	20	TEN	0.00404	<i>Mame</i> insertion
GgPt1-16	3.46	2.5	DIR	0.0029	<i>Mptv</i> origin
GgPt17-32	3.435	2	DIR	0.00424	<i>Mptv</i> origin
GgP17-32	1.975	3	DIR	0.00166	<i>Mame</i> origin
GgF1-16	5.65	3	NON	0	Frontal bone
GgF17-32	3.32	2	NON	0	Frontal bone
IiC1-16	4.035	22.5	TEN	0.00237	<i>Mame</i> insertion
IiSa1-16	3.14	0	DIR	0.00237	<i>Mame</i> insertion
IiArt1-16	4.54	16	TEN	0.000965	<i>Mptv</i> insertion
IiP1-16	3.665	2	DIR	0.000601	<i>Mame</i> origin
IiF1-16	3.95	15	NON	0	Frontal bone
FsD1-16	3.36	18	TEN	0.00131	<i>Mpss</i> insertion
FsP1-16	1.72	6	NON	0	Frontal bone
FsP17-32	2.07	2	DIR	0.000166	<i>Mpsp</i> origin
FsA1-16	3.85	1	DIR	0.000983	<i>Mames</i> insertion
FsA17-32	2.83	1.5	DIR	0.00427	<i>Mptv</i> insertion
FsPt1-16	2.895	11.5	DIR	0.00395	<i>Mptv</i> origin
AmSa1-16	2.9	2	DIR	0.00514	<i>Mames</i> insertion
AmArt1-16	3.51	1	DIR	0.000579	<i>Mptv</i> insertion
AmP1-16	3.61	2.5	DIR	0.000246	<i>Mamep</i> origin
AmQ1-16	5.94	14	TEN	—	<i>Mamp</i> origin
AmF1-16	3.7	3	NON	0	Frontal bone

APPENDIX B

PHYLOGENETICALLY INDEPENDENT CONTRASTS

PHYLOGENETICALLY INDEPENDENT CONTRASTS

The derivation of PIC values from raw species data is a process of scaling the difference between two species values by the expected variance of those values, as determined by a random or Brownian motion model of character change. Figure 31 shows a generalized phylogeny to be used as an example.

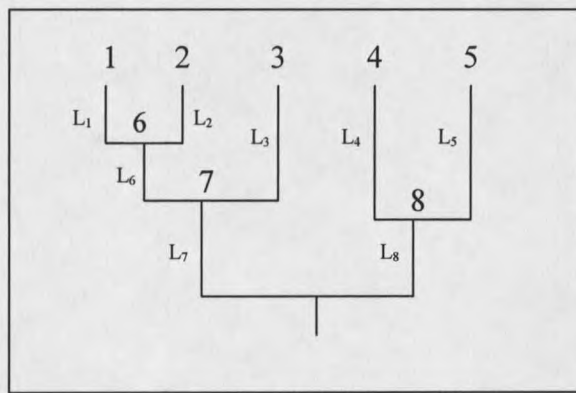


Figure 31. A phylogeny showing the relationship of species 1-5, their proposed ancestors 6, 7, and 8, and the intervening branch lengths L_1 - L_8 .

The contrast between the values of variable X in taxa one and two, $C_{(X_1 - X_2)}$, can be determined as per Felsenstein (1985) as:

$$C_{(X_1 - X_2)} = (X_1 - X_2) / \sqrt{v_1 + v_2}$$

where X_1 and X_2 are species values of variable X , and v_1 and v_2 are variances proportional to branch lengths L_1 and L_2 . After the determination of $C_{(X_1 - X_2)}$, species 1 and 2 are collapsed into their parent node 6, which is given the value:

$$X_6 = ((1/v_1)X_1 + (1/v_2)X_2) / (1/v_1 + 1/v_2)$$

which is the weighted average of X_1 and X_2 . This allows node 6 to be used as a nominal species value in comparison with other values on the phylogeny. The branch below node 6, L_6 , is lengthened by the value $(L_1 L_2) / (L_1 + L_2)$, as the estimation of X_6 introduces a margin of error that can be accounted for by including the proportional variance $(v_1 v_2) / (v_1 + v_2)$ (Felsenstein 1985).

PIC values can also be viewed as estimates of the rate of change of a trait over time. This study uses PIC values of extrinsic fiber density as a qualitative check for adaptive bias in extrinsic fiber morphology related to the phylogeny of the specimens studied.

APPENDIX C

CASTING TECHNIQUE

HIGH FIDELITY SILICONE/EPOXY CASTING TECHNIQUE

1. Stabilize fossil specimen and construct a plasticine dam around the area to be cast.
Dam walls should be higher than the highest point of the surface to be cast. The specimen information can be inscribed in negative on inner wall of the plasticine dam to produce a permanent description on the side of the silicone mold.
2. Pipette a small amount of acetone into the plasticine dam. Gently agitate with a soft brush to remove any plastic preservative. Pipette off acetone. Repeat until the surface to be cast is free of plastic residue.
3. Mix enough silicone to apply a thin layer to the fossil surface. Thorough mixing of the silicone and catalyst will ensure high mold fidelity.
4. Clean the surface with gentle application of compressed air.
5. Apply a thin layer of silicone to the fossil surface. Allow this first layer to set completely.
6. With a second batch of silicone, fill up the plasticine dam to create a solid backing for the silicone mold. Allow this second layer to set.
7. Remove the plasticine dam first, then carefully peel away the silicone mold. Re-apply plastic preservative to return the fossil to its original state.
8. Mix low-viscosity plastic resin, such as Spurr's epoxy or Quetol. Again, thorough mixing will ensure high fidelity.
9. Clean the silicone mold using compressed air.
10. Pour resin into silicone mold. Care should be taken to prevent the formation of bubbles.

11. Place the silicone mold under light vacuum for five to ten minutes.
12. Cure the plastic resin in an oven at 60° C overnight. Complete curing will preserve finer scale structures; it is best to err on the side of curing the cast for longer than is necessary.
13. Carefully peel the silicone mold away from the cured plastic cast.
14. Mount plastic cast on SEM and gold-coat for conductivity.

Technique after Bozzola and Russel 1991, pp. 54-55.

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