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VARIABLE LEVELS OF ATMOSPHERIC OXYGEN DO NOT AFFECT
MICROANATOMY AND LIMB BONE GEOMETRY IN
ALLIGATOR MISSISSIPPIENSIS

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Susan Lee Lujan
March 2013

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Approved by:


Dr. Kevin M. Middleton, Chair, Biology

8 March 2013
Date


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ABSTRACT

Striking changes in atmospheric oxygen levels have occurred since the beginning of the Phanerozoic 542 million years ago. Oxygen may have reached concentrations as high as 35% O₂ (hyperoxic conditions) during the Carboniferous and Permian when many clades of terrestrial and semi-aquatic vertebrates originated. Millions of years later, declining levels of ambient oxygen are thought to have contributed to the mass extinction of many members of these groups at the end of the Permian as atmospheric oxygen dropped to 15% O₂ (hypoxic conditions) by the early Triassic.

The morphological and physiological effects of chronic exposure to conditions other than the current normoxic levels of atmospheric oxygen (21% O₂) have been little studied. It is likely that adaptations to the substantial changes in oxygen availability over geologic time affected the course of vertebrate evolution.

Alligators belong to a clade of vertebrates with a long evolutionary history spanning much of this variability in atmospheric oxygen. An investigation into the effects of hypoxia and hyperoxia on skeletal growth and physiology in alligators may help to reconstruct the life history of archosaurs, explain patterns in the fossil record, and broaden our understanding of the processes underlying the long term success of this clade.

American alligator (*Alligator mississippiensis*) eggs were incubated and hatchlings raised in hypoxia or hyperoxia for up to two years. The femur was

used to assess the effects of non-normoxic ambient oxygen on skeletal growth and biomechanical performance by examining bone microstructure and cross-sectional geometric properties.

Variable levels of atmospheric oxygen were found to have no effect on the physiology and biomechanical performance of the alligator skeleton. In contrast to predictions of impaired growth in response to hypoxia, based on previous findings in many vertebrate clades, alligator femora did not exhibit a reduction in size, changes in shape or resistance to axial, bending or torsional stresses. Similarly, hyperoxic conditions did not facilitate an increase in the size of alligator femora, and the cross-sectional geometric properties of the bone were unaffected.

It is possible that the unique cardiovascular system in alligators and other crocodiles allows them to survive non-normoxic environments that would be detrimental to mammals and birds, and may be instrumental in the survival of this clade through the course of evolution.

Although differences in growth and performance among alligators in different oxygen treatments were not found, several interesting allometric relationships for alligators as a group were uncovered. The femora of these animals were considerably more robust and resistant to axial compression and tension than expected for their size, whereas the bones were significantly weaker in resistance to bending and torsion. These findings have implications for the ontogenetic changes in locomotor performance in these animals.

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CHAPTER ONE
VARYING LEVELS OF ATMOSPHERIC OXYGEN OVER EVOLUTIONARY
TIME AND IMPLICATIONS FOR THE GROWTH AND DEVELOPMENT OF
THE VERTEBRATE SKELETON

Introduction

Atmospheric oxygen is one of the most important abiotic factors underlying the physiological processes of living organisms (Fluck et al., 2007). In turn, biological activity influences the composition of the Earth's atmosphere (McAlester, 1970; Graham et al., 1995; Berner et al., 2003). As a key event in the evolution of life on Earth, the origin and initial rise of oxygen levels in the atmosphere have been the focus of decades of research (McAlester, 1970; Dudley, 1998; Canfield, 2005; Falkowski et al., 2005), although subsequent changes in atmospheric composition have received considerably less attention (Berner, 1999; Kump, 2008). Recent theoretical models have hypothesized that the concentration of oxygen varied widely during the past several hundred million years (Berner, 2006). Estimates suggest that atmospheric oxygen levels may have ranged from a high of 35% O₂ during the Permo-Carboniferous to 15% O₂ or lower in the Triassic (Berner et al., 2007; Figure 1). Insights into the morphological and physiological effects such variations in available oxygen may exert on animals during ontogeny will allow a better understanding of the evolutionary history of vertebrates.

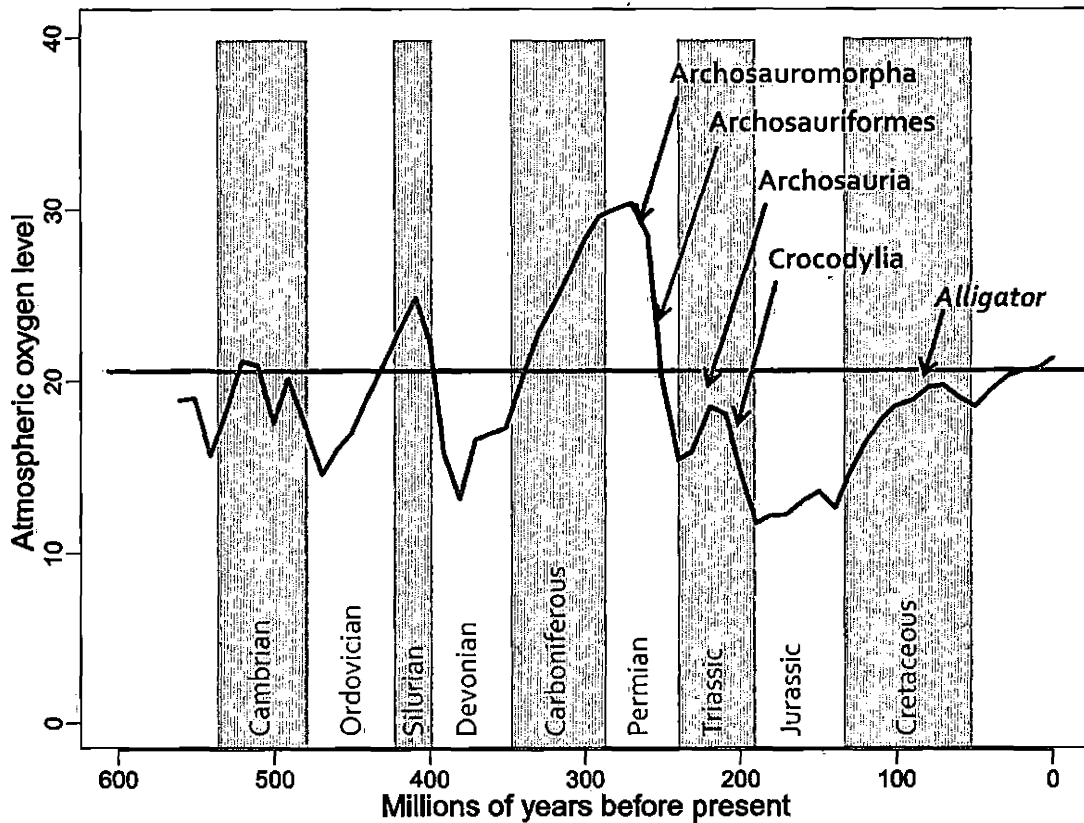


Figure 1. Atmospheric Oxygen in the Phanerozoic. A model for changes in atmospheric oxygen over the past 542 million years. The red line indicates present-day atmospheric levels (PAL) of oxygen while the blue curve represents the extent and magnitude of periods of hyperoxia (above PAL) and hypoxia (below PAL) through geologic time. The emergence of *Alligator* and clades ancestral to this genus (indicated by teal arrows) illustrate the evolutionary success of these vertebrates spanning extremes in atmospheric oxygen. Data from Berner, RA., VandenBrooks, JM., Ward, PD. (2007) Oxygen and evolution. *Science*, 316:557-558.

Atmospheric Oxygen in the Phanerozoic

Research has begun to focus on the rather neglected topic of atmospheric conditions during the Phanerozoic ca. 542 million years ago (Ma)

to the present, a time-frame corresponding to the evolution and spread of the major vertebrate phyla (Berner et al., 2003; Canfield, 2005; Fluck et al., 2007).

Instead of a steady rise to present levels, atmospheric oxygen fluctuated significantly during the Phanerozoic. Much of this variation was associated with altered climatic conditions and seems to be correlated with significant events in the history of life on Earth (McAlester, 1970; Berner, 1999; Fluck et al., 2007; Knoll et al., 2007).

Hyperoxia in the Carboniferous and Permian

Hyperoxia prevails when atmospheric oxygen concentrations are greater than current levels of 21% O₂ (normoxia). Several extended occurrences of hyperoxic conditions early in the Phanerozoic are thought to have contributed to increased size, complexity, and locomotor strategy in marine, and then later in terrestrial animals (Dahl, et al., 2010).

Early in the Paleozoic, levels of oxygen in the atmosphere initially ranged between 15% O₂ and 20% O₂ with a large, rapid increase beginning around 425 Ma in the late Ordovician, peaking as high as 25% O₂ in the Silurian and early Devonian before dropping back down to 15% O₂ (Graham et al., 1995; Berner et al., 2003; Figure 1). This hyperoxic time period corresponds to the initial terrestrialization of small arthropods (Graham et al., 1995) and the evolution of early aquatic stem tetrapods such as *Tiktaalik*, *Ichthyostega*, and *Acanthostega* (Clack, 1988; 2002).

A subsequent, much larger increase in atmospheric oxygen over a greater span of time occurred during the late Carboniferous and early Permian, with atmospheric oxygen hypothesized to have reached 35% O₂ approximately 280 Ma (Berner et al., 2003, 2007; Figure 1) which coincides with the terrestrialization and radiation of the early tetrapods (Ward, et al., 2006; Fluck, et al., 2007).

The emergence of new arthropod clades, and the evolution of insect flight occurred during this hyperoxic interval (Dudley, 1998; Harrison, et al., 2010). The increased availability of oxygen may have resulted in improved respiratory efficiency at decreased metabolic cost contributing to the extreme size seen in fossil insects such as *Meganeura monyi*, a giant dragonfly with a wingspan of 70 cm (Graham, et al., 1995; Harrison, et al., 2010). Very large size also evolved in many groups of previously rather small terrestrial vertebrates, possibly facilitated by similar respiratory and metabolic considerations (McAlester, 1970; Falkowski, et al., 2007).

These early terrestrial tetrapods evolved into several large amphibian clades, the Temnospondyli, Lepsospondyli, as well as groups closer to the origin of Amniota such as Reptilomorpha (Reisz, 1997; Germain & Laurin, 2005; Sanchez et al., 2010). Within the amniotes, two major clades, Synapsida and Sauropsida arose, eventually leading to Mammalia and Reptilia (including Archosauria and Aves), respectively.

Hypoxia in the Permian and Triassic:

Hypoxic conditions prevail when levels of atmospheric oxygen are less than normoxia. Periods of hypoxia accompanied several mass extinctions and may have contributed to the evolution of more effective respiratory function underlying the success of newly emerging taxa (Berner, et al., 2007). Late in the Permian, levels of atmospheric oxygen declined steeply; after a brief rebound, levels continued to drop to as low as 15% O₂, remaining low through the Triassic and Jurassic until beginning to rise again during the early Cretaceous (Berner et al., 2003; Bergman et al., 2004; Falkowski et al., 2005). The late Permian decline in oxygen to well below current atmospheric levels may have contributed to the largest known mass extinction event, during which as many as 78% of terrestrial species (and 92% of marine species) may have been become extinct (McAlester, 1970; Erwin, 1993, Graham et al., 1995; Knoll et al., 2007). In concert with other environmental factors such as increasing temperatures, climate change, and high levels of carbon dioxide, hypoxic conditions likely contributed to background extinctions and the delayed recovery from the extinction event as well (McAlester, 1970; Graham et al., 1995; Huey & Ward, 2005; Knoll et al., 2007).

Many small sauropsids survived the end-Permian mass extinction event as did members of Archosauromorpha, a clade of much larger stem diapsids that arose during the Carboniferous and persisted through the Permian (Lombard & Sumida, 1992; Reisz, 1997; Brochu, 2003). Although only a minor

element among the Permian fauna, archosauromorphs rapidly radiated to become the dominant terrestrial and semi-aquatic vertebrates in the Triassic, first giving rise to several diverse groups including stem-group Archosauriformes, and later crown-group Archosauria (Sereno, 1991; Brochu, 2003; Benton, 2004).

Archosauria

The two clades within Archosauria are Crurotarsi (the most recent common ancestor of the extant Crocodylia and all its descendants; *sensu* Sereno, 1991) and Ornithodira (the most recent common ancestor of Aves and all its descendants: *sensu* Gauthier, 1986). These divergent clades bracket extinct members of the Dinosauria, highlighting the phylogenetic importance of extant representatives of each group, as well as their relevance to understanding evolutionary patterns of locomotion and other life history strategies (Witmer, 1995; Brochu, 2001b; 2003).

Most of the crurotarsans became extinct during the end-Triassic mass extinction with only a few small terrestrial members of Crocodylomorpha, the line leading to modern crocodiles, surviving to diversify in the Jurassic (Brochu, 2003). Eusuchia, the true crocodiles, evolved from this clade during the Cretaceous beginning 146 Ma, with crown group Crocodylia and the three main groups of modern crocodiles (alligators, crocodiles, and gavials) present by the end of this period (Brochu, 1999, 2001a).

The Physiological Effects of Atmospheric Oxygen

During the course of vertebrate evolution, extended periods of hyperoxia or hypoxia (high or low levels of environmental oxygen, respectively) may have affected the physiology and evolutionary histories of many now extinct forms (McAlester, 1970; Berner et al., 2003; Falkowski et al., 2005). With the exception of several studies in avian embryos, investigations into the effects of hyperoxia in animals have mostly been limited to non-vertebrate taxa, with research often carried out at levels of oxygen availability much higher than those likely to have occurred during geologic time (Berner et al., 2003).

On the other hand, a great deal of work has been done on hypoxia in vertebrates, although mostly based on acute, rather than chronic, exposure. The effects of acute exposure are considerably different than those exerted on animals living under chronic conditions of low ambient oxygen (Ramirez et al., 2007). The developmental stage of the animal as well as the duration of the hypoxic event also contributes to the response to hypoxic stress (Mortola, 2004). For example, newborn mammals in hypoxia may exhibit a lowered metabolism and decreased body temperature but may not exhibit the hypoxic ventilatory response (HVR) or increased pulmonary vasoconstriction (HPV) characteristic of the acute response to hypoxia in an adult mammal (Mortola, 2004). During gradual acclimation to chronic hypoxia, HVR and HPV in adult mammals subside as other systems compensate for the lowered oxygen availability (Hochachka et al., 1998).

Mammalia

Of the studies on the effects of chronic hypoxia, many have focused on mammalian respiratory and cardiovascular systems, often in the context of human adaptation to activity at high altitude (Frisancho, 1970; Hochachka et al., 1999). In addition to the well-characterized incidence of low birth weight and prolonged but low growth rates leading to smaller adult size in human populations at high altitude (Frisancho, 1970), hypoxia also affects the timing of development and growth of various organs differently (Mortola, 2004). In contrast to the general retardation of skeletal growth, an increased rate of growth in the thoracic region is associated with adaptive changes in human cardiovascular and respiratory systems (Frisancho, 1970).

Hypoxia has also been shown to negatively affect growth and development in most small mammals (Matsumoto et al., 2011) although some animals, such as the mole rat, *Spalax ehrenbergi*, and the wombat, *Lasiorchinus latifrons*, are specialized for a fossorial or semi-fossorial mode of life, and are likely more tolerant of low levels of environmental oxygen (Widmer, et al., 1997; Frappell, et al., 2002). Frappell and colleagues (1994) demonstrated metabolic depression, decreased growth rates, and a 25% decrease in body mass accompanied by a significant increase in the mass of the heart in newborn laboratory rats chronically exposed to very low oxygen availability (11% O₂) for the first 21 days of life (compared to the control group raised in 21% O₂). In contrast, none of these effects were exhibited by similarly sized newborn

hamsters, a semi-fossorial species, in response to the same experimental conditions. As part of the same experiment, the authors reported a decline in metabolic rate in both laboratory rats and hamsters on initial acute exposure to 11% O₂, whereas among animals previously acclimated to hypoxic conditions, only the laboratory rat exhibited decreased metabolism; this response was dampened in the hamster (Frappell, et al., 1994).

Hunter and Clegg (1973a; 1973b; 1973c) carried out several studies illustrating the effects of exposure to chronic hypoxia on body mass and skeletal growth and development in laboratory mice (*Mus musculus domesticus*) and laboratory rats (*Rattus norvegicus*) at different stages of development. In the first experiment, juvenile and adult mice chronically exposed to severe (11% O₂) or mild (15% O₂) hypoxia exhibited an initial decrease in mass. Juveniles in severe hypoxia were affected the most, losing 14% of body mass in only 24 hours, nearly twice as much as the adults in the same environment. Juvenile and adult mice in mild hypoxia lost 1% of body mass in the same time period. After several days, juveniles resumed growth at a lower rate, however in adults, resumption of growth was delayed, but then occurred at a *higher* rate. Mice recovering in a normoxic environment demonstrated 'catch-up' growth, an increase over normal growth rates, which allowed all of the animals in mild hypoxia to reach the same or greater mass as that of controls, but only partial catch-up occurred in mice exposed to severe hypoxia (Hunter & Clegg, 1973a). A second experiment determined changes in

limb bones as well as whole-body growth in laboratory rats during chronic exposure to severe hypoxia (11% O₂). All of the bones studied exhibited a significant decrease in length and delayed maturity. The average body mass of the laboratory rats decreased by 36%, and body length by 14% compared to control animals (Hunter & Clegg, 1973b). That the effects of hypoxia may not only be permanent as suggested by the previous experiment, but result in an altered phenotype is shown by uneven 'catch-up' growth; experimental animals remained significantly smaller and with relatively longer limbs than control laboratory rats (Hunter & Clegg, 1973b). A final study found a significant decrease in the cross sectional area and size of the medullary cavity in the bones of mice and laboratory rats chronically exposed to severe hypoxia (11% O₂) for up to four weeks followed by a four week recovery period. Interestingly, mice responded to the experimental stress with increased development of the vasculature of the bone marrow whereas laboratory rats did not (Hunter & Clegg, 1973c) indicating species specific variation in phenotypic plasticity.

A more recent investigation on laboratory rats raised from birth in slightly milder hypoxic conditions (13% O₂) for four or eight weeks also resulted in severely retarded growth (Matsumoto et al., 2011). In contrast to the laboratory rats discussed in the previous experiment, tibial cross-sections in these animals showed an increase in the size, number and connectivity of the vascular canals in the cortex as well as an enlargement of the medullary canal from which most of the intracortical blood-vessels arise (Matsumoto, et al. 2011).

Reptilia (including Aves)

Embryos of the domestic chicken have been extensively studied as an animal model for investigation into the effects of oxygen availability in avians (Chan & Burggren, 2005). Eggs incubated under hypoxic conditions are frequently reported to result in decreased growth of the embryos and a significantly lower embryonic mass at hatching compared with normoxic controls. In contrast, only exposure to very high levels of hyperoxia (60% O₂) led to significantly increased embryo mass (Metcalf et al., 1981; Richards et al., 1991-1992; Burton & Palmer, 1992). Although hyperoxic conditions may occur locally, models suggest that atmospheric O₂ never exceeded 40% O₂ or dipped below 13% O₂ (Bernier, 2006; Belcher & McElwain, 2008).

In recent work aimed at identifying the time periods in embryonic development most susceptible to moderate levels of hypoxia, growth deficits in the chicken were compensated for by longer incubation time or an increased growth rate during subsequent recovery in normoxia, resulting in a normal phenotype at hatching, echoing the previously mentioned results of Hunter and Clegg (Dzialowski et al., 2002; Chan & Burggren, 2005).

Chan & Burggren (2005), noting the increased lethality of oxygen levels lower than 15% in chickens, suggest this level as an experimental standard for investigations into the effects of hypoxia in avians. However, ectotherms such as fish, amphibians, and non-avian reptiles may tolerate episodes of hypoxia that are of much greater magnitude and/or duration than endothermic mammals

and birds (Bickler & Buck, 2007). Some reptiles may curtail activity to endure extreme conditions; for example some turtles (*Chelydra*, *Chrysemys*) have been shown to survive extended periods in oxygen-free environments (Bickler & Buck, 2007). During embryonic development under natural conditions, lizards, turtles, and alligators may be exposed to hypoxic nesting environments, possibly as low as 7% O₂ which may alter the duration of egg incubation (Deeming & Ferguson, 1989; Kam, 1993; Crossley & Altimiras, 2005).

Andrews (2002) maintained eggs obtained from wild-caught lizards, *Sceloporus undulatus*, in chronic hypoxia (7% O₂) or hyperoxia (28% O₂), exposing them for either the first 21 days of embryonic development ('early' group) or the last 23-25 days ('late' group) with the remainder of incubation time in normoxic conditions. Lizard embryos exposed to the hypoxic environment delayed hatching and exhibited a higher mortality, especially late in development. Hatchlings were significantly smaller than controls, with body mass 10% and 15% ('early' and 'late' groups respectively) less than that of controls (Andrews, 2002). Decreased incubation time and larger hatchling size might be expected from lizard embryos incubated in hyperoxia, but only those in the 'early' group hatched sooner than controls, and there were no significant differences in hatchling mass in either of the groups (Andrews, 2002). Further highlighting the ability of these animals to tolerate low oxygen availability, Andrews (2002) also documented similar locomotor performance (running

speed) of lizard hatchlings among all groups regardless of size, levels of oxygen availability or duration of exposure.

In alligators, hatching was delayed by an average of 12 days beyond the normal development period of 72 days when eggs were incubated under mildly hypoxic conditions of 17% O₂. The newly hatched animals were significantly smaller, with a 14% decrease in length, and a 41% decrease in mass as compared with control animals (Warburton et al., 1995). In a similar study, Crossley & Altimiras (2005) incubated alligator eggs under more severe conditions (15% O₂ and 10% O₂) to determine the response of the developing embryos to hypoxic stress at regular intervals. Differences in growth were not apparent until the last 20% of the incubation period, when the mass of the alligator embryos developing in 10% O₂ dropped significantly lower than in other treatments. By the end of the experiment, the average mass of these embryos was 33% less than that of normoxic controls while embryos in 15% O₂ showed a similar trend, but mass was not significantly different than that of controls (Crossley & Altimiras, 2005). These animals also exhibited a relative increase in heart mass, a phenomenon also observed in turtles and avians (Kam, 1993; Chan & Burggren, 2005), but perhaps not seen in the previous study due to the nature of the hypoxic stimulus (Warburton et al., 1995).

These findings were later corroborated by further work on alligators in which eggs were incubated and hatchlings maintained under hypoxic (12% O₂) or hyperoxic (30% O₂) conditions (Owerkowicz, Elsey, et al., 2009). After a

normal incubation period, hatchlings which had developed in hypoxia exhibited an enlarged heart, significantly decreased body length (15% less) and mass (33% less) compared with that of normoxic controls. After three months of continued exposure to hypoxia, a dramatically lower growth rate in these alligators resulted in length 21% less and mass nearly 50% less than that of controls (Owerkowicz, Elsey, et al., 2009; Table 1).

Table 1. Results of Selected Experiments on Oxygen Availability. For duration C indicates chronic exposure and A indicates acute exposure.

	Species	O ₂	Duration	Results (compared to normoxia)
Andrews (2002)	Lizard	7	C	Decreased mass, delayed incubation, increased mortality
Burton et al., (1992)	Chicken Embryo	14, 40	C	Decreased mass of embryo in hypoxia, no effects in hyperoxia
Chan & Burggren (2005)	Chicken Embryo	15	C	Abnormal development, normal hatchling
Crossley & Altimiras (2005)	Alligator Embryo	10	C	Decreased mass, delayed incubation
Dzialowski et al., (2002)	Chicken Embryo	14	C	Decreased mass, recover by hatching
Frappell & Mortola (1994)	Hamster Adult	11	C	Maintain growth and metabolism
Harrison et al., (2010)	Insects	10, 40	C	Smaller in hypoxia, Larger in hyperoxia w/tracheal decrease
Hunter & Clegg (1973)	Mouse & Rat (Juvenile)	11	C	Severely retarded growth, partial recovery
Kam (1993)	Turtle Embryo	10	C	Retarded growth and metabolism
Matsumoto et al., (2011)	Rat Juvenile	13	C	Decreased growth, increased vascularity
Metcalfe et al., (1981)	Chicken Embryo	60	C	Increased mass
Owerkowicz, Eelsey, et al., (2009)	Alligator Embryo and post-hatchling	12-30	C	Decreased mass in hypoxia, increased mass of some organs
Richards et al., (1991-1992)	Chicken Embryo	15, 60	C	Decreased/Increased mass organs respectively
Warburton et al., (1995)	Alligator Embryo	17	C	Decreased mass, delayed incubation
Widmer et al., (1997)	Mole Rat Adult	4.6	A	Active in hypoxia, metabolism depressed at rest
Williams & Swift (1988)	Quail Embryo	60	C	No change in growth rate

Phylogenetic Context

As the clade including dinosaurs and birds, the Archosauria has been a major focus of paleontological research for over 150 years (Brochu, 2003). With

ancestors among basal reptilian taxa, and Aves and Crocodylia as extant representatives, early archosaurs are of central importance in the history of vertebrate evolution (Parrish 1987; Sereno, 1991). Early work was descriptive, but more recently, an increased emphasis on discovering the phylogenetic relationships among members of this clade has allowed a better understanding of the biology of these animals. Researchers wishing to reconstruct the physiology of fossil vertebrates often base their inferences on comparisons with related extant animals, an idea formalized as the Extant Phylogenetic Bracket (EPB) by Witmer (1995). This approach is often based on the assumption that the physiology of the extinct organism is similar to that of taxa living in the present-day environmental conditions of 21% atmospheric oxygen, but may need to be tempered with the consideration of the effects on physiology in the context of atmospheric oxygen variation over geologic time (McAlester, 1970; Berner et al., 2003). It has been appreciated at least since the 1960's that the availability of environmental oxygen profoundly influenced the course of evolution (McAlester, 1970), but the effects and timing of hyperoxic and hypoxic events on the physiology of extinct animals are only recently beginning to be considered (Owerkowicz, Elsey, et al., 2009; Harrison, et al., 2010). Investigation into the chronic effects of exposure to non-normoxic oxygen levels in a wider range of extant species will facilitate our understanding of form and function in extinct animals as well as evolutionary patterns evident in the fossil record (McAlester, 1970).

The fossil record of stem crocodylians and their Permian and early Triassic ancestors spans most of the oxygen extremes of the Phanerozoic (Brochu, 2001a; 2001b; 2003; Owerkowicz, Eusey, et al., 2009). With this long evolutionary history, retention (or secondary acquisition) of the 'primitive' body type and ancestral sprawling posture and gait, the extant crocodylian *Alligator mississippiensis* is an appropriate model for the study of the anatomical and physiological consequences exerted by varying levels of atmospheric oxygen during vertebrate evolution (Owerkowicz, Eusey, et al., 2009).

As a well-studied poikilothermic reptile with a life history strategy typified by relatively slow growth to very large size, a long reproductive period, high longevity, and an enormous size range (45-500+ kg), alligators are also capable of altering their growth rate in response to environmental cues and are excellent subjects for growth studies (Wilkinson, 1997; Andrews, 1982; 2002). In this study, I use analyses of histological features and cross-sectional geometry to investigate possible effects of long-term experimentally controlled alterations in oxygen environment on skeletal growth and biomechanical performance in the limb bones of *Alligator mississippiensis*.

Inferring Physiology from Histology

Many aspects of the biology of extinct vertebrates can be inferred from well preserved fossil remains (Benton, 2004). When skeletal material is fairly complete the size and morphology of the animal can often be reconstructed

based on joint anatomy, evidence of muscle attachments, and structural comparisons with related extant taxa (Witmer, 1995; Erickson et al., 2000; Padian et al., 2004; Chinsamy-Turan, 2005; Benton, 2010). Resemblance to living forms may help to explain posture, mode of locomotion, and adaptations for terrestrial, aquatic/marine, or aerial lifestyles in extinct animals. Such fossil finds are rare. Far more common in the fossil record are disarticulated specimens, partial skeletons and isolated skeletal elements, making such deductions much more speculative (Lauder, 1995).

Bone microstructure contains a great deal of information that complements traditional macro-scale description and may help elucidate other *features of the life history of extinct animals* such as growth rate, life-span and locomotor performance. Fortunately, these microstructural analyses can be carried out on incomplete or fragmentary remains (e.g., Chinsamy, 1995; Horner et al., 2001; de Ricqlès et al., 2003; Botha-Brink & Smith, 2011).

In Chapter 2, I will present a brief overview of bone formation and the mechanisms underlying the range of bony tissue types before introducing the first part of my project, an investigation into the effects of oxygen availability on skeletal growth and development in alligators as assessed by potential differences in the microstructural features of the femur.

Inferring Skeletal Performance from Biomechanical Properties

The preparation of histological specimens for microstructural analyses also affords an opportunity to examine the biomechanical properties of bone. The strength of bone depends not only on the properties of the internal material, but on the size and shape of this material as well, both of which can be altered in response to stress (Currey, 2002; Erickson, et al., 2002). Cross-sectional geometry reveals the loading environment that the bone has experienced, and has been used to estimate biomechanical performance in extant animals, as well as infer lifestyle, posture, and locomotor regimes in fossil vertebrates (Demes et al., 1991; Demes & Jungers, 1993; Chinsamy et al., 1995). Cross-sectional geometry may be especially useful in animals with limb bones lacking distinguishing external features, such as those of many crurotarsans (Meers, 2002).

In Chapter 3, I will review the biomechanical function of the vertebrate skeleton in terms of the ability of bone to resist stresses imposed during locomotion before describing the second part of my project, an examination of the cross-sectional geometric properties of the femur related to the strength of bone in alligators raised in different oxygen environments.

CHAPTER TWO

AN INVESTIGATION INTO THE EFFECTS OF VARYING LEVELS OF AMBIENT OXYGEN ON SKELETAL PHYSIOLOGY THROUGH AN ANALYSIS OF MICROANATOMY OF LIMB BONES IN ALLIGATORS

Introduction

The microstructure and material properties of bone are often of interest in experimental or paleontological work, and result from the presence or absence of various types of osseous tissues in different combinations, as well as their characteristic patterns of deposition (Francillon-Vieillot et al., 1990). Osteohistology is a destructive process that involves the sectioning of bone for microscopic analysis of internal architecture, growth processes and tissue composition. Each tissue type exhibits a characteristic collagen fiber orientation and vascular patterns that may correlate with the local rate of deposition, degree of mineralization and strength of bone (de Margerie et al., 2002; Currey, 2003; Jee, 2001; de Ricqlès et al., 2003). Relative rates of bone formation as well as ontogenetic changes in these rates and/or the type of bone being deposited have been assessed in numerous modern taxa (e.g., Castanet et al., 2000; de Margerie et al., 2002). The application of this method to fossil bones originated in the nineteenth century and is a field which has recently been the focus of renewed interest, allowing inferences into the growth strategies of extinct animals (Horner et al., 2001; Padian et al., 2001).

Bone Formation

Bones of the limb skeleton are formed through the process of endochondral ossification (EO) by replacement of a cartilage model by bone during development (Hall, 2005). Bone only forms by apposition on existing surfaces (Francillon-Viellet et al., 1990). EO is initiated by the invasion of cartilage by blood vessels, leading to the destruction of the original extracellular matrix and the establishment of a primary center of ossification in the diaphysis (bone shaft). Bone formation by cells of the osteoblastic lineage then proceeds from this center toward the epiphyses (Carter & Beaupre, 2001). In mammals and lizards, a secondary center of ossification often forms within the epiphyses (bone ends) establishing a growth plate responsible for elongation of the bone until fusion of the ossification fronts occur, resulting in determinate growth (Haines, 1942; Carter & Beaupre, 2001). In other animals, such as crocodiles and turtles, the ancestral pattern of EO is retained; no secondary ossification center is produced, allowing for possible indeterminate growth throughout life (Haines, 1938; 1939; Carter & Beaupre, 2001; Reno et al., 2007).

The cortical diaphysis of a long bone is initially formed without a cartilage model through intramembranous ossification (IO) and further growth in circumference occurs by deposition of new bone under the periosteal membrane surrounding the bone (Francillon-Vieillot et al., 1990). The medullary canal houses the bone marrow and is formed by erosion of internal cancellous bone and the formation of an inner membrane, the endosteum (Hall, 2005).

Primary and Secondary Bone

As the formation of primary bone proceeds, osteoblasts secrete osteoid, a collagen containing ground substance (Francillon-Vieillot et al., 1990).

Collagens are structural proteins; the packing of collagen molecules into microfibrils and their self-aggregation into fibrils provides the framework for mineralization of the extracellular matrix (Martin, et al., 1998). The mineral phase of bone imparts hardness to the bone, while collagen contributes to its resilience (Martin & Ishida, 1989; Turner, 2006).

During normal growth, modeling allows for external changes in size and shape of the bone through a combination of new tissue formation by bone by osteoblasts as well as the removal of bone by osteoclasts (Jee, 2001).

Remodeling, or secondary reconstruction, replaces primary bone and requires the coordinated action of both cell types (Frost, 2003). Pre-existing bone is eroded by the activity of osteoclasts prior to the deposition of new bone by osteoblasts (Hall, 2005). Whereas both modeling and secondary reconstruction may occur in response to biomechanical demands placed on the skeleton, remodeling allows for changes not only to the external surface, but the internal structure of bone as well (Currey, 2002). Secondary reconstruction is common among large birds, mammals and dinosaurs; whereas traditionally this has been thought to occur to a rather limited extent in other vertebrates (Padian & Horner, 2004; Chinsamy-Turan, 2005), recent work suggests that remodeling may occur to a greater extent than previously realized (Middleton,

personal communication, unreferenced). In addition, such remodeling has also been noted in basal synapsids, such as the Permian *Dimetrodon* (Huttenlocker et al., 2010).

Types of Bone

The presence of various types of growth marks can be used to identify whether growth is cyclic, in response to seasonal environmental cues as is typical for crocodylians and many other reptiles (Chinsamy et al., 1995).

Seasonal growth processes result in lamellar-zonal bone (LZB, Francillon-Vieillot et al., 1990). Transverse sections may reveal a pattern of alternating bands of different tissue types: wide zones of vascular bone representing the period of active growth followed by narrow avascular annuli indicating much slower growth (Reid, 1984; Horner et al., 2000; de Ricqlès et al., 2008). Lines of arrested growth (LAGs) representing a temporary cessation of growth may also be present (Padian & Horner, 2004).

To keep pace with the accelerated development and early growth of the tetrapod skeleton, woven bone, a type of fibrolamellar bone (FLB) consisting of a loose, disorganized arrangement of collagen fibers and osteocytes in a porous yet highly mineralized matrix forms first (Currey, 2003). FLB is a weak, but very rapidly formed tissue that may later be replaced by lamellae, layers of less vascular slowly deposited tissue, characterized by more organized osteocytes and highly ordered collagen fibers which contribute to the increased strength of this bone (Martin et al., 1998). The various subtypes of FLB are

classified by the vascular pattern exhibited by the bone (de Ricqlès et al., 1991; Francillon-Vieillot et al., 1990). As blood vessels proliferate and become enclosed during bone formation, simple vascular canals of varying size, orientation, and connectivity result and appear to be correlated with growth rate and the biomechanical properties of the bone (de Margerie et al., 2002; Castanet et al., 1996).

Amprino's Rule

The hypothesis that rates of bone formation, and hence, rate of growth, may be inferred from the vascular patterns of primary bone tissue types is known Amprino's Rule. In the simplest interpretation of this idea, a high density and extensive branching of vascular canals is expected in rapidly growing bone (Castanet et al., 2000; de Margerie, 2002, de Ricqlès et al., 1991). This hypothesis is intuitive, based on the greater blood supply required in tissues exhibiting higher metabolic rates, and is supported by the work of Cubo et al., (2008). Using *in-vivo* fluorochrome bone labeling, the rate of bone formation in the tibia of the Nile crocodile (*Crocodylus niloticus*), an ectotherm typified by poorly vascularized LZB, was found to be only 12% that of the mallard (*Anas platyrhynchos*), an endotherm characterized by highly vascular FLB (Cubo, et al., 2008). The rapid growth associated with the continual, uninterrupted deposition of FLB is suggested by the absence of regularly spaced growth marks and is often seen in extant large birds and mammals as well as many dinosaurs (Reid, 1984; Chinsamy et al., 2009).

Functional Interpretation of Bone Types

LZB with little secondary remodeling of primary tissue predominates in extant ectothermic animals, and FLB with extensive secondary replacement is fairly common among extant large endothermic animals (Reid, 1984). Highly vascular FLB indicative of rapid growth has been shown in the terrestrial archosauriform *Erythrosuchus* and several other groups ancestral to Archosauria, suggesting that LZB and the slower growth strategy of modern crocodylians may be a secondarily derived feature associated with a reversion to a semi-aquatic ecology (Parrish, 1987; Seymour et al., 2004; de Ricqlès et al., 2008). FLB and rapid growth is also suggested for *Maiasaura*, a very large iguanodontid thought to have reached adult size in only a few years (Horner et al., 2000), while the much smaller thyreophoran *Scutellosaurus* grew more like crocodiles (Padian et al., 2004). Although many similar studies have used these traits to infer the life history and categorize extinct animals, the differences are not absolute. For instance, many small mammals do not exhibit FLB, which may reflect a growth strategy related to size, lifespan, or locomotor performance (Erickson, 2005). On the other hand, extensive FLB has been demonstrated in a population of wild, adult *Alligator mississippiensis*, a phenomenon that had previously been assumed to occur in these animals only in juveniles or under optimal conditions in captivity (Reid, 1996; Tumarkin-Deratzian, 2007).

Another unexpected finding, documented by Kohler and Moya-Sola (2009), is the presence of LZB in a fossil mammal, *Myotragus*, an extinct dwarf bovine also exhibiting LAGs. True LAGs are marked by the erosion of bone during cessation of growth, and a reversal line when growth resumes (Reid, 1996). According to Reid (1996) these markings seem to be restricted to ectothermic animals, but not all researchers agree with this assessment (Padian & Horner, 2004). Rest lines (RL) are growth lines corresponding to pauses in otherwise continuously growing bone but are unaccompanied by resorption (Chinsamy-Turan, 2005). They are sometimes also referred to as LAGs and are known to form in some hibernating mammals, cold-adapted marine mammals, and more recently in deer and kangaroos (Horner et al., 2000; Chinsamy-Turan, 2005). Despite the prevalence of references to LAGs in the literature, there is a lack of consensus regarding the definition of these and other growth lines (Padian et al., 2004), and underlying mechanisms leading to their formation are not fully known (Klein et al., 2009).

In this study, I investigate the potential effects of variable atmospheric oxygen levels on histological features related to skeletal growth in the alligator.

Experimental Design

To gain insight into the consequences of varying levels of oxygen on skeletal physiology, I chose an ectothermic model organism: *Alligator mississippiensis*. In order to test for possible effects on skeletal growth, I used alligators raised under differing levels of ambient oxygen, simulating the range

of atmospheric oxygen availability during the origin and radiation of archosaurs and their ancestors. I used the femur (the most commonly used skeletal element in comparative studies of limb bones) and standard histomorphometric techniques to investigate the effects of hyperoxia and hypoxia on skeletal growth in alligators. I hypothesized that the limited oxygen available to alligators reared in a hypoxic environment will result in reduced growth reflected by a decrease in osteocyte density and the extent and rates of bone formation compared with alligators reared under conditions of hyperoxia.

Materials and Methods

Specimens of *Alligator mississippiensis* preserved in formalin were obtained from Professor James Hicks at University of California, Irvine. The alligators had been incubated and raised under different oxygen concentrations: 16% (n=8), 26% (n=8), 31% (n=11), and 36% O₂ (n=11), mirroring Phanerozoic atmospheric oxygen conditions. The animals received injections of a fluorochrome dyes (calcein and alizarin) at four weeks and twelve weeks post-hatching before sacrifice (at 16 weeks) to ascertain rate of bone growth. The dyes bind to calcium and become incorporated into the newly forming bone as it becomes mineralized and can be visualized with a microscope using UV illumination.

Specimen Preparation

The right femur of each specimen was dissected and prepared for histological analysis as in An, et al. (2003), by removal of soft tissue, brief formalin fixation (1 hour) to ensure adequate preservation, followed by a graded ethanol dehydration series (1 hour each in 30%, 50%, 70%, 80%, 90%, 95%, 100% ethanol) and air drying for 24 hours. Femoral length was measured with digital calipers, and the bones were then processed as undecalcified ground sections to preserve the vital dyes. Each femur was positioned in the same anatomical orientation (dorsal aspect facing up) in a labeled foil tray lined with hardened resin and then covered with epoxy resin, (EpoThin; Buehler Ltd.) and allowed to cure for two days. During the polymerization process, the femora were simultaneously impregnated with resin and embedded in a hardened block which supported the bone and subsequently allowed for even thin-sectioning. The anatomical orientation of each bone (indicating dorsal and medial surfaces) was marked by labeling the corresponding sides of the block with different colors of permanent ink, and three ~1mm serial transverse sections were removed from the mid-diaphysis of each specimen with a low-speed saw (IsoMet; Buehler, Ltd.).

The proximal surface of each section was indicated by a small dot of permanent ink in the corner as it was removed from the saw. This mark, together with the colored labels from the block now visible along the dorsal and medial edges of the section, allowed the original anatomical orientation to be

preserved as the sections were mounted onto a labeled slide with resin. After a second round of curing, sections were ground to a thickness of approximately 125 μm (checked with calipers) using a graded series (600 to 1200 grit) of abrasive paper on a grinder-polisher (MetaServ, Buehler, Ltd.) and finally polished with 2400 grit abrasive paper.

Image Analysis

Each section was viewed and photographed using a polarizing microscope (Nikon Eclipse LV100) fitted with a digital camera and image capture software (Spot Imaging Systems). Images were taken of the entire cross section at 40X, as well as of each quadrant of the cross section (dorsal, ventral, anterior, and posterior aspects) at higher magnifications of 100X and 200X. The images were then analyzed using NIH ImageJ (<http://rsbweb.nih.gov/ij/>).

The extent of bone formation was assessed using 100X images, and the distance between the fluorochrome labeled bands corresponding to the vital dyes, calcein (yellow) and alizarin (red) was measured using the 'straight-line' selection tool in NIH ImageJ. Bone formation rate (BFR) was calculated as the extent of bone formation divided by the number of days between administration of the fluorochrome dyes (60) and reported as $\mu\text{/day}$ for each quadrant (dorsal, ventral, anterior, posterior) of the cross-section (Ebner, 2003; Figure 2).

Osteocyte density was determined from 200X images by choosing a randomly selected square area ($5\mu \times 5\mu$) free of vascular canals in each

quadrant of the cross-section and manually counting the number of osteocyte lacunae present using the 'point selector' tool in NIH ImageJ. Density was reported as the number of cells/unit area (Figure 3).

Statistical Analysis

All measurements were \log_{10} transformed prior to analysis. Analysis of variance (ANOVA) was used to test for differences in femur length and osteocyte density between the treatment groups. Analysis of covariance (ANCOVA) was used to test for differences in bone formation rates between the treatment groups. In the latter analysis, femur length was included as a covariate to account for variation of body size. Body mass was not available for the alligator specimens, and FL has been shown to be a suitable proxy for body size in crocodylians (Farlow et al., 2005). All statistical tests were carried out using R (R Development Core Team, 2012) and were evaluated at $\alpha = 0.05$.

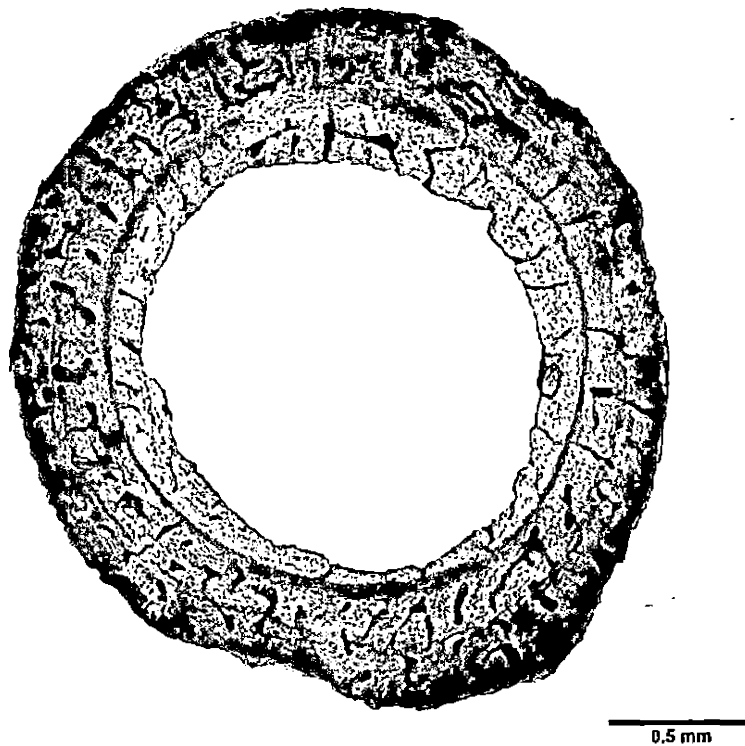


Figure 2. Fluorochrome Labeling of Bone. Calcein (yellow) and alizarin (red) administered at 4-weeks and 12-weeks of age during alligator growth become incorporated into newly forming bone and mark the extent of bone formation. Bone formation rates are determined by measuring the distance between labeled bands and dividing by the time between injection of the dyes. Bone deposition occurs on both the outer (periosteal) as well as on the inner (endosteal) surfaces of bone, but as endosteal bone is removed as the medullary canal enlarges during growth, only periosteal BFR was determined. Scale bar = 0.5mm.

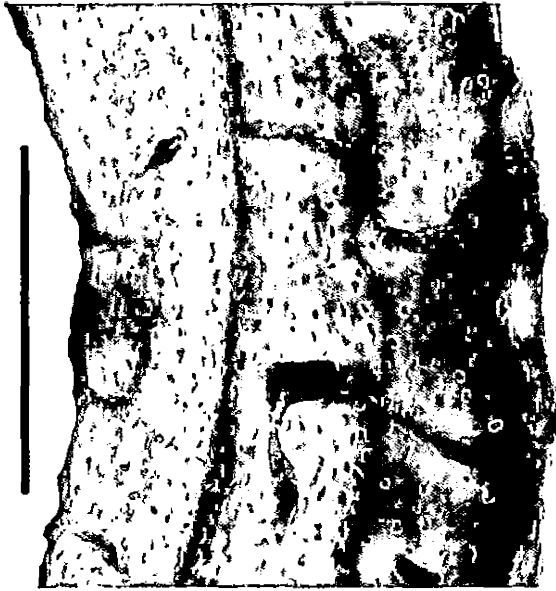


Figure 3. Osteocyte Density. The posterior quadrant of the same cross-section seen in Figure 3. The small cavities are osteocyte lacunae, and the large irregular openings are vascular canals. Osteocytes represent the terminal differentiation stage of the osteoblast lineage, the cells responsible for forming bone. Scale bar = 0.5 mm.

Results

No significant differences in mean femur length between the four oxygen treatment groups were found (ANOVA, Table 2). Similarly, mean osteocyte density in each of the four quadrants did not differ significantly between the oxygen treatment groups (ANOVA, Table 2).

No significant interaction effects of oxygen treatment with femur length were found. Regression slopes were compared and were not significantly different. When slopes were combined into a single slope, intercepts were significantly different for dorsal BFR only, but only barely ($P = 0.04$; ANCOVA, Table 2; Figures 4, 5, 6, 7).

Table 2. ANOVA and ANCOVA Analyses of Microanatomical Traits. Results (F statistics and P values) are shown for femur length (FL) and osteocyte density in the dorsal, ventral, anterior and posterior quadrants (ODD, ODV, ODA, and ODP, respectively) versus oxygen treatment in ANOVA and periosteal bone formation rates in dorsal, ventral, anterior and posterior quadrants (BFRD, BFRV, BFRA, and BFRP, respectively) versus oxygen treatment in ANCOVA. Significant P values are indicated by *.

Trait	df	F	P
FL	3, 33	1.93	0.14
ODD	3, 33	1.51	0.23
ODV	3, 33	1.77	0.17
ODA	3, 33	0.064	0.92
ODP	3, 33	0.704	0.40
BFRD	3, 32	3.2	0.04*
BFRV	3, 32	0.828	0.49
BFRA	3, 32	1.03	0.39
BFRP	3, 32	0.258	0.86

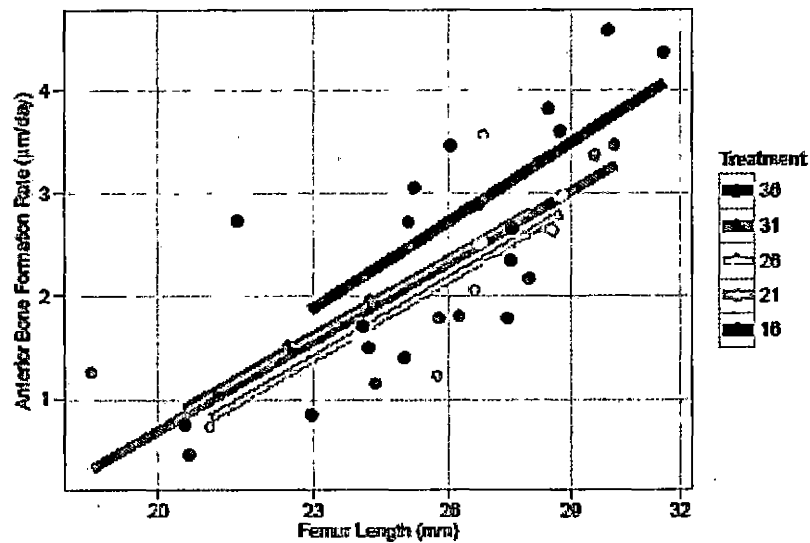


Figure 4. Anterior Bone Formation Rate. The slopes of the regression lines between the different oxygen treatment groups were not significantly different. A model combining the slopes into a single slope and testing for differences in intercepts between the different oxygen treatment groups was also not significantly different. (Table 2).

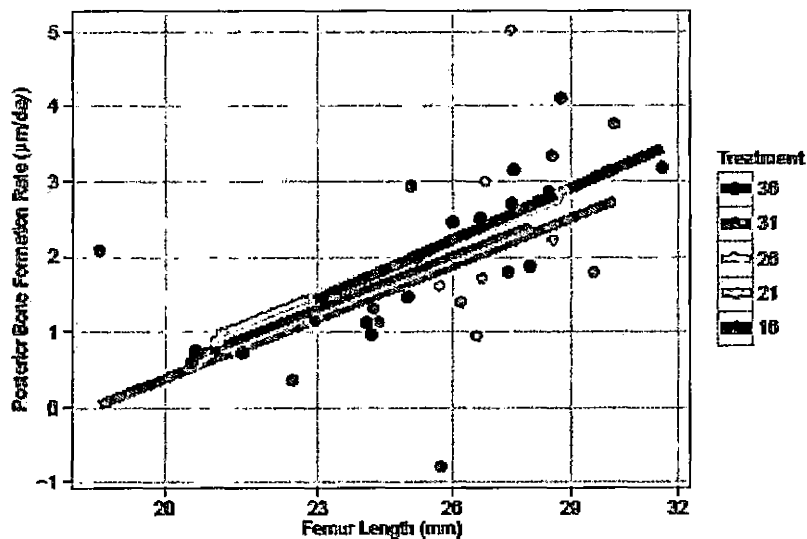


Figure 5. Posterior Bone Formation Rate. No significant differences in slope or intercept between oxygen treatment groups. (Table 2)

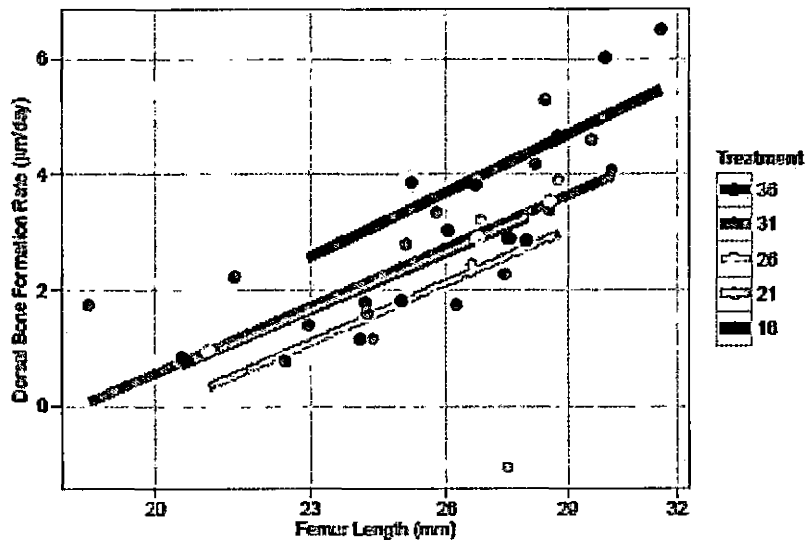


Figure 6. Dorsal Bone Formation Rate. The regression slopes between the treatment groups were not significantly different, but intercepts were significantly different when slopes were combined. (Table 2)

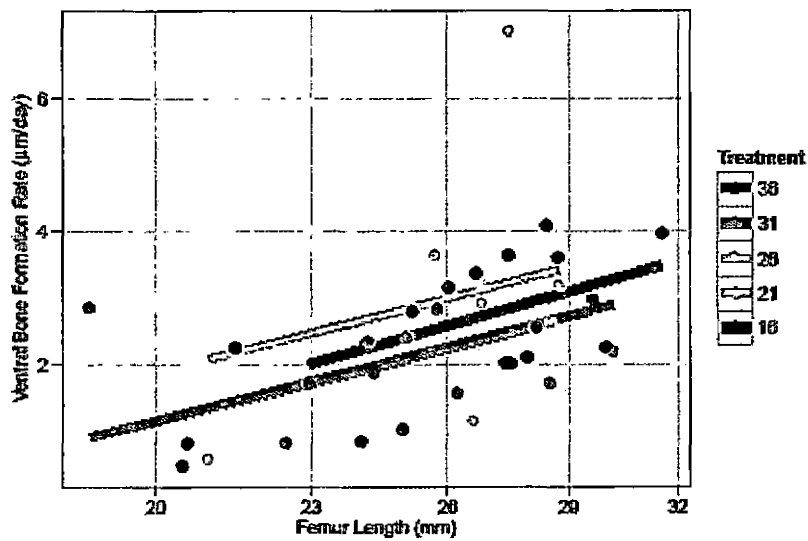


Figure 7. Ventral Bone Formation Rate. No significant differences in slope or intercept between oxygen treatment groups. (Table 2)

Discussion

Studies of the histological features of bone have been an important part of paleontological research since the development of ground sectioning in the 1800's, with the techniques later finding application in clinical investigations into pathologies of the human skeleton (de Ricqlès, 2007). Early work attempted to use the characteristics of various types of bone tissue to categorize taxonomic differences between vertebrate clades, as well as to infer thermophysiology in extinct forms with ambiguous results (Chinsamy-Turan, 2005). Continued investigation has revealed the complexity underlying the process of bone formation and has fostered an increased focus on the relationship between form and function in the skeleton (Erickson, 2005; de Ricqlès, 2007). Bone exhibits a high degree of phenotypic plasticity. During development and later growth, many factors act to modify the original genetic template of bone; internal homeostatic mechanisms are affected by nutrition, health, activity and biomechanical demands, as well as environmental influences (Hall, 2005). Environmental oxygen is thought to have been one of the most important evolutionary forces in vertebrate history, yet the effects of oxygen on skeletal growth and function are poorly known. In this study, I examined potential variations in the osteohistological characteristics of the femur engendered by exposure to different environmental oxygen regimes.

The results reported here, of little to no significant differences in osteocyte density and bone formation, were unexpected in light of previous

studies describing decreased growth and body mass in response to hypoxia, and possible permissive effects of hyperoxia leading to increased size and body mass in alligators and other reptiles (Andrews, 2002; Owerkowicz, Eelsey, et al., 2009, Crossley & Altimiras, 2005; Warburton et al., 1995). Stunted growth in alligators reared under 17% O₂ was reported by Warburton and colleagues (1995) with similar findings for alligator hatchlings in more severe hypoxic conditions of 10% O₂ and 12% O₂ (Crossley & Altimiras, 2005; Owerkowicz, Eelsey, et al., 2009) as well as lizards in 7% O₂ (Andrews, 2002). I expected that the experimental conditions of 16% O₂ in this study would have resulted in a decrease in femur length for alligators reared under hypoxia, and a corresponding decrease in osteocyte density and bone formation rate.

Owerkowicz and colleagues (2009) observed an increased rate of growth in juvenile alligators reared in a hyperoxic environment of 30% O₂. I expected an increase in femur length for alligators reared in hyperoxic conditions of 26% O₂, 31% O₂ and 36% O₂ in this study, accompanied by increase in osteocyte density and bone formation rate.

Osteocyte Density

Osteocytes are destroyed during specimen preparation, but the lacunae in which the cells reside are preserved and provide evidence of size, shape and orientation of the cells during life, and allow for cell density to be determined. Contrary to my predictions, osteocyte density was not affected by either hypoxia or hyperoxia (Table 2). I hypothesized a lower density of osteocyte

lacunae in the femora of alligators in the hypoxic treatment groups, because hypoxia decreases the numbers and activity of osteoblasts, at least *in vitro* (Utting et al., 2006). Osteocytes represent the end-stage of the life cycle of cells in the osteoblast lineage. The fraction of cells enclosed in the bone matrix are thought to provide a reasonable means of comparing the metabolic activity of the bone tissue, at least within a given species (Bromage et al., 2009). Osteocyte density has been shown to vary between bone types, with rapidly deposited woven bone having a greater concentration of cell lacunae than slower growing lamellar bone (Ferretti et al., 1999).

At a critical threshold of hypoxia in tissue, cell metabolism shifts from aerobic to anaerobic in response to the activity of the transcription factor Hypoxia Inducible Factor, HIF-1 α which decreases energy expenditure by the cell and upregulates genes for glycolysis to allow survival of low oxygen stress (Schipani et al., 2001). Glycolysis increases the acidity of the bone microenvironment due to lactate production which has also been shown to decrease bone formation rate *in vitro* (Brandao-Burch et al., 2005). It seems reasonable to predict a similar response to hypoxia and acidity for osteoblasts *in vivo*, although it is also likely that hypoxia induced angiogenesis (promoted by HIF-1 α) may offset some of the deleterious effects by increasing blood flow, and delivery of oxygen, nutrients and growth factors.

I am not aware of any studies directly relating exposure to hyperoxia to bone formation, but I predicted a greater number of osteocytes in the femora of

alligators reared in hyperoxia on the basis of a few studies suggesting increased size and growth rate for animals reared in conditions of increased oxygen availability which may allow for a greater allocation of resources to growth as opposed to respiration (Owerkowicz, Elsey, et al., 2009). An increase in the number of osteoblasts actively forming bone might be expected under conditions of enhanced growth; hence, a greater proportion of these cells later transforming to osteocytes. However, no effects on either growth or osteocyte density occurred in response to hyperoxia in this study.

Bone Formation

Bone formation rates did not differ between the oxygen treatment groups for the anterior, posterior and ventral quadrants of the femoral cross-sections. Only the dorsal quadrant exhibited a significant difference in bone formation rate between the oxygen treatment groups, with slightly increased BFR in the hyperoxic treatment compared with the hypoxic group. This may be the result of a statistical artifact due to small sample size or reflect an actual difference in bone deposition due to growth or biomechanical considerations, although further testing is necessary. I hypothesized that bone formation rates would be lower in the femora of alligators reared under hypoxia because limited oxygen availability would decrease the number of and activity of osteoblasts responsible for matrix synthesis. An increase in resorption might also contribute to lowered bone formation because *in vitro* studies have shown that fusion of osteoclasts leading to higher rates of activity occur in response to hypoxia

(Arnett, et al., 2003). Similar conditions result from the Pasteur effect (shift to anaerobic metabolism) are known to inhibit osteoblasts and enhance the activity of osteoclasts (Brandao-Burch et al., 2005). Osteoclasts are controlled by osteoblasts, and both cell types are regulated by many potentially overlapping pathways controlling a complex process of induction, proliferation, differentiation, activation and senescence (Frost, 2000; Hall, 2005). For this reason, even if osteocyte density is not affected by hypoxia, it might still be possible for bone formation to be decreased due to increased resorption by osteoclasts, although this possibility has not yet been assessed for this sample. My hypothesis predicted that the extent of bone formation would be greater in the femora of alligators raised under hyperoxic conditions for reasons similar those for osteocyte density. I expected exposure to hyperoxia to result in increased growth rate and size, supported by an increased extent and rate of bone formation presumably requiring an increase in cell number and/or activity.

The lack of a response to the 16% O₂ in hypoxia might be partly explained by Crossley and Altimiras' (2005) suggestion that alligators may be able to tolerate much lower levels of ambient oxygen before experiencing effects detrimental to growth. In their study, significantly decreased growth of alligator embryos was only evident late in development during incubation, and the hypoxic stress was considerably greater (10% O₂) than in the current experiment (Crossley & Altimiras, 2005). Differences in experimental design may also account for seemingly contradictory results. For example, in the

investigation into the effects of 17% O₂ on growth and development of alligators, only two clutches of eggs were utilized (Warburton et al., 1995) whereas greater genetic variation was achieved in Crossley & Altimiras' 2005 study examining over 200 eggs from ten different clutches, as well as in the current study in which a similar number of eggs from eight different clutches were used.

Another likely possibility is the increased angiogenesis that has been observed in response to hypoxia in the mammalian growth plate that has been shown to be controlled by hypoxia inducing factors (HIFs) and other regulators that are part of a set of genes involved in oxygen sensing common to all organisms (Schipani, 2006; Fluck et al., 2007). Improved delivery of blood and circulating factors may be responsible for the increased growth rate observed in chick embryos and laboratory rats and mice exposed to hypoxic conditions (Hunter & Clegg, 1973; Chan & Burggren 2005). If a similar mechanism is responsible for the normal growth of alligators reared in 16% O₂ in this study, an investigation into the size and numbers of vascular canals present in the femoral cortices might help to explain these differences as well species-related differences in tolerance to hypoxia.

Several studies have suggested that the effects of hyperoxia are modest except at very high levels (60% O₂) in birds (Metcalf et al., 1981; Burton & Palmer, 1992). Only a few studies have investigated the results of chronic hyperoxia on growth and development in reptiles. Andrews (2002) reported no

difference in the size of hatchlings or post-hatching growth rates for lizards incubated in 28% O₂ compared with those incubated under normoxic conditions. In alligators, hatchling mass was similarly unaffected by incubation in 30% O₂ but continued exposure for three months resulted in a higher growth rate and larger size of juveniles (Owerkowicz, Elsey, et al., 2009) whereas Berner and colleagues have suggested 27% O₂ as a threshold for beneficial effects of hyperoxia, above which mortality increases in alligator embryos (Berner et al., 2007).

Factors other than oxygen delivery *per se* may also affect growth and skeletal development. Growth hormone (GH) and Insulin-like growth factor (IGF-1) affect the growth plate during EO and are critical for growth. Delivery of hormones and circulating factors depends on an adequate blood supply, so hypoxia and/or resulting angiogenesis may alter these pathways (Serrat et al., 2007).

Temperature also affects activity in the growth plate responsible for increased length during growth in long bones; warmer temperatures led to enhanced growth in mice femora both *in vivo* (with vascular support) and *in vitro* (without vascular support) (Serrat et al., 2009). Nutrient delivery to the avascular growth plate is dependent on diffusion from peripheral vasculature (Schipani, 2006). Temperature may compensate for decreased vascular competence by facilitating the movement of dissolved substances to the growth plate in the femora of mice with experimentally altered limb temperature (Serrat

et al., 2009). With body temperature of the mice held constant, Serrat and colleagues (2009) demonstrated that an injected fluorochrome entered the growth plate of a warmer limb nearly twice as fast as in the cooler limb.

Temperature exerts a significant effect on the metabolic rates of reptiles and other ectotherms (Coulson, et al., 1989). The alligators in the current study were held at constant temperature (30°C) for the entire duration of the experiment, so it is possible that negative effects of hypoxia were mitigated due to temperature induced changes in blood flow, or by changes in metabolic rate (and oxygen demand) during growth.

In this study, no effects on size, as estimated by femur length in response to chronic levels of hyperoxia were found in four-month old alligators. In this respect, the results reported here are not in agreement with the findings of decreased mass and growth rate in alligators by Owerkowicz and colleagues (Owerkowicz, Elsey, et al., 2009) although their study focused on whole body measurements. It is possible that limb length, or specific skeletal elements of the limb may not be affected by oxygen availability. Osteocyte density and bone formation rates might be expected to increase in concert with femur length if skeletal growth in alligators is enhanced by increased oxygen availability, although this was not seen here. Several potential mechanisms have already been discussed, but an intriguing explanation put forth by Andrews (2002) is that additional levels of oxygen would have no effect on growth and metabolism in lizards if the hemoglobin in arterial blood was already completely saturated.

with oxygen. This may be possible in alligators as well; the unique cardiovascular system and potential for cardiac shunting of crocodilians may allow these animals to regulate hemoglobin saturation in arterial blood. This topic will be discussed more fully in the next chapter.

CHAPTER THREE

EFFECTS OF VARYING ATMOSPHERIC OXYGEN ON CROSS-SECTIONAL GEOMETRY AND BIOMECHANICS IN LIMB BONES OF ALLIGATORS

Introduction

Wolff's Law informally states that the microstructure of bone as well as modifications in external morphology are the result of stresses to which the bone has been subjected to during biomechanical loading (Cubo & Casinos, 1998; Meers, 2002). Cross-sectional geometry is often used to evaluate the biomechanical performance of long bones by modeling the skeletal element as a beam and using theoretical formulæ to estimate resistance to stress (Demes et al., 1991; Demes & Jungers, 1993; Cubo & Casinos, 1998).

The most significant source of stress in long bones is due to loading, the application of forces during terrestrial locomotion (Biewener, 2005). Loading is the result of ground reaction forces (GRFs) generated during the stance phase of locomotion that are countered by torque moments produced by muscle activity acting at joints, which are then transmitted to the bone (Reilly et al., 2005). The direction and magnitude of these forces depend on the position of the limb bones, limb posture, muscle attachments, and loading regime (Biewener, 1983; Demes & Jungers, 1993; Carrano, 1998; 2001).

Extant crocodylians are unusual in that they employ a range of terrestrial locomotor strategies, from the fully abducted "belly-walk" similar to the primitive sprawling condition to a more adducted semi-erect "high-walk" which lifts the body and part of the tail off of the ground, while some are even capable of galloping, at least early in ontogeny (Gatesy, 1991; Meers, 2002; Allen, et al., 2010). Blob and Biewener (1999; 2001) demonstrated that torsion is the principal mode of locomotor loading in alligators. In addition to bending, the femur experiences high levels of torsion due to the rotation of the femur by the main propulsive muscle, the caudofemoralis longus and the orientation of the GRF perpendicular to the bone (Gatesy, 1990). An unexpected finding reported by Blob and Biewener (1999; 2001) was that the strain measured in the femur increased in response to a more upright (adducted) stance in these animals. This is in contrast to the decrease in strain that occurs in mammals where axial compression and bending rather than torsion are the dominant loading patterns on the femur and other limb bones (Biewener, 1989; Blob & Biewener, 2001).

Long bones are designed to not only be strong enough to withstand the forces encountered in support and movement with minimal deformation, but with an additional 'safety-margin' to lessen the probability of failure due to repetitive loading or unexpected strain (Blob & Biewener, 2001; Currey, 1999; Biewener, 2005). Strength in weight bearing skeletal elements is defined as the load required to cause fracture, and can be determined for different loading modes through mechanical testing of whole bone or machined segments

(Martin & Ishida, 1989; Currey, 2002). Bone strength depends on the size and shape of the skeletal element as well as the material properties of the internal components of the bone (Guo, 2001; Erickson et al., 2002). Because these characteristics of bone may be modified in response to stress, the structure of bone conveys the biomechanical environment it has experienced. Cross-sectional geometry offers a way to estimate the strength of cortical bone in resisting stress and evaluate the intensity of force acting on it (Erickson et al., 2002; Lieberman, 2004).

Cross-Sectional Geometry

Cross-sectional geometry has been used in many studies of primate and hominid locomotion. Trinkaus and Ruff (1999) used cross-sectional limb bone geometry to test for hypothesized differences in the robustness of femora between Neandertal and early modern humans during evolution, finding instead an evolutionary shift in loading patterns. In prosimian primates subject to different loading modes, a trend toward increasing strength with size was identified in the forelimb bones of animals limited to climbing, or in the hindlimb for leaping forms, respectively, suggesting the usefulness of the analysis of cross-sectional geometry for the prediction of locomotor strategies in fossil forms (Demes & Jungers, 1993).

In one example of the application of this method to fossils, Heinrich et al. (1993) analyzed femoral cross-sections in a growth series of an iguanodontid dinosaur (*Dysalotosaurus* [*Dryosaurus*] *lettowvorbecki*) to suggest an

ontogenetic change from a quadrupedal juvenile stage to an adult bipedal stance.

Cross-sectional geometry has also been used to compare the loading patterns of wing bones with those of the hind-limbs in birds. Cubo and Casinos (1998) found that the wing bones as well as the femur were more resistant to torsion than bending stresses. A study of the wing bones in pelicans and related birds revealed that those utilizing flapping or flap-gliding flight were more resistant to bending stress, while soaring birds were more resistant to torsion (Simons et al., 2011).

Cross-sectional geometric properties are commonly measured at the mid-diaphysis, a region considered to be the site of maximal stresses and free of muscular attachments (Rubin & Lanyon, 1982). The following parameters are determined in cross-sectional analyses (following McFarlin, 2006). Based on beam theory, cross sectional area (*CSA*) of the cortex is an indication of the resistance of the bone to axial compression or tension. For bending loads, shape as well as size is important in evaluating bending rigidity, the resistance of bone to deformation in response to being loaded in bending. The second moment of area (*I*), is a measure of the quantity of bone and its distribution around a defined axis and denotes bending rigidity in the plane perpendicular to that axis. A value for I_x defined in the anteroposterior (may also be indicated as I_{AP}) direction estimates bending rigidity in the dorsoventral plane; similarly I_y

(I_{DV}) defined in the dorsoventral direction represents resistance to anteroposterior bending.

An elliptical distribution of material is the most favorable shape for a bone loaded in unidirectional bending, whereas a circular shape is optimal for a bone subjected to torsional loading and multidirectional or unpredictable bending. The maximum and minimum second moments of area (I_{max} and I_{min}) may not correspond to the anatomically defined anteroposterior and dorsoventral axes, and estimates the direction of the greatest and least resistance to unidirectional bending. Ellipticity (I_{max}/I_{min}) illustrates the non-circularity of the section and therefore provides an estimate of maximum bending resistance. The polar moment of inertia (J) is an estimate of torsional rigidity, or resistance to shear stress as well as average bending strength in all directions and implies a more circular cross-section. As in other aspects of bone biology, most studies of cross-sectional geometric properties have been limited to interspecific analyses among extant mammals and birds (Meers, 2002; Blob, 2006). Expanding these comparisons to cover a wider range of animals more representative of the diversity within the vertebrate clade, as well as including analyses of ontogenetic changes will augment our current understanding of the biomechanical constraints underlying the structure and function of bones and help inform evolutionary trends in posture and locomotion, leading to more accurate interpretation of fossil remains.

Allometric Relationships

Allometry, the effects of size on structure and function in animal design, can be studied using the allometric equation, $y = ax^b$ where the variable x is often designated as mass or length of the body or a component, and y is a related variable such as a measure of length or area in another component or the rate of a physiological process. Data are log transformed and regressed. The slope of the regression is compared to an experimentally derived value or a theoretical null hypothesis, often based on geometric similarity (Garcia et al., 2006). Geometric similarity (isometry) implies a proportional increase (or decrease) in the linear dimensions of similarly shaped animals as size changes (Schmidt-Nielsen, 1984).

It has been appreciated since the time of Galileo Galilei that isometric growth may not be consistent with the reduced capability of the skeleton to support greater loads with increasing size (Gould, 1966; Garcia et al., 2006). Changes in limb orientation and posture leading to a more erect stance are sufficient to decrease skeletal stress in mammals with a mass less than 300 kg, while the columnar limbs of larger mammals must rely on positive allometry of the diameter of skeletal elements as mass increases (Biewener, 2005) although Christiansen suggests that in many animals, the increasing robustness of limb bones is actually due to the negative allometry of length with respect to diameter (1999a; 1999b).

Allometry of cross-sectional geometric properties have been used to predict biomechanical strength, loading patterns and characterize changes in locomotor forces accompanying size increases in both fossil and living animals. In a study of indriid primates, Demes et al. (1991) found isometric scaling of limb bone geometry in agreement with a decrease in performance evident in larger animals.

Scaling patterns in interspecific comparisons usually involve adult animals (Alexander, 1979) and do not necessarily agree with ontogenetic changes. Main & Biewener (2007) found CSA of the femur scaled with negative allometry and I scaled near isometry during growth to adult size in the very large emu, which may reflect an increased safety factor in young birds. Similarly, in ontogenetic scaling of limb bone geometry in goats, negative allometry of CSA has been shown to occur in the radius, resulting in relatively weaker, less robust bones in adults, perhaps related to running performance or unpredictable loading conditions in younger animals (Main & Biewener, 2004; Main, 2007).

Fewer scaling analyses have been carried out among reptilian taxa. Blob (2000) has suggested that strong positive allometry of the diameter of limb bones would be required to offset increased torsional stress during growth in alligators, as has been shown for varanid lizards. Alligators exhibit an ontogenetic decrease in relative limb length, with the radius and ulna of the forelimb and the tibia of the hindlimb scaling with negative allometry against the

more proximal skeletal elements (humerus and femur, respectively), which likely contributes to the purported decrease in terrestrial locomotor performance seen in larger adult animals (Dodson, 1975; Livingston et al., 2009; Allen et al., 2010;).

In mammals, the forelimbs support a greater proportion of the locomotor load (Christiansen, 1999b). In crocodiles, the hindlimbs support greater loading due to the presence of a large heavy tail and the forelimbs are relatively shorter than the hindlimbs (Allen et al., 2010). Livingston et al. (2009) used a scaling analysis to investigate possible changes in proportions between the fore and hindlimbs during alligator ontogeny reflecting an evolutionary inheritance from bipedal ancestors. However, they found that the original proportions were maintained and most of the limb bones scaled isometrically with femur length, perhaps as a consequence of loading and locomotor mode. Meers (2002) failed to find support for Wolff's Law in an interspecific comparison of the cross sectional properties in which the humeri of crocodiles distinguished by different locomotor habits scaled with unexpected isometry.

Experimental Design

To investigate the effects of varying levels of atmospheric oxygen on biomechanical function in the skeleton, I analyzed the cross-sectional geometric properties in the alligator femur. I hypothesized that the reduced availability of oxygen in a chronically hypoxic environment will negatively impact skeletal development and the ability of bone to resist locomotor forces whereas

chronic exposure to hyperoxia will facilitate the development of bone with increased resistance to stress.

Materials and Methods

American alligator (*Alligator mississippiensis*) eggs representing eight clutches (n=179) were incubated, and hatchling and juvenile alligators raised under chronic conditions of hypoxia (16% O₂), hyperoxia (26% 31% and 36% O₂), or normoxia (21% O₂) simulating Phanerozoic atmospheric oxygen conditions. Alligators were sacrificed at 2, 4, 16, 24, 52, and 104 weeks of age.

Specimen Preparation

The right femur of each specimen was dissected and prepared for histological analysis by removal of soft tissue, brief formalin fixation (1 hour), followed by a graded ethanol dehydration series (1 hour each in 30%, 50%, 70%, 80%, 90%, 95%, 100% ethanol) and air drying for 24 hours. The following measurements were made for each bone: femoral length, width and length of the proximal and distal articular surfaces, mid-diaphyseal circumference, and dorsoventral and anteroposterior diameters (Farlow et al., 2005).

Undecalcified ground sections were prepared according to An et al. (2003; also see Chapter 2). Small femora, less than 44 mm long, were placed in a labeled plastic tray lined with hardened resin. All of the bones were positioned in the same anatomical orientation (dorsal aspect up) and anchored in place with a few drops of resin. After being allowed to set for a few hours,

bones were covered with epoxy resin (EpoThin; Buehler Ltd.) and allowed to cure for two days. For femora more than 45 mm in length, the epiphyses were removed with a band saw, leaving approximately 1.4 cm of the diaphysis to be embedded. Before cutting, the dorsal surface, mid-diaphysis and proximal end of the bone was marked in pencil to ensure correct anatomical orientation, and then labeled and embedded. During the polymerization process, the femora were simultaneously impregnated with resin and embedded in a hardened block which supported the bone and allowed for sections to be evenly cut. The anatomical orientation of each bone (indicating dorsal and medial surfaces) was marked by labeling the corresponding sides of the block with different colors of permanent ink, and three ~1mm serial transverse thick sections were removed from the mid-diaphysis of each specimen with a low-speed saw (IsoMet; Buehler, Ltd.).

The proximal surface of each section was also marked with a small dot of permanent ink in the corner as it was removed from the saw. This dot, together with the colored labels from the block now visible along the dorsal and medial edges of the section, allowed the original anatomical orientation to be preserved as the sections were mounted onto a labeled slide with resin. After a second round of curing, sections were ground to a thickness of approximately 125 μm using a graded series (280 to 600 grit) of abrasive paper on a grinder-polisher (MetaServ, Buehler, Ltd.). The slides were then cover-slipped using Permunt (Fisher Scientific) mounting medium thinned with xylenes.

Image Analysis

Each section was viewed and photographed using a polarizing microscope (Nikon Eclipse V100) fitted with a digital camera and image capture software (Spot Imaging Systems). Images were taken of the entire cross section at 40X , as well as of each quadrant of the cross section (dorsal, ventral, anterior, and posterior aspects) at higher magnifications of 100X and 200X. For larger femoral cross-sections in which the entire cross-section did not fit in the microscope field of view, additional images were taken using a Zeiss Discovery stereo microscope at 15X. Images were then analyzed using NIH ImageJ (<http://rsbweb.nih.gov/ij/>). The internal (medullary) and external dorsoventral and anteroposterior diameters were measured from images of the entire cross-section using on-board analytical tools. The thickness of the cortex in each quadrant was also determined.

The images were converted to binary silhouettes of the cortex (black for bone, white for non-bone) and analyzed using MomentMacro (<http://www.hopkinsmedicine.org/fae/mmacro.htm>), a plug-in for ImageJ for the calculation of the cross sectional geometric properties of each specimen: CSA estimates resistance of the bone to axial compression, I_{AP} and I_{DV} (I_x) and (I_y) estimate bending resistance about the anteroposterior and dorsoventral axis, respectively, I_{max} and I_{min} denote maximum and minimum resistance to unidirectional bending, respectively, and $J (I_{max}+I_{min})$ signifies torsional rigidity and resistance to multidirectional bending. Ellipticity (I_{max}/I_{min}) is a ratio

illustrating the deviation from circularity of the cross-section and estimates maximum bending (Figure 8). Together these estimate the biomechanical strength of the bone in resistance to stresses due to compression, tension, bending and shear forces encountered during locomotion.

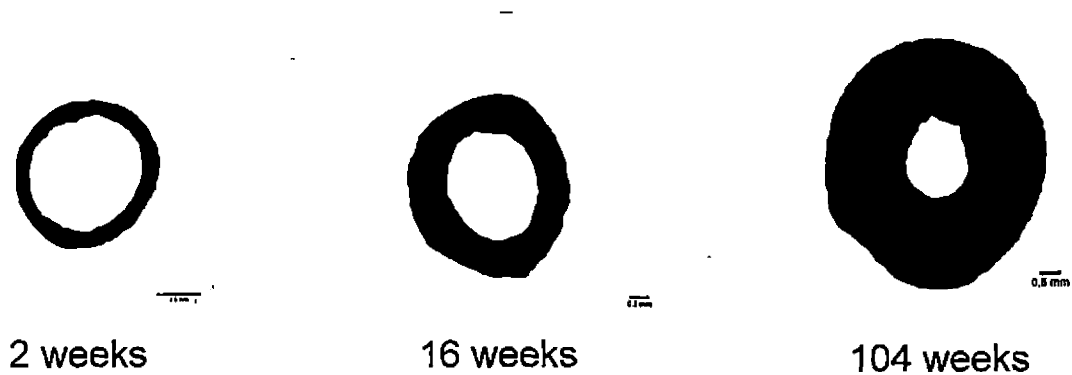


Figure 8. Cross-Sectional Geometry. Binary images representing a growth series of *Alligator mississippiensis* for analysis of biomechanical properties and ontogenetic changes in shape and size of cortex. Limb bones of alligators are typically dense with extremely thick cortices, presumably as an aid to counteract buoyancy during dives. Scale bar = 0.5 mm.

Statistical Analysis

All measurements were analyzed with ANCOVA using R (R Development Core Team, 2012). Femur length (FL) was used as a covariate to

account for differences in size between the different treatment groups. Slopes were compared to isometric predictions using reduced major axis (RMA) regression implemented in the R package *smatr* (Warton et al., 2012). Statistical tests were evaluated at $\alpha = 0.05$.

Results

No significant differences were found for any of the whole-bone morphometric measurements (FL, width and length of proximal and distal articular surfaces, mid-diaphyseal circumference and DV and AP diameters) or for cortical thickness between oxygen treatment groups using ANCOVA .

Similarly, significant differences were not found for CSA between oxygen treatment groups using ANCOVA (Table 3, Figure 9). As slopes were not significantly different, all groups were pooled for allometric slope comparisons. Slopes were then compared to isometric predictions using reduced major axis (RMA) regression implemented in the R package *smatr* (Warton et al., 2012). Because measurements of area are predicted to increase in proportion to the square of a linear dimension, the null hypothesized slope is 2. The RMA slope for CSA was found to be 2.17, significantly different from 2 ($P < 0.001$; Table 3).

No significant differences were uncovered between treatment groups for diameters in either the anteroposterior or dorsoventral directions with ANCOVA. As for CSA, slopes were compared to isometric predictions using RMA.

Diameters are traits with linear dimensions, so are predicted to scale proportionately with FL, with a null hypothesized slope of 1. The RMA slopes were 0.85 and 0.88, for anteroposterior and dorsoventral diameters respectively, both significantly different from 1 ($P < 0.001$; Figures. 10, 11; Table 3).

Table 3. RMA Analyses of Biomechanical Traits. Results (slope, P -values, variance and confidence intervals) for RMA regression of biomechanical traits versus oxygen treatment. All P -values are significant.

Trait	b	P	R^2	95% CI
Cross-section area (CSA)	2.17	<0.001	0.98	2.13--2.22
Maximum Second Moment of Area (I_{max})	3.76	<0.001	0.98	3.66--3.85
Minimum Moment of Area (I_{min})	3.79	<0.001	0.97	3.69--3.90
Polar Moment of Inertia (J)	3.77	<0.001	0.97	3.67--3.87
Second Moment of Area (I_x)	3.78	<0.001	0.98	3.69--3.87
Second Moment of Area (I_y)	3.77	<0.001	0.96	3.66--3.89
Ellipticity (I_{max}/I_{min})	-0.67	<0.001	0.04	-0.58--0.78
Anteroposterior Diameter	0.88	<0.001	0.96	0.86--0.90
Dorsoventral Diameter	0.85	<0.001	0.97	0.83--0.88

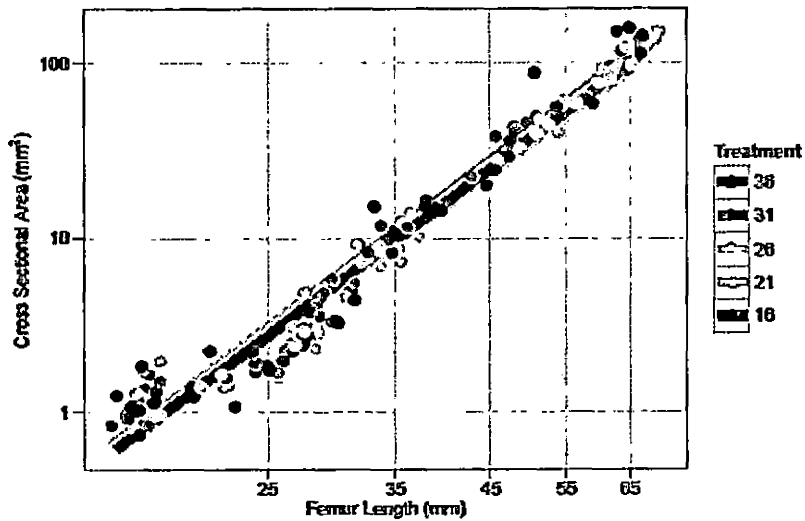


Figure 9. Cross-Sectional Area, CSA. CSA plotted against FL is not significantly different among treatment groups. Pooled RMA regression slopes are higher than expected from isometry (Table 3).

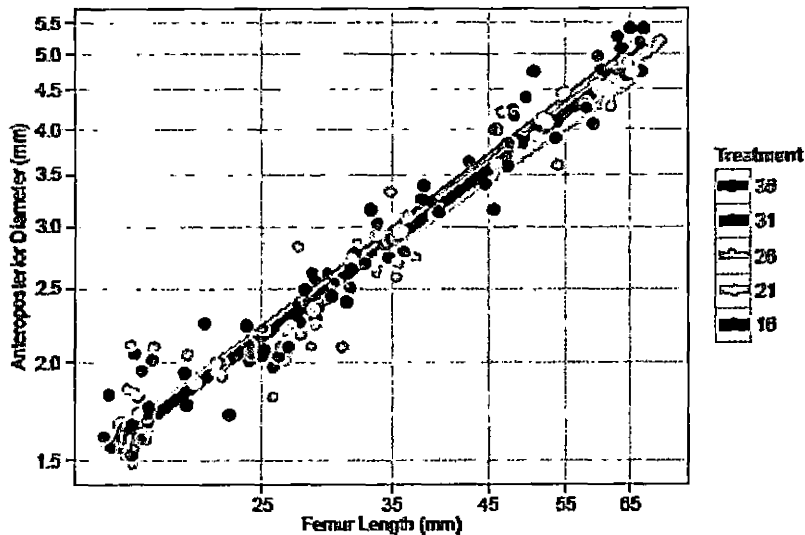


Figure 10. Anteroposterior Diameter. Anteroposterior diameter plotted against FL is not significantly different among treatment groups. Pooled RMA slopes are lower than expected from isometry (Table 3).

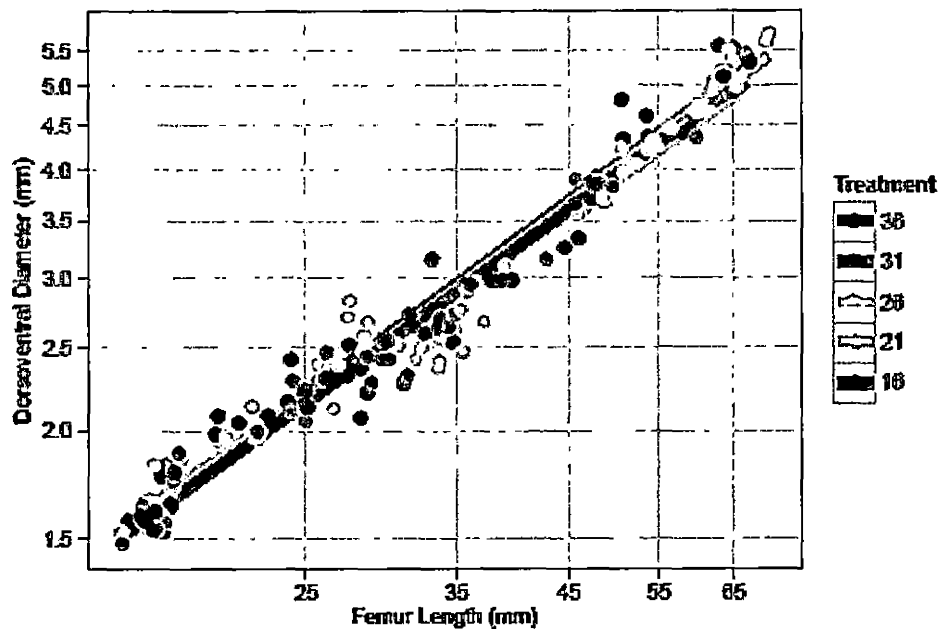


Figure 11. Dorsoventral Diameter. Dorsoventral diameter plotted against FL is not significantly different among treatment groups. Pooled RMA regression slopes are lower than expected from isometry (Table 3).

For the second moments of area, I_x , I_y , I_{max} , and I_{min} , and polar moment of inertia J , no significant differences were found between the oxygen treatment groups using ANCOVA. RMA was used to compare the slopes to isometric predictions. Because all of these traits are measured as the square of squared distances, they are expected to increase proportionally with the fourth power of FL, and the null hypothesized slope is 4. For I_x and I_y the RMA slopes were found to be 3.78 and 3.77, respectively, both significantly different from 4 ($P < 0.001$; Figures 12, 13; Table 3). For I_{max} and I_{min} RMA slopes were 3.76 and 3.79 both significantly different from 4 ($P < 0.001$; Figures 14, 15; Table 3).

An RMA slope of 3.77, significantly different from 4, was obtained for J ($P < 0.001$; Figure 16, Table 3).

Ellipticity (I_{max}/I_{min}) was not found to be significantly different among the oxygen treatment groups. (Figure 17; Table 3). As a ratio of perpendicular inertial moments, ellipticity is expected to scale in proportion with FL, and the null hypothesized slope is 1. The RMA slope is -0.67, significantly different from 1 ($P < 0.001$).

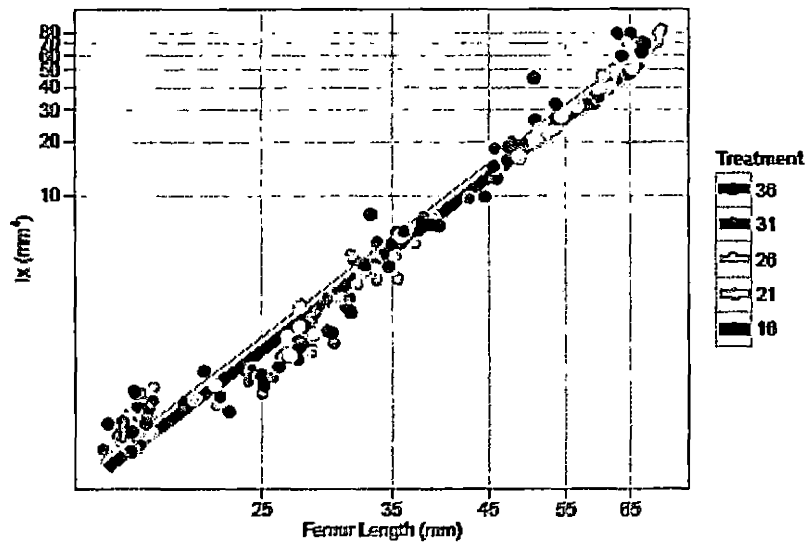


Figure 12. Second Moment of Area, I_x . I_x plotted against FL is not significantly different among treatment groups. Pooled RMA regression slopes are lower than predicted from isometry (Table 3).

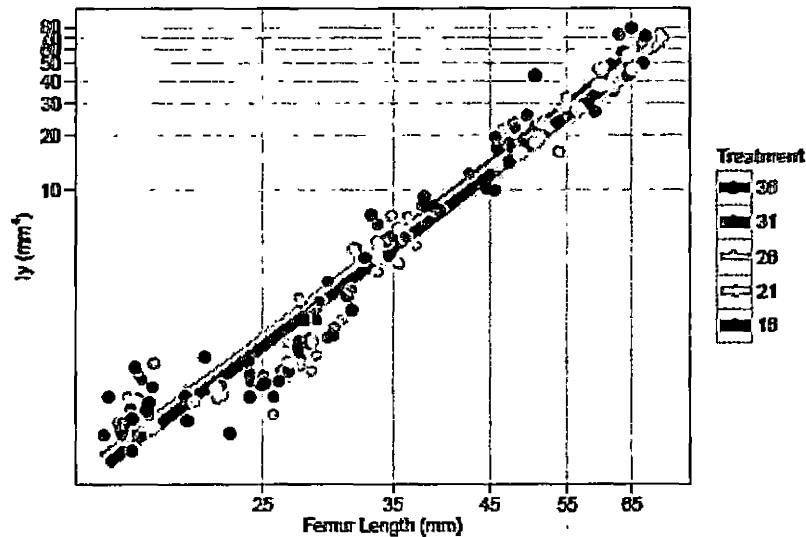


Figure 13, Second Moment of Area, I_y . I_y plotted against FL is not significantly different among treatment groups. Pooled RMA regression slopes are lower than expected from isometry (Table 3).

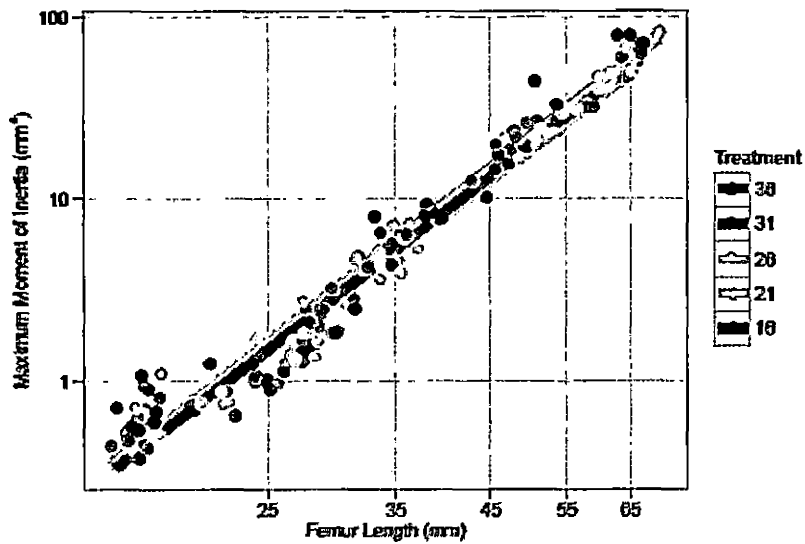


Figure 14. Second Moment of Area, I_{max} . I_{max} plotted against FL is not significantly different among treatment groups, however RMA regression of pooled slopes are lower than expected from isometry (Table 3).

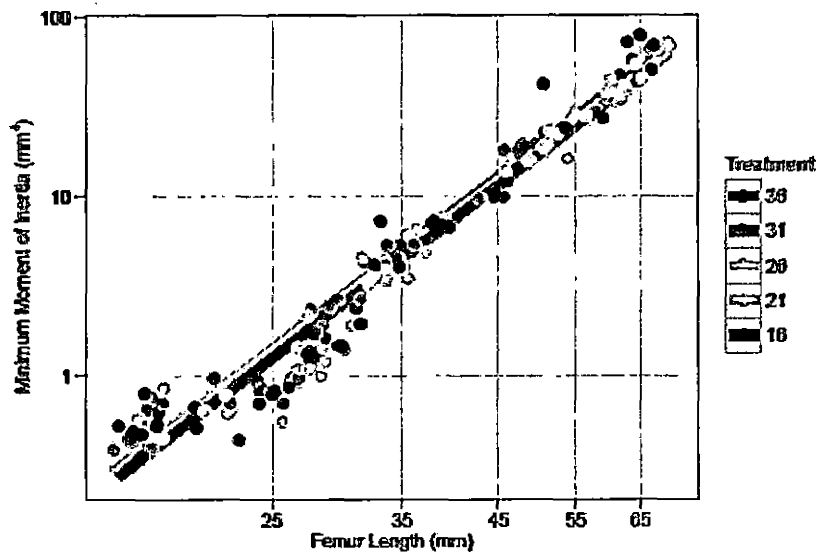


Figure 15. Second Moment of Area, I_{min} . I_{min} plotted against FL is not significantly different among treatment groups, however RMA regression of pooled slopes are lower than expected from isometry (Table 3).

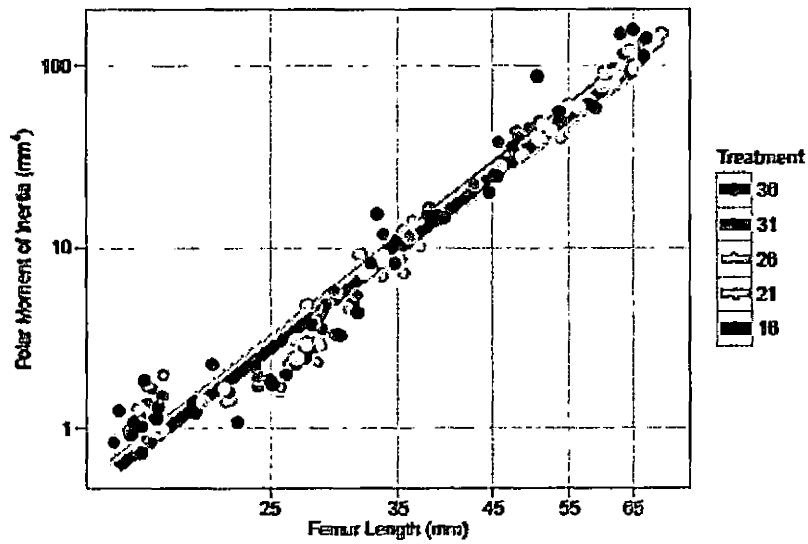


Figure 16. Polar Moment of Inertia, J . J plotted against FL is not significantly different among groups. Pooled RMA regression slopes are lower than expected from isometry (Table 3).

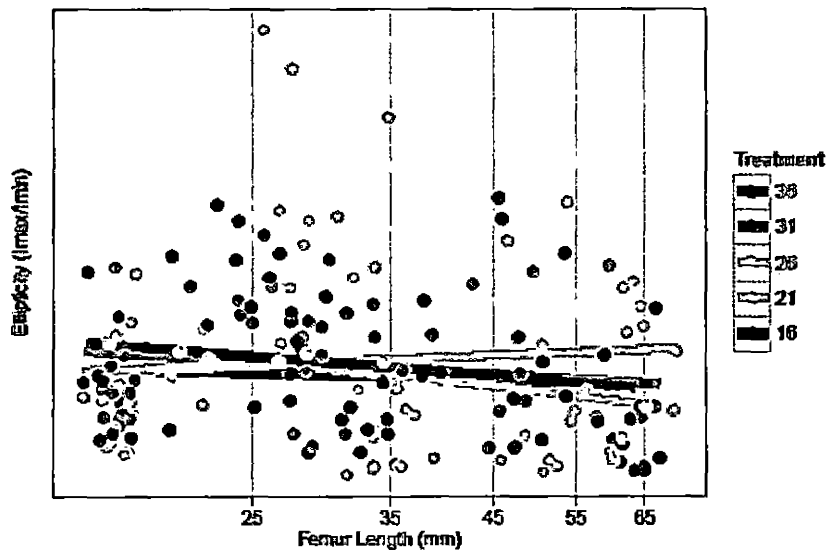


Figure 17. Ellipticity. Ellipticity plotted against FL is not significantly different among treatment groups. Pooled RMA regression slopes are lower than expected from isometry (Table 3).

Discussion

Extensive research into the influence of external loading conditions as well as the internal biomechanical environment of bone have illustrated the capacity of bone to alter internal structure, as well as external shape and size, in response to the demands of development, growth and activity (Martin et al., 1998). Intermittent loading generated by locomotion is the primary mechanism leading to these structural alterations (Hall, 2005) and analyses of these changes using cross-sectional properties of bone allow for reconstructing the loading pattern in extinct vertebrates and uncovering patterns in the evolution of locomotor activity (Thomason, 1995). Oxygen availability has been shown to affect growth and development in many vertebrates, although some species are considerably more tolerant of non-normoxic conditions than others. Very few studies have investigated skeletal responses to hypoxia or hyperoxia. However, if growth is either negatively or positively impacted, perhaps these changes were also accompanied by modifications in locomotor loading or biomechanical performance. Potential deficits in the intrinsic material properties affecting the rigidity of bone may be the result of effects of oxygen on cell activity or nutrient availability whereas changes in the amount and distribution of bone might elicit changes in locomotor loading or patterns of activity and lead to changes in the extrinsic properties (rigidity as well as shape and size) of bone (Wainwright et al., 1982). In this study, I used cross-sectional geometry to identify potential

differences in biomechanical properties of the femur in response to altered oxygen availability.

Whole-Bone Morphometrics

Measurements included traits describing the size and shape of the femur. If oxygen availability affects skeletal growth, changes in the proportions as well as size of the femur may be reflected by alterations in one or more of these traits; such a change may affect loading or posture, and have implications for locomotor function as well as scaling patterns between the different oxygen treatment groups. Under the geometric similarity (GS) hypothesis, all of these traits would be expected to increase linearly in proportion to femur length with increasing size of alligators during ontogeny.

My findings of no significant differences in whole-bone morphometric traits were surprising in light of previous studies documenting a decreased rate of growth and smaller body size in alligators reared in hypoxic conditions (Warburton et al., 1995; Crossley & Altimiras, 2005), as well as one study demonstrating an increased rate of growth and larger body size in alligators reared in hyperoxia (Owerkowicz et al., 2009). A possible explanation is that the experimental level of hypoxia (16% O₂) is within a range well tolerated by these animals; Crossley and Altimiras (2005) reported significantly smaller alligator embryos incubated at 10% O₂ compared to 15% O₂. In hyperoxia, a relaxation of respiratory constraints due to increased oxygen availability may

allow for the reallocation of resources and may be permissive for growth (Owerkowicz, Elsey, et al., 2009).

Cross-Sectional Area (CSA)

The outer cortex of a limb bone is subject to considerable stress during locomotion as a result of external ground reaction forces as well as internal muscle forces acting to move the skeleton (Currey, 2002). Bones must be able to support the mass of the body during locomotion and bear locomotor stresses without exceeding a failure threshold for deformation (Turner & Burr, 1993). CSA measures the area of cortical bone in the cross-section and estimates the strength of bone loaded in tension and compression.

The similarity of CSA between the oxygen treatment groups shows that the strength of bone in resisting tensile and compressive forces acting in the direction of the long axis of the bone are neither compromised nor enhanced by chronic exposure to hypoxia or hyperoxia. As a limb bone grows in length, CSA must also increase to support added weight and prevent breakage due to higher stresses. Relationships between linear, areal and volumetric measurements are often compared to the isometric model of geometric similarity, GS. In this experiment, CSA increased more rapidly than FL, exceeding the predictions of GS (Figure 10, Table 3) and exhibiting positive allometry. Thus, the alligator femora of all oxygen treatment groups are more robust than predicted, with greater resistance to axial loading than expected based on their size. This result is of note because the femur and other limb

bones in the alligator are known to exhibit negative allometry for length with respect to increasing size through ontogeny (Dodson, 1975; Allen et al., 2010). Perhaps the augmented resistance to axial compression and tension afforded by an increase in CSA is important for biomechanical function in these animals, especially as terrestrial locomotor capability seems to become more limited in larger alligators (Livingston et al., 2009; Allen et al., 2010).

Diameter (AP, DV)

Anteroposterior and Dorsoventral diameters were not significantly different between the oxygen treatment groups. In contrast to CSA, for alligators in all of the treatment groups, diameters did not scale in accord with GS (Figures 11, 12; Table 3); both diameters exhibited negative allometry (increasing more slowly than FL). This might seem unexpected both from a biomechanical viewpoint as well as in light of the positive allometry exhibited by CSA, however measurements for AP and DV diameter were taken along the x and y axes of the cross-section respectively and therefore do not reflect changes in overall size and shape of the cross-section. Cortical thickness and CSA vary with size and shape of the medullary canal and may have a functional basis in the positioning of bone material in regions experiencing greatest stress, for example, during cortical drift which results in an eccentric medullary cavity in response to bending (Frost, 2003).

Second Moments of Area (I_{max} , I_{min} , I_x and I_y)

The second moments of area (SMAs) I_{max} , I_{min} , I_x and I_y account for the distribution as well as amount of bone present in a cross-section, and describe the elliptical shape of the cross-section in terms of the direction of maximum (I_{max}) and minimum (I_{min}) resistance to unidirectional bending. I_x and I_y estimate resistance to bending in the anteroposterior and dorsoventral directions, respectively. All limb bones experience stress due to bending during locomotion, the magnitude and direction of which may vary depending on posture (Blob & Biewener, 1999).

No significant differences in resistance to bending as measured by the SMAs between the oxygen treatment groups were found. In contrast to findings for CSA, but similar to those for AP and DV diameter, I_{max} , I_{min} , I_x , and I_y each exhibit negative allometry (increasing more slowly than FL) indicating resistance to bending in alligator femora is decreased in larger animals regardless of oxygen treatment (Figures 13,14,15,16; Table 3). Alligators are capable of a range of postures and gaits and it might be expected that bending would become more, not less important as size increases, especially since it has been suggested that in tandem with the previously mentioned decline in locomotor performance with increasing size, larger alligators adopt a greater variety of postures (Allen et al., 2010)

Polar Moment of Inertia (J) and Ellipticity (I_{max}/I_{min})

Polar moment of inertia is the sum of I_{max} and I_{min} and describes the circularity of the cross section and estimates the resistance of bone to torsion and multidirectional bending. Torsion has been shown to be the major mode of locomotor loading on the limb bones in alligators due to posture and the muscle forces acting on the femur (Blob and Biewener, 1999, 2001). Similar to *SMA*s, J did not vary significantly between the oxygen treatment groups, and exhibited negative allometry relative to FL (Figure 17; Table 3). This indicates decreasing resistance to torsion as alligators grew larger. Whereas negative allometry of *SMA*s can perhaps be explained as the decreased importance of an elliptical cross-section optimized for unidirectional bending resistance in an animal with limbs loaded primarily in torsion, it may also signify that resistance to bending in a preferred direction imparted by I_{max} and I_{min} is of secondary importance to bending in all directions in the femora of these alligators. A more circular shape of the cross-section affords the greatest resistance to both torsion and multidirectional bending. Although J might be expected to be important in alligators which are capable of both sprawling as well as a semi-erect posture and gait, the finding of negative allometry in this trait may be associated with the ontogenetic decrease in limb length and locomotor performance observed in these animals..

Ellipticity (I_{max}/I_{min}) is a ratio that illustrates the changes from a circular cross-sectional shape toward a more elliptical shape and estimates maximum

resistance to bending in a preferred direction. Results for ellipticity parallel those of *SMA*s and *J* (Fig. 18; Table 3) and seem to support the decreased reliance on resistance to unidirectional bending implied by the negative allometry in I_{max} and I_{min} .

The limb bones of extant alligators have extremely thick, dense cortices, which may be an adaptation to a semi-aquatic ecology, facilitating submergence and diving (Currey, 1985). Blob and Biewener (1999, 2001) have suggested that the increased robusticity relative to length of the limb bones in these animals may confer an advantage in unpredictable loading environments. If so, the strength of these bones may mean that they are less likely to exhibit the expected scaling relationships for cross-sectional geometric properties. This may partly explain the positive allometry for *CSA*, found here, whereas the negative allometry of ellipticity, polar moment of inertia and the *SMA*s suggest that biomechanical factors affecting cross-sectional shape become less important during growth.

Variable Atmospheric Oxygen Does Not Affect Limb Bone Geometry

The findings presented here are not in agreement with many studies documenting severe growth deficits associated with chronic exposure to hypoxia in many species. In addition, while permissive effects on growth in response to hyperoxia were not seen, neither were there any deleterious consequences of exposure to extremely high levels of ambient oxygen at the upper range of experimental conditions (31% O₂ and 36% O₂) as previously

seen in embryonic alligators (Bernier, et al., 2007) and suggested by work in other ectothermic animals in regard to the production of damaging reactive oxygen species (Bickler & Buck, 2007).

Differences in experimental design may explain some of the lack of accord between this study and others. Many investigations into the effects of chronic exposure to hypoxia are conducted for only a few days to weeks and involve mammals and birds. With the exception of turtles, only a few long term investigations into the effects of hypoxia utilize reptiles and because of the time involved or difficulty in maintaining experimental conditions, may only last a few months, at most (Owerkowicz, Elsey, et al., 2009). In this study, animals were raised under specific experimental conditions for up to two years.

The degree of hypoxic exposure may explain some differences between my results and those of previous studies. 16% O₂ may not have been low enough to elicit the expected negative effects on growth in hypoxia as seen in other species (Andrews, 2002). It is also possible that the magnitude of the effects of hypoxic exposure vary to a greater or lesser extent at different stages of an animal's development, so the timing as well as duration is important. Only one long-term study has examined the chronic effects of hyperoxia in reptiles using alligator eggs incubated and hatchlings raised for 3 months in 30% O₂; (Owerkowicz, Elsey, et al., 2009). In contrast to the results described here, their findings demonstrated no effects of hyperoxia on growth of embryos or time of hatching, but higher growth rates in these animals after hatching.

Another aspect of the experimental protocol that may impact the interpretation of the results presented here is activity. Although alligators are semi-aquatic, the performance as well duration of terrestrial locomotion decreases through ontogeny, with young animals more active on land, and larger animals spending an increasing amount of time in the water (Dodson, 1975). In this experiment animals were confined to an aquatic environment for the duration of the study. Therefore locomotor stresses on the femur and other limb bones will be different than those experienced by animals in the wild, with the extent of terrestrial activity dependent on social hierarchy and behavior (Dodson, 1975). Owerkowicz, Tsai, et al. (2009) documented positive effects of exercise training on bone deposition in alligators using treadmills but not in swim flumes, while a similar study showed a decrease in size and mass of alligators exercised to exhaustion (Eme et al., 2010).

Crocodylian Cardiovascular System

The anatomy and physiology of the crocodylian heart may provide some clues to the lack of response to variable oxygen environments reported in this study. A four chambered heart is present in both extant lineages of Archosauria, the crocodiles and the birds (Eme, et al., 2010). Both groups partition oxygenated and deoxygenated blood into separate circulatory systems. Crocodiles, however, are similar to other reptiles in that they possess two aortae serving the systemic circulation, compared with the single aorta in avians (Eme, et al., 2010).

In crocodiles, the right aorta exits from the left ventricle delivering oxygenated blood to the body, and the left aorta and the pulmonary artery both exit from the right ventricle (Hicks, 2002). This pattern allows for deoxygenated blood to be directed toward the lungs (via the pulmonary artery) or into the systemic circulation (left aorta), bypassing the lungs altogether to develop a cardiac shunt (Hicks, 2002).

Recent work suggests that alligators utilize this right-left (R-L) shunt to actively control the level of oxygen saturation of hemoglobin in the blood (Owerkowicz, personal communication, unreferenced). Ectotherms such as alligators rely on anaerobic metabolism for burst activity, and demand for oxygen is much lower than it is for mammals and birds (Coulson et al., 1989). By increasing the R-L shunt, oxygen saturation in systemic blood is reduced, preventing unnecessary reoxygenation. In low levels of ambient oxygen, the shunt is decreased, and blood travels through the pulmonary artery to the lungs for more complete saturation of arterial blood (Skovgaard, et al., 2005). Alligators may be able maintain a preferred level of oxygen saturation independent of ambient oxygen levels (Owerkowicz, personal communication, unreferenced).

This mechanism may help to explain the observations of normal growth in alligators reared under hypoxic conditions because desaturation of arterial oxygen will trigger a decrease in the normal shunting patterns by opening the valve controlling pulmonary blood flow. It is estimated that shunting remains

operational in levels of ambient oxygen as low as 12% O₂ (Owerkowicz, personal communication, unreferenced). Conversely, growth in normoxia or hyperoxia may be limited by factors other than oxygen availability such as temperature, acid-base regulation or nutritional resources, and excess oxygen in arterial blood is either limited by the cardiac shunt or simply not utilized.

Conclusions

Skeletal growth and development has been extensively studied in *Alligator mississippiensis* (Rieppel, 1993), although this is the first investigation to specifically address the effects of chronic non-normoxic environmental conditions on the alligator skeleton. This study has shown that chronic exposure to hypoxia does not lead to diminished growth or a reduction in biomechanical performance in alligators, as might be expected from previous studies. On the other hand, chronic hyperoxia was not associated with a relaxation of growth constraints and did not lead to an increase in the size and strength of the skeleton.

Whether the capability of alligators to maintain similar skeletal growth and biomechanical properties in variable levels of atmospheric oxygen is primarily due to cardiac shunting or in tandem with other physiological mechanisms is an avenue of research that may lead to a greater understanding of the factors leading to the impressive evolutionary success of these animals.

In this work, I have found that the histological and morphometric traits, as well as the biomechanical properties tested in this study, cannot be used to infer potential effects of oxygen regime on skeletal growth and development or locomotor performance in fossil animals. However, interesting allometric relationships were uncovered. Alligator femora are significantly more robust and resistant to axial tension and compression than expected for their size, yet the bones are weaker in respect to torsion and bending. This is in direct contrast to expectations based on previous *in-vivo* work on stress and strain in these animals (Blob and Biewener, 1999; 2001).

Future Work

This study has shown that cross-sectional geometric properties estimating biomechanical performance relating to the capability of the skeleton to resist stress are not affected by hypoxia and hyperoxia. Other effects of exposure to non-normoxic oxygen variability may occur at a finer scale. The osteocyte density, rate and extent of bone formation reported in Chapter 2 consisted of a small sample of femora (n=37) from a cohort of four-month old alligators. These same traits will be investigated using the cross-sectional images created for the study reported in Chapter 3 (n=179) which also covers a much greater age/size range (femora ranging in size from 17mm to 70 mm in length from alligators 2-weeks to 104-weeks old).

Porosity of the bone due to vascular canals and osteocyte lacunae has important implications for strength of bone (Schaffler & Burr, 1988). An investigation into this trait can be determined using ImageJ from the binary cross-sectional images already obtained. A study of this nature would complement the biomechanical findings which are concerned with the amount and distribution of the bone, but do not account for the composition of the bone tissue. Crocodiles and other semi-aquatic vertebrates generally have long bones with thick cortices, little secondary reconstruction and are considerably lower in porosity than those of terrestrial animals (Houssaye, 2009, Habib et al., 2010). Using mid-diaphyseal cross sections, Horner et al. (2001), showed that the porosity of the tibia in a late-stage embryonic alligator, *Alligator mississippiensis*, was 14% of the total area of the cortex, as compared to only 4% in a yearling animal, whereas Wink and Elsey (1986) demonstrated substantial increases in the porosity of femora of large alligators of both sexes that have reached reproductive maturity (more so in females, as a result of egg shell production).

Collagen fiber orientation (CFO) is also correlated with bone strength. The alignment of collagen fibers relative to the long axis of a bone dictates the direction of the mineral phase which confers resistance to various biomechanical forces. Transverse CFO is predicted to increase resistance to compressive loading, while longitudinal CFO increases resistance to tension. As a birefringent material, CFO can be visualized under the microscope with

circularly polarized light (Bromage, 2003). Lee (2004) found partial support for this hypothesis in a small sample of alligators. During the stance phase of a stride, the dorsal and ventral quadrants of alligator femora are subjected to compressive and tensile forces, respectively. Contrary to expectations, CFO was predominantly longitudinal throughout the femur. However the dorsal quadrant did contain a greater concentration of transverse CFO than the ventral quadrant in accordance with theory. It would be interesting to apply this method to our much larger sample to determine if CFO varies between the different oxygen treatment groups.

A final goal for future research using the materials already prepared from this study is the characterization of potential LAGs. There is some disagreement regarding the annual nature of these growth marks as well as whether they form during periods of rapid growth early in ontogeny, or under aseasonal laboratory conditions (Padian, et al., 2001). A subset of alligators in this study were not sacrificed until two years old. Histological slides from this cohort will be ideal for investigating the incidence of LAGs and if present, whether they were affected by variable levels of atmospheric oxygen.

The greater scope of these potential studies in conjunction with the present work will lead to a greater understanding not only of the effects of variable atmospheric oxygen in extant taxa, but will improve interpretation of fossil remains.

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