# ECOMORPHOLOGICAL IMPLICATIONS OF PRIMATE DIETARY VARIABILITY: AN EXPERIMENTAL MODEL

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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a candidate for the degree of doctor of philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

The evolution and function of the mammalian skull and feeding apparatus is intimately related to the mechanical demands imposed by food items. The diets of wild species are often seasonal and thus individuals may experience multiple masticatory loading regimes across their ontogeny. However, despite the temporal complexity of many mammalian diets, it remains poorly understood how such long-term dietary variability affects the growth and form of the craniomandibular complex.

This experimental research evaluated the effects of longitudinal variation in dietary mechanical properties on craniomandibular morphology and on the biological processes that underlie functional adaptation in this region. Results suggest that the skeletal morphology of adults, particularly those characters associated with the mandibular joint and muscle insertion sites, best reflect an individual's diet. Furthermore, variations in bone physiology and growth rates were observed to be influenced more by an individual's masticatory loading history than by absolute levels of loading.

This study emphasizes the character- and age-specific nature of phenotypic plasticity related to variation in dietary properties. Furthermore, this work also highlights the importance of long-term, ontogenetic studies for assessing the impact of diet on craniomandibular form. This enhanced understanding is critical for evaluating ecomorphological reconstructions of feeding behavior in living and fossil mammals.

# CHAPTER 1: INTRODUCTION

The evolution of the human craniomandibular complex is intricately related to the evolution of the human diet and the processing demands imposed by the mechanical properties of food items. Indeed, the assessment of primate craniofacial morphology in relation to feeding behavior is a well-established area of biological anthropology, fundamental to ecological and evolutionary interpretations of extant and extinct species. However, despite the large volume of ecological and evolutionary research that underscores the spatial and temporal complexity of primate diets, there exists a dearth of work addressing the morphological impact of such changes in dietary composition experienced within an individual's lifetime. This significantly hinders our understanding of functional morphology in primates experiencing cyclical fluctuations in dietary composition, as may occur with the consumption of seasonal, mechanically challenging "fallback foods" (Robinson and Wilson, 1998; Marshall and Wrangham, 2007).

Prior research concerning the role of fallback foods and dietary variability in human and non-human primate evolution has often relied on dental tissues to infer the composition of an individual's diet (Teaford and Ungar, 2000; Laden and Wrangham, 2005; Stanford, 2006; Antón, 2008; Cerling et al., 2011; Dominy, 2012). For example, the current knowledge concerning dietary variability in the extinct hominin *Paranthropus* is largely

derived from dental microwear and enamel-based dietary isotope data (Grine, 1981; Sponheimer et al., 2006; Ungar et al., 2008; Cerling et al., 2011; Ungar and Sponheimer, 2011; Dominy, 2012; Grine et al., 2012). These studies have sufficiently demonstrated that the ecology of robust australopithecines was characterized by seasonal dietary variability, yet it remains unclear how this variation in dietary composition affected the craniomandibular skeletal morphology of these Plio-Pleistocene hominins. Since primate fallback foods are often more mechanically challenging than preferred foods (Rosenberger, 1992; Wright, 2005; Marshall and Wrangham, 2007), and since the skeletal elements of the masticatory apparatus are known to dynamically respond to variation in dietary properties (Beecher and Corruccini, 1981; Beecher et al., 1983; Bouvier and Zimny, 1987; Bouvier, 1988; Yamada and Kimmel, 1991; Bouvier and Hylander, 1996b; Kiliaridis et al., 1996; Nicholson et al., 2006; Ravosa et al., 2006; Ravosa et al., 2007b; Ravosa et al., 2008b; Menegaz et al., 2009; Menegaz et al., 2010; Ravosa et al., 2010), this study investigates whether the craniomandibular skeleton, similar to dentition, can provide valuable ecomorphological information related to dietary variability in fossil hominins and non-human primates.

While the concept of fallback foods has permeated the study of human and non-human primate evolution and ecology over the previous decade, issues of dietary variability and seasonality are hardly exclusive to primates. Indeed, the theory that craniodental adaptations may be related to the seasonal consumption of mechanically challenging food items is derived from the study of cichlid fish (Liem, 1980; Robinson and Wilson, 1998). This theory has recently been adopted for the study of craniodental morphology and

dental microwear in non-primate mammals, such as cervid artiodactyls (Merceron et al., 2010), rodents (Firmat et al., 2010), and macropodid marsupials (Christine Janis, personal communication). Thus, while this research examines craniomandibular morphology and dietary variability in the well-studied context of primates, these topics are additionally relevant to many non-primate mammals.

The surprising and unfortunate deficiency of evidence concerning the morphological and physiological responses to ontogenetic variation in dietary properties impedes our understanding of the origins of craniomandibular variation and diversification in primates and non-primate mammals. This is especially relevant for populations experiencing significant variation in dietary behaviors. This variation may manifest as short-term cyclical changes related to environmental variability, such as seasonal fluctuations in the consumption of fallback foods, or more long-term changes in subsistence strategies as with human groups transitioning to agriculturalism (Carlson and Van Gerven, 1977; Armelagos et al., 1989). In order to address the critical role of dietary variability in primate and mammalian evolution, this experimental research tests a series of hypotheses regarding the effect of ontogenetic variation in the mechanical properties of food items on craniomandibular growth and form. These hypotheses are categorized into two principal objectives.

**Objective I:** Elucidate how variation in dietary mechanical properties and post-weaning dietary shifts affect the ontogeny of craniomandibular form. These hypotheses will test whether dietary variability can be recognized via skeletal

morphology, and which morphological characters are most useful for classifying an individual by its dietary behavior. This objective will assess ecomorphological theory regarding seasonality, fallback food use, dietary shifts, and phenotypic variation in hominin and primate evolution.

**Objective II:** Investigate the biological processes underlying skeletal functional adaptation, how they vary postnatally, and how they are influenced by postnatal shifts in dietary behavior. This objective will evaluate the use of data derived from bone physiology and microstructure to enhance our understanding of diet-induced phenotypic plasticity, and will illuminate the mechanistic basis of functional adaptation throughout the skull and feeding apparatus.

The long-term goal of these analyses is to develop an experimental model of mammalian craniomandibular growth that encompasses the complexities of adaptive plasticity related to the seasonality of dietary resources. In this regard, this study will elucidate functional adaptation in the craniomandibular complex related to shifts in dietary composition and attendant masticatory loading regimes. An ontogenetic approach will examine how stable versus temporally dynamic diets affect the skeletal form and physiology of growing organisms, and the variation in adult phenotypes that result from these respective diets. While this experimental design will not replicate all aspects of wild diets or all possible patterns of variation in feeding behaviors, it provides a novel opportunity to evaluate fundamental skeletal responses to variation in dietary properties and composition. This basic information has been heretofore neglected and is requisite for more refined

characterizations of the relationships among morphological plasticity, dietary seasonality, and skull form in primates and non-primate mammals.

## APPROACHES TO STUDYING FUNCTIONAL MORPHOLOGY

The fundamental goal of laboratory- and field-based studies of functional morphology is to understand the diversity of morphological forms in light of their environmental and/or behavioral roles. A broad understanding, established across multiple extant taxa, of the morphology associated with a given functional role provides a more stable base from which to make inferences of that role in extinct species (Kay and Cartmill, 1977; Gans, 1988). In recent years, phenotypic plasticity has been highlighted in the biological sciences for its potential to shed light on such form-function relationships (Galis, 1996). Phenotypic plasticity refers to the ontogenetic modulation of a phenotype across an environmental gradient (Bradshaw, 1965; Stearns, 1989; West-Eberhard, 1993; Via et al., 1995; Pigliucci and Hayden, 2001; DeWitt and Scheiner, 2004; Pigliucci, 2005; West-Eberhard, 2005) and can function as a mechanism for the fine-tuning of form-function relationships across an individual's lifespan (Grant and Grant, 1989; Losos, 1990). In skeletal tissues, phenotypic plasticity is accomplished through functional adaptation, or the dynamic processes by which bone tissue is modeled and remodeled in order to maintain a skeletal element's structural integrity in a given loading environment (Lanyon and Rubin, 1985; Biewener, 1993; Bouvier and Hylander, 1996b, a; Vinyard and Ravosa, 1998; Hamrick, 1999). Thus, plasticity studies may be employed in musculoskeletal biology to provide insight into morphology, growth, performance, and evolution

(Biewener, 2002; Garland and Kelly, 2006; Ravosa et al., 2007a; Ravosa et al., 2007b; Ravosa et al., 2008b; Ravosa et al., 2008a; Menegaz et al., 2009; Menegaz et al., 2010; Ravosa et al., 2010).

In experimental approaches to functional morphology, it is critical to observe an organism under naturalistic conditions. Such studies should strive to replicate relevant variables as they would be experienced in the wild, while simultaneously minimizing environmental or developmental noise. Thus, to the extent possible, experimental analyses induce normal behavioral and physiological ranges in an attempt to better understand form-function relationships within an organism's biology (Bock and von Wahlert, 1965). As the biological roles of many behaviors and anatomical structures vary across an organism's lifetime (Bock and von Wahlert, 1965), this naturalistic approach must necessarily include an ontogenetic perspective. Furthermore, an organism's capacity to respond to environmental fluctuations through phenotypic plasticity is likely related to its ontogenetic stage and life history, and different organisms – indeed, even different traits within a single organism – may demonstrate variation in plastic responses to environmental changes (West-Eberhard, 1993; Via et al., 1995; Nylin and Gotthard, 1998; Sultan, 2000; West-Eberhard, 2005). Thus, a comprehensive, laboratory-based approach to functional morphology must account for species-normative behavior and physiology across representative ontogenetic stages.

## Phenotypic Plasticity Across Ontogeny

The environmental conditions encountered by an individual early in its postnatal life may shape the nature of its plasticity responses and growth trajectories in later life (Schlichting and Pigliucci, 1998; Taborsky, 2006). Weaning is an important landmark in a life cycle, after which growth rates may change (Bowman and Lee, 1995; Helm and German, 1996) and be influenced more readily by environmental and epigenetic factors (Atchley, 1993; Helm and German, 1996). Post-weaning juvenile feeding performance may influence growth rates and body size (Schluter, 1995) which, later in life, are correlated with reproductive success (Roff, 1992; Taborsky, 2006). Furthermore, individuals that experience stressful environments as juveniles may demonstrate growth compensation later in their ontogeny (Metcalfe and Monaghan, 2001; Ali et al., 2003) to reach optimal adult size, but this carries the risk of decreased fitness (Gotthard and Nylin, 1995; Metcalfe and Monaghan, 2001). For instance, vertebrate taxa which experience prenatal or early postnatal nutritional stress may accelerate growth/maturation rates or extend the growth period in order to compete with less-stressed conspecifics. However, these compensatory strategies may, in the long-term, negatively affect characters related to fitness such as physiological parameters (e.g. cardiovascular health or insulin regulation), lifespan and survival prospects, and ultimately offspring size and survival (Metcalfe and Monaghan, 2001). The environment inhabited early in the life of an organism may significantly contribute to adult morphology and performance, and selection may shape the plasticity of important life history variables such as the timing of weaning and growth rate (Roff, 1992; Gotthard and Nylin, 1995; Abrams et al., 1996; Arendt, 1997; Nylin and Gotthard, 1998; Di Bitetti and Janson, 2000).

While the phenotypic plasticity of behavioral characters is often considered to be flexible given changes in environment over ontogeny, the extent to which morphological plasticity is flexible (i.e. reversible) or inflexible is unclear (Stearns, 1989; Via et al., 1995). Functional studies of skeletal and cartilaginous tissues indicate that growing individuals may be capable of such flexible plasticity, particularly for positive growth (Bouvier and Hylander, 1984; Bouvier, 1987, 1988; Yamada and Kimmel, 1991). However, it remains to be seen whether there is continuous and bidirectional capacity for plasticity as an individual ages. Indeed, the ability of an organism to respond to the environment by means of morphological plasticity may decrease as growth and the rate of bone modeling slow (Hinton and McNamara, 1984; Meyer, 1987; Bertram and Swartz, 1991; Rubin et al., 1992; Pearson and Lieberman, 2004; Hoverman and Relyea, 2007; Ravosa et al., 2008b). Furthermore, adaptive plasticity during early growth stages is thought to have an additive influence on underlying growth allometries (Bernays, 1986). In such cases, adult morphology would be strongly affected by the environmental conditions experienced during early life, and modified to a lesser degree by changes in habitat and diet experienced after skeletal maturity. Thus, the question of whether or not morphological plasticity, and particularly skeletal plasticity, is flexible has important ramifications for mammalian taxa inhabiting variable environments. At present, our ability to make behavioral and ecological inferences about species living in seasonal environments is impeded by a dearth of knowledge regarding the ontogenetic nature of plasticity in the relevant morphological structures and anatomical tissues.

## Phenotypic Plasticity and Evolution

Plasticity is a significant source of morphological, physiological, and behavioral variation in populations (Stearns, 1989) and the capacity for plasticity may improve fitness in novel or variable environments (Stearns, 1989; Travis, 1994; Sultan, 1995; Agrawal, 2001; Ghalambor et al., 2007). Furthermore, character-specific plasticity itself may be subject to selection (Bradshaw, 1965; Berrigan and Scheiner, 2004; Garland and Kelly, 2006) and is posited to be one origin of morphological adaptations. Through the process of genetic assimilation, environmentally induced phenotypic variation may facilitate shifts towards new adaptive peaks and eventually result in heritable variation (Waddington, 1953; Schlichting, 2003; Pigliucci, 2005). Indeed, plasticity may play a key role in morphological differentiation and speciation (West-Eberhard, 1993, 2005).

The study of phenotypic plasticity has become integral for understanding the functional and adaptive significance of morphological variation and life history trends (Bouvier and Hylander, 1981, 1982, 1984; Gotthard and Nylin, 1995; Bouvier and Hylander, 1996a, b; Nylin and Gotthard, 1998; Agrawal, 2001; Nicholson et al., 2006; Ravosa et al., 2007a; Ravosa et al., 2007b; Ravosa et al., 2008b; Ravosa et al., 2008a; Lambert, 2009; Menegaz et al., 2009; Menegaz et al., 2010). The details and subtleties of interactions among environment, ontogeny, and morphology can be teased apart via the use of model organisms in long-term laboratory-based studies coupled with multi-disciplinary analyses that can be difficult if not impossible to perform on wild populations. These experimentally-derived data can then be used to evaluate evolutionary models pertaining to functional morphology in comparative and fossil populations (Pigliucci, 2005; Garland

and Kelly, 2006; Ravosa et al., 2008b; Ravosa et al., 2008a; Menegaz et al., 2009; Menegaz et al., 2010; Copes, 2012). Indeed, a refined appreciation for the variation possible within a species is necessary for interpretation of the fossil record and precise morphology-based identification of species (Roth, 1989; West-Eberhard, 1993, 2005). Considerable concern has been raised among paleoanthropologists regarding the use of phenotypically plastic characters in reconstructing phylogenetic relationships (Wood and Lieberman, 2001; Collard and Wood, 2007; Collard and Lycett, 2008, 2009; von Cramon-Taubadel, 2009). However, the hypothesis that plastic, functional morphological characters are inherently non-informative with regards to evolutionary systematics makes two basic and perhaps flawed assumptions, namely: that functional diversification is not related to evolutionary diversification; and that there is a simple predictive relationship between strain levels and plasticity values across morphological structures and tissue types. Laboratory-based studies represent a unique opportunity to investigate patterns of phenotypic plasticity and the biological processes underlying functional adaptation across taxa, morphological regions, and tissue types. Thus, this approach may provide crucial insight into form-function links that, in turn, can be applied to both fine- and broad-scale evolutionary questions such as the effect of environmental variability on morphology and adaptation at the level of the individual, population, and species.

## MASTICATORY MORPHOLOGY & PLASTICITY

Dietary composition and behavior has long been a central consideration of work on human and non-human primate cranial function (Hrdlička, 1930; Weidenreich, 1941; Hylander, 1975). Notably, our current knowledge of the morphological and behavioral significance of dietary composition is the product of a vast synthesis of field and laboratory studies (Vinyard et al., 2008). Through this synthesis, the material properties of food items are understood to influence jaw adductor activity, jaw kinematics, and feeding behaviors (Crompton, 1986; Weijs et al., 1989; Hylander et al., 1992; Hylander et al., 2000; Hylander et al., 2005). Increased jaw muscle activity associated with mechanically resistant food items results in elevated peak and cyclical strains in the craniomandibular skeleton (Weijs and de Jong, 1977; Hylander, 1979, 1988, 1992; Hylander et al., 1992; Herring and Teng, 2000; Ravosa et al., 2007b; Ravosa et al., 2008b) and, in turn, differential growth and remodeling of hard and soft tissues in the cranium and mandible (Beecher and Corruccini, 1981; Beecher et al., 1983; Bouvier and Zimny, 1987; Bouvier, 1988; Yamada and Kimmel, 1991; Bouvier and Hylander, 1996b; Kiliaridis et al., 1996; Nicholson et al., 2006; Ravosa et al., 2006; Ravosa et al., 2007b; Ravosa et al., 2008b; Menegaz et al., 2009; Menegaz et al., 2010; Ravosa et al., 2010). Indeed, our experimental understanding of plasticity in the size and form of craniomandibular elements (e.g. mandible, zygoma, hard palate), cross-sectional bone distribution and cortical thickness, and masticatory muscle skeletal attachments has considerably informed the use of these characters in ecomorphological and systematic reconstructions of human and non-human primate evolution (Du Brul, 1977; Walker,

1981; Rak, 1983; Demes and Creel, 1988; Hylander, 1988; Daegling, 1989; Ravosa et al., 2006; Constantino and Wood, 2007; Ravosa et al., 2007b; Ravosa et al., 2008b; Menegaz et al., 2009; Menegaz et al., 2010).

A large body of experimental work has demonstrated that the basic principles of functional adaptation of the masticatory skeleton are remarkably similar across mammalian taxa (e.g. primates, suids, lagomorphs, rodents, and carnivorans, to name a few). Increased masticatory loading related to the consumption of mechanically resistant food items is known to influence craniomandibular joint size and shape, as well as the morphology and material properties of the joint cartilage (Bouvier and Hylander, 1981, 1984; McFadden and McFadden, 1986; Bouvier, 1987, 1988; Yamada and Kimmel, 1991; Nicholson et al., 2006); mandibular corpus dimensions and cortical bone distribution (Watt and Williams, 1951; Bouvier and Hylander, 1984; Kiliaridis et al., 1985; McFadden and McFadden, 1986; Yamada and Kimmel, 1991); symphyseal dimensions and cortical thickness (Ravosa et al., 2007b; Ravosa et al., 2008a); tooth row length and placement relative to the joint (McFadden and McFadden, 1986; Menegaz et al., 2010); the size, shape, and cortical bone morphology of muscle attachment sites such as the sagittal crest and temporal fossa, coronoid process, zygomatic arches, angular process, and pterygoid plates (Kiliaridis et al., 1985; Bouvier and Hylander, 1996a; Kiliaridis et al., 1996; He and Kiliaridis, 2003; Nicholson et al., 2006; Menegaz et al., 2010); and hard palate dimensions, cortical thickness, and trabecular density (Beecher and Corruccini, 1981; Beecher et al., 1983; He and Kiliaridis, 2003; Menegaz et al., 2009).

Indeed, within the masticatory apparatus, larger skeletal dimensions coupled with increased cortical thickness and trabecular density are the standards by which elevated levels of masticatory loading are recognized in comparative and fossil samples (Jolly, 1970; Kay, 1981; Rak, 1983; Hylander, 1988)**.** Individually, each of these morphologies suggest specific adaptations to increased masticatory loading, such as elongation of a muscle effort arm by modification of the attachment sites or the resistance of complex loading regimes through cortical bone distribution. When viewed in aggregate, these individual characters combine to give the overall impression of a masticatory apparatus well-adapted to the high masticatory loads associated with the consumption of a mechanically resistant diet composed of hard/tough food items. Such is the case with the robust australopithecine *Paranthropus*, which exhibits a unique mixture of masticatory characters including a well-developed sagittal crest, flared zygomatic arches, a dished and buttressed face, a tall mandibular ramus, a high craniomandibular joint relative to the occlusal plane, megadontia of the premolars and molars, thickened enamel, large dimensions of the mandibular corpus and symphysis, thickened cortical bone in the mandibular corpus, and a thickened hard palate (Rak, 1983; Hylander, 1988; Daegling, 1989; McCollum, 1997; Wood and Aiello, 1998; McCollum, 1999; Constantino and Wood, 2007; Strait et al., 2007; Rak and Hylander, 2008; Villmoare and Kimbel, 2011; Voss et al., 2013). Thus, the experimental evidence for diet-induced phenotypic plasticity provides a basis by which the functional significance of masticatory characters – both individually and in aggregate – can be understood in wild and fossil taxa.

Despite this broad synthesis of experimental and comparative work pertaining to the functional morphology of the craniomandibular complex, the issue of phenotypic plasticity as it relates to dietary variability has been largely overlooked (Lambert, 2009). The diets of wild primates are complex and often show seasonal variation (Conklin-Brittain et al., 1998; Lambert et al., 2004), and thus this gap in our understanding of masticatory plasticity greatly hinders our ability to assess the role of dietary seasonality, niche partitioning, and subsistence shifts underlying phenotypic variation and diversification. Furthermore, our lack of knowledge concerning the extent to which plasticity varies ontogenetically throughout the cranium prevents us from understanding the effects of aging on functional adaptation in the skeleton (Bertram and Swartz, 1991; Hoverman and Relyea, 2007) and intraspecific norms of reactions that may impact phylogenetic analyses (West-Eberhard, 2005).

As discussed above, much of our current knowledge regarding the effects of masticatory loading on skull form from experimental research on primate and non-primate mammals fed stable, homogenous diets for the duration of a study (Beecher and Corruccini, 1981; Beecher et al., 1983; Bouvier and Hylander, 1996b; Kiliaridis et al., 1996; Nicholson et al., 2006; Ravosa et al., 2007b; Ravosa et al., 2008b; Ravosa et al., 2008a; Menegaz et al., 2009; Jašarević et al., 2010; Menegaz et al., 2010). A select number of studies of joint mechanobiology in rats have addressed shifts in dietary composition, and found that the craniofacial skeleton (e.g. subchondral bone) and its associated soft tissues (e.g. articular cartilage) may be capable of significant morphological plasticity in response to dietary shifts in growing individuals (Bouvier and Hylander, 1984; Bouvier and Zimny, 1987;

Yamada and Kimmel, 1991). These studies were localized to a single structure or tissue type and short-term in the duration of the loading stimulus (see Appendix I), such that the broader nature of morphological plasticity throughout the craniomandibular skeleton remains poorly understood.

Our ability to discern the subtleties of morphological variation related to dietary shifts and seasonality in extant and extinct primates is constrained by our understanding of how dietary composition modulates inter- and intra-individual variation across ontogeny. This effectively limits our capacity to address questions such as the importance of seasonal diets and the use of fallback foods for niche separation and evolution among early hominins (Teaford and Ungar, 2000; Laden and Wrangham, 2005; Stanford, 2006; Antón, 2008) and non-human primates (Rosenberger, 1992; Conklin-Brittain et al., 1998; Lambert et al., 2004; Stanford, 2006; Yamashita, 2008). The majority of our knowledge concerning the effects of masticatory loading on cranial growth is derived from protocols thatF employ stable, homogenous dietary treatments. These protocols expose experimental animals to a single diet for the duration of the study (Watt and Williams, 1951; Beecher et al., 1983; Kiliaridis et al., 1985; McFadden and McFadden, 1986; Kiliaridis et al., 1996; He and Kiliaridis, 2003; Ravosa et al., 2007b; Ravosa et al., 2008a; Menegaz et al., 2009; Menegaz et al., 2010). While providing data necessary for understanding the basic processes of functional adaptation in the skull, this is ill-suited for modeling the naturalistic variation in dietary composition which characterizes many primate populations.

This study represents a novel attempt to model the temporal complexity of primate diets in a laboratory setting. In addition to two treatment groups representing the stable diet models found in the majority of previous experiments, this work also includes two variable diet cohorts that experience a mid-experiment shift in dietary composition. The inclusion of these variable diet cohorts makes it possible to test hypotheses pertaining to the ability of paleoanthropologists to identify dietary variability (e.g. seasonality and the use of fallback foods) in the fossil record, and to evaluate the relative value of morphological characters in these paleoecological analyses. Finally, the inclusion of two variable-diet treatment groups that experience multiple behavioral modalities during the course of the experiment allows examination of ontogenetic variation in phenotypic plasticity and functional adaptation in the craniomandibular skeleton. While previous studies have focused primarily on *inter-individual* variation in masticatory loading and craniomandibular form, this work also examines the significance of *intra-individual* variation (e.g. variation within an individual's lifespan). Thus this study aims to model diet-induced phenotypic plasticity more naturalistically, in an attempt to improve our understanding of the complex relationship between dietary variability and craniomandibular form.

## DIETARY VARIABILITY

A commonly encountered difficulty in ecomorphology is that dietary predictions based solely on skeletal morphology are not always confirmed by observations of individuals in the wild. Indeed, species identified as morphological specialists based upon skeletal traits

often are observed acting as ecological generalists. This problem, first observed in African cichlids (Liem, 1980), has become known as Liem's Paradox. Upon broader investigation, this paradox has been interpreted across vertebrates as the adaptation to the consumption of "fallback foods," dietary items which are consumed during periods when more preferred food items are unavailable or scarce due to seasonality and/or competition (Table 1.1) (Robinson and Wilson, 1998; Marshall and Wrangham, 2007). Fallback foods may be either less nutritionally valuable than primary foods (Conklin-Brittain et al., 1998; Marshall and Wrangham, 2007) and/or more mechanically difficult to consume (Rosenberger, 1992; Wright, 2005; Marshall and Wrangham, 2007). While traditional dietary categorization among primates assumed that masticatory adaptations were related to the food type consumed most often by a species (Kay, 1975), the contemporary optimal foraging view suggests that craniodental morphology may be primarily adapted to those fallback foods which require specialization for effective processing (Rosenberger, 1992; Conklin-Brittain et al., 1998; Wright, 2005; Marshall and Wrangham, 2007). In the primate diet, fallback foods may include items such as seeds, nuts, leaves, and terrestrial herbaceous vegetation (THV). Due to the use of fallback foods, the craniofacial morphology of primate species living in environments subject to seasonal or semi-routine fluctuations in food availability may reflect the use of dietary items consumed periodically rather than regularly.

The relative significance of fallback foods for an animal's overall diet is dependent upon environmental conditions, e.g. the seasonality of preferred resources such as fruit, as well as competition for food resources with conspecific and allospecific individuals (Robinson

and Wilson, 1998; Laden and Wrangham, 2005). Some species may consume non-preferred fallback foods year-round as staples, with seasonal decreases in use during periods of preferred food availability (Table 1.1) (Laden and Wrangham, 2005; Marshall and Wrangham, 2007; Marshall et al., 2009). The diets of lowland gorillas are often cited for an example of stable fallback foods (Marshall and Wrangham, 2007), where foliage (e.g. THV and pith) is consumed year-round and comprises 100% of the diet when preferred fruit is unavailable. As closely related species often compete for similar preferred resources (Harper et al., 1961), the use of fallback foods is thought to alleviate the competitive stresses that arise when sympatric species inhabit resource-limited environments (Stanford, 2006). For example, sympatric cercopithecine species *Lophocebus albigena* and *Cercopithecus ascanius* in Kibale National Park, Uganda, consume a similar frugivorous diet, except during periods of low food availability (e.g. the late dry season) when *L. albigena* consumes more mechanically resistant food items such as bark and hard seeds (Lambert et al., 2004). This seasonal divergence in dietary composition is though to reduce competition among these sympatric cercopithecine species, and the mechanical properties of the fallback foods consumed by *L. albigena* are hypothesized to be the selective pressure behind the evolution of thickened dental enamel in the species. Thus, this seasonal divergence in feeding behaviors may play a key role in the sympatric diversification of vertebrate species.

Among primates, the use of fallback foods has been posited to increase dietary flexibility, reduce resource competition between sympatric species, and even promote speciation in such taxa as early hominins (Teaford and Ungar, 2000; Laden and Wrangham, 2005;

Stanford, 2006; Antón, 2008), African apes and cercopithecines (Conklin-Brittain et al., 1998; Lambert et al., 2004; Stanford, 2006), platyrrhines (Rosenberger, 1992; Wright, 2005), and Malagasy strepsirrhines (Yamashita, 2008). The use of underground storage organs (USOs; e.g., rhizomes, tubers, and corms) as fallback foods is hypothesized to have promoted the adaptive radiation of hominins (Hatley and Kappelman, 1980; Teaford and Ungar, 2000; Laden and Wrangham, 2005; Stanford, 2006; Lambert, 2007; Dominy et al., 2008). Indeed, USOs are known to be valuable fallback foods for modern humans on multiple continents (Marshall, 1976; Lee, 1979; Gott, 1982; Thoms, 2008). Raw USOs and other fallback foods utilized by hominoids (e.g. THV and seeds) exhibit mechanical properties which make them difficult to consume (Dominy et al., 2008) and thus specialized craniodental morphologies may be necessary to exploit these fallback resources. While comparative studies of masticatory biomechanics support the hypothesis that early hominins may have utilized USOs as fallback foods (Walker, 1981; Demes and Creel, 1988; Hylander, 1988; Wright, 2005), there is a paucity of work regarding the inconstant use of these resources and the morphological significance of dietary shifts within the lifetime of an individual. Such a gap in the understanding of the relationship between functional morphology and dietary variability stands as an impediment to the ability to understand the role of fallback foods in the ecology and diversification of extinct and extant primate populations.

# THE HOMININ DIET

Since the discovery of *Paranthropus* (Broom, 1938), a considerable effort has been made to decipher the diet of this hominin genus based on a large collection of craniodental remains. The conventional wisdom, as first put forth by Robinson (Robinson, 1954a, b), focused on a generalist-specialist dichotomy. *Paranthropus*, with its robust masticatory apparatus and megadontia, was viewed as a dietary specialist. Conversely, *Australopithecus* and *Homo* were viewed as dietary generalists, capable of a considerable degree of behavioral flexibility. The persistence of *Homo* through the climatically volatile Pleistocene was attributed to this generalist strategy, while specialization was thought to have doomed *Paranthropus* to extinction (Wood and Strait, 2004).

The concept of *Paranthropus* as a specialist is especially complicated by the various definitions of "generalist" and "specialist". Wood and Strait (2004) defined generalists as species with the ability to adapt to seasonal changes in their environment. This definition encompasses so-called "seasonal specialists," such that species exhibiting morphological specializations to non-preferred foods are considered generalists. The introduction of the concept of fallback foods (Laden and Wrangham, 2005) has helped considerably to explain this occurrence of Liem's paradox (Liem, 1980; Robinson and Wilson, 1998) in hominin paleobiology. Indeed, the use of fallback foods is often cited to resolve apparent discrepancies between dietary interpretations based on craniomandibular morphology and those interpretations drawn from other lines of evidence, such as dental microwear and

comparative isotope analyses (Teaford and Ungar, 2000; Laden and Wrangham, 2005; Stanford, 2006; Antón, 2008; Cerling et al., 2011; Dominy, 2012).

It is generally accepted in the current literature that *Paranthropus* utilized fallback foods, and the focus of the debate has shifted to identify what resources were used as fallback foods and how often these resources were consumed. Exactly what food item(s) *Paranthropus* specialized upon has been the source of considerable debate for over a half century. The robusticity of the jaws and the megadontia of the postcanine teeth led Robinson (1954b:328) to conclude that the masticatory apparatus of *Paranthropus* was well adapted for "crushing and grinding." This led to the inference that *Paranthropus* consumed, at least periodically, a tough diet of terrestrial herbaceous vegetation (THV) and/or underground storage organs (USOs) (Robinson, 1954b; Du Brul, 1977; Hatley and Kappelman, 1980; Lucas et al., 1985; Laden and Wrangham, 2005). Furthermore, observations of enamel chipping in *P. robustus* (Robinson, 1954b) and early microwear studies (Grine, 1981) contributed to the idea that the robust australopithecines fed on gritty food items, such as USOs or vegetation found in riparian environments adjacent to lakes or rivers. Comparative morphological and isotopic studies have found dietary signals similar to that of *Paranthropus* in primate species such as the extinct lemur *Hadropithecus* (Dumont et al., 2011; Godfrey et al., 2011), the Chacma baboon (*Papio ursinus)* (Robinson, 1954b), the extinct baboon *Theropithecus oswaldi* (Dunbar, 1976; Cerling et al., 2011), and the extant great apes (Stanford, 2006; Constantino et al., 2009). These studies support the idea that the robust australopithecines may have been adapted

to consume a low-quality, high-quantity diet composed of tough and gritty vegetal material.

Phillip Tobias, in discussing the *P. boisei* specimen OH5, referred to the large teeth as an "enormous set of nutcrackers", giving rise to *Paranthropus*' popular nickname of the "Nutcracker Man" (Tobias, 1973; Tobias, 2009). Despite this nickname (which Tobias considered "unfortunate"), the hard object specialist hypothesis is discussed in a relatively small section of the paleoecological literature. A few researchers have interpreted the dental morphology and microwear found in *Paranthropus* as adaptations to consuming small, hard items such as seeds and nuts (Jolly, 1970; Grine, 1981; Lucas et al., 1985). Grine (1981) concluded that the postcanine teeth of robust australopithecines were adapted for puncture-crushing movements, and thus may have been used to process small, hard objects. However, he also considered gritty and/or fibrous items to be possible primary or fallback foods for *Paranthropus*. More recent analyses of dental microwear have found that *P. robustus* groups with extant primates known to consume hard items as fallback foods, such as *Lophocebus* and *Sapajus* (Scott et al., 2005; Ungar et al., 2008), while *P. boisei* has low microwear complexity similar to that in *A. afarensis* (Ungar and Sponheimer, 2011). Thus, it is possible that at least *P. robustus* exhibits dental adaptations for the infrequent consumption of small, hard items as fallback foods (Ungar et al., 2008; Ungar and Sponheimer, 2011).

In recent years, the concept of *Paranthropus* as the hard-object feeding "Nutcracker Man" has been used often as a "straw man" introduction to the discussion of a diet

composed of tough items (e.g. THV and USOs) (Lee-Thorp, 2011; Ungar and Sponheimer, 2011). Frequently, hard *and* tough foods are discussed more broadly as a group, since these items are not necessarily mutually exclusive within the primate diet (Grine, 1981; Lucas et al., 1985; Hylander, 1988). Furthermore, recent studies have suggested that despite their morphological similarities, variation may have existed between the diets of *P. robustus* and *P. boisei*.

Intrageneric variation in the diet of *Paranthropus* may be related to habitat variation. A climatic shift towards more dry, xeric environments around the Plio-Pleistocene boundary coincides with both the rise of African grasslands and the appearance of *Paranthropus* (Vrba, 1985, 1988; Cerling, 1992; Reed, 1997). In east Africa, *P. boisei* inhabited open woodlands and water-logged edaphic grasslands adjacent to plentiful water sources (Reed, 1997). Isotopic studies have shown that the diet of *P. boisei* was characterized by a higher percentage of $C_4$ plants (e.g. tropical grasses and sedges) than that of any other known hominoid (van der Merwe et al., 2008; Cerling et al., 2011). Instead, $C_4$ values observed in *Paranthropus* are similar to those of *Theropithecus oswaldi*, an extinct baboon which specialized on grasses (Cerling et al., 2011). Sedges, a $C_4$ plant found in wetlands and riparian environments, have been identified as a possible food source for *P. boisei* (Lee-Thorp, 2011; Ungar and Sponheimer, 2011; Dominy, 2012). Sedges have a fibrous pith and a gritty, starchy USO (the corm). Chimpanzees have been known to consume sedge pith as a fallback food, and the corms have been cultivated by historic human populations (Dominy, 2012). Finally, enamel isotope data suggest the presence of low seasonal variation in the diet of *P. boisei*, such that these

tough sedges and USOs may have been consumed consistently (Cerling et al., 2011; Lee-Thorp, 2011). Nevertheless, based on the nutritional and mechanical properties of sedges, it is still possible to define them as staple fallback foods even if they were consumed across seasons (Table 1.1) (Laden and Wrangham, 2005; Marshall and Wrangham, 2007; Marshall et al., 2009).

In South Africa, *Paranthropus robustus* inhabited more arid open or wooded grasslands (Reed, 1997). Enamel isotope studies have shown that unlike *P. boisei*, *P. robustus* consumed a diet that was up to 70% $C_3$ plants. This suggests a diet much more like that of extant chimpanzees, which prefer to feed on $C_3$ foods such as tree fruits (Cerling et al., 2011). The diet of *P. robustus* was also likely highly seasonally variable (Cerling et al., 2011; Lee-Thorp, 2011) and supplemented by seasonal consumption of $C_4$ fallback foods (Sponheimer et al., 2006). Dental microwear studies have also found higher textural complexity in the teeth of *P. robustus* compared to *P. boisei*, supporting the idea that the former might have consumed more hard-object fallback foods than its east African counterpart (Ungar and Sponheimer, 2011). Despite its high temporal dietary variability, *P. robustus* likely consumed a narrow range of food items, including woody $C_3$ plants and fibrous $C_4$ plants (Sponheimer et al., 2006; Balter et al., 2012).

Although dental microwear and isotopic data suggest dietary variation within their genus, *Paranthropus boisei* and *P. robustus* share many morphological similarities in the craniomandibular complex (Robinson, 1954a; Rak, 1983; Wood and Constantino, 2007; Villmoare and Kimbel, 2011). This suggests that the masticatory apparatus of the robust

australopithecines functions to resist high levels of masticatory strain associated with the consumption of generally mechanically resistant (e.g. hard *and/or* tough) food items (Ungar and Sponheimer, 2011). While recent advances in paleoanthropology, particularly the increasing popularity of comparative isotope studies, have provided valuable information about dietary breadth and resource use in *Paranthropus*, the effects of interspecific and intergeneric variation in fallback food usage on craniomandibular morphology is still unclear. This research addresses the morphological correlations of seasonal versus year-round consumption of mechanically resistant foods in an effort to better understand dietary variation between *P. boisei* and *P. robustus*, and between *Paranthropus* and genera (e.g. *Australopithecus* and *Homo*) that are not thought to have specialized, seasonally or otherwise, on these hard and/or tough foods.

**Table 1.1.** The difference between preferred foods and fallback foods. Adapted from Marshall and Wrangham (2007; Table 1).

| | | **Preference** | |
|---|---|---|---|
| | | **High** | **Low** |
| **Importance** | **High** | **Staple Preferred Foods**<br><br>High quality foods that comprise a substantial part of the diet during seasons of availability. | **Staple Fallback Foods**<br><br>Low quality foods that comprise a substantial part (up to 100%) of the diet seasonally and at least a small part (<0%) of the diet annually. |
| | **Low** | **Filler Preferred Foods**<br><br>High quality foods that comprise a small portion of the diet; may be eaten only seasonally. | **Filler Fallback Foods**<br><br>Low quality foods that comprise a small portion of the diet; may be eaten only seasonally. |

# CHAPTER 2: EXPERIMENTAL DESIGN

## MODEL SPECIES

A number of practical and ethical constraints prevent the use of primates as subjects. Thus, the proposed study uses a well-established mammalian laboratory model, the Sprague-Dawley rat (*Rattus norvegicus*), to investigate the effects of dietary variability on craniofacial growth and morphology. From an experimental standpoint, the rat is an ideal model species due to a short lifespan that is easily observed and manipulated in a laboratory setting. Thus, the choice of this taxon facilitates a study of plasticity covering the *entire* postweaning period of development, previously unavailable data vital for understanding long-term plasticity in the wild. As an experimental model, the skeletal growth patterns of rats are well documented (Moss and Baer, 1956; Roach et al., 2003) and Sprague-Dawley rats are known to experience similar processes of intracortical remodeling as larger mammals (Bentolila et al., 1998). Rats also conform to the pattern of early, rapid brain growth followed by prolonged facial growth characteristic of primate and non-primate mammals (Moore, 1966). Furthermore, preliminary imaging studies noted the presence of diploë within the bones of the rodent neurocranium, confirming that the layered arrangement of dense tabular bone and spongy diploë that exists in larger-bodied mammals also exists in small-bodied rodents. Thus, despite differences in absolute cranial size, the configuration of the neurocranial skeleton is similar between

rodents and larger mammals like primates. These findings are thus directly relevant for understanding primate patterns of craniomandibular phenotypic plasticity and growth.

This research will explore the fundamental responses of the craniomandibular complex to shifts in dietary composition and attendant masticatory loading regime. Indeed, rats have been used extensively for research concerning skull growth related to differential biomechanical loading and diet (Beecher and Corruccini, 1981; Bouvier and Hylander, 1984; Bouvier and Zimny, 1987; Bouvier, 1988; Yamada and Kimmel, 1991; Kiliaridis et al., 1996) and their efficacy as an experimental model species for primate biology is well supported by fundamental similarities in skull growth, masticatory behavior and diet-induced plasticity (Washburn, 1947; Watt and Williams, 1951; Moss and Baer, 1956; Beecher and Corruccini, 1981; Beecher et al., 1983; Bouvier and Hylander, 1984; Bouvier and Zimny, 1987; Bouvier, 1988; Yamada and Kimmel, 1991; Bouvier and Hylander, 1996b; Kiliaridis et al., 1996). This study will build upon the existing body of research through an ontogenetic approach to understanding both local and systemic craniofacial responses to dietary shifts. The experimental design of this research will also result in more detailed data regarding functional modularity and masticatory biomechanics than achieved by previous studies. Although a certain amount of discretion is required in the extrapolation of results from model species, consistent patterns of functional adaptation and skeletal growth among mammals have validated repeatedly the efficacy of their use in experimental biology. In sum, due to a series of important similarities in the feeding apparatus between rats and primates, this study provides a unique opportunity to understand diet-induced plasticity responses in the skull and

masticatory complex of living and fossil primates. A major benefit of this animal model is that dynamic, postweaning changes in rat feeding behaviors can be related directly to variation in the form and function of primate craniomandibular elements. Indeed, this project will offer novel biological data on long-term plasticity responses to fallback foods highly relevant to seasonal loading conditions experienced by primates in the wild.

## MODELING DIETARY VARIABILITY

To model the effects of fluctuations in dietary composition on craniomandibular morphology, this study employs the use of two stable diet cohorts and two variable diet cohorts. The stable diet cohorts are raised on a homogenous diet of either solid pellets or powdered pellets, while the variable diets are weaned onto either the solid or powdered pellets for the first half of the experimental period and then switched to the alternate diet for the second half of the experiment. This mid-experiment shift serves as a theoretic shift in dietary composition, such as may be experienced by primates in seasonally variable environments. Though this study encompasses only a single shift in dietary properties, rather than repetitive shifts such as individuals might experience over a longer lifespan, it represents an opportunity to examine how a marked change in dietary composition affects the skeletal growth and form of individuals during the important period of growth between weaning and maturity.

In this study, whole pellets comprise the mechanically resistant diet for experimental individuals. Indeed, the mechanical properties of these compressed pellets fall within the

range of toughness and elastic modulus values for underground storage organs (USOs), a known category of primate fallback food (Figure 2.1) (Laden and Wrangham, 2005; Dominy et al., 2008). The inclusion of a powdered form of these sample pellets permits the modification of masticatory behavior and loading regimes while ensuring comparable nutrition among all cohorts. While there is no direct wild analog to the powdered diet, its use allows for the magnification of differences in dietary properties and thereby produces a wider, more realistic range of resultant phenotypes. As such, this study examines morphological and physiological plasticity due to the biomechanical demands of feeding independent of nutritional factors.

This study models the response of the craniomandibular skeleton related to the mastication of dietary items processed along the cheek teeth, rather than those foods requiring greater incisal preparation (e.g. fruit sclerocarp). Thus, this model is most applicable to species using fallback resources such as underground storage organs (USOs), leaves, terrestrial herbaceous vegetation (THV), and small nuts or seeds. These mechanically-resistant food items are or may have been consumed as fallback foods by many primate taxa, including extant African apes (Conklin-Brittain et al., 1998; Stanford, 2006) and Plio-Pleistocene hominins (Teaford and Ungar, 2000; Laden and Wrangham, 2005; Antón, 2008).

# EXPERIMENTAL METHODS

## Experimental Sample

All procedures for this project were conducted in accordance with the University of Missouri ACUC-approved protocol number 6622. A total of 42 male Sprague-Dawley rats were obtained from Harlan Laboratories (Haslett, MI) as weanlings (22 days). All animals were housed in AALAC-accredited Office of Animal Resources facilities at the Harry S. Truman VA Hospital/University of Missouri for a period of 13 weeks. Weaning was chosen as the starting point for the experimental period because this approximates a shift in masticatory function in the wild and to minimize the confounding influences of postweaning diets other than those included in this study. The early period of postweaning growth is also when the capacity for phenotypic plasticity is predicted to be greatest (Goldspink, 1970; Hinton and McNamara, 1984; Meyer, 1987; Bouvier, 1988; Bertram and Swartz, 1991; Rubin et al., 1992; Pearson and Lieberman, 2004; Hoverman and Relyea, 2007; Ravosa et al., 2008a). As Sprague-Dawley rats reach skeletal maturity between 80-91 days (Roach et al., 2003), the sample was raised to the age of 110 days to ensure the completion of skeletal growth. All animals were housed in individual cages to ensure adequate food intake (Bouvier and Hylander, 1984). Body mass for all animals (Figure 2.2) was measured at least twice weekly to monitor intra- and inter-cohort variation in growth and feeding performance. During weeks of dietary shift (weeks 4 and 10), body mass was measured daily. Body mass analyses and behavioral observations confirmed that none of the animals failed to thrive nor did they develop incisor

malocclusions at any point during the experimental period. Under anesthesia (see μCT Imaging section, below), right-side linear postcranial measures were collected weekly to monitor body and limb growth rates. These measures included the lengths of the head-body, tail, upper arm, forearm, forepaw, thigh, shank, and hindpaw (Table 2.1). At the end of the experimental period, all animals were euthanized via inhalation of 100% $CO_2$ from a compressed tank using a $CO_2$ chamber. Bilateral thoracotomy was used as a secondary means of assuring death.

Animals were randomly sorted into four dietary treatment cohorts for the duration of the experimental period (Table 2.2). All cohorts were fed *ad-lib* comparable amounts of diets consisting of LabDiet 5001 Rodent Diet (PMI Nutrition International, St. Louis, Mo.) in either solid pellet or meal/powdered pellet form. The use of the same diet presented in two different consistencies allows for the modification of masticatory behavior and the frequency/intensity of loading while offering comparable nutrition for all animals. Two cohorts were raised on a stable diet of either pellets (cohort 1) or meal (cohort 3). The remaining two cohorts were raised on a variable diet consisting of either pellets (cohort 2) or meal (cohort 4) for weeks 4-9 and then switched to the opposite diet for weeks 10-16. This schedule models a shift in dietary composition as may be experienced due to seasonal variation in the wild, and allows the evaluation of phenotypic plasticity during the optimal growth period.

## Material Properties of Experimental Foods

A portable food tester (Darvell et al., 1996; Lucas et al., 2001) was used to assess the material properties of pellets (Figure 2.1; Table 2.3) (Wainwright et al., 1976; Vincent, 1992; Lucas, 1994; Currey, 2002). The elastic, or Young's, modulus (E) is the stress/strain ratio at small deformations, characterizing the stiffness or resistance to elastic deformation. Toughness (R) is an energetic property describing the work performed propagating a crack through an item. Hardness (H) is used to quantify indentation.

Due to the specifications of the food tester, it was possible only to measure the material properties of the whole pellets. The meal diet, comprised of ground pellets, primarily differs from whole pellets in the scale of the food particles. Such differences in dietary consistency are known to evoke differences in ingestion behavior, masticatory muscle recruitment, and biomechanical loading in the masticatory apparatus (Bouvier and Hylander, 1982, 1984; Kiliaridis et al., 1985; Kiliaridis, 1989; Ravosa et al., 2007c; Ravosa et al., 2008b; Ravosa et al., 2008a). Thus, a meal diet represents a shorter preparation/ingestion time with a decrease in masticatory peak and cyclical loads relative to a diet of whole pellets.

## Micro-Computed Tomography (μCT) Imaging

Between the ages of 4-16 weeks, all animals were imaged weekly using μCT to produce a longitudinal series of three-dimensional images of the craniofacial skeleton (Figure 2.3). The Siemens Micro-SPECT/CT unit was operated at 80 kv and 500 mA, with

reconstruction using 0.126 $mm^3$ voxels. During imaging, animals were anesthetized via inhalation anesthesia using an isoflurane non–rebreathing anesthetic system at 3.0% per minute induction rate, and maintained at the 2.5-3.0% level for the duration of the scan. Body temperature during anesthesia induction, imaging procedure, and recovery period was supported using heating pads and a heat lamp.

**FIGURE 2.1.** Mechanical properties of common experimental foods (+), USOs and fruits in the diets of *Pan* and *Pongo* (Δ), and data (◊) and ranges (O) for African USOs and other potential primate foods (Lucas, 2004; Williams et al., 2005; Ravosa et al., 2007b; Dominy et al., 2008). 1 MPa (megapascal) = .001 GPa (gigapascal). The mechanical properties of the rat pellets used in this study fall within the range of toughness and elastic modulus values for USOs, a group of known primate fallback foods.

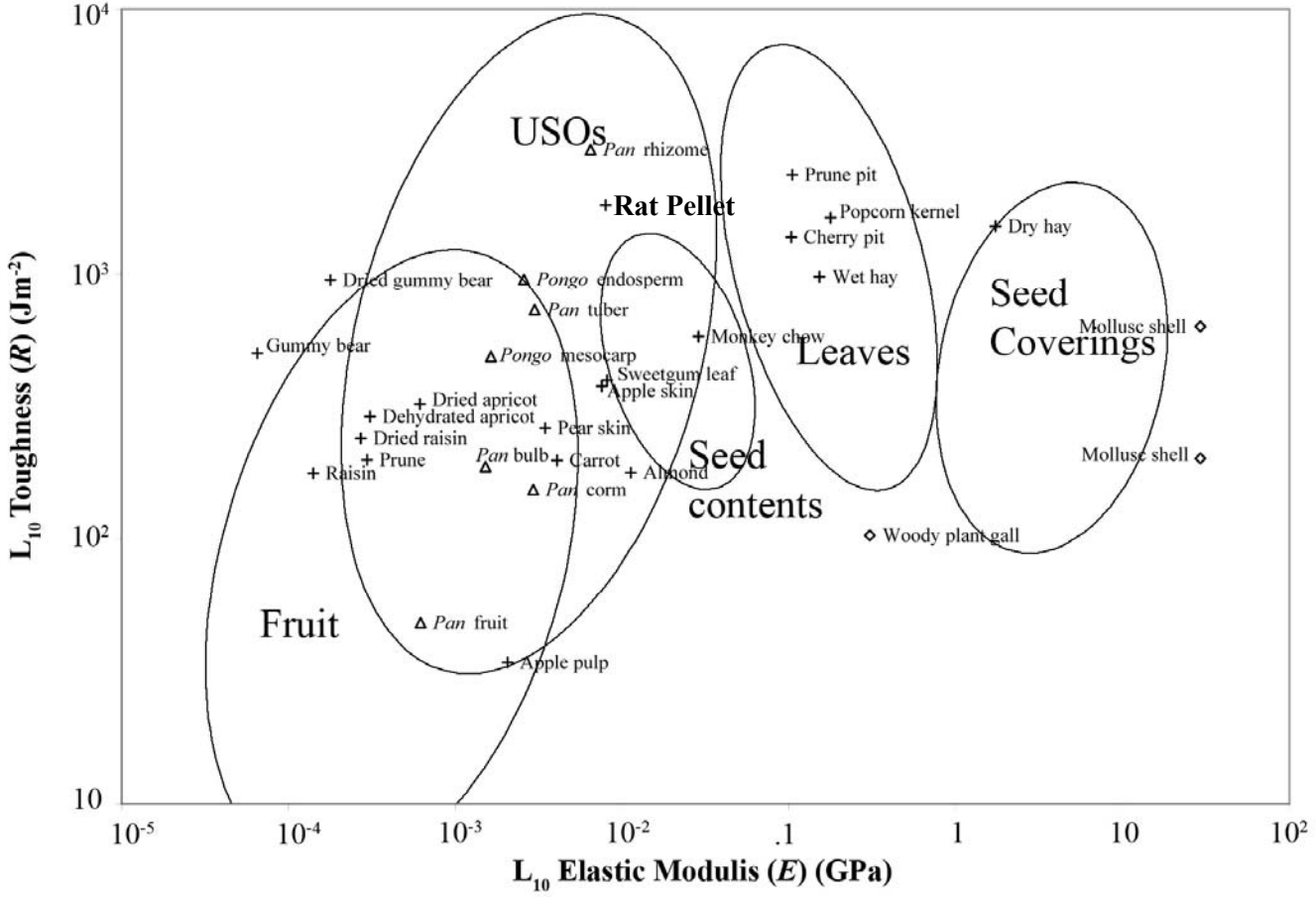


**FIGURE 2.2.** Average body mass for dietary cohorts across the experimental period.

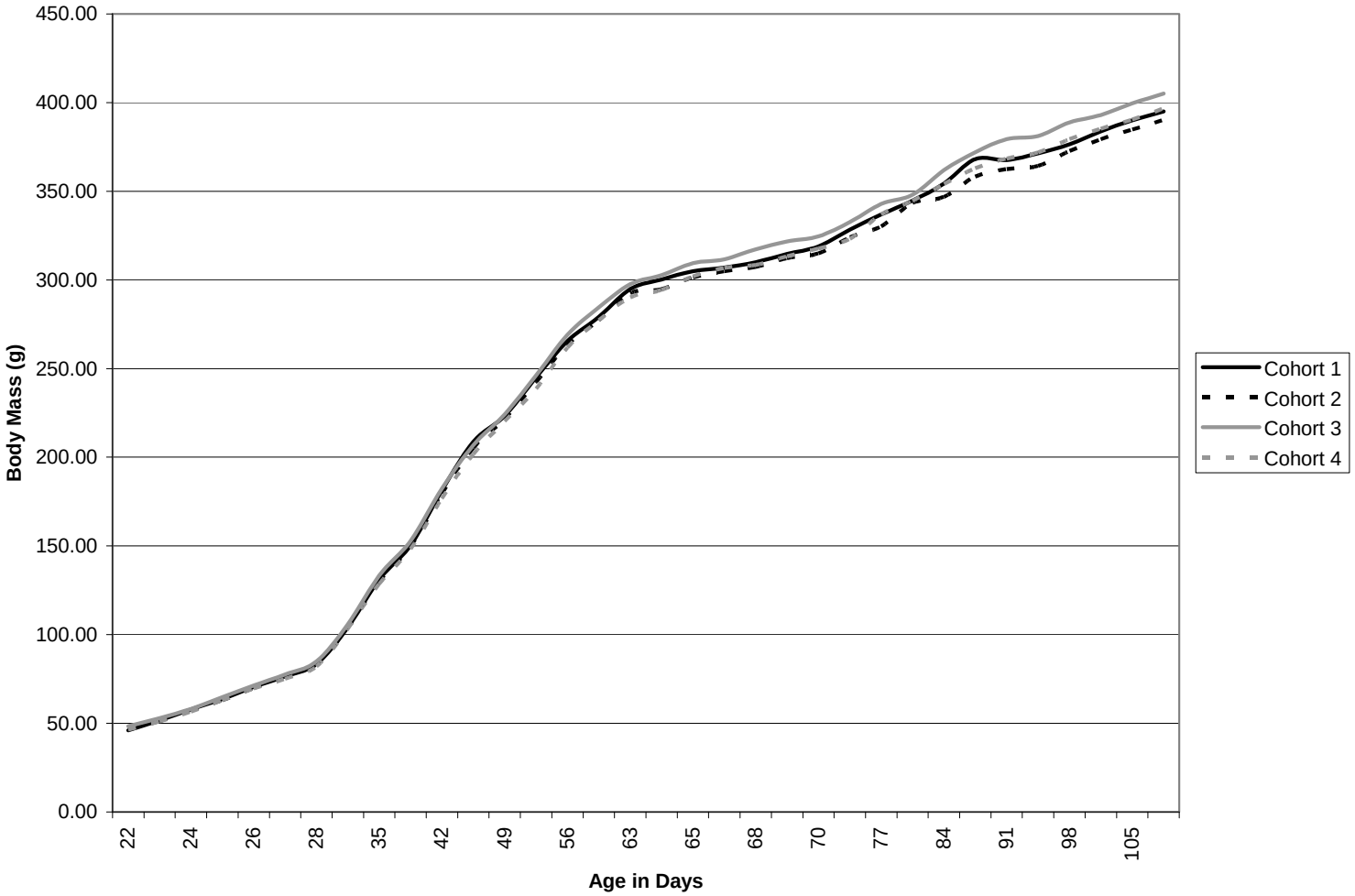


**FIGURE 2.3**. Lateral 3D reconstructions of longitudinal μCT scans of a rat between 4 and 16 weeks. This individual was chosen at random from the study sample and is representative of the data collected. Image provided by Ms. Ashley Szczodroski of the Harry S. Truman VA Biomolecular Imaging Center.

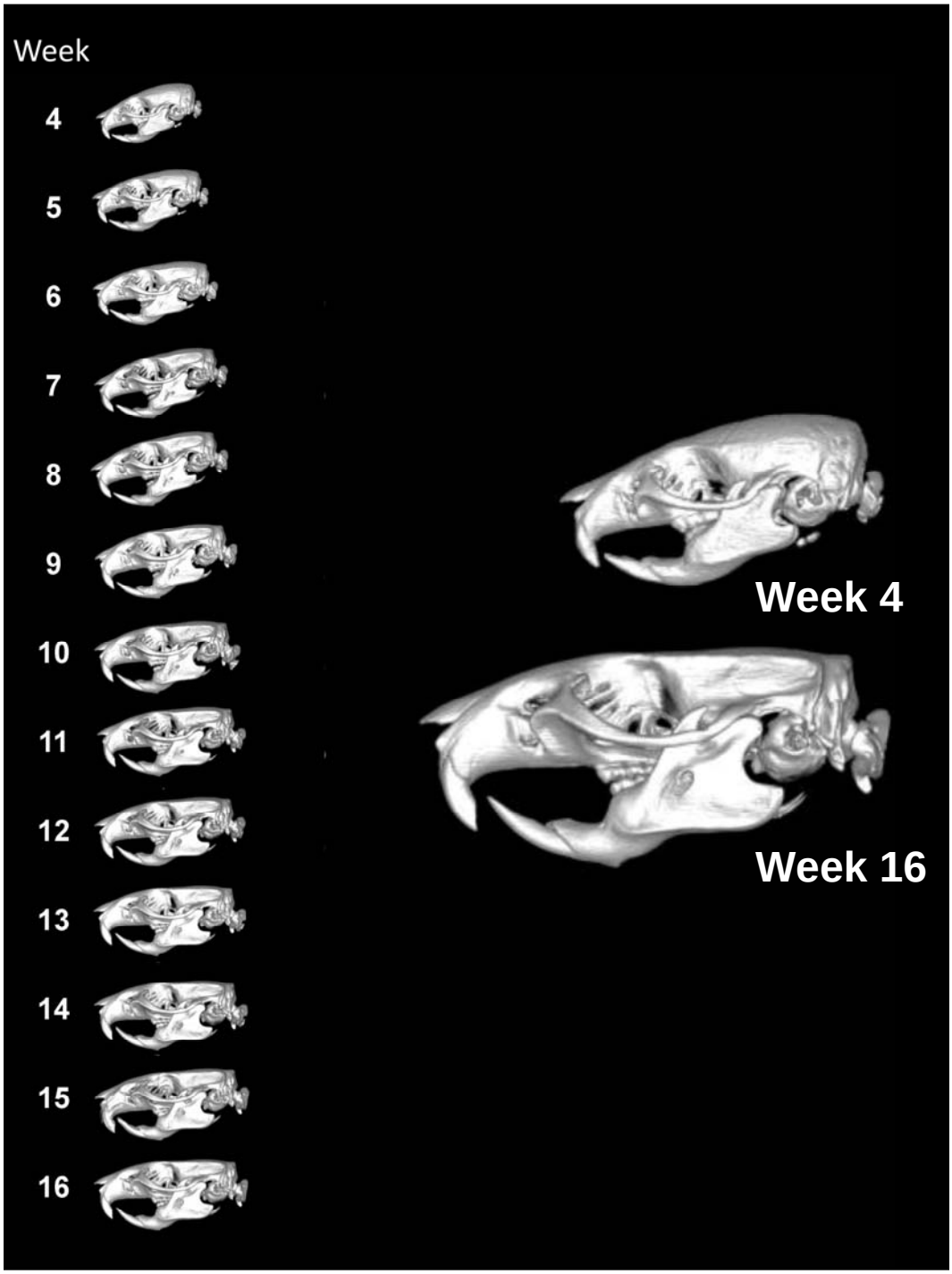


**TABLE 2.1.** Linear post-cranial measurements used to monitor growth.

| Measure | Distance |
|---|---|
| Head-body | Most rostral point of snout to base of tail |
| Tail | Base to tip of tail |
| Upper arm | Glenohumeral joint to lateral epicondyle |
| Forearm | Olecranon to radiocarpal joint |
| Forepaw | Radiocarpal joint to end of longest phalanx |
| Thigh | Greater trochanter to tibiofemoral joint |
| Shank | Tibiofemoral joint to lateral malleolus |
| Hindpaw | Calcaneus to end of longest phalanx |

**TABLE 2.2.** Dietary treatment groups for the experimental period. Where methodological issues caused n/cohort to vary from numbers listed in this table, it is noted in the affected analyses. Diet key: m, meal; p, pellets.

| | **Diet** | |
|---|---|---|
| **Cohort** | **Weaning (day 22/week 4) to mid-juvenile (day 64/week 9)** | **Mid-juvenile (day 65/week 10) to skeletal maturity (day 110/week 16)** |
| **1 (P) (n=10)** | Pellets | Pellets |
| **2 (P/M) (n=10)** | Pellets | Meal |
| **3 (M) (n=11)** | Meal | Meal |
| **4 (M/P) (n=11** | Meal | Pellets |

**TABLE 2.3.** Dietary material properties of the experimental rat pellets measured with a portable food tester.

| **LabDiet 5001 Rat Pellets (n=10)** | **Young's Modulus (E in MPa)** | **Toughness (R in $Jm^{-2}$)** | **Hardness (H in MPa)** |
|---|---|---|---|
| **Mean** | 13.61 | 3,325.12 | 7.25 |
| **Range** | 7.49 - 21.50 | 1,446.00 - 5,002.00 | 5.26 – 9.91 |

# CHAPTER 3: BONE MACROSTRUCTURE

## GEOMETRIC MORPHOMETRICS

### Aims

Geometric morphometric analyses are used here to analyze variation in mandibular form related to dietary composition and variability across ontogenetic stages. The goal of these analyses is to evaluate whether skeletal morphology reflects the presence of dietary variability in a population, and if so, which morphological characters are most informative for such an ecomorphological analysis. In order to achieve these goals, two statistical approaches are used. One, canonical variates analysis of the Procrustes-transformed 3D landmark data, is used to assess whether variable diet cohorts can be distinguished from stable diet cohorts based on mandibular morphology. The second, principal components analysis of the Procrustes-transformed 3D landmark data, is used to describe variation in mandibular morphology within the experimental sample and to identify morphological features which best sort individuals into their correct dietary categories.

As these analyses evaluate the ability of skeletal-based morphological studies of the mandible to detect dietary seasonality, the experimental design is intended to model the

range of feeding regimes that could potentially be employed by wild primate species. The extent to which a species includes mechanically-resistant fallback foods in its diet will fall along a gradient of little/no inclusion (e.g. the powdered diet) to variable/seasonal inclusion (e.g. the variable diet cohorts) to year-round/consistent inclusion (e.g. the pellet diet). Paleoecological studies have suggested that this gradient of fallback food usage may have occurred in Plio-Pleistocene hominins, such that genera such as *Australopithecus* and early *Homo* may have rarely if ever consumed hard/tough fallback food items, while *Paranthropus robustus* relied on these food items seasonally and *P. boisei* did so year-round (Cerling et al., 2011; Lee-Thorp, 2011).

Furthermore, in an assessment of how dietary variability affects mandibular growth and form, consideration must be given to life history factors such as the timing of weaning and post-weaning dietary shifts. The environment in which an individual exists during the important growth stage between weaning and skeletal maturity may have profound impacts on growth trajectories, body size at maturation, musculoskeletal performance, reproductive success, and survival (Roff, 1992; Schluter, 1995; Schlichting and Pigliucci, 1998; Taborsky, 2006). Additionally, an organism's capacity for morphological plasticity may decrease with age as musculoskeletal growth rates slow (Hinton and McNamara, 1984; Meyer, 1987; Bertram and Swartz, 1991; Rubin et al., 1992; Pearson and Lieberman, 2004; Hoverman and Relyea, 2007; Ravosa et al., 2008a). Thus, when discussing the role of variation in dietary composition and mechanical properties in determining craniomandibular form, the ontogenetic stage during which these diets are consumed may be an important component. If an animal is engaging in the seasonal

consumption of mechanically resistant fallback foods, this behavior may affect adult morphology differently if it occurs earlier (e.g. immediately post-weaning) versus later (e.g. closer to maturity) in ontogeny. Through the inclusion of two variable diet cohorts, each weaned onto a different diet, this study seeks to elucidate how ontogenetic variation in diet and masticatory loading affect adult masticatory morphology. Three possible scenarios are explored: first, that adult morphology most strongly reflects the diet encountered post-weaning due to the high growth rates observed in young individuals (Hinton and McNamara, 1984; Rubin et al., 1992; Pearson and Lieberman, 2004; Hoverman and Relyea, 2007); second, that adult morphology is related to the most recent feeding regime, suggesting that the masticatory skeleton is functionally adapted to its most immediate biomechanical requirements (Lanyon and Rubin, 1985)**;** and third, that a morphological gradient exists among animals fed stable non-resistant diets, variable diets, and stable resistant diets. This final scenario would suggest that an organism's adult morphology is tied to the sum of biomechanical demands encountered throughout its life history, and is reflective of both current and historical feeding behaviors.

The second goal of these morphometric analyses is to identify potentially useful morphological characters for recognizing dietary variability in wild and fossil populations. A functional matrix view of morphology predicts that skeletal regions subject to elevated strain levels will display greater levels of interspecific variation and phenotypic plasticity than those regions that experience lower strain values (Wood and Lieberman, 2001; Daegling, 2004; Ravosa et al., 2008b). Applied to the craniomandibular complex, this would suggest that the greatest levels of diet-related

plasticity will be observed in those structures experiencing elevated strains during mastication (e.g. the mandible) (Bouvier and Hylander, 1996b; Ravosa et al., 2000a; Ravosa et al., 2000c ; Ravosa et al., 2007c). At a finer scale, diet-related morphological plasticity may vary within an individual skeletal element due to strain gradients and proximity to features such as joints and muscle insertion points (Bouvier and Hylander, 1996b). Prior experimental work has identified substantial levels of morphological plasticity related to masticatory loading in the posterior region of the mandible (e.g. the ramus and temporomandibular joint) (Barber et al., 1963; Bouvier and Hylander, 1984; McFadden and McFadden, 1986; Bouvier, 1987, 1988; Yamada and Kimmel, 1991; Nicholson et al., 2006; Ravosa et al., 2008b). This is particularly true of those features in the posterior mandible related to the attachment of masticatory muscles, such as the coronoid and angular processes (Moore, 1965; Whiteley et al., 1966; Kiliaridis et al., 1996). Comparative studies support the hypothesis that morphological variation of the mandibular ramus and temporomandibular joint is related to diet in primates (Taylor, 2002; Terhune, 2013). Relatively lower levels of plasticity are observed in the anterior mandible (e.g. the corpus), perhaps due to spatial restraints related to housing the dentition (Daegling, 1996). When observed, diet-related plasticity in the anterior mandible tends to manifest as variation in the cross-sectional anatomy (e.g. width, length, cortical bone distribution) of the corpus and symphysis (Watt and Williams, 1951; Beecher et al., 1983; Bouvier and Hylander, 1984; Ravosa et al., 2008a). These analyses will describe morphological variation within the experimental sample in order to elucidate those regions and characters of the mandible that are most informative with

reference to the variability and mechanical composition of an individual's diet during ontogeny.

**Hypothesis 1:** At week 10 (mid-experiment), cohorts with similar diets during the first half of the experimental period will be most similar morphologically. Cohort 1 (P) and cohort 2 (P/M) will group together, and cohort 3 (M) and cohort 4 (M/P) will group together based on morphological characters.

**Hypothesis 2**: At week 16 (end-experiment), a gradient in mandibular morphology will be observed due to post-dietary shift processes of functional adaptation and incomplete growth rate compensation. In this gradient, cohorts with similar diets during the second half of the experimental period will be morphologically similar (cohorts 1 (P) and 4 (M/P), and cohorts 2 (P/M) and 3 (M).

**Hypothesis 3:** The morphological features most useful for classifying individuals into their correct dietary category will be those directly associated with masticatory muscles (e.g. insertion sites) and joints (e.g. the temporomandibular joint and mandibular symphysis). These are predicted to experience relatively high levels of strain during mastication.

## Methods

In order to assess morphological differences among cohorts at different ontogenetic stages, 3D landmark data were collected from μCT scans for weeks 4, 10, and 16. These weeks represent the beginning, middle, and end of the experimental period, respectively. They also represent the three ontogenetic stages encompassed by the experimental period: prepubescence (weeks 4-7), adolescence (weeks 8-12), and subadulthood/young adulthood (weeks 13-16). 3D landmarks for the right hemimandible (Figure 3.1; Table 3.1) were collected for each ontogenetic point using the landmark placement plugin for eTDIPS (Mullick et al., 1999). A repeatability study (n=4, trials=4) was conducted to ensure precision in right-side mandibular landmark placement with resulting standard errors (0.05-0.57 mm) below 5% of mean skull length during week 10 (mean = 44.0 mm, 5% of mean = 2.2 mm). Visual inspection of landmark accuracy was also performed on individual wireframe models after Procrustes superimposition in Morphologika v2.5 (O'Higgins and Jones, 1998).

## Statistics

Size variation was assessed using ln-transformed centroid sizes for the mandibular data set from each ontogenetic point obtained after Procrustes translation using the Morphologika v2.5 software package (O'Higgins and Jones, 1998). Kruskal-Wallis tests ($\alpha$=0.05) were employed to statistically compare ln (centroid sizes) among cohorts for each longitudinal point. When a statistically significant difference was detected among cohorts within a given longitudinal point, individual pairwise comparisons were made

using the Mann-Whitney *U* test with Bonferroni-adjusted *p*-values (α=0.0083, 6 inter-cohort comparisons).

Two levels of shape analysis were conducted on each 3D landmark data set. First, a canonical variates analysis (CVA) of the Procrustes-transformed 3D landmark coordinates was used to identify the shape differences that best distinguished the dietary cohorts. CVA combines multiple shape variables to produce a small number of canonical variates (CVs) that maximize the differences among cohorts (Albrecht, 1980). The CVs generated by this test are those with the greatest ratios of among-group to within-group variance. CVAs were performed on each data set using the MorphoJ software package (Klingenberg, 2011). 3D landmarks were subjected to Procrustes superimpositions, and a covariance matrix was generated from the resultant coordinate data set. CVAs were then performed on the covariance matrix using the dietary cohort as the classification variable. Wireframe models are used to illustrate mean and target shapes. Here, the mean shape is a reference shape representing a Procrustes distance value of zero (CV score of 0.0), and the target shape is derived from the mean shape plus the shape change corresponding to an increase of 10.0 units of Procrustes distance along the CV axis (CV score of +10.0). Morphological descriptions are given as the differences between the target and mean shapes, using lollipop graphs as a guide for regions where substantial differences occur. Overall differences in shape configurations among cohorts were assessed using Procrustes distances (Klingenberg et al., 2003a). Pairwise comparisons of Procrustes distances were made using a permutation method (10,000 permutations) to test the null

hypothesis of no difference among the cohort means (Bonferroni-adjusted $\alpha$=0.0083, 6 inter-cohort comparisons).

Secondly, principal components analyses (PCA) of the Procrustes-transformed 3D landmark coordinates were used to characterize the contribution of main shape components to the observed variation within the experimental sample. PC scores were then subjected to a stepwise discriminant function analysis (DFA) to determine which PCs best classified individuals into their correct dietary cohort. PCA differs from CVA in that the aim of the former is not to separate treatment groups but to describe the main components of shape variation. Thus the two analyses are used to complement one other, with CVA used to define overall shape differences among the cohorts and PCA used for a finer analysis of shape variation. Finally, a DFA of PC scores is used to assess the ability of these individual components of shape variation to classify individuals to their correct dietary cohort.

PCA was performed on the mandibular data set from each ontogenetic point using Morphologika. 3D landmarks were first subjected to Procrustes superimpositions, then a PCA was conducted on the transformed data set. Visualization of the shape changes represented by each PC was performed using both Morphologika and MorphoJ, with the latter program used to generate figures. Wireframe models are used to illustrate mean shapes (PC score of 0.0) and target shapes (a mean shape plus the shape change corresponding to an increase of 0.10 units of Procrustes distance along the PC axis, or a PC score of +0.10). Morphological descriptions are given as the differences between the

target and mean shapes, using lollipop graphs as a guide for regions where substantial differences occur. A stepwise DFA was performed in NCSS statistical software (Hintze, 2007) using the PCs that accounted for the first ~75% of variance in that data set. PCs were classified as "in" (included) or "out" (excluded) by the DFA, with the included PCs contributing to the model which maximized correct classification of individuals to their dietary cohorts. Only included PCs are presented in wireframe figures and PC score scatter plots. Allometric scaling of PC scores was assessed using a least squares regression for each PC against the ln-transformed centroid size.

## Results

**Week 4 mandible.** A Kruskal-Wallis test identified a significant difference ($p$=0.015) among cohorts in mandibular centroid size during week 4 (Table 3.2). Pairwise analyses show that during week 4, cohort 1 (P) demonstrates a smaller ln(centroid size) for the mandible than cohort 4 (M/P) ($p$=0.003) (Table 3.3).

Pairwise comparisons of Procrustes distances revealed significant differences in average mandibular shape between cohort 1 (P) versus cohorts 2 (P/M) ($p$=0.006) and 4 (M/P) ($p$=0.001) during week 4 (Table 3.4). These shape differences are borne out by the CVA, which maximized the distance among cohorts along three CV axes (Table 3.5; Figures 3.2-3.3). Cohort 1 (P) separates from cohorts 2 (P/M) and 4 (M/P) along CV1 (52.33% of variance), which describes change in diastema length, incisal alveolus height, coronoid process orientation, and preangular notch curvature. Cohort 1 (P) also separates from all other cohorts along CV2 (31.84%), which describes changes in TMJ length, angular

process length, coronoid process height, and mandibular corpus height. CV3 (15.84%) describes changes in the coronoid process orientation, TMJ height, and incisal ramus length. Cohorts 1 (P) and 4 (M/P) separate from cohorts 2 (P/M) and 3 (M) along CV3, although the separation is weaker than along previous CVs and some overlap occurs among cohorts.

A PCA of week 4 mandibular shape described the first 77.8% of morphological variation in the experimental sample along 8 PC axes (Tables 3.6-3.7; Figures 3.4-3.5). Of these, 5 PCs were included by a stepwise DFA in a model that resulted in an average of 61% of individuals being classified in their correct dietary cohort (Table 3.8). These 5 PCs describe TMJ height (PC2), diastema length (PC3), incisal alveolar height (PC4), mandibular corpus height (PC7), and preangular notch curvature (PC8) (Tables 3.6-3.7) (Figure 3.5). Pairwise Mann-Whitney *U*-tests revealed that cohorts 1 (P) and 2 (P/M) differ significantly (Bonferroni-adjusted $p$=0.001) in PC2 scores (Figure 3.4), with cohort 1 tending towards negative PC2 scores (mean=-0.012; SD=0.007) and cohort 2 tending towards positive scores (mean=0.008; SD=0.008) (Figure 3.4. Kruskal-Wallis tests revealed no significant differences ($p$-values>0.05) in PC scores among cohorts for PCs 3, 4, 7, and 8.

Regression of individual PC scores against ln(centroid) revealed slopes significantly different from zero for PC1 ($p$=0.021) and PC2 ($p$=0.001) (Table 3.6), indicating that angular process size decreases (PC1) and TMJ height increases (PC2) as mandibular size increases.

**Week 10 mandible.** No significant differences in mandibular centroid size were observed among cohorts during week 10 (Table 3.2).

Pairwise comparisons of Procrustes differences reveal no significant differences among cohorts in average mandibular shape (Table 3.9) during week 10. A CVA maximized the distance among cohorts along 3 CV axes (Table 3.10; Figures 3.6-3.7). CV1 separates all cohorts into discrete groups, with cohorts 1 (P) and 2 (P/M) falling in the negative range of the axis and cohorts 3 (M) and 4 (M/P) along the positive range. CV1 accounts for 88.23% of the variance observed in the sample and describes changes in mandibular corpus length and subcondylar notch curvature. CV2 (9.97%) describes changes in angular process length and TMJ length, and separates cohort 2 (P/M) from all other cohorts. Finally, CV3 (1.81%) describes changes in coronoid process orientation and incisal alveolus height. Minimal separation of cohorts occurs along the CV3 axis, with the CV score means for cohorts 1 (P) (mean=0.75; SD=0.55) and 2 (P/M) (mean=0.36; SD=1.10) falling close to zero, while cohort 3 (M) tends to have positive CV3 scores (mean=1.56; SD=1.12) and cohort 4 (M/P) tends to have negative CV3 scores (mean=-2.01; SD=1.03).

A PCA of week 10 mandibular shape describes the first 75.56% of morphological variance in the experimental sample along 7 PC axes (Tables 3.11-3.12; Figures 3.8-3.9). Of these, 2 PCs were included by a stepwise DFA in a model that resulted in an average of 54% of individuals being classified in their correct dietary cohort (Table 3.13). These 2

PCs describe mandibular corpus height (PC3) and the distance between the coronoid and angular processes (PC5) (Tables 3.11-3.12). Kruskal-Wallis tests identified significant differences among cohorts in both PC3 and PC5 ($p<0.05$). Pairwise comparisons revealed that cohorts 1 (P) and 2 (P/M) differ significantly (Bonferroni-adjusted $p=0.003$) in PC3 scores (Figure 3.8), with cohort 1 tending towards negative PC3 scores (mean=-0.015; SD=0.006) and cohort 2 tending towards positive scores (mean=0.007; SD=0.014) (Figure 3.8). Pairwise comparisons did not confirm any intercohort differences in PC5 scores (Bonferroni-adjusted $p$-values>0.008).

Regression of individual PC scores against ln(centroid) revealed no slopes significantly different from zero for PCs 1-7 (Table 3.11).

**Week 16 mandible.** A Kruskal-Wallis test identified a significant difference ($p=0.025$) among cohorts in mandibular centroid size during week 16 (Table 3.2). Pairwise analyses show that during week 16, cohort 2 (P/M) demonstrates a smaller ln(centroid size) for the mandible than cohort 4 (M/P) ($p=0.003$) (Table 3.3).

Pairwise comparisons of Procrustes differences reveal no significant differences among cohorts in average mandibular shape (Table 3.14) during week 16. A CVA maximized the distance among cohorts along 3 CVA axes (Figures 3.10-3.11). CV1 describes 57.32% of variance in the experimental sample and describes changes in the distance between the coronoid and angular processes (Tables 3.15). CV1 separates cohorts 1 (P) and 2 (P/M) from cohorts 3 (M) and 4 (M/P), with the first two falling in the positive

range and the latter two in the negative range of the CV1 axis. CV2 (37.46%) describes changes in mandibular notch depth, and separates cohorts 1 (P) and 4 (P/M) from cohorts 2 (P/M) and 3 (M). The first two fall in the negative range of the CV2 axis, while the latter two cohorts are predominantly in the positive range. CV3 (5.22%) describes changes in TMJ height and width, mandibular notch depth, coronoid process width, preangular notch curvature, and mandibular corpus length. Cohort separation along CV3 is less pronounced, with cohorts 1 (P) (mean=1.38; SD=1.06) and 3 (M) (mean=1.41; SD=0.87) tending to have positive CV3 scores and cohorts 2 (P/M) (mean=-1.00; SD=0.96) and 4 (M/P) (mean=-1.76; SD=1.10) tending to have negative CV3 scores.

A PCA of week 16 mandibular shape describes the first 74.84% of morphological variance in the experimental sample along 8 PC axes (Tables 3.16-3.17; Figures 3.12-3.13). Of these, 4 PCs were included by a stepwise DFA in a model that resulted in an average of 69% of individuals being classified in their correct dietary cohort (Table 3.18). These PCs describe mandibular ramus size (PC3), TMJ length (PC6), coronoid process size (PC7), and TMJ orientation (PC8) (Tables 3.16-3.17). Kruskal-Wallis tests identified significant differences among cohorts in PC3, PC6, and PC8 ($p<0.05$). A pairwise comparison of PC3 scores revealed that cohort 1 (P) differs significantly from both cohort 3 (M) (Bonferroni-adjusted $p=0.005$) and cohort 4 (M/P) (Bonferroni-adjusted $p=0.007$). Along the PC3 axis, cohort 1 (P) (mean=-0.008; SD=0.011) tends towards negative scores while cohorts 3 (M) (mean=0.006; SD=0.006) and 4 (M/P) (mean=0.006; SD=0.006) tend towards positive scores (Figure 3.12). For PC6, cohort 3 (M) differs significantly from cohort 4 (M/P) (Bonferroni-adjusted $p=0.008$), with the former cohort

tending towards the positive range of the axis (mean=0.003; SD=0.008) and the latter cohort towards the negative (mean=-0.004; SD=0.005) (Figure 3.12). For PC8, a significant difference was observed between cohort 1 (P) and cohort 3 (M) (Bonferroni-adjusted $p$=0.004). Here, cohort 1 (P) (mean=0.003; SD=0.005) tended to fall along the positive range of the PC8 axis and cohort 3 (M) (mean=-0.003; SD=0.008) along the negative range.

Regression of individual PC scores against ln(centroid) revealed slopes significantly different from zero for PC1 ($p$=0.004), PC2 ($p$=0.032), PC3 ($p$=0.038), and PC7 ($p$=0.005) (Table 3.16). This suggests that coronoid-angular distance (PC1), alveolar process length (PC2), mandibular ramus size (PC3) all increase as mandibular size increases, while coronoid process size (PC7) decreases as mandibular size increases.

## Conclusions

Results from the week 4 geometric morphometric analyses suggest that individuals were not randomly sorted into four dietary cohorts (see Appendix III, Random Sorting Analysis). Cohort 1 (P) is distinguished from the remaining cohorts by a small mandibular centroid size, anteroposteriorly short jaw and mandibular diastema (linear measurements and CV1), a tall TMJ process with an anteroposteriorly long condyle (linear measurements and PC2). This is likely due to the inclusion of a disproportionate number of littermates in cohort 1 (P). However, statistical differences in mandibular

centroid size and linear mandibular measurements are no longer observed during week 10, suggesting that post-weaning growth equalized mandibular size among the cohorts.

Hypothesis 1, that cohorts with similar diets during the first half of the experiment will be most morphologically similar, is partially supported by results from week 10. At this ontogenetic point, the canonical variates analyses separated those cohorts raised on pellets [1(P) and 2 (P/M)] from those raised on meal [3(M) and 4 (M/P)] along a variate that described anteroposterior mandibular corpus length and subcondylar notch angle (CV1). However, the pellet diet cohorts [1(P) and 2 (P/M)] were dissimilar along CV2 (angular process and TMJ condyle length) as well as PC3 (mandibular corpus height). It is possible that these results may be related to the non-random sorting of cohorts at the start of the experiment (Appendix III). However, the fact that it is cohort 2 (P/M) and not cohort 1 (M) that is isolated along CV2 suggests that other factors may also contribute to the morphological differences seen between these pellet-fed cohorts. The meal-fed cohorts [3(M) and 4 (M/P)] were found to be similar in all geometric morphometric analyses during week 10. That morphological differences are observed between the pellet-fed cohorts but not the meal-fed cohorts may be related to the idea that that elevated masticatory loading can contribute to increased morphological variation (Wood and Lieberman, 2001; Daegling, 2004; Ravosa et al., 2008b).

Hypothesis 2 postulated that adult mandibular morphology would be most similar between cohorts fed the same diet during the second half (post-shift) of the experiment, but that these cohort pairs would still show some morphological differences due to

incomplete growth rate compensation or incomplete bone remodeling in the variable diet cohorts. A canonical variates analysis provides only weak support for this hypothesis. CV1, which describes the distance between the coronoid and angular processes, instead groups cohorts by their pre-shift diet. However, CV2 (mandibular notch depth) does group cohorts by their post-shift diet, although it explains less of the sample variance than does CV1. A third distinct trend is exhibited along CV3, where the cohorts separate weakly along a stable versus variable diet dichotomy. Thus, only the observed distribution along CV2 supports the hypothesis that adult morphology reflects the most recent diet. Likewise, a principal components analysis of mandibular morphology during week 16 provides incomplete support for $H2_A$. A weak gradient is observed for PC3 (mandibular ramus size), such that cohort 1 (P) falls along the negative values of the axis while cohorts 3 (M) and 4 (M/P) fall along the positive values, with values for cohort 2 (P/M) distributed across both ranges. Other significant differences in PC scores are seen in TMJ length (PC6), which groups cohort 4 (M/P) with the similar end-diet cohort 1 (P) rather than cohort 3 (M). TMJ orientation (PC8) also separates the two stable diet cohorts [1(P) and 3(M)], while grouping cohorts sharing similar end-diets [cohort 1 (P) and 4 (M/P), and cohorts 2 (P/M) and 3 (M)]. Thus, while some mandibular morphologies (e.g. mandibular notch depth, TMJ condyle length and orientation) may reflect an individual's most recent diet, a stronger signal seems to originate from the diet onto which the individual was weaned. This may be related to the high growth rates associated with the early post-weaning growth period. Interestingly, at week 16 cohort 2 (P/M) has a significantly smaller mandibular centroid size than cohort 4 (M/P), suggesting that a mid-

ontogeny shift onto a less mechanically challenging diet results in a smaller mandible in these adults.

One of the principal goals of these morphometric analyses is to characterize the morphological variation in the mandible related to diet variability, and to identify those characters which best classify individuals into their correct dietary categories. Results from these analyses indicate that, except for those morphological characters observed in cohort 1 (P) likely due to non-random sorting (Appendix III), mandibular morphology is comparable among the cohorts at weaning (week 4). Weaning-age variation in mandibular morphology is distributed relatively evenly between posterior and anterior mandibular characters. At mid-experiment (week 10), variation in anterior mandibular characters (e.g. the incisal ramus and mandibular corpus) still accounts for the first 50% of morphological variation, while posterior mandibular characters account for the next 26%. Corpus size contributes significantly to morphological variation in the sample at this ontogenetic stage. At week 10, discriminant function analyses identified both anterior (e.g. corpus size) and posterior (e.g. coronoid-angular distance) mandibular characters that reduced the classification error incurred in attempts to assign individuals to their correct dietary cohort. Finally, by the end of the experiment (week 16), variation in the posterior mandible accounted for a full 52% of the first 75% of morphological variation in the sample. Indeed, all of the morphological characters identified in the week 16 analysis for their ability to reduce classification error were posterior mandible characters. Thus, the morphology observed during the young adult stage in this study supports hypothesis $3_A$, that the features most useful for classifying individuals into their correct

dietary category are those in the posterior mandible. In this population, those features include mandibular ramus size, TMJ condyle length, coronoid process size, and TMJ orientation.

In sum, results from these geometric morphometric analyses reveal that the presence of dietary variability increases the difficulty of correctly classifying plastic morphotypes within a single species. This may translate into difficulty in recognizing the presence of dietary variability in the fossil record, particularly in closely related taxa. Noticeably, these analyses did not document any emergent trend for individuals raised on temporally variable diets to fully resemble individuals raised on either their post-weaning or post-shift diet. Rather, the plasticity documented in this study appears to be character-specific. The relative importance of individual morphological features for dietary inferences may be related to numerous factors, such as location-specific growth rates and masticatory strain gradients.

To overcome the difficulties in morphological-based behavioral inferences created by dietary variability, this study identified mandibular characters that were the most useful for assigning individuals to their dietary cohort. In prepubescent and adolescent individuals, significant levels of morphological variation were observed throughout the mandible. However, in young adults, morphological variation was largely confined to the posterior mandible. Indeed, among the young adults, all characters identified for their ability to reduce classification error were located in the posterior mandible. These results are consistent with previous work which has suggested that the posterior mandible,

particularly those features related to muscle insertion sites and joint structures, is more plastic with respect to variation in feeding behavior as compared to the anterior mandible, which may be influenced by early growth processes and spatial factors (McFadden and McFadden, 1986; Daegling, 1996; Taylor, 2002; Terhune, 2013). Laboratory rats are monophyodont, with molar eruption occurring along the following schedule: first molar, day 16 (pre-weaning); second molar, day 21 (peri-weaning); third molar, week 6 (post-weaning). The molars and their roots continue to develop through 18 weeks of age (Schour and Massler, 1949). It is possible that this process of dental growth contributes to the observed variation in the anterior mandible in immature individuals. The functional differences observed herein along an anterior-posterior gradient in the mandible are also consistent with identified developmental and genetic modules in the mandible (Atchley et al., 1985; Leamy, 1993; Cheverud et al., 1997; Mezey et al., 2000; Klingenberg et al., 2003b; Fish et al., 2011), which separate the "hinge" region (mandibular ramus) from the "cap" region (mandibular corpus) (Fish et al., 2011). Thus, the regional functional variation noted in this study may be associated with genetic and developmental variation between the temporomandibular joint and masticatory muscle insertion sites in the posterior mandible, and the tooth-bearing structures in the anterior mandible.

Finally, this research highlights the importance of ontogenetic studies for understanding species behavior and dietary history. The relative contribution of intrinsic versus extrinsic factors to an individual's phenotype is dependent on many factors, including but not limited to the following: pre-weaning influences (e.g. early development and maternal effects); ontogenetic stage; and behavioral history (e.g. historical and current dietary

regimes). Although behavioral variation during early ontogeny may not be the predominant factor influencing morphology in young individuals, it may play a significant role in determining adult morphology by means of influencing growth trajectories and plasticity responses (Schlichting and Pigliucci, 1998; Taborsky, 2006).

**Future Directions.** These geometric morphometric analyses focused on mandibular shape and size across the ontogeny of this experimental population. While the mandible is often the focus of morphological-based analyses of dietary behavior, feeding is also known to influence the broader cranial complex (Watt and Williams, 1951; Kiliaridis et al., 1985; Kiliaridis et al., 1996; He and Kiliaridis, 2003; Menegaz et al., 2009; Menegaz et al., 2010). Future analyses will examine ontogenetic variation in facial and neurocranial form in relation to dietary variability. Similar methods will be employed, with the number of collected 3D landmarks significantly increased to account for the challenge of assessing cranial morphology in three-dimensional space.

# CROSS-SECTIONAL MANDIBULAR MORPHOLOGY

## Aims

In order to evaluate the effect of dietary variability on the internal structure of the mandible, longitudinal μ-CT images were used to collect linear data on cross-sectional mandibular morphology, including cortical thickness. Experimental work has demonstrated that the cross-sectional morphology of the mandible is influenced by ontogenetic variation in masticatory loading (Bouvier and Hylander, 1981, 1984; Ravosa

et al., 2008b; Ravosa et al., 2008a) This is further supported by comparative work observing an increase in cross-sectional cortical bone area and/or regional cortical bone thickness in species observed consuming hard/tough dietary objects, such as robust cebids (Daegling, 1992; Wright, 2005). The mandibles of *Paranthropus robustus* are also described as having relatively more cortical bone in cross-section as compared to *Australopithecus africanus*, although regional thickness values do not differ significantly (Daegling and Grine, 1991). Indeed, skeletal strain gradients may affect internal morphology in ways not readily appreciated by external measures alone (Koyabu and Endo, 2009).

The variation in regional thickness of cortical bone observed in primates has been attributed to both masticatory strain patterns (Demes et al., 1984) and the gross structure of the primate mandible (Daegling and Grine, 1991). Daegling and colleagues have observed no interspecific differences in regional cortical thickness in both hominoids (Daegling and Grine, 1991) and cercopithecines (Daegling, 2001). However, within hominoids, all species exhibit thicker cortical bone on the buccal aspect and thinner cortical bone on the lingual aspect (Demes et al., 1984; Daegling and Grine, 1991). Demes et al. (1984) attributed this pattern of cortical distribution to combined torsion and shearing stresses experienced in the primate mandible during mastication, under which the buccal aspect of the mandible experiences greater strains than the lingual aspect. Daegling and Grine (1991) more directly attributed the thicker cortical bone on the buccal aspect to the structural junction of the mandibular corpus and ramus on that side of the bone.

This component of the project investigates the association between cross-sectional morphology of the mandible and ontogenetic variation in masticatory loading related to dietary properties. Due to the fact that the chewing cycle in rodents is predominantly propalinal[1] (Hiiemäe and Ardran, 1968; Weijs and Dantuma, 1975), compared to the transverse movements observed during primate mastication (Luschei and Goodwin, 1974; Hylander and Crompton, 1986; Hylander et al., 1987), it is not assumed that the masticatory stress scenario described above underlies patterns of regional cortical thickness variation in the experimental rodent sample. However, as much attention has been paid to regional cortical thickness patterns in the primate literature, this project also tests the hypothesis (H3) that such a pattern may exist in the Sprague-Dawley rat.

**Hypothesis 1:** Cross-sectional mandibular dimensions (e.g., width and height) will be greater in adult rats that have experienced greater masticatory loads during their ontogeny.

**Hypothesis 2**: Regional cortical bone thickness in the mandibular corpus will be greater in adult rats which have experienced greater masticatory loads during their ontogeny.

**Hypothesis 3**: Regional cortical bone thickness will be greater on the buccal aspect than the lingual aspect across all cohorts.

[1] Propalinal refers to forward and backward motion of the mandible relative to the cranium.

## Methods

Cross-sectional measures of the mandible (Figure 3.14) were collected from the longitudinal series of μCT scans using ImageJ (Rasband, 2011). Maximum superoinferior height and buccolingual width of the right mandibular corpus were measured at the level of the incisor/mandibular symphysis (Figure 3.14A) and the second molar (Figure 3.14B). Cortical bone thickness was measured at the level of the second molar for the buccal, lingual, and inferior aspects of the mandibular corpus (Figure 3.14C). For each individual, multiple measures of cortical thickness were taken along each aspect of the corpus (4 measures for buccal and lingual aspects, 2 measures for inferior aspect) and then averaged to produce a measure of mean cortical thickness per corporal aspect per individual.

## Statistics

Cross-sectional measures (height, width, and individual averages of regional cortical thickness) were statistically compared among cohorts via Kruskal-Wallis tests ($\alpha$=0.05). When a statistically significant difference was detected among cohorts for a given cross-sectional measure, individual pairwise comparisons were made using the Mann-Whitney *U* test with Bonferroni-adjusted *p*-values ($\alpha$=0.0083, 6 inter-cohort comparisons).

Regional cortical thickness measures (buccal, inferior, and lingual) were compared statistically within each cohort via Kruskal-Wallis tests ($\alpha$=0.05). As above, Mann-

Whitney *U* tests with Bonferroni-adjusted *p*-values (α=0.0166, 3 intra-cohort comparisons) were used to make further pairwise comparisons.

## Results

During week 4, the beginning of the experiment, a Kruskal-Wallis test suggested differences in the corpus height at the level of the second molar (Table 3.19). However, pairwise comparisons (Table 3.20) showed no significant differences among cohorts in this measure.

No significant differences in cross-sectional mandibular metrics were found during week 10 (Table 3.21).

During week 16, Kruskal-Wallis tests suggested significant differences in both buccal and lingual cortical thickness (Table 3.22). Pairwise comparisons identified significant differences only in lingual cortical thickness (Table 3.23). By the end of the experiment, cohort 3 (M) showed thicker cortical bone along the lingual margin of the mandibular corpus compared to cohorts 2 (P/M) and 4 (M/P) (Figure 3.15 – Week 16 Lingual).

Across all cohorts, regional cortical thickness was greater along the buccal and inferior aspects of the mandible as compared to the lingual aspect during week 4 (Table 3.24) and week 10 (Table 3.25). These significant differences were also found during week 16, except in cohort 3 (M) (Table 3.17). In cohort 3 (M) during week 16, cortical bone along

the buccal aspect of the mandible was found to be thicker than the cortical bone on both the inferior and the lingual aspects (Table 3.26).

## Conclusions

Results from this analysis showed no significant differences in cross-sectional mandibular dimensions (e.g. width and height) among dietary cohorts at any ontogenetic stage ($H1_0$). Significant differences in regional cortical bone thickness were noted in young adult rats during week 16. Cohort 3 (M) was observed to have thicker cortical bone along the lingual aspect of the mandibular corpus as compared to cohorts 2 (P/M) and 4 (M/P) ($H2_A$). Cohort 3 (M) was raised on a stable diet of powdered pellets and was the only cohort to never consume the fracture-resistant whole pellet diet. Thus, this would suggest that the thinner lingual cortex present in the other cohorts is associated with elevated levels of masticatory loading in this sample.

In this sample of laboratory rats, as in primates, cortical bone on the buccal aspect of the mandibular corpus was observed to be thicker than the lingual cortex at all ontogenetic stages ($H3_A$). Cortical bone along the inferior aspect was also noted to be thicker than the lingual cortex at all time points. The exception to this observation occurred in cohort 3 (M) during week 16, when the cortex bone along the buccal aspect was thicker than both the inferior and lingual cortices. Thus, the pattern of regional cortex variation in the mandibular corpus in this experimental sample of rats is consistent with the pattern observed in primates. It is unknown whether other mammalian taxa share this pattern of regional cortical thickness in the mandibular corpus. Future *in vivo* studies of regional

bone strain in the mandibular corpus would help to clarify whether this regional pattern of cortical thickness is related to the distribution of masticatory stresses (Demes et al., 1984) or the structural junction of the alveolar and ramus regions of the mandible (Daegling and Grine, 1991).

Unlike previous experimental work utilizing small-bodied mammalian models (Bouvier and Hylander, 1984; Ravosa et al., 2008b; Ravosa et al., 2008a), this research did not find any significant inter-cohort differences in the cross-sectional dimensions of the rat mandible. However, results from these previous studies suggest that articular regions of the mandible (e.g. the temporomandibular joint and mandibular symphysis) may be more plastic than the mandibular corpus in response to variation in masticatory loading. Further work is needed to elucidate the effects of dietary variability on joint cross-sectional morphology and cortical thickness in this sample.

The experimental rodents used in this sample were found to have a primate-like pattern of regional cortex variation (Demes et al., 1984; Daegling and Grine, 1991). It is unknown how common this pattern of regional cortex thickness is among all mammalian taxa. However, the presence of buccal cortical thickening in primates consuming a mechanically resistant diet (Daegling, 1992; Wright, 2005) was not observed in this study. Rather than a thicker buccal cortex thickness, rats raised on a mechanically resistant diet demonstrated a thinner lingual cortex. However, the end result would appear to be the same: a large buccal-to-lingual cortex ratio in animals experiencing elevated masticatory loads. Finally, although the examination of cortical bone distribution can

provide some insight into masticatory plasticity, this type of analysis is not an efficient stand-alone approach to functional adaptation in the mandible (Daegling, 2002). Instead, it is best supplemented with studies of trabecular distribution and density, comparisons of relative corpus size, and, perhaps most crucially, analyses of relative mandibular size and shape (Daegling, 2002).

**Figure 3.1.** Mandibular landmarks used in the analyses. See Table 3.1 for key.

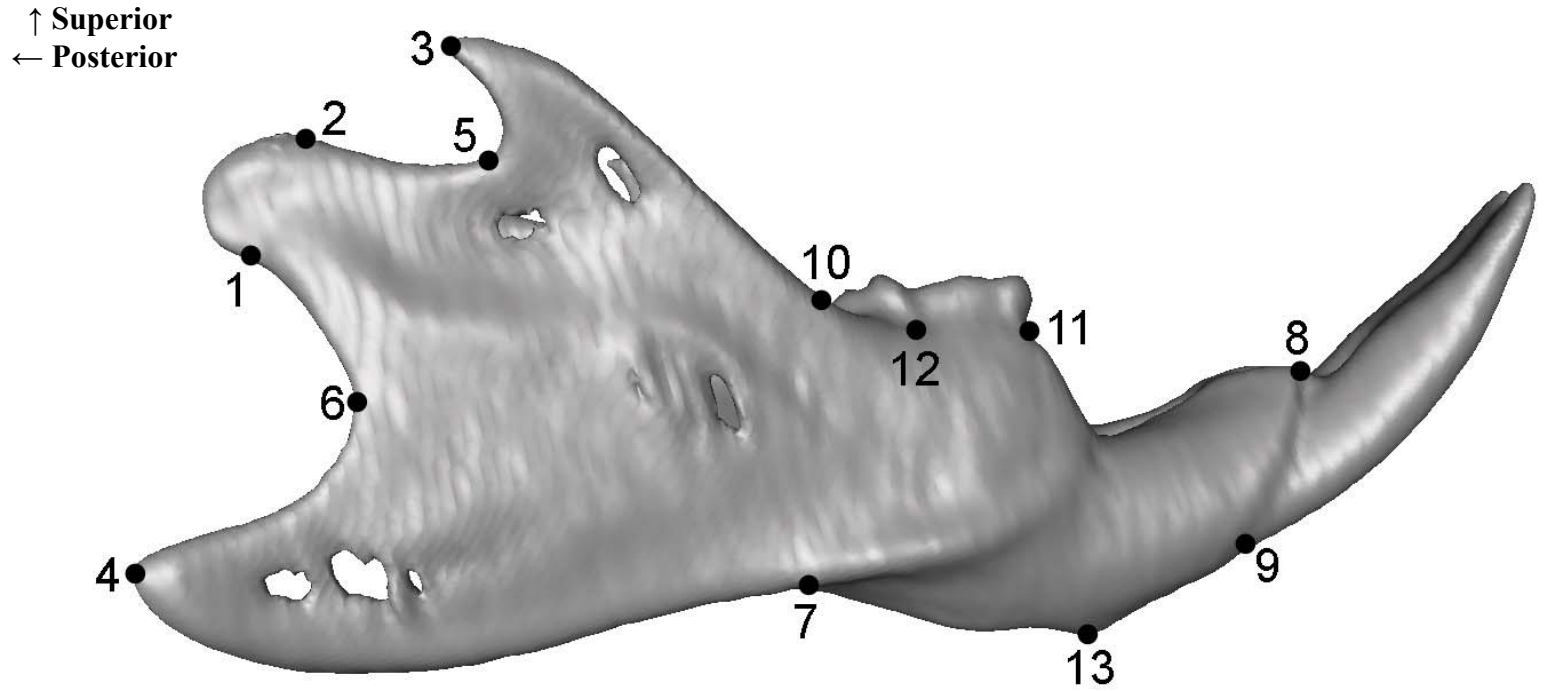


**Figure 3.2.** Canonical variates scores for the week 4 mandibular analysis.

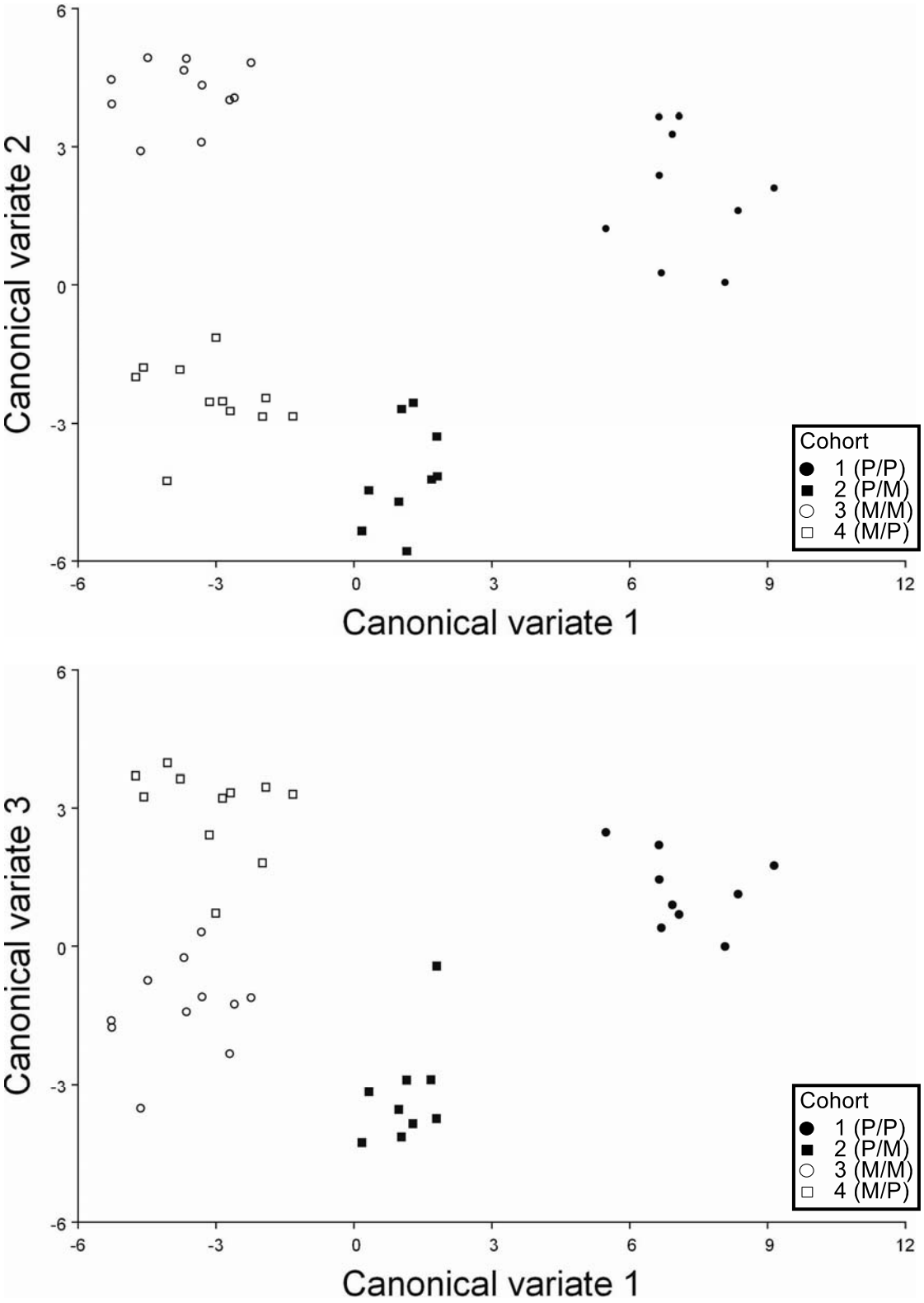
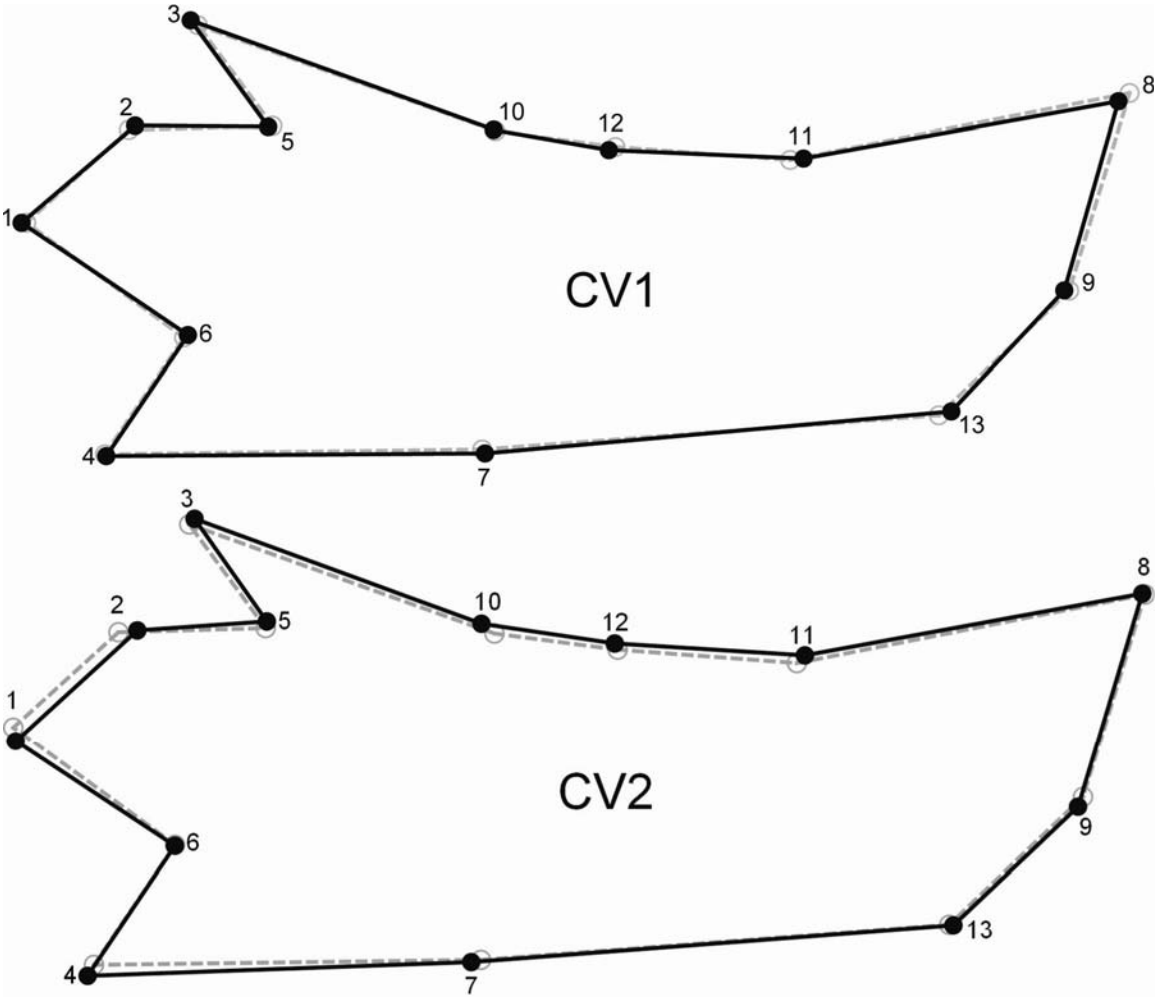


**Figure 3.3.** Wire-frame representations of the canonical variates from the week 4 mandibular analysis described in table 3.5. Black outline is the target shape (CV score of +10.0), grey outline is the mean shape (CV score of 0.0).



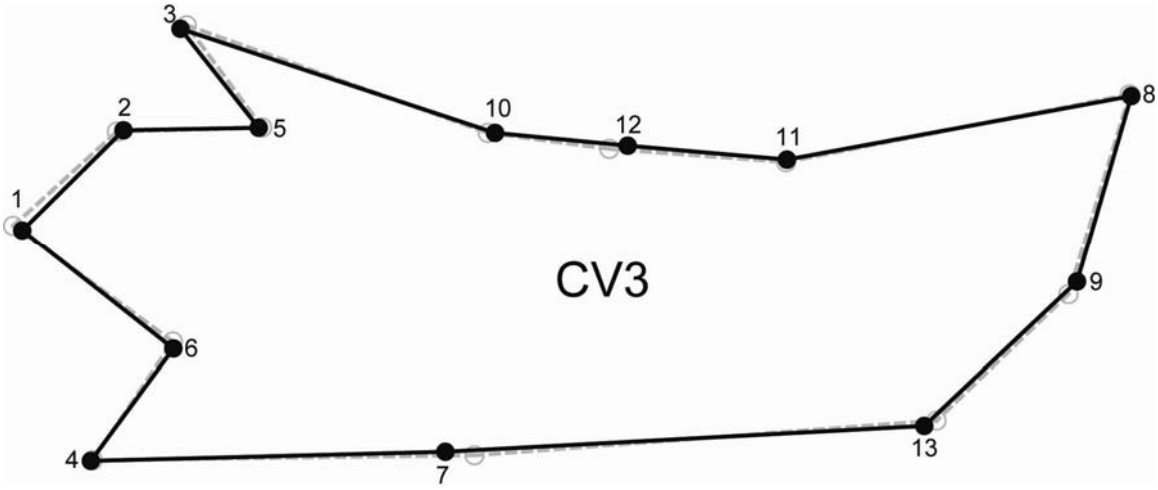
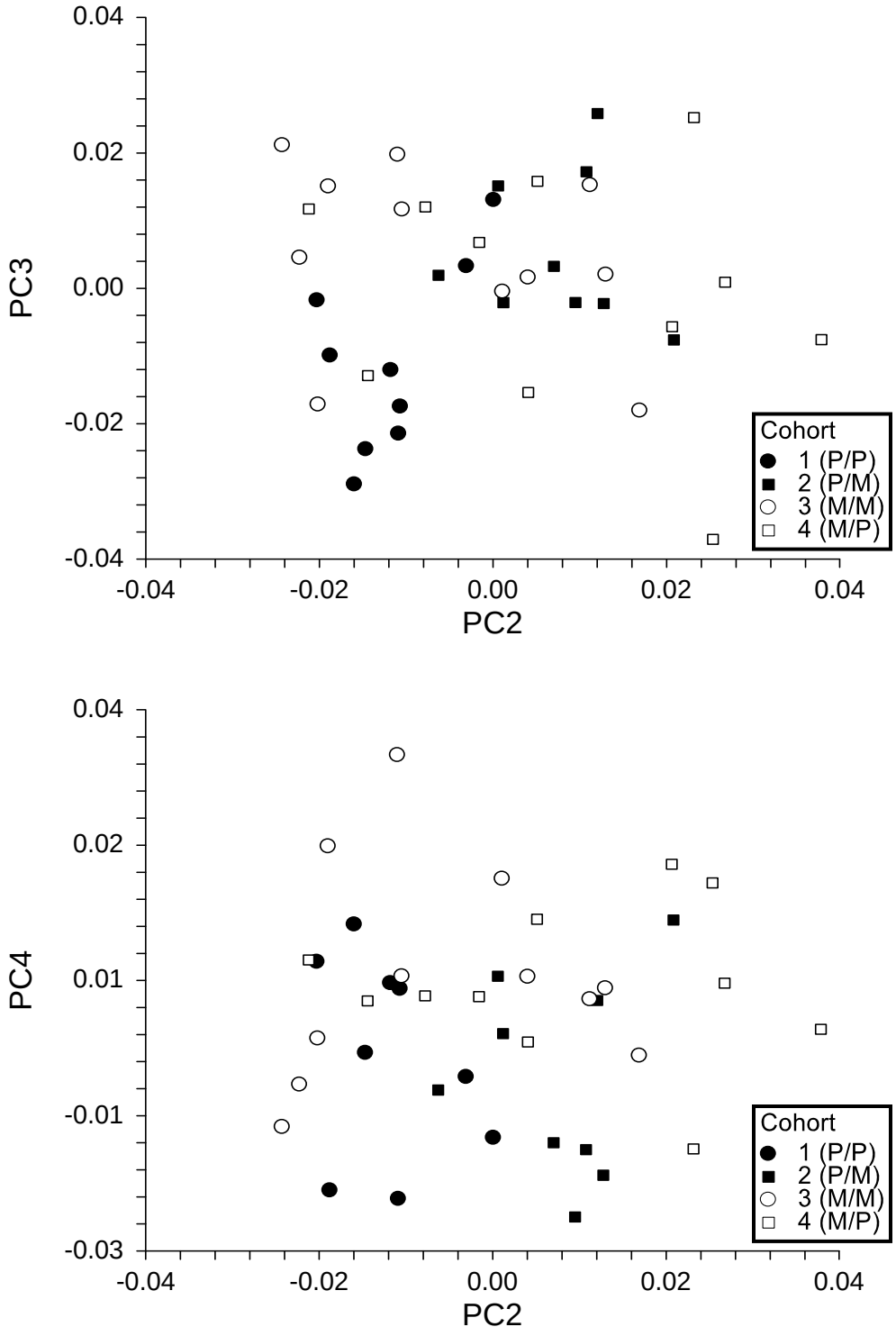

3
2
5
10
12
11
8
1
CV3
9
6
4
7
13

**Figure 3.4.** Principal components scores for included PCs for week 4 mandibular analysis.



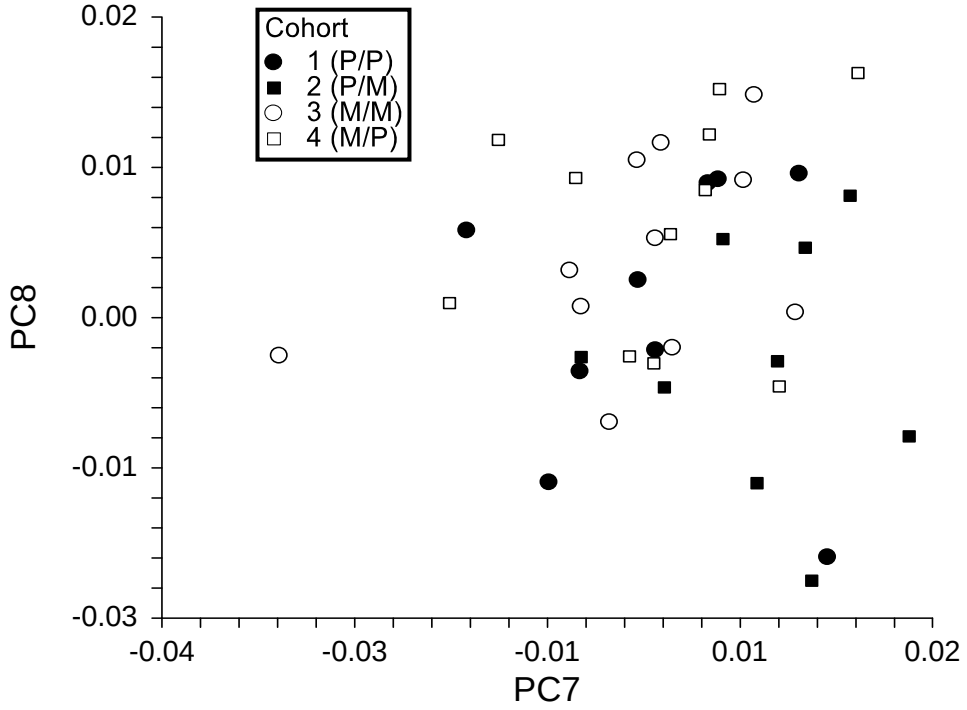
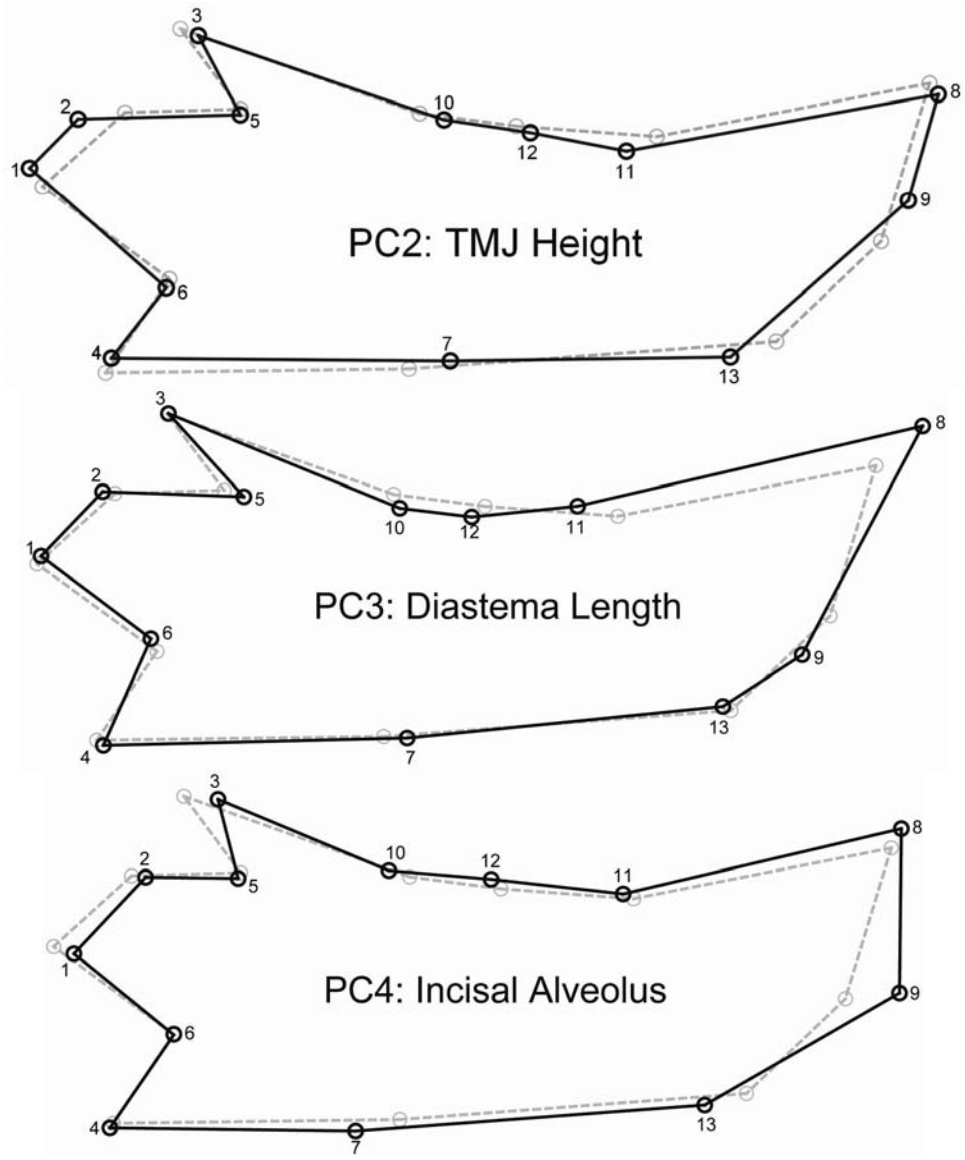
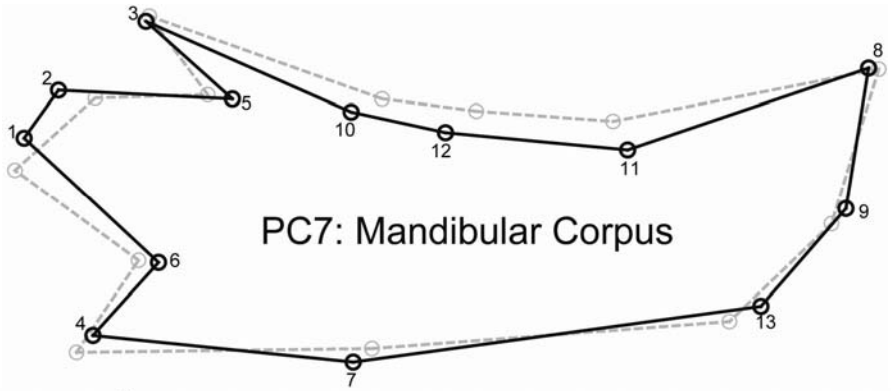

Cohort
1 (P/P)
2 (P/M)
3 (M/M)
4 (M/P)
0.02
0.01
0.00
-0.01
-0.03
PC8
-0.04
-0.03
-0.01
0.01
0.02
PC7

**Figure 3.5**. Included principal components identified by step-wise discriminant function analysis on the mandibular landmark set for week 4. See Table 3.7 for descriptions. Black outline is the target shape (PC score of +0.1), grey outline is the mean shape (PC score of 0.0).




PC7: Mandibular Corpus
1
2
3
4
5
6
7
8
9
10
11
12
13

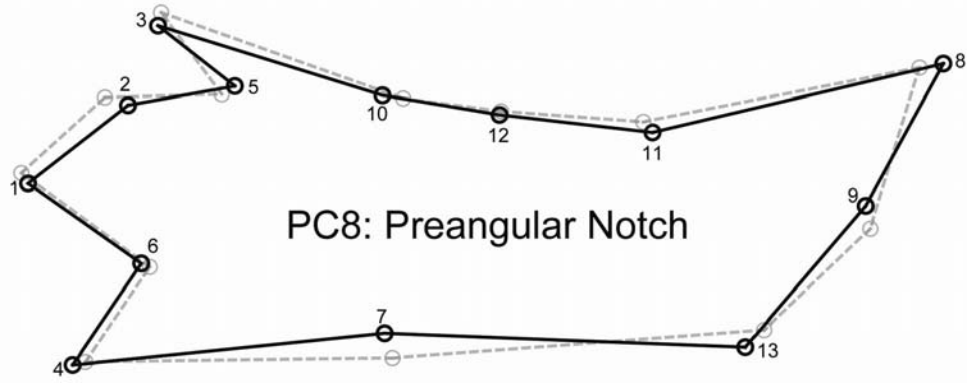
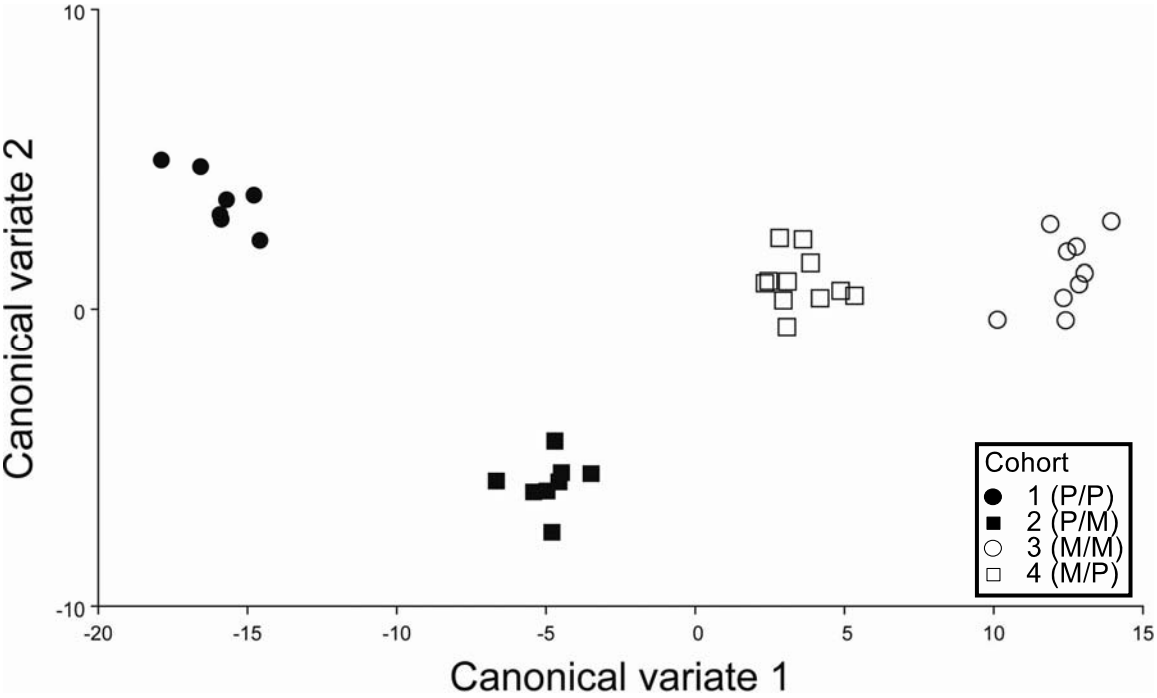

PC8: Preangular Notch
1
2
3
4
5
6
7
8
9
10
11
12
13

**Figure 3.6.** Canonical variates scores for week 10 mandibular analysis.



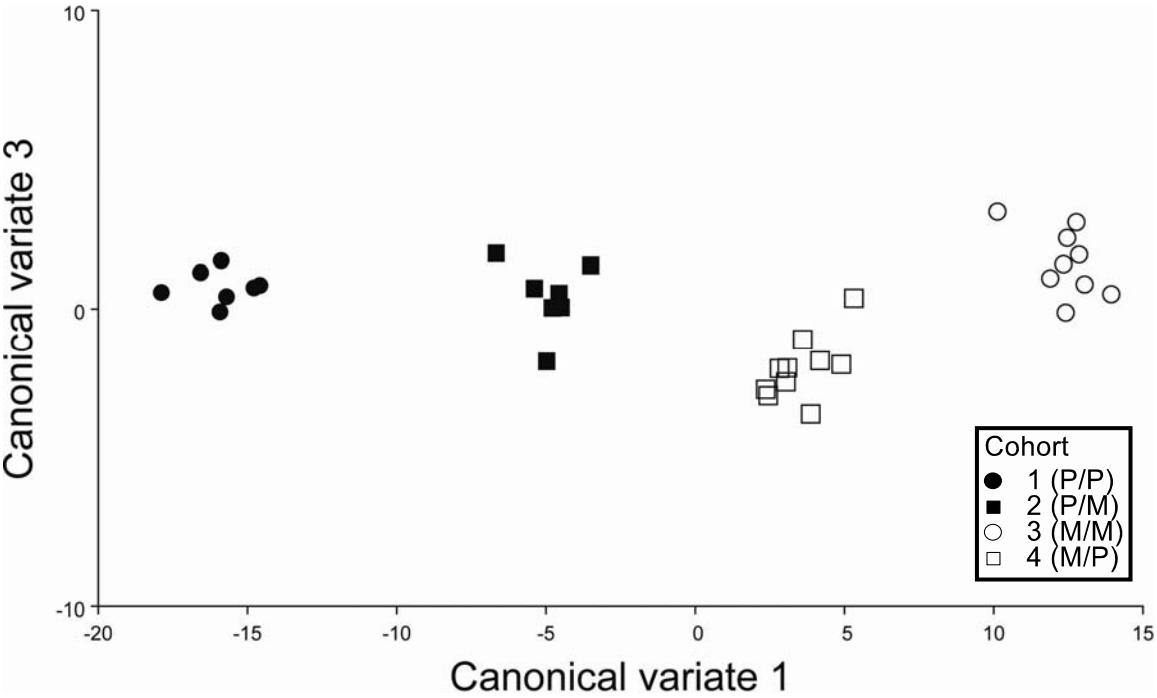



**Figure 3.7.** Wire-frame representations of the canonical variates from the week 10 mandibular analysis described in table 3.10. Black outline is the target shape (CV score of +10.0), grey outline is the mean shape (CV score of 0.0).

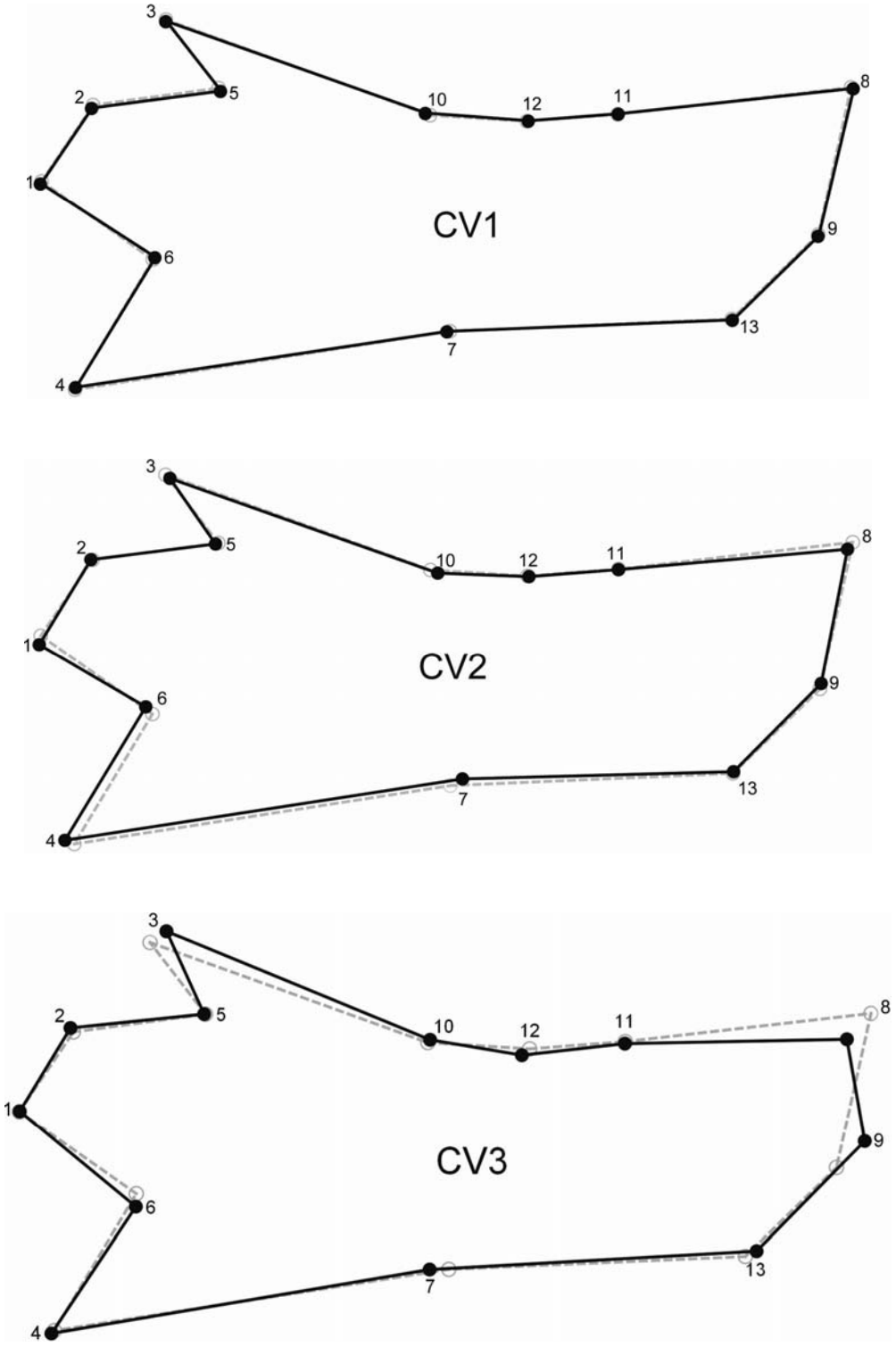


**Figure 3.8.** Principal components scores for included PCs for week 10 mandibular analysis.

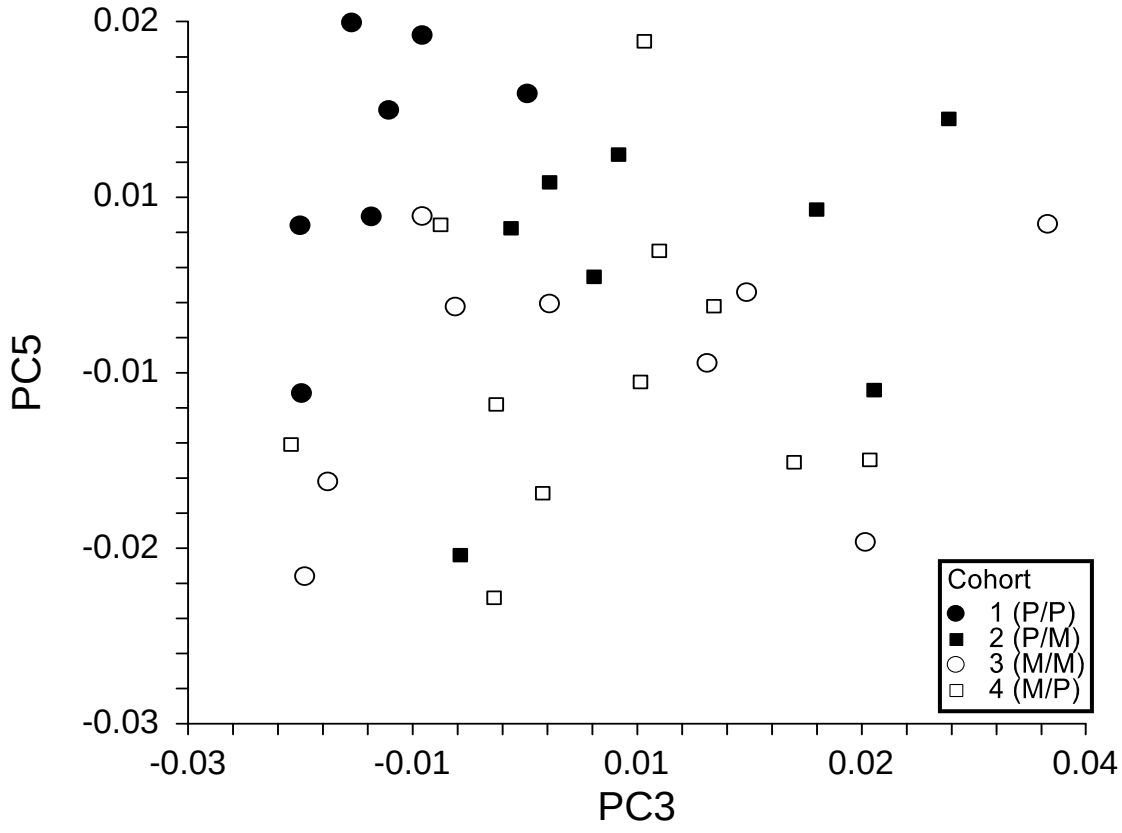
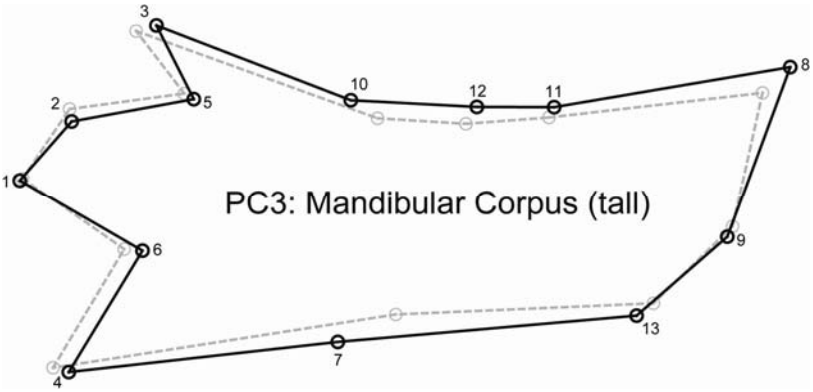


**Figure 3.9**. Included principal components identified by step-wise discriminant function analysis on the mandibular landmark set for week 10. See Table 3.12 for descriptions. Black outline is the target shape (PC score of +0.01), grey outline is the mean shape (PC score of 0.0).



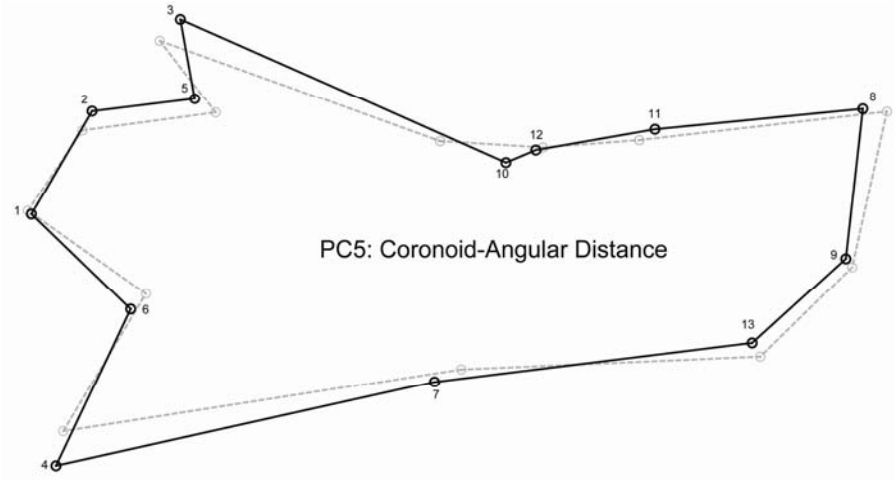



**Figure 3.10.** Canonical variates scores for week 16 mandibular analysis.

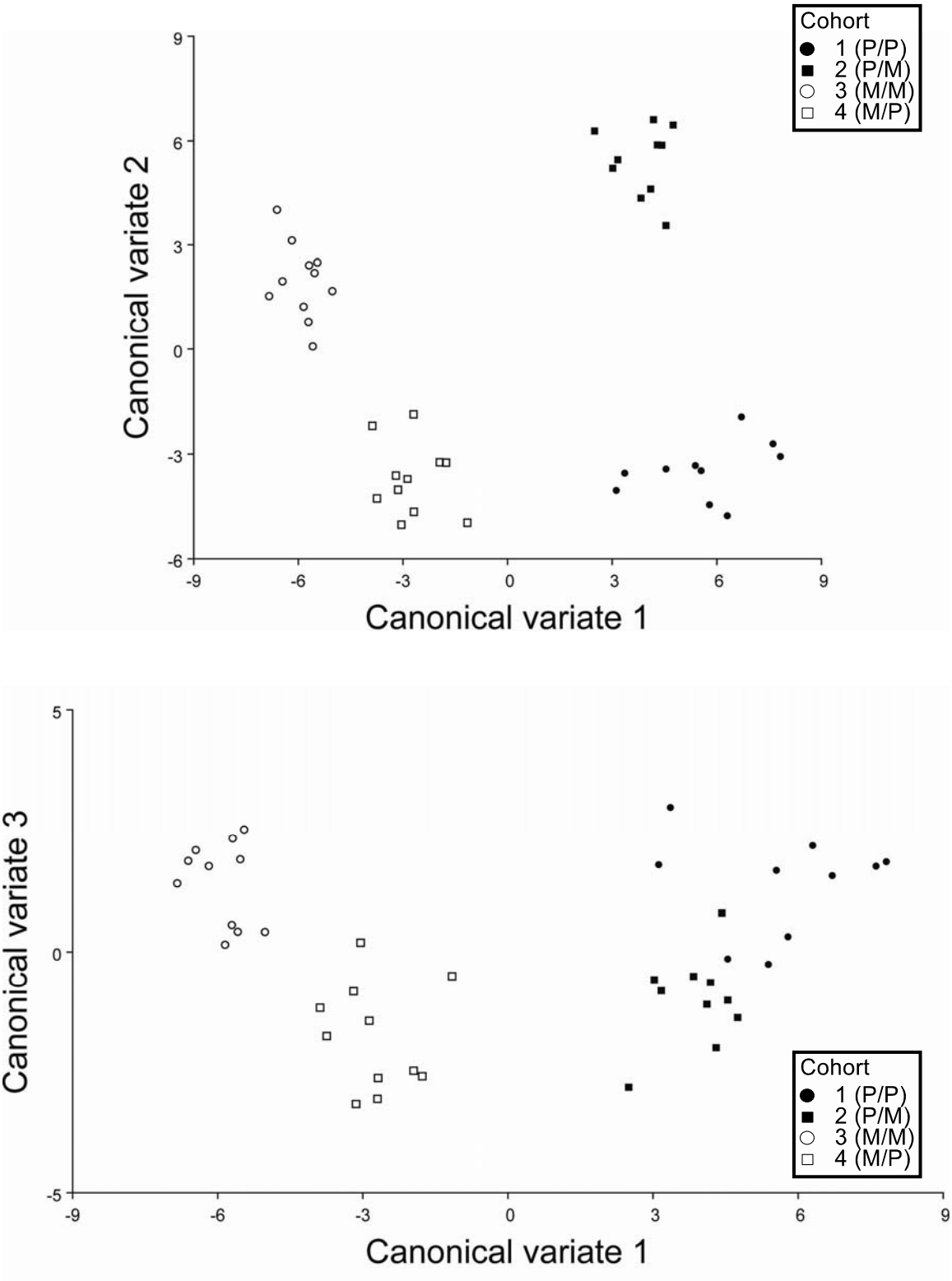
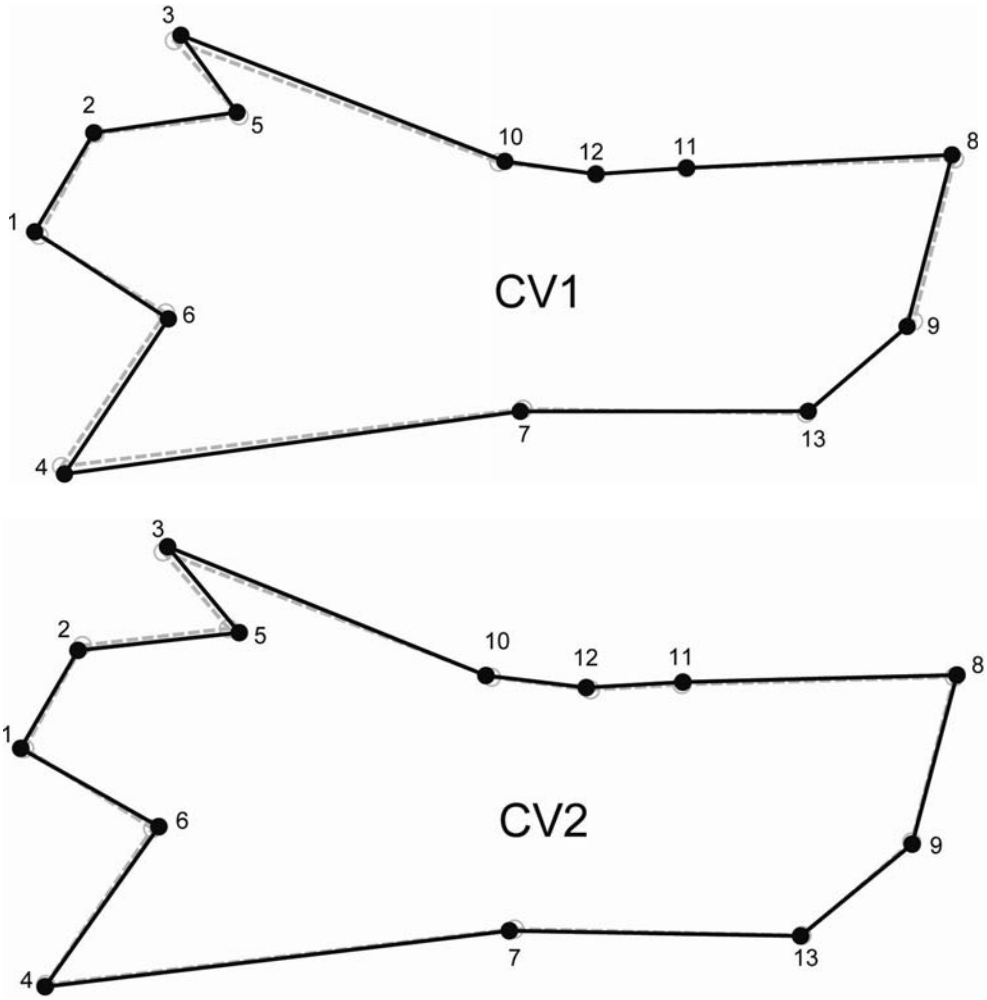


**Figure 3.11.** Wire-frame representations of the canonical variates from the week 16 mandibular analysis described in table 3.15. Black outline is the target shape (CV score of +10.0), grey outline is the mean shape (CV score of 0.0).



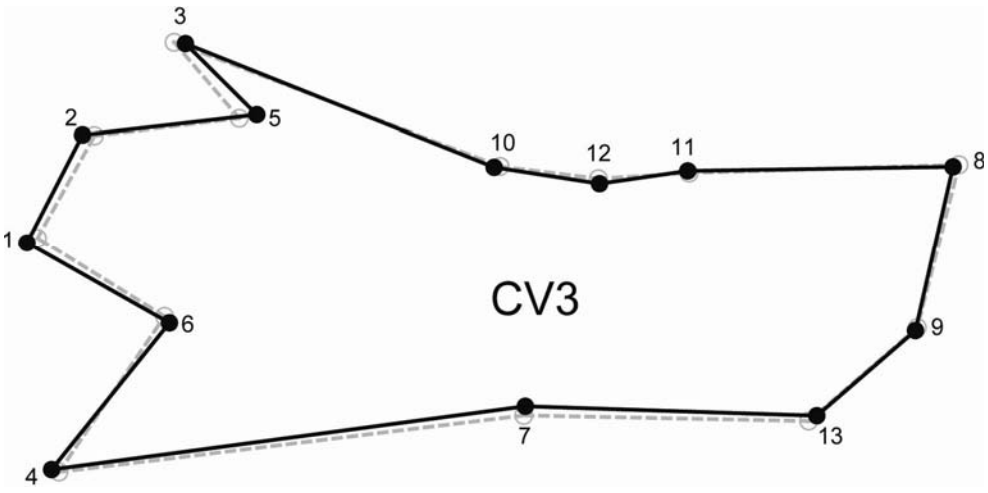
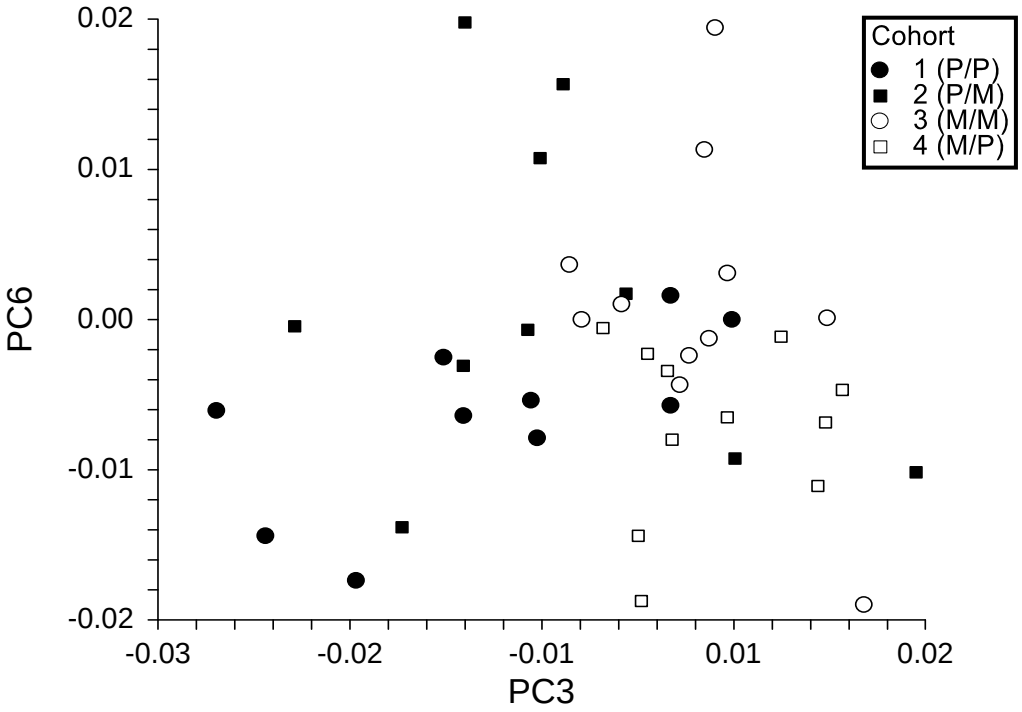

3
2
5
10
12
11
8
1
CV3
6
9
7
13
4

**Figure 3.12.** Principal components scores for included PCs for week 16 mandibular analysis.



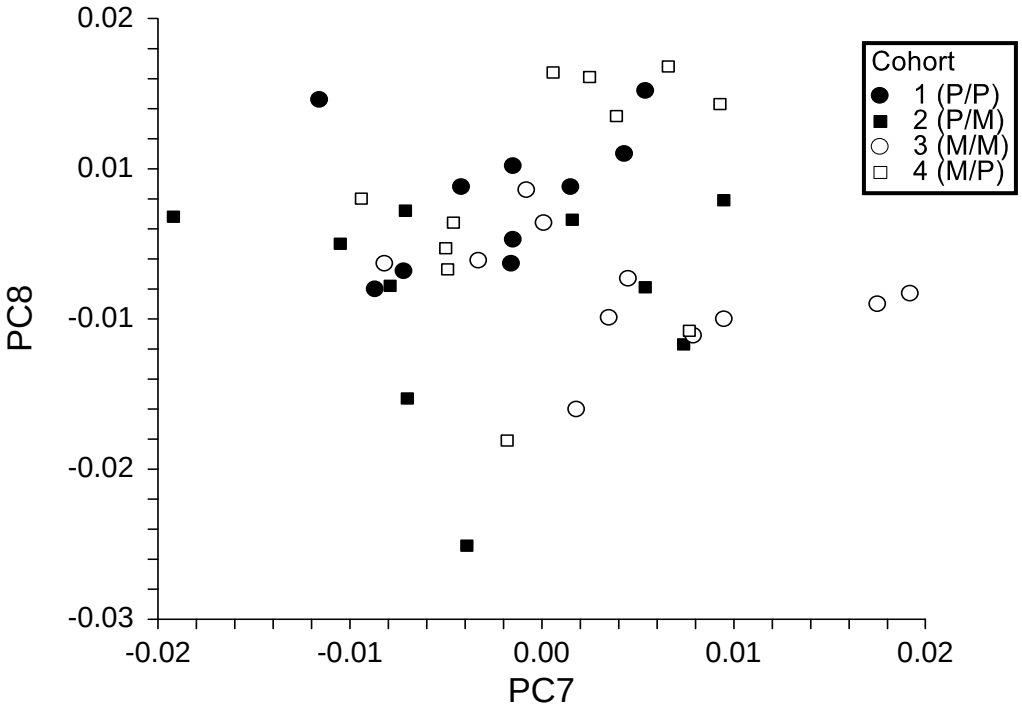



**Figure 3.13**. Included principal components identified by step-wise discriminant function analysis on the mandibular landmark set for week 16. See Table 3.17 for descriptions. Black outline is the target shape, grey outline is the mean shape.

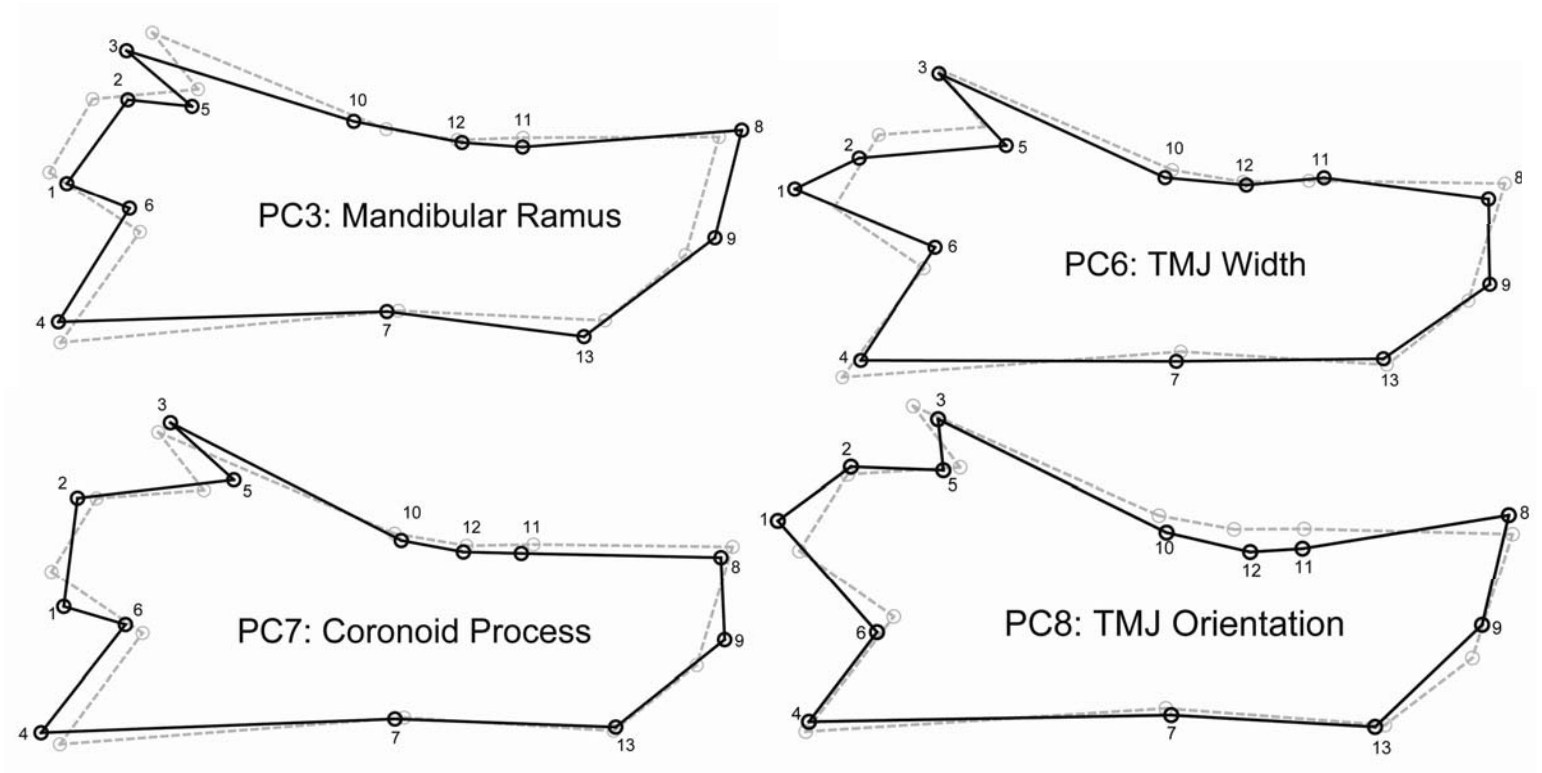


**Figure 3.14**. Cross-sectional measures of the right mandibular corpus. A: cross-section through the mandibular corpus at the level of the incisor/mandibular symphysis. B-C: cross-section through the mandibular corpus at the level of the second molar. Key: B, buccal cortical bone; H, superoinferior height; I, inferior cortical bone; IR, incisor root; L, lingual cortical bone; M2, second molar; MC, mandibular corpus; W, buccolingual width.

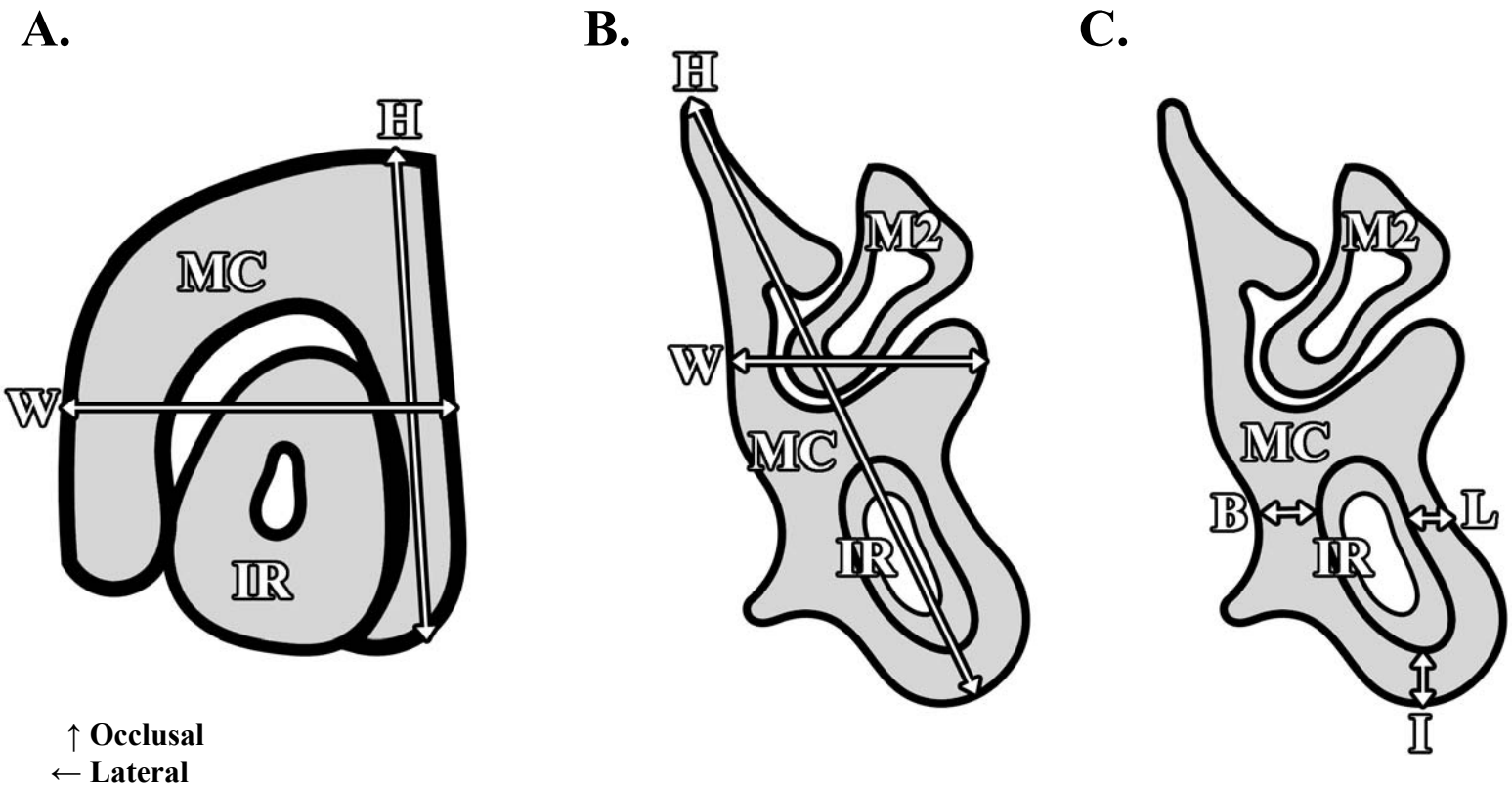


**Figure 3.15.** Regional cortical thickness by week for all cohorts.

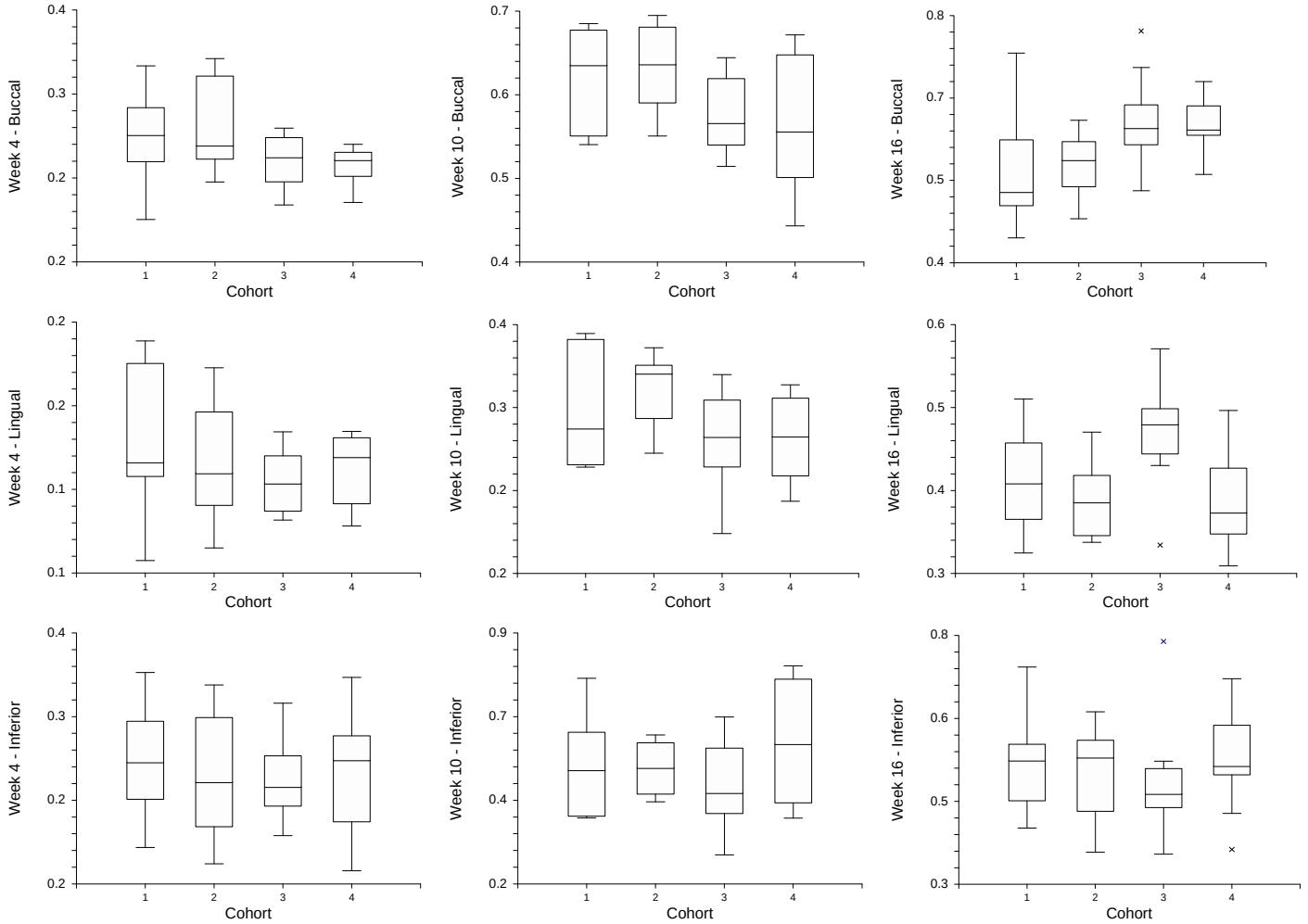


**Table 3.1.** 3D landmarks collected in eTDIPS. All mandibular landmarks were collected from the right side.

| **Mandibular landmarks** | |
|---|---|
| **1** | Posterior point on the temporomandibular condyle |
| **2** | Anterior point on the temporomandibular condyle |
| **3** | Coronoid process |
| **4** | Angular process |
| **5** | Mandibular notch |
| **6** | Subcondylar notch |
| **7** | Preangular notch |
| **8** | Superior aspect of incisal alveolus |
| **9** | Inferior aspect of incisal alveolus |
| **10** | Ramus-alveolar rim intersection |
| **11** | Mandibular molar 1 |
| **12** | Mandibular molar 2 |
| **13** | Incisal ramus |

**Table 3.2.** Ln-transformed centroid sizes (mean and standard deviations) with Kruskal-Wallis *p*-values ($\alpha$=0.05) for the mandibular landmark set used in the geometric morphometric analyses.

| **Cohort** | **Week 4** | | **Week 10** | | **Week 16** | |
|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P)** | 3.163 | 0.016 | 3.507 | 0.021 | 3.577 | 0.016 |
| | n = 9 | | n = 7 | | n = 10 | |
| **2 (P/M)** | 3.181 | 0.019 | 3.497 | 0.011 | 3.570 | 0.011 |
| | n = 9 | | n = 8 | | n = 10 | |
| **3 (M)** | 3.175 | 0.02 | 3.507 | 0.015 | 3.582 | 0.012 |
| | n = 11 | | n = 9 | | n = 11 | |
| **4 (M/P)** | 3.193 | 0.019 | 3.508 | 0.013 | 3.585 | 0.010 |
| | n = 11 | | n = 11 | | n = 11 | |
| ***P*** | **0.015*** [A] | | 0.399 | | **0.020*** [A] | |

[A] See Table 3.3 for pairwise comparisons. * $p \leq 0.05$

**Table 3.3.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of ln(centroid size) for the mandibular landmark set during weeks 4 and 16.

| **Week 4** | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | x | x | x | X |
| **2 (P/M)** | 0.047 | x | x | X |
| **3 (M)** | 0.087 | 0.470 | x | X |
| **4 (M/P)** | **0.003*** | 0.210 | 0.071 | X |
| **Week 16** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | x | x | x | X |
| **2 (P/M)** | 0.290 | x | x | X |
| **3 (M)** | 0.181 | 0.020 | x | X |
| **4 (M/P)** | 0.121 | **0.006*** | 0.250 | X |

* Bonferroni-adjusted $p \leq 0.008$

**Table 3.4.** Procrustes distances (*p*-values) among cohorts for the week 4 mandibular analysis.

| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
|---|---|---|---|---|
| **1 (P)** | x | x | x | x |
| **2 (P/M)** | 0.0287 (**0.006***) | x | x | x |
| **3 (M)** | 0.0238 (0.070) | 0.0248 (0.042) | x | x |
| **4 (M/P)** | 0.0304 (**0.001***) | 0.0254 (0.027) | 0.0230 (0.080) | x |

* Bonferroni-adjusted $p \leq 0.008$

**Table 3.5.** Canonical variates and descriptions for the week 4 mandibular analysis.

| CV | % Variance | Description of Target Shape (Change in PC score by +10.0) |
|---|---|---|
| 1 | 52.326 | Anteroposteriorly shorter diastema and superoinferiorly shorter incisal alveolus; posteriorly elongated coronoid process; inferiorly shallow preangular notch. |
| 2 | 31.839 | Anteroposterior elongation of the TMJ; inferior elongation of the angular process; anteroposterior orientation of the coronoid process; increased posteroinferior height of the mandibular corpus. |
| 3 | 15.835 | Posterosuperior orientation of the coronoid process; shortening of the posterior aspect of the TMJ; anteroposteriorly longer incisal ramus. |
| **Total** | **100.00%** | |

**Table 3.6.** Results of the step-wise discriminant function analysis on the mandibular landmark set for week 4. Results of least squares regression analyses [PC score vs ln(centroid)] are also presented.

| Step-wise DFA | | | | | | | | | Least Squares Regression: vs ln(centroid) | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Status** | **PC** | **Variance** | **Morphological Variable** | **F-value** | ***p*-value** | **Canonical Variates[a]** | | | | |
| | | | | | | **CV1** | **CV2** | **CV3** | **Slope** | ***p*-value** |
| Out | 1 | 16.96 | Angular Process | 1.300 | 0.291 | | | | 0.306 | **0.021*** |
| **In** | **2** | 13.58 | **TMJ Height** | 5.480 | **0.004*** | -0.302 | -0.669 | 0.551 | 0.387 | **0.001*** |
| **In** | **3** | 12.41 | **Diastema Length** | 2.850 | 0.053 | -0.119 | -0.412 | -0.837 | 0.091 | 0.439 |
| **In** | **4** | 9.81 | **Incisal Alveolus** | 3.730 | **0.021*** | -0.406 | 0.312 | 0.171 | 0.024 | 0.820 |
| Out | 5 | 8.80 | Coronoid Process | 1.370 | 0.269 | | | | -0.050 | 0.612 |
| Out | 6 | 6.32 | Incisal Ramus | 0.770 | 0.517 | | | | 0.056 | 0.504 |
| **In** | **7** | 5.12 | **Mandibular Corpus** | 3.310 | **0.032*** | 0.439 | 0.037 | -0.029 | 0.064 | 0.395 |
| **In** | **8** | 4.86 | **Preangular Notch** | 3.640 | **0.023*** | 0.410 | -0.274 | 0.303 | 0.016 | 0.825 |
| | **Total** | **77.85%** | | | | | | | | |

[a] Variable-variate correlations

* $p < 0.05$

**Table 3.7.** Morphological variables and descriptions for the week 4 mandibular PCA.

| PC | Morphological Variable | Description of Target Shape |
|---|---|---|
| PC1 | Angular Process | Angular process is shortened by posterior location of the preangular notch. |
| PC2 | TMJ Height | TMJ is posterosuperiorly taller. |
| PC3 | Diastema Length | Longer diastema formed by anteroposterior extension of incisal alveolus. |
| PC4 | Incisal Alveolus | Anterior extension and increased superoinferior height of the incisal alveolus. |
| PC5 | Coronoid Process | Posterosuperior extension of coronoid process. |
| PC6 | Incisal Ramus | Ramus is posteroinferiorly shorter, mediolaterally wider |
| PC7 | Mandibular Corpus | Posteroinferiorly shortened mandibular corpus due to inferior placement of molar alveolus. |
| PC8 | Preangular Notch | Superiorly deepened preangular notch. |

**Table 3.8.** Classification count table produced by the step-wise DFA on the mandibular landmark set for week 4.

| | Predicted | | | | | |
|---|---|---|---|---|---|---|
| Actual | **1** | **2** | **3** | **4** | Total | % Correct |
| **1** | 6 | 1 | 2 | 0 | 9 | 67% |
| **2** | 0 | 7 | 0 | 2 | 9 | 78% |
| **3** | 2 | 0 | 5 | 4 | 11 | 45% |
| **4** | 1 | 0 | 4 | 6 | 11 | 55% |
| Total | 9 | 8 | 11 | 12 | 40 | 61% |

Reduction in classification error due to X's (PC2, PC3, PC4, PC7, and PC8): 46.7%

**Table 3.9.** Procrustes distances (*p*-values) among cohorts for the week 10 mandibular analysis.

| Cohort | 1 (P) | 2 (P/M) | 3 (M) | 4 (M/P) |
|---|---|---|---|---|
| **1 (P)** | x | x | X | x |
| **2 (P/M)** | 0.0284 (0.114) | x | X | x |
| **3 (M)** | 0.0279 (0.122) | 0.0227 (0.243) | X | x |
| **4 (M/P)** | 0.0283 (0.066) | 0.0204 (0.325) | 0.0121 (0.215) | x |

Bonferroni-adjusted α=0.008.

**Table 3.10.** Canonical variates and descriptions for the week 10 mandibular analysis.

| CV | % Variance | Description of Target Shape (Change in PC score by +10.0) |
|---|---|---|
| 1 | 88.227 | Longer mandibular corpus as a result of posterior placement of the coronoid-alveolar rim intersection and the preangular notch; anterior deepening of the subcondylar notch. |
| 2 | 9.967 | Posterior elongation of the angular process; posterior elongation of the TMJ. |
| 3 | 1.806 | Anterior placement of the coronoid process; posteroinferiorly shortened incisal alveolus. |
| **Total** | **100.00%** | |

**Table 3.11.** Results of the step-wise discriminant function analysis on the mandibular landmark set for week 10. Results of least squares regression analyses [PC score vs ln(centroid)] are also presented.

| Step-wise DFA | | | | | | | | Least Squares Regression: vs ln(centroid) | |
|---|---|---|---|---|---|---|---|---|---|
| **Status** | **PC** | **Variance** | **Morphological Variable** | **F-value** | ***p*-value** | **Canonical Variates[a]** | | | |
| | | | | | | **CV1** | **CV2** | **Slope** | ***p*-value** |
| Out | 1 | 20.06 | Incisal Ramus | 0.480 | 0.697 | | | 0.413 | 0.061 |
| Out | 2 | 16.31 | Mandibular Corpus (short) | 1.540 | 0.226 | | | 0.025 | 0.901 |
| **In** | **3** | **13.21** | **Mandibular Corpus (tall)** | 4.180 | **0.014*** | -0.578 | 0.816 | 0.027 | 0.882 |
| Out | 4 | 7.94 | Mandibular Notch Angle | 0.700 | 0.561 | | | -0.241 | 0.084 |
| **In** | **5** | **6.81** | **Coronoid-Angular Distance** | 4.840 | **0.007*** | 0.648 | 0.762 | 0.003 | 0.981 |
| Out | 6 | 6.19 | Coronoid Process | 1.320 | 0.285 | | | -0.065 | 0.604 |
| Out | 7 | 5.04 | Angular Process | 1.210 | 0.323 | | | 0.001 | 0.992 |
| | **Total** | **75.56%** | | | | | | | |

[a] Variable-variate correlations

* $p < 0.05$

**Table 3.12.** Morphological variables and descriptions for the week 10 mandibular analysis.

| **PC** | **Morphological Variable** | **Description of Target Shape (Change in PC score by +0.01)** |
|---|---|---|
| PC1 | Incisal Ramus | Anterior placement and superior orientation of incisal ramus. |
| PC2 | Mandibular Corpus (short) | Posteroinferiorly shorter mandibular corpus. |
| PC3 | Mandibular Corpus (tall) | Posteroinferiorly taller mandibular corpus. |
| PC4 | Mandibular Notch Angle | Coronoid process is more anteriorly placed, forming a more oblique angle between the anterior aspect of the TMJ and the posterior aspect of the coronoid process. |
| PC5 | Coronoid-Angular Distance | Greater distance between coronoid and angular processes. Coronoid process is superiorly positioned; angular process is more inferiorly positioned. |
| PC6 | Coronoid Process | Coronoid process is more anteriorly placed and its intersection with the alveolar rim is more superior. |
| PC7 | Angular Process | Angular process is anteroinferiorly positioned, projecting below the line formed by the inferior border of the mandibular corpus. |

**Table 3.13.** Classification count table produced by the step-wise DFA on the mandibular landmark set for week 10.

| | Predicted | | | | | |
|---|---|---|---|---|---|---|
| Actual | **1** | **2** | **3** | **4** | Total | % Correct |
| **1** | 6 | 0 | 0 | 1 | 7 | 86% |
| **2** | 1 | 5 | 1 | 1 | 8 | 63% |
| **3** | 1 | 2 | 3 | 3 | 9 | 33% |
| **4** | 2 | 2 | 3 | 4 | 11 | 36% |
| Total | 10 | 9 | 7 | 9 | 35 | 54% |

Reduction in classification error due to X's (PC3 and PC5): 35.2%

**Table 3.14.** Procrustes distances (*p*-values) among cohorts for the week 16 mandibular analysis.

| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
|---|---|---|---|---|
| **1 (P)** | x | x | x | x |
| **2 (P/M)** | 0.0146 (0.455) | x | x | x |
| **3 (M)** | 0.0205 (0.014) | 0.0176 (0.073) | x | x |
| **4 (M/P)** | 0.0185 (0.117) | 0.0216 (0.019) | 0.0158 (0.230) | x |

Bonferroni-adjusted $\alpha$=0.008.

**Table 3.15.** Canonical variates and descriptions for the week 16 mandibular analysis.

| CV | % Variance | Description of Target Shape (Change in PC score by +10.0) |
|---|---|---|
| 1 | 57.321 | Increase in coronoid-angular distance due to superior placement of coronoid process and inferior placement of angular process. |
| 2 | 37.463 | Anterior deepening of mandibular notch. |
| 3 | 5.216 | Increase in TMJ height (anteroposterior) and length (posteroinferior); anterior deepening of mandibular notch with a anteroposteriorly narrower coronoid process; superior deepening of preangular notch with a posteroinferiorly shorter mandibular corpus. |
| **Total** | **100.00%** | |

**Table 3.16.** Results of the step-wise discriminant function analysis on the mandibular landmark set for week 16. Results of least squares regression analyses [PC score vs ln(centroid)] are also presented.

| Step-wise DFA | | | | | | | | | Least Squares Regression: vs ln(centroid) | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Status** | **PC** | **Variance** | **Morphological Variable** | **F-value** | ***p*-value** | **Canonical Variates[a]** | | | | |
| | | | | | | **CV1** | **CV2** | **CV3** | **Slope** | ***p*-value** |
| Out | PC1 | 23.80 | Coronoid-angular process distance | 1.480 | 0.237 | | | | -0.542 | **0.004*** |
| Out | PC2 | 12.71 | Alveolar Process | 1.000 | 0.404 | | | | 0.297 | **0.032*** |
| **In** | **PC3** | **9.94** | **Mandibular Ramus** | 7.790 | **0.000*** | 0.560 | -0.549 | 0.614 | 0.255 | **0.038*** |
| Out | PC4 | 6.99 | Angular Process | 1.210 | 0.321 | | | | 0.046 | 0.667 |
| Out | PC5 | 6.52 | Subcondylar Angle | 0.460 | 0.711 | | | | -0.044 | 0.671 |
| **In** | **PC6** | **5.44** | **TMJ Length** | 4.100 | **0.014*** | -0.304 | -0.477 | 0.005 | -0.063 | 0.496 |
| **In** | **PC7** | **5.33** | **Coronoid Process** | 3.560 | **0.024*** | -0.358 | 0.192 | 0.908 | 0.250 | **0.005*** |
| **In** | **PC8** | **4.11** | **TMJ Orientation** | 4.930 | **0.006*** | 0.349 | 0.522 | 0.186 | -0.055 | 0.499 |
| | **Total** | **74.84%** | | | | | | | | |

[a] Variable-variate correlations

* $p < 0.05$

**Table 3.17.** Morphological variables and descriptions for the week 16 mandibular analysis.

| PC | Morphological Variable | Description of Target Shape |
|---|---|---|
| PC1 | Coronoid-angular process distance | Greater distance between coronoid and angular processes. Coronoid process is superiorly positioned; angular process is more inferiorly positioned. |
| PC2 | Alveolar Process | Anteroposteriorly shorter alveolar process. |
| PC3 | Mandibular Ramus | Mandibular ramus is smaller, with a more posteriorly oriented coronoid process, superiorly shortened angular process, and anteroposteriorly shortened TMJ. |
| PC4 | Angular Process | More inferiorly oriented angular process. |
| PC5 | Subcondylar Angle | More oblique angle of subcondylar notch formed by superiorly positioned TMJ and inferiorly positioned angular process. |
| PC6 | TMJ Length | Shorter anteroposteior width of TMJ. |
| PC7 | Coronoid Process | Coronoid process is anteroposteiorly narrowed by anterior placement of mandibular notch. |
| PC8 | TMJ Orientation | Posterior border of TM is elevated so that the surface of the TMJ is oriented more superiorly. |



**Table 3.18.** Classification count table produced by the step-wise DFA on the mandibular landmark set for week 16.

| | Predicted | | | | | |
|---|---|---|---|---|---|---|
| Actual | **1** | **2** | **3** | **4** | Total | % Correct |
| **1** | 7 | 1 | 0 | 2 | 10 | 70% |
| **2** | 1 | 5 | 3 | 1 | 10 | 50% |
| **3** | 0 | 1 | 9 | 1 | 11 | 82% |
| **4** | 0 | 1 | 2 | 8 | 11 | 73% |
| Total | 8 | 8 | 14 | 12 | 42 | 69% |

Reduction in classification error due to X's (PC3, PC6, PC7, and PC8): 58.7%

**Table 3.19.** Cross-sectional mandibular measurements (mm) for week 4 with with Kruskal-Wallis *p*-value (α=0.05).

| Cohort | Height at incisor | | Width at incisor | | Height at molar | | Width at molar | | Buccal cortical thickness | | Lingual cortical thickness | | Inferior cortical thickness | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. |
| **1 (n=10)** | 2.096 | 0.089 | 1.533 | 0.099 | 5.863 | 0.196 | 2.085 | 0.156 | 0.274 | 0.043 | 0.174 | 0.038 | 0.248 | 0.045 |
| **2 (n=10)** | 2.16 | 0.097 | 1.601 | 0.093 | 6.065 | 0.168 | 2.132 | 0.095 | 0.283 | 0.044 | 0.162 | 0.032 | 0.236 | 0.049 |
| **3 (n=9)** | 2.176 | 0.11 | 1.669 | 0.135 | 6.044 | 0.409 | 2.133 | 0.098 | 0.251 | 0.025 | 0.152 | 0.017 | 0.232 | 0.031 |
| **4 (n=11)** | 2.112 | 0.092 | 1.583 | 0.083 | 6.235 | 0.165 | 2.175 | 0.094 | 0.246 | 0.017 | 0.159 | 0.019 | 0.238 | 0.049 |
| ***p*-value** | 0.205 | | 0.117 | | **0.012*** [A] | | 0.384 | | 0.074 | | 0.546 | | 0.854 | |

[A] See Table 3.20 for pairwise comparisons. * $p \leq 0.05$

**Table 3.20.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of cross-sectional mandibular measurements at week 4.

| Molar Width | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | x | x | x | x |
| **2 (P/M)** | 0.290 | x | x | x |
| **3 (M)** | 0.327 | 1.000 | x | x |
| **4 (M/P)** | 0.151 | 0.273 | 0.391 | x |

**Table 3.21.** Cross-sectional mandibular measurements (mm) for week 10 with Kruskal-Wallis *p*-value (α=0.05).

| Cohort | Height at incisor | | Width at incisor | | Height at molar | | Width at molar | | Buccal cortical thickness | | Lingual cortical thickness | | Inferior cortical thickness | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. |
| **1 (n=7)** | 3.063 | 0.215 | 2.007 | 0.112 | 8.531 | 0.942 | 2.600 | 0.131 | 0.568 | 0.065 | 0.317 | 0.059 | 0.539 | 0.136 |
| **2 (n=6)** | 3.041 | 0.149 | 2.051 | 0.158 | 8.462 | 0.848 | 2.648 | 0.098 | 0.583 | 0.052 | 0.337 | 0.037 | 0.523 | 0.073 |
| **3 (n=9)** | 2.917 | 0.132 | 1.964 | 0.185 | 8.953 | 0.713 | 2.570 | 0.105 | 0.529 | 0.046 | 0.286 | 0.049 | 0.474 | 0.118 |
| **4 (n=10)** | 3.057 | 0.123 | 2.024 | 0.110 | 8.488 | 0.773 | 2.616 | 0.122 | 0.515 | 0.079 | 0.287 | 0.041 | 0.594 | 0.156 |
| ***p*-value** | 0.089 | | 0.658 | | 0.509 | | 0.490 | | 0.135 | | 0.150 | | 0.419 | |

**Table 3.22.** Cross-sectional mandibular measurements (mm) for week 16 with Kruskal-Wallis *p*-value (α=0.05).

| Cohort | Height at incisor | | Width at incisor | | Height at molar | | Width at molar | | Buccal cortical thickness | | Lingual cortical thickness | | Inferior cortical thickness | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. |
| **1 (n=10)** | 3.389 | 0.148 | 2.249 | 0.159 | 7.955 | 0.642 | 2.722 | 0.195 | 0.547 | 0.097 | 0.395 | 0.050 | 0.542 | 0.089 |
| **2 (n=9)** | 3.409 | 0.128 | 2.313 | 0.153 | 8.016 | 0.211 | 2.637 | 0.098 | 0.559 | 0.050 | 0.370 | 0.039 | 0.524 | 0.098 |
| **3 (n=11)** | 3.319 | 0.157 | 2.300 | 0.169 | 7.907 | 0.226 | 2.664 | 0.131 | 0.631 | 0.069 | 0.445 | 0.051 | 0.501 | 0.111 |
| **4 (n=11)** | 3.279 | 0.141 | 2.349 | 0.195 | 8.192 | 0.369 | 2.783 | 0.132 | 0.622 | 0.041 | 0.373 | 0.043 | 0.547 | 0.092 |
| ***p*-value** | 0.063 | | 0.426 | | 0.104 | | 0.065 | | **0.009*** [A] | | **0.009*** [A] | | 0.324 | |

[A] See Table 3.23 for pairwise comparisons. * $p \leq 0.05$

**Table 3.23.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of cross-sectional mandibular measurements at week 16.

| **Buccal cortical thickness** | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | x | x | x | x |
| **2 (P/M)** | 0.253 | x | x | x |
| **3 (M)** | 0.020 | 0.014 | x | x |
| **4 (M/P)** | 0.024 | 0.017 | 0.818 | x |
| **Lingual cortical thickness** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | x | X | x | x |
| **2 (P/M)** | 0.253 | X | x | x |
| **3 (M)** | 0.035 | **0.007*** | x | x |
| **4 (M/P)** | 0.260 | 0.970 | **0.004*** | x |

***** Bonferroni-adjusted $p \leq 0.008$



**Table 3.24.** Results (*p*-values) from Kruskal-Wallis tests (α=0.05) and pairwise Mann-Whitney *U* tests (α=0.0166) of cross-sectional mandibular measurements at week 4. See Table 3.10 for raw data.

| **Cohort** | **Kruskal-Wallis *p*-values** | **Mann-Whitney *p*-values** | | |
|---|---|---|---|---|
| | | **Buccal vs Lingual** | **Buccal vs Inferior** | **Lingual vs Inferior** |
| **1** | **<0.001*** | **0.001$^{+}$ (B>L)** | 0.175 | **0.001$^{+}$ (I>L)** |
| **2** | **<0.001*** | **<0.001$^{+}$ (B>L)** | 0.082 | **0.002$^{+}$ (I>L)** |
| **3** | **<0.001*** | **<0.001$^{+}$ (B>L)** | 0.171 | **<0.001$^{+}$ (I>L)** |
| **4** | **0.001*** | **<0.001$^{+}$ (B>L)** | 1.000 | **0.006$^{+}$ (I>L)** |

***** $p \leq 0.05$

**+** Bonferroni-adjusted $p \leq 0.016$

**Table 3.25.** Results (*p*-values) from Kruskal-Wallis tests (α=0.05) and pairwise Mann-Whitney *U* tests (α=0.0166) of cross-sectional mandibular measurements at week 10. See Table 3.12 for raw data.

| Cohort | Kruskal-Wallis *p*-values | Mann-Whitney *p*-values | | |
|---|---|---|---|---|
| | | Buccal vs Lingual | Buccal vs Inferior | Lingual vs Inferior |
| **1** | **0.002*** | **0.002$^{+}$ (B>L)** | 0.565 | **0.006$^{+}$ (I>L)** |
| **2** | **0.002*** | **0.004$^{+}$ (B>L)** | 0.150 | **0.004$^{+}$ (I>L)** |
| **3** | **<0.001*** | **<0.001$^{+}$ (B>L)** | 0.171 | **0.002$^{+}$ (I>L)** |
| **4** | **0.001*** | **<0.001$^{+}$ (B>L)** | 0.257 | **<0.001$^{+}$ (I>L)** |

* $p \leq 0.05$
+ Bonferroni-adjusted $p \leq 0.016$

**Table 3.26.** Results (*p*-values) from Kruskal-Wallis tests (α=0.05) and pairwise Mann-Whitney *U* tests (α=0.0166) of cross-sectional mandibular measurements at week 16. See Table 3.13 for raw data.



| Cohort | Kruskal-Wallis *p*-values | Mann-Whitney *p*-values | | |
|---|---|---|---|---|
| | | Buccal vs Lingual | Buccal vs Inferior | Lingual vs Inferior |
| **1** | **<0.001*** | **<0.001$^{+}$ (B>L)** | 0.762 | **0.001$^{+}$ (I>L)** |
| **2** | **0.001*** | **<0.001$^{+}$ (B>L)** | 0.353 | **0.007$^{+}$ (I>L)** |
| **3** | **<0.001*** | **<0.001$^{+}$ (B>L)** | **0.002$^{+}$ (B>I)** | 0.108 |
| **4** | **<0.001*** | **<0.001$^{+}$ (B>L)** | 0.028 | **<0.001$^{+}$ (I>L)** |

* $p \leq 0.05$
+ Bonferroni-adjusted $p \leq 0.016$

# CHAPTER 4: BONE PHYSIOLOGY

## Aims

This component of the project investigates how the biological processes underlying functional adaptation vary ontogenetically and are affected variably throughout the skeleton by masticatory behavior. Current ecomorphological studies are limited by our understanding of the significance of environmental variation on regional vs. systemic scales (Lieberman, 1996; Stock and Pfeiffer, 2001). In order to evaluate the roles of growth and masticatory behavior on postnatal variation in bone physiology, a series of enzyme-linked immunoabsorbent assays (ELISA) were used to measure ontogenetic changes in three serum markers of bone modeling and resorption. The first, serum procollagen I N-terminal extension peptide (PINP) is a byproduct of type I collagen synthesis by osteoblasts (Hale et al., 2007). The second, osteocalcin, is a noncollagenous protein secreted by osteoblasts during bone formation (Fu and Muller, 1999) and released from the bone matrix by osteoclasts during bone resorption (Ivaska et al., 2004). Therefore, osteocalcin may be considered an indicator of bone turnover. The third and final marker, tartrate-resistant acid phosphatase form 5b (TRACP 5b) is expressed in high quantities by osteoclasts and thus is used as measure of bone resorption (Halleen et al., 2006).

**Hypothesis 1:** The concentrations of serum markers related to bone growth/osteoblast activity (i.e. PINP and osteocalcin) will be highest in younger animals.

**Hypothesis 2**: Markers of bone (**A**) growth (i.e. PINP) and (**B**) turnover (i.e. osteocalcin) will be higher in animals experiencing greater masticatory loads.

**Hypothesis 3**: Following the dietary shift, older animals switched from a meal to a pellet diet (Cohort 4) will undergo greater amounts of bone modeling and/or remodeling compared to animals switched from a pellet to meal diet (Cohort 2).

## Methods

Blood samples were collected into Microvette EDTA-coated tubes (Sarstedt Inc., Newton, NC) at four longitudinal points during the experimental period: 7, 10*, 13, and 16 weeks old. The beginning of the experiment (week 4) was not considered for serum analysis as it was a period of acclimation to the housing environment. Serum was obtained through centrifugation at 4˚C and stored at -20˚C until analyzed. Serum markers were analyzed using commercial enzyme-

* The dietary shift occurred at the beginning of week 10 (d. 64). The blood draw occurred at the end of week 10 (d. 70). See Appendix II for experimental schedule.

linked immunosorbant assays for serum N-terminal propeptide of type I procollogen (PINP) (Rat/Mouse PINP EIA, ImmunoDiagnostic Systems Ltd, Arizona, USA), osteocalcin (Rat-MID™ Osteocalcin EIA, ImmunoDiagnostic Systems Ltd), and tartrate-resistant acid phopsphatase form 5b (TRACP 5b)(RatTRAP™ Assay, ImmunoDiagnostic Systems Ltd). All samples were tested in duplicate. Absorbance readings of each test were performed with a SpectraMax® Gemini™ EM fluorescent plate reader (Molecular Devices Corp, Sunnyvale, CA).

## Statistics

Kruskal-Wallis tests ($\alpha$=0.05) were used to statistically compare serum marker concentrations among cohorts for each longitudinal point. When a statistically significant difference was detected among cohorts within a given longitudinal point, individual pairwise comparisons were made using the Mann-Whitney *U* test with Bonferroni-adjusted *p*-values ($\alpha$=0.0083, 6 inter-cohort comparisons).

## Results

The concentrations of all quantified serum markers of bone activity (i.e. PINP, osteocalcin, and TRACP 5b) decrease with age, reflecting the ontogenetic decrease in combined cranial and postcranial skeletal growth rate (Figure 4.1). This ontogenetic decrease in skeletal growth is illustrated by linear humeral

growth, as measured by percentage length increase from the previous week [($length_{week\ b}$-$length_{week\ a}$)/$length_{week\ b}$] (Figure 4.1 D).

During week 13, following the dietary shift at the beginning of week 10, significant differences ($p$=0.01) were observed in serum PINP levels among cohorts (Table 4.1). Pairwise comparisons indicated that type I collagen production was significantly higher ($p \leq 0.0083$) in cohort 4 (M/P) compared to cohorts 1 (P) and 2 (P/M) (Figure 4.1A; Table 4.2).

No significant differences in the concentration of serum osteocalcin were observed among cohorts at any time period sampled (Figure 4.1B; Table 4.3).

A Kruskal-Wallis test suggested significant differences in serum concentrations of TRACP 5b among cohorts during weeks 10, 13, and 16 (Table 4.4). However, further pairwise comparisons revealed significant differences ($p \leq 0.0083$) only during weeks 10 and 13 (Figure 4.1C; Table 4.5). Immediately following the dietary shift (Week 10) and still detectable three weeks later (week 13), cohort 4 (M/P) had decreased osteoclast activity compared to cohorts 1 (P) and 2 (P/M).

## Conclusions

Serum markers of bone physiology closely track an ontogenetic decrease in skeletal growth rate. The serum concentrations of markers related to bone

growth/turnover (i.e. PINP and osteocalcin) and resorption (i.e. TRACP 5b), like rates of postcranial bone growth, also decrease with age ($H1_A$). During the later ontogenetic stages sampled, significant differences in these serum markers related to variation in masticatory behavior can be detected. It is possible that these differences are not detectable during earlier ontogenetic stages (e.g., week 7) due to the intensity of the combined cranial and postcranial growth signal. Furthermore, the differences in serum markers detected following the dietary shift (weeks 10 and 13) do not persist into week 16, suggesting a rapid period of functional adaptation related to changes in feeding behavior.

Following the dietary shift, the cohort switched from meal to pellets (cohort 4) shows increased type I collagen production ($H2A_A$, $H3_A$) and decreased bone resorption ($H3_A$). While elevated production of type I collagen is first detected in week 13, increased levels of bone resorption are detectable within a week following the dietary shift (week 10) and persist for over three weeks (week 13). Interestingly, no significant differences in serum osteocalcin are demonstrated at any time point. Thus, the null hypothesis ($H2\text{-}B_0$) that levels of bone turnover are equivalent among all cohorts cannot be rejected. However, this work does support the hypothesis that an increase in masticatory loading related to dietary shifts results in increased bone modeling, e.g. an increase type I collagen production and decrease in bone resorption ($H3_A$).

At least two possibilities exist to explain why variation exists in the production of type-I collagen (i.e. PINP) but not noncollagenous bone protein (i.e. osteocalcin). Type-I collagen composes 95% of the collagen in bone, but also over 80% of the collagen in non-mineralized connective tissues (Viguet-Carrin et al., 2006). Therefore, it is possible that increased fibroblast activity (responsible for the production of tendons, ligaments, cartilage, etc.) rather than osteoclast activity could explain the differences in serum PINP seen among cohorts. Alternatively, type I collagen plays an important role in bone strength by increasing the material toughness, while the mineral component of bone is related to its stiffness (Viguet-Carrin et al., 2006). Thus, increases in relative concentration of serum PINP could also be related to a need to increase bone strength in the presence of recently elevated levels of masticatory loading.

Results from this analysis of bone physiology suggest that intra-individual behavioral variation related to diet can be detected at the systemic level through circulating serum markers. As the examined markers of growth and resorption are uncoupled, this suggests that the physiological response to a dietary shift is one of increased bone modeling rather than remodeling. This is additionally supported by the lack of significant results related to a serum marker of bone turnover (osteocalcin). It is important to remember that these results are derived from a study of juvenile rats, and thus may apply only to individuals who have not yet achieved skeletal maturity. Older individuals may exhibit different physiological

patterns and are likely to show signals of more bone remodeling rather than modeling. Further studies are needed to address the physiological patterns related to dietary variability in aging individuals.

**FIGURE 4.1.** Results of longitudinal assays of serum markers of bone physiology (A-C), and longitudinal growth of the humerus (D). Pairwise comparisons are made using the Mann-Whitney U-test with Bonferroni-adjusted p-values (α=0.008). **Axes**: X, week; Y (A-C), concentration (ng/mL); Y (D), % increase. **Line Key**: Cohort 1 (P), solid black line. Cohort 2 (P/M), dashed black line. Cohort 3 (M), solid gray line. Cohort 4 (M/P), dashed gray line. **Symbol Key:** *[a] Cohort 4 (M/P) > Cohorts 1 (P) and 2 (P/M). *[b,c] Cohort 4 (M/P) < Cohorts 1 (P) and 2 (P/M).

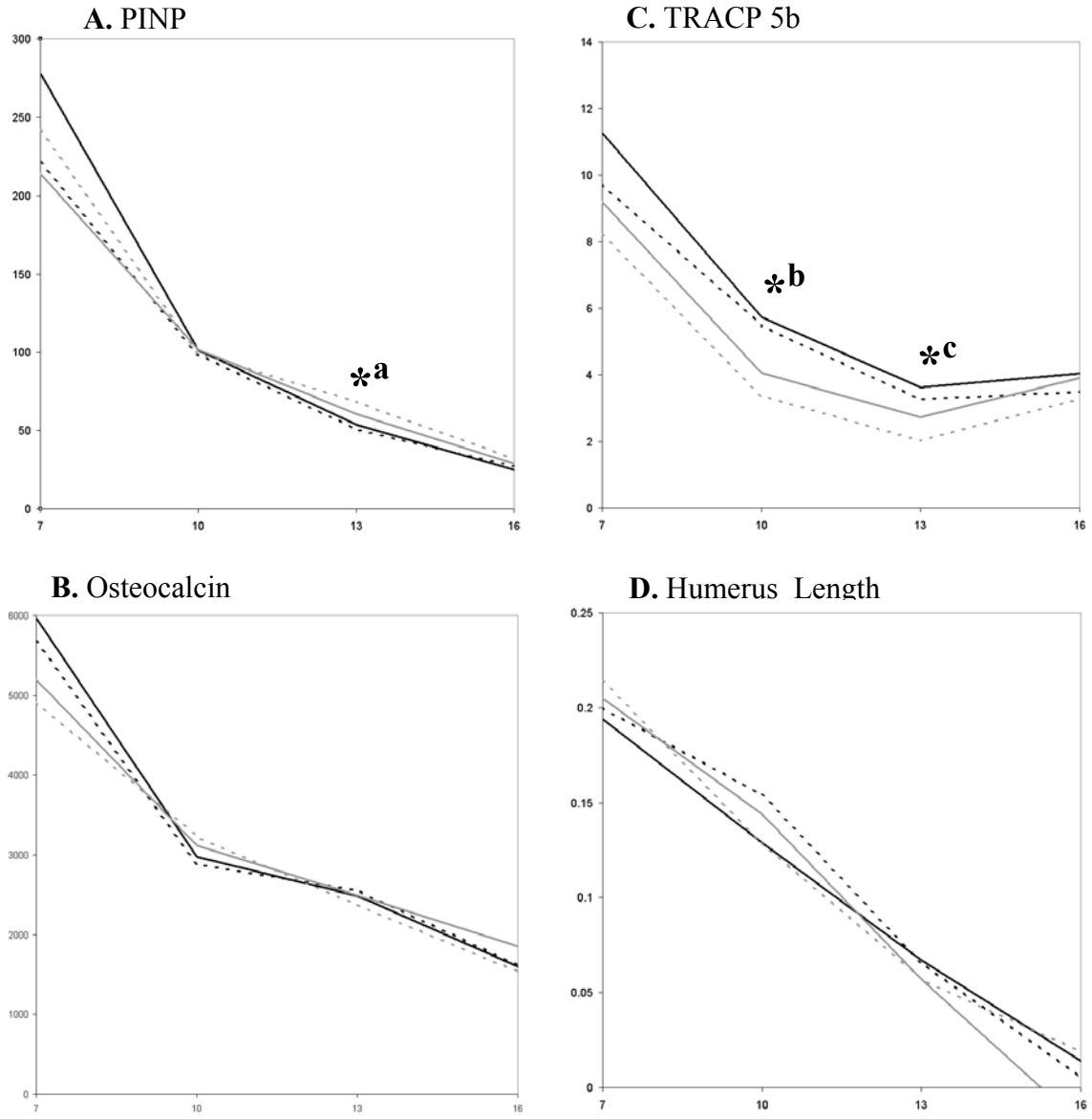


**TABLE 4.1.** Concentrations of serum PINP (ng/mL) with Kruskal-Wallis *p*-value (α=0.05).

| **Cohort** | **Week 7** | | **Week 10** | | **Week 13** | | **Week 16** | |
|---|---|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P)** (n=10) | 277.880 | 88.975 | 101.187 | 14.509 | 53.745 | 13.369 | 25.027 | 4.480 |
| **2 (P/M)** (n=10) | 221.263 | 28.319 | 98.501 | 22.427 | 51.041 | 7.056 | 27.365 | 6.149 |
| **3 (M)** (n=10) | 213.891 | 60.194 | 101.797 | 8.425 | 60.366 | 16.965 | 28.816 | 8.688 |
| **4 (M/P)** (n=10) | 241.517 | 83.675 | 99.268 | 30.161 | 68.636 | 11.150 | 32.140 | 12.145 |
| ***p*-value** | 0.061 | | 0.605 | | **0.010***[A] | | 0.516 | |

[A] See Table 4.2 for pairwise comparisons.

* $p \leq 0.05$



**TABLE 4.2.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of serum PINP for week 13.

| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
|---|---|---|---|---|
| **1 (P)** | x | X | x | x |
| **2 (P/M)** | 0.940 | X | x | x |
| **3 (M)** | 0.364 | 0.131 | x | x |
| **4 (M/P)** | **0.008*** | **0.003*** | 0.059 | x |

* Bonferroni-adjusted $p \leq 0.008$

**TABLE 4.3.** Concentrations of serum osteocalcin (ng/mL) with Kruskal-Wallis *p*-value (α=0.05).

| Cohort | Week 7 | | Week 10 | | Week 13 | | Week 16 | |
|---|---|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P)** (n=10) | 5968.612 | 1216.455 | 2972.990 | 357.392 | 2487.211 | 524.587 | 1603.347 | 538.291 |
| **2 (P/M)** (n=10) | 5675.324 | 1084.213 | 2890.243 | 340.529 | 2557.677 | 273.685 | 1619.704 | 328.064 |
| **3 (M)** (n=10) | 5194.260 | 871.738 | 3121.521 | 419.373 | 2494.999 | 222.849 | 1855.271 | 654.773 |
| **4 (M/P)** (n=11) | 4916.747 | 1074.669 | 3229.348 | 419.373 | 2377.507 | 565.789 | 1536.239 | 487.272 |
| ***p*-value** | 0.122 | | 0.337 | | 0.943 | | 0.748 | |

**TABLE 4.4.** Concentrations of serum TRACP 5b (ng/mL) with Kruskal-Wallis *p*-value (α=0.05).

| Cohort | Week 7 | | Week 10 | | Week 13 | | Week 16 | |
|---|---|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P)** (n=10) | 11.267 | 3.952 | 5.734 | 1.533 | 3.634 | 0.749 | 4.041 | 0.972 |
| **2 (P/M)** (n=10) | 9.735 | 2.805 | 5.472 | 1.316 | 3.262 | 0.830 | 3.493 | 0.590 |
| **3 (M)** (n=11) | 9.188 | 1.603 | 4.059 | 1.631 | 2.735 | 1.033 | 3.914 | 0.483 |
| **4 (M/P)** (n=11) | 8.197 | 1.482 | 3.382 | 1.132 | 2.046 | 0.604 | 3.286 | 0.523 |
| ***p*-value** | 0.092 | | **0.001***[A] | | **0.001***[A] | | **0.039***[A] | |

[A] See Table 4.5 for pairwise comparisons.

* $p \leq 0.05$

**TABLE 4.5.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of serum TRACP 5b for weeks 10-16.

| **Week 10** | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.597 | X | X | X |
| **3 (M)** | 0.017 | 0.045 | X | X |
| **4 (M/P)** | **0.002*** | **0.002*** | 0.309 | X |
| | | | | |
| **Week 13** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.257 | X | X | X |
| **3 (M)** | 0.041 | 0.181 | X | X |
| **4 (M/P)** | **0.001*** | **0.002*** | 0.108 | X |
| | | | | |
| **Week 16** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.151 | X | X | X |
| **3 (M)** | 0.725 | 0.067 | X | X |
| **4 (M/P)** | 0.049 | 0.291 | 0.016 | X |

***** Bonferroni-adjusted $p \leq 0.008$

# CHAPTER 5: BONE MICROSTRUCTURE

## Aims

*In vivo* fluorochrome labeling of actively mineralizing bone surfaces was used to generate longitudinal comparisons of daily mineral apposition rate (MAR) among cohorts (Erben, 2003). Calcein is a fluorochrome that adheres to newly formed apatite crystals in bone with excitation/emission wavelengths of 494/517 nm (Pautke et al., 2005). Thus, calcein-labeled bone can be visualized using fluorescent microscopy. MARs were examined in multiple skeletal locations, both within a masticatory element (mandible) and within a postcranial control element (femur). These data are used to identify skeletal regions undergoing mineralization significantly affected by masticatory loading and to compare ontogenetic rates of cortical bone apposition.

**Hypothesis 1**: In all skeletal elements, MARs will decrease with age.

**Hypothesis 2**: Individuals experiencing higher masticatory loads will exhibit higher MARs in masticatory elements.

**Hypothesis 3**: Variation in masticatory loading will not significantly affect MARs in postcranial non-masticatory elements.

## Methods

A series of five injections of calcein, diluted with sterile saline to a concentration of 10 mg/mL, was administered to all animals via intraperitoneal injection at a dosage of 1.5 mL/kg. These injections were given during weeks 4, 7, 10, 13, and 16, with the last injection (week 16) occurring 2 days prior to euthanasia. Subsequent to euthanasia, right-side cranial and femoral tissues were dissected and fixed for 72 hours in 4% paraformaldehyde at 4˚C. Tissues were then stored in 70% ethanol at 4˚C until used for histological analyses.

Histological slides were prepared at the Comparative Orthopaedics Research Laboratory at the University of Wisconsin School of Veterinary Medicine in Madison, WI. For processing, bones were dehydrated in a graded series of ethanol (70%, 100%) and embedded in methylmethacrylate. Transverse calcified sections, 125 μm thick, were made and mounted on standard microscope slides. All sections used for histomorphometry were unstained.

Three regions of interest were chosen for histological analysis of bone growth. Within the mandible, two sections were chosen at the level of the mandibular symphysis (hereafter referred to as the "anterior corpus") and at the level of the mandibular corpus adjacent to the molars (hereafter referred to as the "posterior corpus"). In both the anterior and posterior corpus, histological analyses were performed on the lateral periosteal surface of

the bone. More caudal sections, such as those taken at the level of the coronoid process and temporomandibular joint, proved uninformative as bone remodeling had resulted in the loss of the calcein label. A third section through the midshaft of the femur served as a control, here defined as a skeletal element for which the loading regime had not been experimentally modified.

Sections of calcein-labeled bone were imaged via fluorescent microscopy using an Olympus IX81 motorized inverted microscope. The microscope and Hamamatsu EM-CCD digital camera were controlled by SlideBook 4.2.0.10 software (Intelligent Imaging Innovations, Inc.). Calcein was excited by a 488 nm argon laser and its signal was detected through a widefield FITC filter. All sections were imaged using a 4x dry objective lens. Slide images were saved as 512x512 16-bit TIFF files at a scale of 4.032 microns/pixel.

ImageJ (Rasband, 2011) was used to gather linear measurements of the bone between fluorescent calcein bands (Figure 5.1). These linear measures were taken at 90-degree angles to the calcein bands. Each inter-band space was measured four times spaced evenly across the slide and then averaged to arrive at a mean linear distance for each inter-band space. Measures of bone growth were standardized among individuals by the use of a daily bone growth rate (μm/day), calculated as the mean inter-band distance divided by the number of days between calcein injections. Daily bone growth rate was measured for each individual over four periods: period 1 (weeks 4-7), period 2 (weeks 8-

10), period 3 (weeks 11-13), and period 4 (weeks 14-16). For the section through the femur midshaft, only periods 2-4 were able to be visualized as the calcein label for period 1 had been degraded by bone remodeling.

## Statistics

Kruskal-Wallis tests ($\alpha$=0.05) were used to compare daily bone growth rates statistically among cohorts for each histological region. When a statistically significant difference was detected among cohorts within a given region, individual pairwise comparisons were made using the Mann-Whitney *U* test with Bonferroni-adjusted *p*-values ($\alpha$=0.0083, 6 inter-cohort comparisons).

## Results

During period 1 (weeks 4-7), a significant difference ($p$=0.004) was observed among cohorts in MAR in the anterior mandibular corpus (Figure 5.2). Pairwise comparisons indicated that anterior corpus MAR is significantly ($p$=0.005) greater in cohort 1 (P) compared to cohort 3 (M) (Figure 5.2; Table 5.2). That the anterior corpus MAR in cohort 2 (P/M) does not differ significantly from the MAR of cohorts 3 (M) and 4 (M/P) is likely related to issues of sample size.

While a Kruskal-Wallis test indicated a significant difference ($p$=0.003) among cohorts in anterior mandible MAR during period 2 (Table 5.1), this was not borne out by pairwise comparisons (Table 5.2).

A significant difference ($p$<0.001) in anterior corpus MAR was also observed among cohorts during period 3 (weeks 11-13), following the dietary shift (Table 5.1). During this period, cohort 2 (P/M) was found to have significantly lower MAR in the anterior corpus compared to all other cohorts: cohort 1 (P) ($p$=0.001), cohort 3 (M) ($p$<0.001), and cohort 4 (M/P) ($p$<0.001) (Table 5.2). Additionally, cohort 4 (M/P) was found to have significantly greater MAR in the anterior corpus during period 3 compared to all other cohorts: cohort 1 (P) ($p$=0.001), cohort 2 (P/M) ($p$<0.001), and cohort 3 (M) ($p$=0.003) (Table 5.2).

No significant difference ($p$=0.186) was observed among cohorts in anterior mandible MAR during period 4 (Table 5.1).

No significant difference ($p$=0.159) was observed among cohorts in posterior mandible MAR during period 1 (Table 5.3).

A significant difference ($p$=0.022) in MAR in the posterior mandible was observed among cohorts during period 2 (weeks 8-10) (Table 5.3). Pairwise tests indicated that posterior corpus MAR was significantly lower in cohort 3 (M) compared to cohort 4 (M/P) ($p$=0.006) (Table 5.4). This is attributed to the fact that the calcein injection during week 10 occurred 24-48 hours *following* the dietary shift (Appendix II), and thus the

significant difference in posterior corpus MAR during period 2 may be related to a scheduling artifact.

A significant difference ($p$<0.001) in MAR in the posterior mandible was observed among cohorts during period 3 (week 11-13), following the dietary shift (Table 5.3). Pairwise comparisons indicated that posterior corpus MAR is significantly greater in cohort 4 (M/P) compared to all other cohorts: cohort 1 (P) ($p$=0.007), cohort 2 (P/M) ($p$<0.001), and cohort 3 (M) ($p$=0.001) (Table 5.4).

No significant difference ($p$=0.659) was observed among cohorts in posterior mandible MAR during period 4 (Table 5.3).

No significant differences were observed among cohorts in MAR at the femoral midshaft during periods 2-4 (Table 5.5).

## Conclusions

Mineral apposition rates (MARs) within the cortical bone of the rat mandible decrease throughout ontogeny (hypothesis $1_A$) (Figure 5.2). Immediately following weaning, absolute MAR in the posterior corpus of the rat mandible exceeds that in the anterior corpus. By week 8, the absolute values of these rates are more equivalent. Prior work by Yamada and Kimmel (1991) observed intact calcein labels in the mandibular ramus and the gonial region through postnatal week 11 in laboratory rats. However, the study

described here observed obliteration of the calcein labels due to remodeling in the mandibular ramus at week 16. Assuming equivalence in the skeletal ontogeny between the unknown strain of female laboratory rats used in the Yamada and Kimmel (1991) study and the Sprague-Dawley male rats examined herein, this suggests a shift from bone modeling to bone remodeling occurs in the mandibular ramus after skeletal maturity is achieved around week 12 (Roach et al., 2003).

The histomorphometric analyses reveal periods of functional adaptation within the mandible induced by changes in masticatory loading associated with two life history events: weaning at the onset of the experimental period (week 4), and the dietary shift during postnatal week 10. Following weaning (week 3), the cohort fed a diet of solid pellets exhibits a higher MAR on the lateral periosteal surface of the anterior mandibular corpus as compared to the cohort fed a meal diet. Lower MAR in the lateral mandible in cohorts fed a meal diet are consistent with the histomorphological results found by Yamada and Kimmel (1991), which the authors related to the development of narrow mandibles in these cohorts. Differences in mandibular MARs disappear by period 2 (weeks 8-10), at which point no significant difference is observed between stable diet cohorts fed pellet (cohort 1) versus meal (cohort 3) diets.

Following the mid-experimental dietary shift (week 10), the variable meal-to-pellet cohort (cohort 4, P/M) exhibits a higher MAR on the lateral periosteal surface of both the anterior and posterior mandibular corpus compared to all other cohorts. Additionally, the

variable pellet-to-meal cohort (cohort 2, M/P) was found to have lower MAR in the anterior corpus compared to all other cohorts. These differences disappeared by period 4 (weeks 14-16), at which point all dietary cohorts – both stable and variable – were found to have statistically similar rates of mineral apposition throughout the mandibular corpus.

In sum, these results suggest that greater masticatory strain related to variation in dietary material properties (Weijs and de Jong, 1977; Hylander, 1979, 1988; Yamada and Kimmel, 1991; Hylander, 1992; Hylander et al., 1992; Williams et al., 2005; Ravosa et al., 2008b; Ravosa et al., 2008a) may result in increased bone growth rates. Thus, these results support hypothesis $2_A$ – that individuals experiencing higher masticatory loads will exhibit higher MARs in masticatory elements. There is, however, a caveat: these elevated rates of mineral apposition are temporary and subside once differential bone modeling has achieved an optimal strain environment (Lanyon, 1984; Rubin, 1984; Yamada and Kimmel, 1991).

The interplay between ontogenetically decreasing growth rates and transient periods of functional adaptation means that rates and patterns of bone modeling are load history dependent. That is, daily bone growth rates in the mandible are not determined by the absolute magnitude of masticatory loading, but rather by the relative increase or decrease of the current load to previously experienced loads. Prepubescent rats (4 weeks old) weaned onto a solid pellet diet and adolescent rats (10 weeks old) experiencing a dietary shift to a solid pellet diet do not demonstrate equivalent absolute rates of bone growth,

but both groups do experience *relative* increases in mandibular MARs. Bone growth rates are thus specific to the loading history in a given skeletal element for that particular individual. These results underscore the importance of longitudinal approaches for understanding the ontogenetic and historical factors that influence plasticity responses at the individual level.

Finally, changes in daily bone growth rates related to variation in masticatory loading were localized to the mandible in this study, supporting hypothesis $3_A$. No changes in MARs related to diet were observed in the femur, which served as a skeletal control. From these data, a further hypothesis can be posited: cranial skeletal elements subject to masticatory loading will exhibit greater MAR in the presence of increased masticatory loading compared to non-loaded cranial elements. Additional histological data for viscerocranial and neurocranial elements are needed to test this hypothesis. Histomorphometric studies utilizing fluorescent labeling represent an opportunity to evaluate *in vivo* the osteogenic response of various cranial skeletal elements to masticatory loading (Hylander et al., 1991a, b; Rawlinson et al., 1995; Ross and Hylander, 1996; Herring and Teng, 2000; Ravosa et al., 2000a; Ravosa et al., 2000b; Ravosa et al., 2000c; Rawlinson et al., 2009; Menegaz et al., 2010). This may prove especially useful for confirming the results of *in vivo* and *in vitro* strain gauge studies, or evaluating skeletal elements inaccessible to such studies.

**FIGURE 5.1.** A histological section of a rat mandible, illustrating calcein labels and inter-band distance. Section is at the level of the mandibular symphysis. Key: IBD, inter-band distance; IR, incisor root; arrowheads point to fluorescent lines denoting calcein injections 1-5 (right to left).

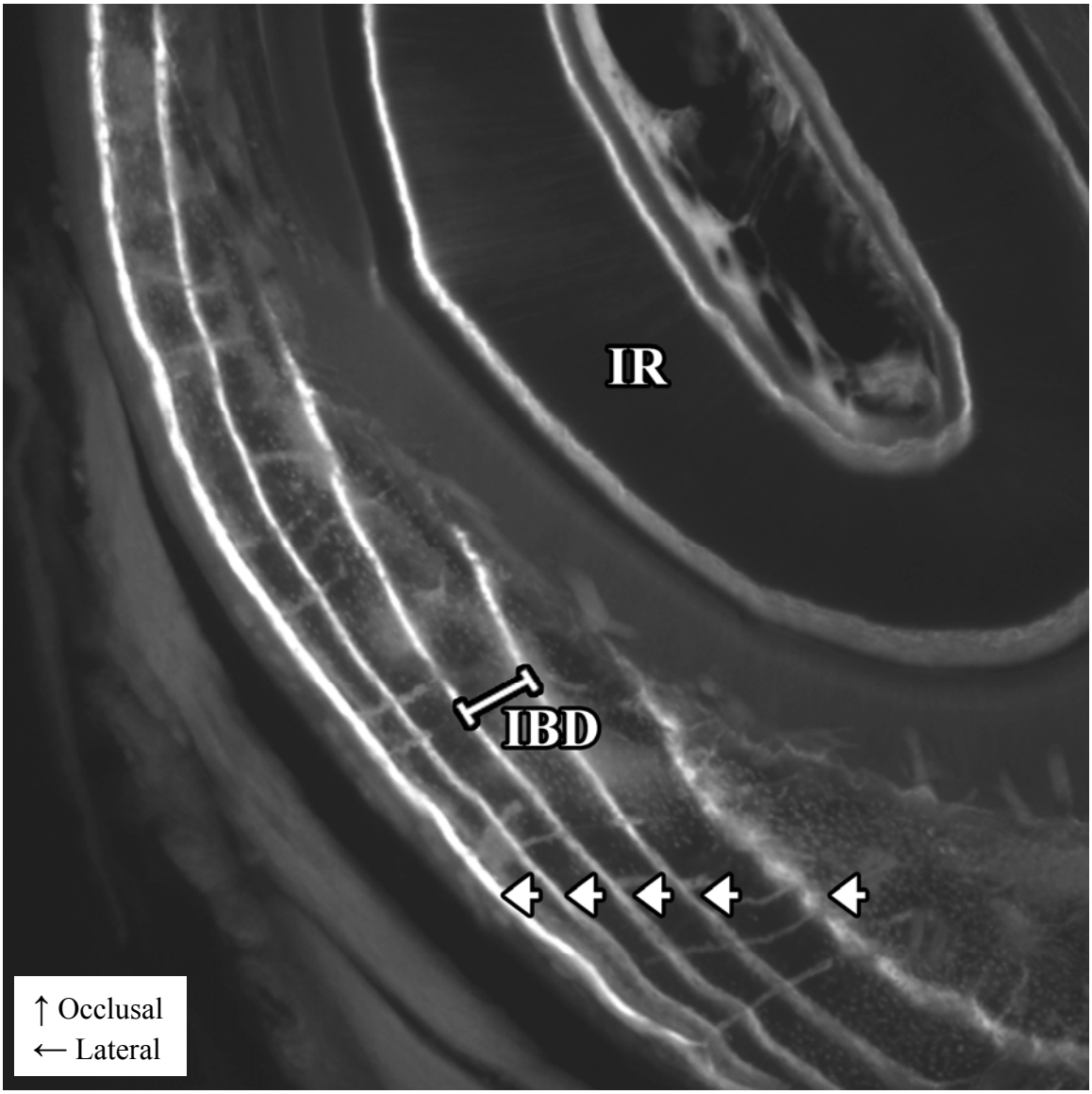
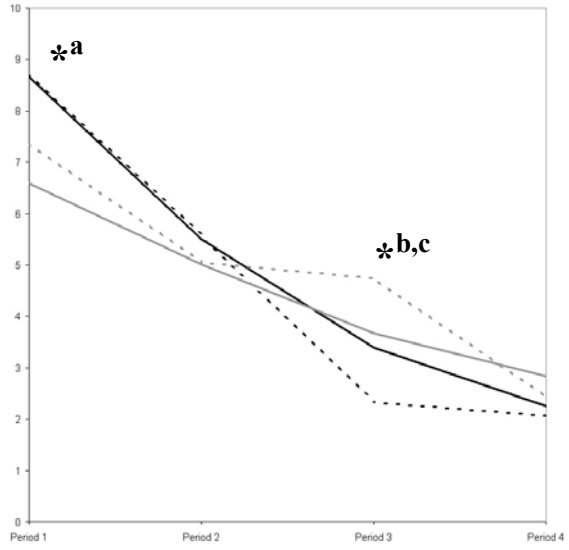
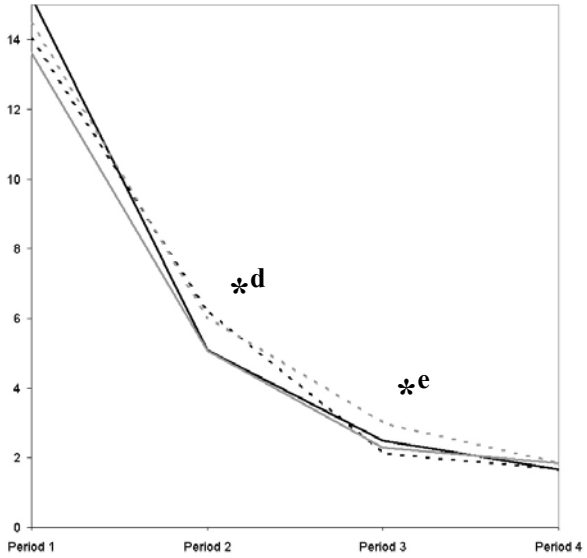


**FIGURE 5.2.** Results of fluorescent histomorphometry of the anterior mandibular corpus (A), the posterior mandibular corpus (B), and the femur (C). Pairwise comparisons are made using the Mann-Whitney U-test with Bonferroni-adjusted p-values (α=0.008). **Axes**: X, growth period; Y (A-C), bone growth rate (μm/day). **Line Key**: Cohort 1 (P), solid black line. Cohort 2 (P/M), dashed black line. Cohort 3 (M), solid gray line. Cohort 4 (M/P), dashed gray line. **Symbol Key:** *[a] Cohort 1 (P) > Cohort 3 (M). *[b] Cohort 2 (M/P) < Cohorts 1 (P), 3 (M), and 4 (M/P). *[c] Cohort 4 (M/P) > Cohorts 1 (P), 2 (P/M), and 3 (M). *[d] Cohort 4 (M/P) > Cohort 3 (M). *[e] Cohort 4 (M/P) > Cohorts 1 (P), 2 (P/M), and 3 (M).

**A.** Anterior mandibular corpus




**B.** Posterior mandibular corpus




**C.** Femur

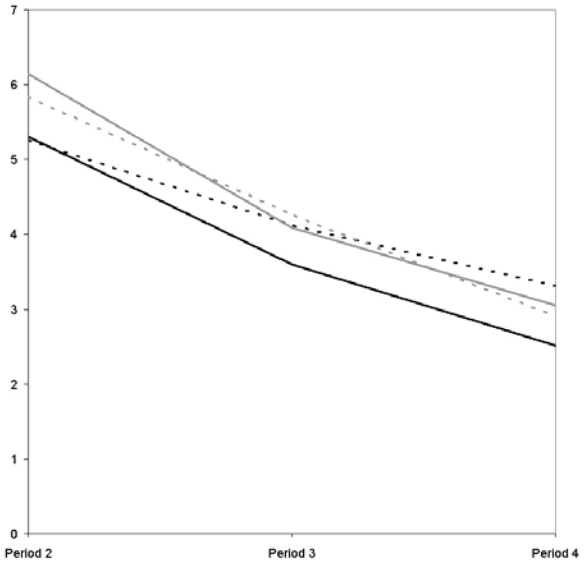


**TABLE 5.1.** Mineral apposition rates (μm/day) in the anterior corpus with Kruskal-Wallis *p*-value (α=0.05).

| Cohort | Period 1 | | Period 2 | | Period 3 | | Period 4 | |
|---|---|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P) (n=10)** | 8.655 | 1.298 | 5.502 | 0.492 | 3.392 | 0.414 | 2.250 | 0.318 |
| **2 (P/M) (n=8)** | 8.730 | 1.078 | 5.577 | 0.265 | 2.336 | 0.473 | 2.067 | 0.605 |
| **3 (M) (n=11)** | 6.593 | 1.532 | 5.012 | 0.555 | 3.669 | 0.355 | 2.834 | 0.908 |
| **4 (M/P) (n=11)** | 7.367 | 1.336 | 5.054 | 0.649 | 4.754 | 0.850 | 2.433 | 0.751 |
| ***p*-value** | **0.004*** [A] | | **0.003*** [A] | | **< 0.001*** [A] | | 0.186 | |

[A] See Table 5.2 for pairwise comparisons.

* $p \leq 0.05$

**TABLE 5.2.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of mineral apposition rates (μm/day) in the anterior corpus for periods 1-3.

| **Period 1** | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.790 | X | X | X |
| **3 (M)** | **0.005*** | 0.010 | X | X |
| **4 (M/P)** | 0.290 | 0.026 | 0.224 | X |
| | | | | |
| **Period 2** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 1.000 | X | X | X |
| **3 (M)** | 0.041 | 0.010 | X | X |
| **4 (M/P)** | 0.091 | 0.048 | 0.974 | X |
| | | | | |
| **Period 3** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | **0.001*** | X | X | X |
| **3 (M)** | 0.078 | **<0.001*** | X | X |
| **4 (M/P)** | **0.001*** | **<0.001*** | **0.003*** | X |

***** Protected $p \leq 0.008$

**TABLE 5.3.** Mineral apposition rates (μm/day) in the posterior corpus with Kruskal-Wallis *p*-value (α=0.05).

| Cohort | Period 1 | | Period 2 | | Period 3 | | Period 4 | |
|---|---|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P) (n=10)** | 15.214 | 2.443 | 5.093 | 0.632 | 2.500 | 0.358 | 1.660 | 0.393 |
| **2 (P/M) (n=9)** | 14.027 | 2.626 | 6.234 | 2.470 | 2.124 | 0.428 | 1.685 | 0.384 |
| **3 (M) (n=11)** | 13.616 | 1.191 | 5.066 | 0.728 | 2.282 | 0.457 | 1.853 | 0.575 |
| **4 (M/P) (n=11)** | 14.474 | 1.081 | 6.038 | 0.860 | 3.010 | 0.321 | 1.866 | 0.258 |
| ***p*-value** | 0.159 | | **0.022*** [A] | | **< 0.001*** [A] | | 0.659 | |

[A] See Table 5.4 for pairwise comparisons.

* $p \leq 0.05$

**TABLE 5.4.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of mineral apposition rates (μm/day) in the posterior corpus for periods 2-3.

| **Period 2** | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.165 | X | X | X |
| **3 (M)** | 0.944 | 0.160 | X | X |
| **4 (M/P)** | 0.014 | 0.342 | **0.006*** | X |
| | | | | |
| **Period 3** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.050 | X | X | X |
| **3 (M)** | 0.181 | 0.621 | X | X |
| **4 (M/P)** | **0.007*** | **<0.001*** | **0.001*** | X |

* Protected $p \leq 0.008$

**TABLE 5.5.** Mineral apposition rates (μm/day) in the femur at the level of the midshaft with Kruskal-Wallis *p*-value (α=0.05).

| Cohort | Period 2 | | Period 3 | | Period 4 | |
|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P) (n=9)** | 5.305 | 2.006 | 3.600 | 0.853 | 2.520 | 0.470 |
| **2 (P/M) (n=10)** | 5.269 | 2.097 | 4.130 | 0.747 | 3.316 | 1.311 |
| **3 (M) (n=11)** | 6.139 | 2.382 | 4.093 | 0.990 | 3.056 | 0.678 |
| **4 (M/P) (n=11)** | 5.843 | 1.820 | 4.267 | 1.022 | 2.911 | 0.714 |
| ***p*-value** | 0.797 | | 0.530 | | 0.310 | |

# CHAPTER 6: DISCUSSION AND CONCLUSIONS

The role of diet in shaping human craniomandibular form has long been a focus of study within physical anthropology (Hrdlička, 1930; Weidenreich, 1941; Hylander, 1975; Carlson and Van Gerven, 1977), inspiring a large body of experimental and comparative work which has elucidated the associations between the mechanical properties of food items, masticatory kinematics and loading patterns, and the differential growth and remodeling of masticatory tissues (Beecher and Corruccini, 1981; Beecher et al., 1983; Bouvier and Hylander, 1984; Hylander and Crompton, 1986; Bouvier, 1988; Hylander et al., 1992; Bouvier and Hylander, 1996b; Hylander et al., 2000; Hylander et al., 2005; Nicholson et al., 2006; Ravosa et al., 2006; Ravosa et al., 2007b; Ravosa et al., 2008b; Ravosa et al., 2008a; Vinyard et al., 2008; Menegaz et al., 2009; Menegaz et al., 2010). However, despite growing awareness of the spatial and temporal complexity that characterizes the primate diet (Conklin-Brittain et al., 1998; Robinson and Wilson, 1998; Lambert, 2007; Marshall and Wrangham, 2007), few studies prior to this one have investigated the effects of dietary variability on craniomandibular growth and morphology. Attempts to understand the role of dietary variability in primate evolution and biology have often focused on dental tissues, using microwear and dietary isotopes to infer the composition of an individual's diet (Teaford and Ungar, 2000; Laden and Wrangham, 2005; Stanford, 2006; Antón, 2008; Cerling et al., 2011; Dominy, 2012). As

the skeletal elements of the masticatory apparatus are known to be plastic in response to dietary composition and masticatory loading, this research asked whether the craniomandibular skeleton could provide similar insight into dietary variability in fossil hominins and non-human primates. The objectives of this research were twofold: to evaluate the utility of skeletal morphology in assessments of dietary variability, and to investigate the biological processes that underlie functional adaptation in a growing organism experiencing shifts in feeding behavior. This chapter will summarize the major results of this experimental research, and discuss these findings in the broader context of the current state of knowledge regarding phenotypic plasticity and hominin evolution.

## Bone Macrostructure

In this study, two approaches were employed to elucidate how variation in dietary mechanical properties and post-weaning dietary shifts affect the ontogeny of craniomandibular form. The first set of analyses, a multivariate approach based on geometric morphometrics (Adams et al., 2004; Klingenberg, 2011) was employed to assess variation in mandibular form among the experimental dietary cohorts. The goal of this approach was to ask whether skeletal morphology reflects the presence of dietary variability in a population, and if so, which morphological characters – or suites of characters – are most informative for this type of ecomorphological analysis. In juvenile rats, preceding the dietary shift, mandibular morphology distinguishes cohorts fed a mechanically resistant diet from their non-resistant diet counterparts. However, a combination of multiple morphological dimensions spanning both the posterior (i.e.

ramus) and anterior (i.e. corpus) regions of the mandible is necessary to make this diet-based distinction. In adult rats, based upon mandibular morphology, dietary cohorts can be grouped by both their early pre-shift diet and late post-shift diet. Unlike the juvenile cohorts, the adult cohorts can be distinguished using a single morphological dimension in the posterior mandible. The distance between the coronoid and angular processes can be used to separate cohorts by their early diet, and the mandibular notch angle can be used to separate cohorts by their late diet. These results emphasize the character-specific nature of morphological plasticity in the mammalian mandible, as this study observed no trend for all mandibular characters or even regional groups of mandibular characters to group dietary cohorts in the same manner. Localized variation in skeletal growth rates and developmental timings may be responsible for this observation that different morphological characters reflect dietary behaviors at different ontogenetic stages.

The distribution of informative morphological characters also varies between the juvenile and adult stages. These characters, which reduce classification error in a discriminant function analysis, were identified in both the posterior and anterior mandible in juvenile rats at prepubescent and adolescent ages. Indeed, in adolescent rats, immediately preceding the dietary shift, the full first 50% of morphological variance identified by a principal components analysis is confined to the tooth-bearing structures in the anterior mandible. After skeletal maturity, informative morphological characters were found solely in the posterior mandible. Additionally, a majority of the described morphological variance in adult rats is also related to structures in the posterior mandible. The trend for

morphological variance in the anterior mandible to recede by adulthood may reflect an ontogenetic shift in the factors that affect mandibular form. Intrinsic factors, such as postnatal growth rates and dental development, may contribute more significantly to mandibular morphology in earlier ontogeny, while extrinsic factors, such as environmental influences, may predominate as growth rates slow and adult behavioral patterns (e.g. masticatory kinematics) are established. The longitudinal approach employed in this study indicates that multiple morphogenic factors may explain variation in mandibular form. Indeed, models which attribute morphological variation differentially to masticatory muscle hypertrophy or tooth growth (Atchley et al., 1992) need not be mutually exclusive, so long as ontogenetic stage is taken into consideration. Furthermore, results from this study are consistent with the observed trend in human populations for mandibular morphology to reflect diet more strongly in adults than in juveniles (Holmes and Ruff, 2011).

The second approach used in this study, quantifying cross-sectional dimensions of the mandibular corpus, identified a pattern of regional cortical thickness in the experimental sample of rodents similar to that which is observed in hominoid primates (Demes et al., 1984; Daegling and Grine, 1991). In this population of Sprague-Dawley rats, all dietary cohorts exhibited thicker cortical bone along the buccal aspect of the mandibular corpus as compared to the lingual aspect. It remains unknown how common this pattern of regional cortical thickness is among mammalian taxa, and further *in vivo* strain work is necessary to clarify whether this pattern is related to the distribution of masticatory

strains in the mammalian mandibular corpus (Demes et al., 1984) or alternatively is the byproduct of the structural junction between the mandibular alveolar process and ramus (Daegling and Grine, 1991). Furthermore, in this study, the long-term stable consumption of a non-resistant meal diet is associated with elevated cortical bone thickness along the lingual aspect of the mandibular corpus. This contrasts with the pattern observed in cebid primates, where the durophageous *Sapajus apella*[2] (Daegling, 1992) has been observed to have thicker cortical bone along the buccal aspect of the mandibular corpus compared to *Cebus* species that do not feed on hard objects. What is consistent between the patterns observed in rats and cebids is a trend for animals feeding on more mechanically resistant food items to have a higher ratio of buccal-to-lingual cortical bone in the mandibular corpus.

In sum, this research has demonstrated that dietary variability may increase the difficulty of classifying individuals by feeding behavior in studies of skeletal morphology. In skeletal-based analyses of masticatory variation, characters associated with joint and muscle attachment structures in the posterior mandible were shown to have a higher utility for classification studies than those structures in the anterior mandible. Finally, these results underscore the hazards of including juvenile individuals in ecomorphological studies, as factors such as high growth rates and tooth development may obscure environmental signals in earlier ontogenetic stages. In the young adults included in this study, posterior mandibular characters proved to be informative with regard to diet. However, these characters did not consistently group individuals by either

[2] Formerly *Cebus apella* (Alfaro et al., 2012).

their pre- or post-shift diet. Future investigations will be required to determine whether this character-specific plasticity persists through later ontogeny and through multiple dietary shifts.

## Bone Physiology

A longitudinal study of serum markers of bone activity was used to assess how aging and masticatory behavior influence skeletal physiology. These physiological processes, such as bone formation and resorption, underlie morphological plasticity and functional adaptation of the skeleton. A suite of three markers was used in order to quantify the various contributions of organic and inorganic bone formation and bone resorption to mastication-induced functional adaptation in the craniomandibular skeleton. This study confirmed that skeletal growth rates, measured here by serum markers and postcranial skeletal dimensions, were highest immediately following weaning and gradually decreased across ontogeny. This is consistent with previous observations that an organism's capacity for morphological plasticity may subside as growth rates decrease with aging (Hinton and McNamara, 1984; Meyer, 1987; Bouvier, 1988; Bertram and Swartz, 1991; Rubin et al., 1992; Pearson and Lieberman, 2004; Hoverman and Relyea, 2007; Ravosa et al., 2008b).

Stable, long-term variation in masticatory loading was not found to affect circulating levels of any of the serum markers measured in this study. No significant differences in markers of bone formation or resorption were observed between the stable resistant and

stable non-resistant dietary cohorts [1 (P) and 3 (M)] at any point during ontogeny. However, longitudinal dietary variation was found to influence serum levels of markers related to type I collagen production (PINP) and bone resorption (TRACP 5b). Following a mid-experiment shift in diet composition, the variable diet cohort moved from a non-resistant diet to a resistant diet [cohort 4 (M/P)] exhibited greater levels of PINP and reduced levels of TRACP 5b as compared to similarly aged individuals in cohorts 1 (P) and 2 (P/M). No significant differences in the marker reflecting noncollagenous bone formation (osteocalcin) were noted in this study. Thus, this study observed that intra-individual variation in dietary behavior, rather than inter-individual variation, more significantly affects serum markers of bone physiology.

In adolescent rats (postnatal week 10), a shift from a non-resistant diet to a resistant diet is accompanied by an increase in type I collagen production and a decrease in bone resorption. The uncoupling of the formation and resorption markers indicates that, at this ontogenetic stage, the skeletal response to a sudden change in masticatory loading is one dominated by modeling rather than remodeling. These results apply to skeletally immature individuals, as the balance between bone modeling and remodeling in response to changing masticatory loads may differ after skeletal maturity (Bertram and Swartz, 1991; Pearson and Lieberman, 2004). Furthermore, the observed increase in type I collagen production may be associated with an increase in the material toughness of the craniomandibular skeleton, which would improve the capacity of these bones to absorb energy produced during mastication (Viguet-Carrin et al., 2006).

The longitudinal nature of this research made it possible to observe the temporal duration of functional adaptation in the craniomandibular skeleton induced by the dietary shift. The dietary shift was imposed on day 64, during the rats' postnatal week 10 (see Appendix II). Elevated levels of serum PINP associated with type I collagen production were detectable 24 days later, during week 13, and had returned to baseline levels by week 16. Significant differences in TRACP 5b, associated with bone resorption, were more immediately apparent. Cohort 4 (M/P) had an observable decrease in bone resorption within 6 days of the dietary shift, and this trend persisted through week 13. Thus, in this sample of adolescent male rats, changes in serum markers related to skeletal functional adaptation were observed to occur within a relatively brief period (approximately 3 weeks) following the dietary shift. The duration of the bone modeling period is known to vary among mammalian species, and these results are consistent with the current (but limited) knowledge regarding bone remodeling periods in small mammalian taxa (Garetto et al., 1995).

This research illustrates the utility of serum markers of bone physiology to complement studies of bone morphology. Here, this minimally invasive technique was used to assess the longitudinal nature of bone formation and resorption, to provide preliminary insight into the relative contributions of the organic and inorganic components of bone in response to variation in masticatory loading, and to approximate the temporal duration of functional adaptation to longitudinal shifts in feeding behavior. These data, which reflect

processes occurring at the systemic level, were then used to inform investigations of regionalized functional adaptation observed within the craniomandibular complex.

## Bone Microstructure

Dynamic histomorphometry was used in this study to assess how age and masticatory loading affect regional rates of bone growth. Within the mandible, daily mineral apposition rates (MAR) were quantified in the anterior corpus at the level of the incisors and in the posterior corpus at the level of the molars. Two distinct periods of functional adaptation were observed in the longitudinal MAR data from the mandibular corpus. Following weaning, a higher daily MAR was noted in the anterior corpus of the cohort weaned onto a resistant diet of pellets [cohort 1 (P)] as compared to the cohort weaned onto a non-resistant diet of meal [cohort 3 (M)]. This elevated daily growth rate was observed only for the period encompassing the 3 weeks immediately following weaning, after which these two cohorts exhibited a similar MAR through the end of the experiment.

A second period of functional adaptation was observed in the mandibular corpus following the mid-experiment diet shift. For the three weeks following the shift, the cohort that had been moved from a resistant diet to a non-resistant diet [cohort 2 (P/M)] exhibited a lower MAR in the anterior corpus as compared to all other cohorts. Conversely, the cohort shifted from a non-resistant diet to a resistant diet [cohort 4 (M/P)] was found to have an increased MAR in the anterior corpus compared to all other

cohorts. This cohort also demonstrated an increased MAR in the posterior corpus when compared to the stable non-resistant diet cohort [3 (M)] immediately following the dietary shift (24-48 hours), and when compared to all other cohorts for the 3 week period following the shift. These data confirm that skeletally immature rodents respond plastically to changes in masticatory loading through the modification of mineral apposition rates in the mandibular corpus. Furthermore, regional variation was observed within the corpus in the MAR-based response to longitudinal changes in masticatory loading. A decrease in MAR related to a reduction in masticatory loading was observed only in the anterior corpus adjacent to the incisors, while the response to a recent elevation in masticatory loading was more immediately evident in the posterior than the anterior corpus.

In the posterior mandible, e.g. the ramus at the levels of the coronoid process and the temporomandibular joint, it was not possible to quantify MAR due to the resorption of the fluorescent bone label. This occurrence suggests that, in this sample of male Sprague-Dawley rats, bone remodeling processes become predominant following skeletal maturity at approximately 12 weeks postnatally. However, the persistence of the fluorescent bone labels in the mandibular corpus through week 16 indicates that bone modeling is still dominant in this region.

Results from these dynamic histomorphometric analyses of mandibular microstructure suggest that daily mineral apposition rates (MAR) are plastic in response to masticatory

loading. Furthermore, this study observed regional variation in the ontogenetic onset of bone remodeling between the mandibular ramus and the mandibular corpus, as well as regional variation in MAR plasticity within the mandibular corpus. These functional distinctions, which occur along a posterior-anterior gradient within the mandible, were also noted in analyses of bone macrostructure and may have important ramifications for the intersection of developmental and functional modularity within the masticatory complex (Atchley, 1993; Fish et al., 2011).

## IMPLICATIONS FOR PHENOTYPIC PLASTICITY

Laboratory-based studies of phenotypic plasticity, the fine-tuning of form-function relationships across an individual's lifespan, have been embraced by experimental biologists for their potential to shed light on morphology, growth, performance, and evolution (Biewener, 2002; Garland and Kelly, 2006; Ravosa et al., 2007a; Ravosa et al., 2007b; Ravosa et al., 2008b; Ravosa et al., 2008a; Menegaz et al., 2009; Menegaz et al., 2010; Ravosa et al., 2010). Indeed, for studies of craniomandibular functional morphology, this approach has proven to be an invaluable resource for understanding the relationships between dietary mechanical properties and masticatory form. The research described here expanded upon the traditional dietary manipulation model (Beecher and Corruccini, 1981; Beecher et al., 1983; Bouvier and Hylander, 1996b; Kiliaridis et al., 1996; Nicholson et al., 2006; Ravosa et al., 2007b; Ravosa et al., 2008b; Ravosa et al.,

2008a; Menegaz et al., 2009; Jašarević et al., 2010; Menegaz et al., 2010) in order to investigate the impact of longitudinal dietary variation on craniomandibular plasticity.

Dissimilar from many previous studies (Watt and Williams, 1951; Moore, 1966; Bouvier and Hylander, 1984; Kiliaridis et al., 1985; McFadden and McFadden, 1986; Kiliaridis et al., 1996; Ravosa et al., 2008b; Ravosa et al., 2008a), this research found minimal association between long-term inter-individual variation in dietary properties and gross dimensions of the mandible. Most geometric morphometrics-derived mandibular centroid sizes and linear measurements of mandibular cross-sectional size were found to be similar among cohorts of adult individuals raised on stable and variable diets of differing mechanical properties. It is conceivable that the lack of significant size-related differences among these cohorts is related to the duration of the experimental period, the ontogenetic stages observed, or the choice of small-bodied mammalian experimental species. However, this study also found that it was indeed possible to distinguish the four dietary cohorts at the subadult/young adult stage using morphological features associated with the posterior mandible, such as the temporomandibular joint and attachment sites for masticatory muscles.

Results from this experimental research highlight the variable nature of phenotypic plasticity among morphological characters and tissue types. A canonical variates analysis performed as part of this study found that, even within a single adult cohort raised on a variable diet, two morphological characters in close structural proximity could reflect two

different dietary modalities (early/pre-shift and late/post-shift). This character-specific nature of morphological plasticity could be related to ontogenetic variation in growth rates. Here, the morphological character that grouped cohorts by their early diet was a gross measure of mandibular ramus size (coronoid-angular process), while the character which grouped cohorts by their late diet was a finer detail of ramus shape (mandibular notch angle). Thus, as hypothesized here, morphological dimensions on a larger scale may be more plastic in younger individuals than older individuals due to the accelerated growth rates present in early ontogeny (Bertram and Swartz, 1991; Mosley and Lanyon, 2002; Pearson and Lieberman, 2004). Indeed, this study showed that absolute rates of daily bone growth differed within regions of the mandibular corpus during early ontogeny, but these differences only persisted for a short period (4 weeks) post-weaning. This hypothesis regarding structure-specific growth rates within the mandibular ramus may be testable in a future experimental sample of immature individuals using dynamic histomorphometry with multiple fluorescent labels.

While this research examined skeletal tissue within the masticatory apparatus, similar studies have demonstrated diet-related plasticity in associated soft tissues such as masticatory muscle (Taylor et al., 2006; Ravosa et al., 2010), joint cartilages (Bouvier and Hylander, 1981, 1984; McFadden and McFadden, 1986; Bouvier, 1987, 1988; Yamada and Kimmel, 1991; Nicholson et al., 2006; Ravosa et al., 2007b), and cranial ligaments (Jašarević et al., 2010). In a study of temporomandibular joint cartilage, Bouvier and Hylander (Bouvier and Hylander, 1984) found that juvenile rodents switched

from a mechanically non-resistant diet to a resistant diet recovered the cartilage phenotype associated with the consumption of a resistant diet. This suggests that within the masticatory apparatus, soft tissues such as cartilage may exhibit a greater degree of phenotypic plasticity related to variation in dietary mechanical properties as compared to calcified tissues, i.e. bone. Future analyses of tissues collected during this experimental protocol will address the tissue-specific nature of phenotypic plasticity in the rodent craniomandibular complex.

## Modularity in the Mandible

In organismal biology, the concept of modularity is often used to discuss the developmental and/or functional associations among morphological structures (Atchley et al., 1992; Atchley, 1993; Klingenberg, 2008). A module is a single morphological unit inside which structures share a close association and are relatively independent from structures outside that module. The mammalian mandible provides an example of a complex structure composed of multiple developmental modules, linked by the cell populations they arise from and the developmental stage during which they differentiate (Atchley et al., 1992; Atchley, 1993; Cheverud et al., 1997; Mezey et al., 2000; Klingenberg et al., 2003b; Zelditch et al., 2008; Fish et al., 2011). Functionally, the mandible is often divided into two regions, the posterior mandibular ramus and the anterior mandibular corpus (Atchley et al., 1985; Leamy, 1993; Cheverud et al., 1997; Mezey et al., 2000; Klingenberg et al., 2003b). These regions can then be further subdivided into functional modules, such as the condylar, coronoid, and angular

processes in the posterior mandible, and the molar and incisal alveoli in the anterior mandible. Furthermore, these functional regions are spatially aligned with developmental modules linked by common gene expression (Fish et al., 2011).

Morphological and histomorphometric analyses conducted in this study support the division into posterior and anterior functional units. In the adult animals examined in this study, morphological characters within the posterior mandible were identified as the most useful for distinguishing dietary cohorts and for classifying individuals by their correct dietary history. This would appear to lend support to the muscle hypertrophy model for the determination of mandibular form (Atchley et al., 1992), wherein bone-muscle interactions related to masticatory activity explain the greatest amount of morphological variance. However, this study also noted a substantial ontogenetic component to the regional distribution of observed morphological variance. In adolescent individuals, characters in the anterior mandible contributed significantly to observed morphological variance, in fitting with the tooth development model (Atchley et al., 1992). The longitudinal results from this study suggest that multiple morphogenic factors may be at work across ontogeny, and thus the muscle hypertrophy and tooth development models proposed by Atchley (Atchley et al., 1992) need not be mutually exclusive. Furthermore, this work also documents differences in the ontogenetic onset of bone remodeling in the posterior and anterior regions of the mandible, as well as differences in the plasticity of daily bone growth rates to masticatory loading *within* the mandibular corpus along posterior-anterior axis. Thus, this research substantiates previous work suggesting that the

mammalian mandible is composed of anterior and posterior functional regions, with multiple functional modules existing within these regions, and that variation in developmental processes may underlie this functional differentiation (Atchley et al., 1985; Leamy, 1993; Cheverud et al., 1997; Mezey et al., 2000; Klingenberg et al., 2003b).

## Functional Adaptation and Masticatory Loading Histories

Phenotypic plasticity, as the ontogenetic modulation of a phenotype across an environmental gradient (Bradshaw, 1965; Stearns, 1989; West-Eberhard, 1993; Via et al., 1995; Pigliucci and Hayden, 2001; DeWitt and Scheiner, 2004; Pigliucci, 2005; West-Eberhard, 2005), is intimately related to the concept of functional adaptation in skeletal tissues. This dynamic process, in which bone tissue is differentially modeled and remodeled, functions to maintain the skeleton's structural integrity in a given loading environment (Lanyon and Rubin, 1985; Biewener, 1993; Bouvier and Hylander, 1996b, a; Vinyard and Ravosa, 1998; Hamrick, 1999). When behavioral shifts change the levels and distribution of strain within a skeletal element, we expect to see the emergence of processes related to functional adaptation within that element. Indeed, the analyses of bone physiology and microstructure conducted in this research identified periods of functional adaptation following the two behavioral shifts occurring at weaning and at the mid-experiment shift in diet composition.

The functional adaptation periods observed in this study were significant but brief. The onset of changes in aspects of bone activity such as serum marker of bone resorption or daily mineral apposition rate was observed as soon as 24 hours to a week following a dietary shift. Signals of functional adaptation tended to persist through the 3-week observational period following a dietary shift, after which they were no longer observed. Thus, this study observed significant plasticity in processes related to bone physiology and microstructural growth of the mandible in response to changes in masticatory loading. These processes are temporary and were observed to subside once differential bone modeling had achieved an optimal strain environment under the new dietary regime (Lanyon, 1984; Rubin, 1984; Biewener et al., 1986; Yamada and Kimmel, 1991; Biewener and Bertram, 1993).

Furthermore, results of this research show that that an organism's masticatory loading history influences the functional adaptation response induced by behavioral shifts during later ontogeny. Post-shift changes in measures of bone physiology and bone microstructure were not determined by the absolute magnitude of masticatory loading, but rather by the relative increase or decrease of the current load to previously experienced loads. This is consistent with the presence of temporally limited periods of functional adaptation, such that only individuals experiencing relative changes in masticatory loading undergo skeletal processes related to maintaining an optimal strain environment. Furthermore, the cohort experiencing a shift from a mechanically non-resistant diet to a resistant diet was noted to display a stronger response than the opposing

shift from a resistant diet to a non-resistant diet. The response in the former cohort [4 (M/P)] was associated with changes in type I collagen production, bone resorption, and daily mineral apposition rates at the microstructural level. Whereas, in the latter case [cohort 2 (P/M)], the response to the dietary shift was confined to changes in daily mineral apposition rate in the anterior mandible. This resistant-to-non resistant cohort displayed no differences in serum markers of bone activity compared to a cohort raised on a stable resistant diet. Thus, plasticity related to bone modeling and remodeling appears to be specific to the loading history in a given skeletal element for a particular individual.

## IMPLICATIONS FOR THE FOSSIL RECORD

Recent advances in the field of primate ecology have led to a growing emphasis placed on the role of seasonality and dietary variability in primate biology and evolution (Conklin-Brittain et al., 1998; Robinson and Wilson, 1998; Lambert, 2007; Marshall and Wrangham, 2007). Fallback foods, the inclusive term for non-preferred and sometimes seasonally consumed food items which may be less nutritious or more mechanically resistant than preferred foods, have been highlighted for their role in reducing interspecific competition and even promoting speciation among taxa, such as early hominins (Teaford and Ungar, 2000; Laden and Wrangham, 2005; Stanford, 2006; Antón, 2008), African apes and cercopithecines (Conklin-Brittain et al., 1998; Lambert et al., 2004; Stanford, 2006), platyrrhines (Rosenberger, 1992; Wright, 2005), and Malagasy

strepsirrhines (Yamashita, 2008). Indeed, the seasonal consumption of mechanically challenging fallback foods has been invoked to explain discrepancies in ecological interpretations of Plio-Pleistocene hominin ecology derived from various sources such as dental microwear, dietary isotope analysis, and skeletal morphology (Teaford and Ungar, 2000; Laden and Wrangham, 2005; Stanford, 2006; Antón, 2008). Previous attempts to resolve these discrepancies have focused largely on dental tissues to infer the presence of intra-individual dietary variation in hominins (Teaford and Ungar, 2000; Laden and Wrangham, 2005; Stanford, 2006; Antón, 2008; Cerling et al., 2011; Dominy, 2012). This goal of this project was to evaluate whether skeletal-based analyses of masticatory morphology could be used in complement to dental-based evidence to assess the presence of dietary variability in Plio-Pleistocene hominin taxa.

The first objective of this research was to appraise the ability of skeletal morphology to distinguish between stable and variable diets. Within the Pleio-Pleistocene genus *Paranthropus*, craniomandibular morphology suggests that the masticatory apparatus of this robust australopithecine genus functioned to resist high levels of masticatory strain associated with consumption of mechanically resistant food items (Robinson, 1954b; Du Brul, 1977; Hatley and Kappelman, 1980; Grine, 1981; Lucas et al., 1985; Laden and Wrangham, 2005; Ungar and Sponheimer, 2011). However, dental microwear and dietary isotopes studies have suggested the presence of interspecific variation within *Paranthropus* in the consumption of fallback foods, with the southern *P. robustus* consuming resistant food items only seasonally and the eastern *P. boisei* consuming

mechanically resistant food items as a stable, year-round fallback strategy (Sponheimer et al., 2006; Cerling et al., 2011; Lee-Thorp, 2011; Ungar and Sponheimer, 2011). Results from this study suggest that mandibular morphology can be used in multivariate morphometric analyses to separate groups of mature individuals based on the stable or variable consumption of mechanically resistant food items. Notably, ontogenetic stage may complicate these dietary analyses. The morphology of immature individuals is likely to be more influenced by intrinsic factors, such as high skeletal growth rates (Mosley and Lanyon, 2002) and dental development (Atchley et al., 1992; Daegling, 1996), and less influenced by external factors such as diet. This study cautions against the use of immature individuals in paleoecological analyses (Alemseged et al., 2006; Berger et al., 2010), particularly so in skeletal regions such as the mandibular corpus where tooth development may obscure dietary signals (Atchley et al., 1992; Daegling, 1996; Cofran, 2012).

The ontogenetic timing of fallback food consumption may also affect mandibular morphology. In this sample, young adult individuals who had been raised on variable diets were found to have morphology that reflected both their post-weaning and their post-shift diets. Additionally, the variable meal-to-pellet diet appeared to have a greater effect than the opposing variable pellet-to-meal diet in terms of mandibular size at maturity[3], bone physiology (type I collagen production and bone resorption levels), and bone macrostructure (daily rates of mineral apposition in the mandibular corpus). This

[3] Ln-transformed mandibular centroid sizes were significantly different between variable diet cohorts at maturity ($p=0.006$) (Table 3.19).

ontogenetic timing, where an individual was weaned onto a less resistant diet than it consumed as an adult, is consistent with the weaning patterns observed in larger bodied primates (Di Bitetti and Janson, 2000; Eckardt and Fletcher, 2013). Mountain gorillas, which live in highly variable environments, may time weaning to coincide with the availability of new bamboo shoots, such that newly weaned individual have greater access to more nutritious and more easily consumed food items (Eckardt and Fletcher, 2013). Indeed, studies of dental wear and mandibular corpus growth suggest that *Paranthropus* may have followed a gorilla-like pattern of early weaning combined with juvenile consumption of low-quality, mechanically resistant food items (Aiello et al., 1991; Cofran, 2012).

The second objective of this research was to identify the morphological characters that are most informative for skeletal-based analyses of diet. In this study, these characters were chosen by their ability to reduce classification error in a discriminant function analysis. As in the previous objective, immature individuals demonstrated a large amount of morphological variance in the mandibular corpus that was likely related to dental development (Atchley et al., 1992; Daegling, 1996). In adults, however, long-term variation in masticatory loading was found to concentrate morphological variance in the posterior mandible (e.g. mandibular ramus) in structures related to the temporomandibular joint and muscle insertion processes. This is consistent with a large body of experimental work which has observed greater morphological plasticity related to diet in the mandibular ramus than in the mandibular corpus (Barber et al., 1963;

Moore, 1966; Whiteley et al., 1966; Bouvier and Hylander, 1984; McFadden and McFadden, 1986; Bouvier, 1987, 1988; Yamada and Kimmel, 1991; Kiliaridis et al., 1996; Nicholson et al., 2006; Ravosa et al., 2008b). Comparative studies have also noted the relationship between primate diet and the morphology of the mandibular ramus and temporomandibular joint (Taylor, 2002; Terhune, 2013). This research suggests that ecomorphological analyses of hominin diet should focus on structures in the mandibular ramus rather than the less informative mandibular corpus. In application, admittedly, this may prove problematic due to the known preservation bias in favor of the corpus and tooth-bearing structure in hominin fossil assemblages (Lague et al., 2008; Cofran, 2012). In sum, morphological characters in the mandibular ramus should be given first consideration in ecomorphological analyses of diet, and caution should be employed with ontogenetically and morphogenically complex structures such as the mandibular corpus.

## FUTURE DIRECTIONS

This project represents the first attempt to naturalistically model the effects of dietary composition and variability on craniomandibular growth and form. The longitudinal, multi-disciplinary results described here highlight the complexity of the interactions among ontogeny, masticatory function, and morphological plasticity. Unique morphological and physiological trends were observed in those cohorts raised on variable diets compared to stable diets, suggesting that static dietary manipulation studies may underestimate the mammalian capacity for masticatory plasticity.

The rodent sample examined in this work was raised from weaning to young adulthood, the period during which skeletal growth rates and the capacity for phenotypic plasticity are greatest (Goldspink, 1970; Hinton and McNamara, 1984; Meyer, 1987; Bouvier, 1988; Bertram and Swartz, 1991; Rubin et al., 1992; Pearson and Lieberman, 2004; Hoverman and Relyea, 2007; Ravosa et al., 2008a). An important next step is to extend the investigation of the effects of dietary variability on masticatory plasticity into later ontogeny. Specifically, subsequent studies should encompass repetitive dietary shifts in order to better understand the morphology associated with the long-term (e.g. weaning to senescence) inhabitation of routinely fluctuating and seasonal environments.

This experimental study generated a large volume of data, including longitudinal μCT images and preserved tissues, which will be utilized to their fullest research potential. The morphological analysis of the rat mandible performed in this study emphasized the character-specific nature of diet-induced phenotypic plasticity. Accordingly, subsequent analyses will examine variation in craniofacial morphology and microstructure related to dietary variability (Watt and Williams, 1951; Kiliaridis et al., 1985; Kiliaridis et al., 1996; He and Kiliaridis, 2003; Menegaz et al., 2009; Menegaz et al., 2010). Future studies will also investigate the tissue-specific nature of phenotypic plasticity in masticatory muscle (Taylor et al., 2006; Grünheid et al., 2009; Ravosa et al., 2010), joint cartilage and subchondral bone (Bouvier and Hylander, 1981, 1984; McFadden and McFadden, 1986; Bouvier, 1987, 1988; Yamada and Kimmel, 1991; Nicholson et al.,

2006), and the organic and inorganic composition of the masticatory skeleton (Viguet-Carrin et al., 2006).

This research focused on the concept of functional adaptation as it pertains to the skeleton's dynamic ability to respond to fluctuating biomechanical demands among individuals within a single generation (Lanyon, 1984; Rubin, 1984; Lanyon and Rubin, 1985). More broadly, phenotypic plasticity is often discussed for its potential contribution to evolutionary adaptation (Stearns, 1989; West-Eberhard, 1993; Gotthard and Nylin, 1995; Sultan, 1995; Via et al., 1995; Sultan, 2000; West-Eberhard, 2005; Ghalambor et al., 2007). Plasticity is a significant source of variation within populations (Stearns, 1989) and may improve fitness in novel or variable environments (Stearns, 1989; Travis, 1994; Sultan, 1995; Agrawal, 2001; Ghalambor et al., 2007). Hypertrophy of craniomandibular musculoskeletal tissues in response to elevated masticatory loading is well documented among mammals (Beecher and Corruccini, 1981; Beecher et al., 1983; Bouvier and Zimny, 1987; Bouvier, 1988; Yamada and Kimmel, 1991; Bouvier and Hylander, 1996b; Kiliaridis et al., 1996; Nicholson et al., 2006; Ravosa et al., 2006; Ravosa et al., 2007b; Ravosa et al., 2008b; Menegaz et al., 2009; Menegaz et al., 2010; Ravosa et al., 2010), and in light of a large body of experimental work on optimal strain environments and functional adaptation (Weijs and de Jong, 1977; Hylander, 1979; Bouvier and Hylander, 1981; Lanyon and Rubin, 1985; Hylander et al., 1987; Hylander et al., 1992; Bouvier and Hylander, 1996b), is considered to be adaptive in terms of improving skeletal integrity and feeding performance (Bock and von Wahlert, 1965; Travis, 1994; Ravosa et al.,

2006; Ravosa et al., 2007b; Ravosa et al., 2008b; Ravosa et al., 2008a; Menegaz et al., 2009). In order to directly evaluate the adaptive nature of masticatory plasticity, a broader consideration of the effect of morphology on feeding performance is required. Behavioral and kinematic data related to feeding efficiency would complement these morphological studies. Additionally, ontogenetic strain gauge and finite element studies in these laboratory models of masticatory plasticity should be used in future work to validate the role of masticatory hypertrophy in maintaining an optimal strain environment in the craniomandibular complex. An enhanced synthesis of behavior, masticatory kinematics and biomechanics, and craniomandibular morphology will shed light on the relationship between individual morphological plasticity and population level morphological evolution.

This project follows a lengthy tradition of applying experimental analyses of non-primates to address outstanding questions in bioanthropology (Washburn, 1947; Moss, 1954, 1961; Bouvier and Hylander, 1984; Lieberman, 1996; Nicholson et al., 2006; Ravosa et al., 2007a; Ravosa et al., 2007b; Ravosa et al., 2008b; Ravosa et al., 2008a; Menegaz et al., 2009; Jašarević et al., 2010; Menegaz et al., 2010). Laboratory-based studies like the one described here represent singular opportunities to collect unique data not always available to field researchers and to better understand the form-function links observed in wild and fossil populations (Bock and von Wahlert, 1965; Kay and Cartmill, 1977; Lauder, 1995). This experimental research should be viewed as impetus for ongoing and future collaborations between laboratory and comparative research. Indeed,

comparative studies on the link between dietary variability and masticatory morphology in wild species are a necessary complement to this mode of research. Extant primates such as the Virunga mountain gorillas (*Gorilla beringei beringei*) should be a priority for comparative functional morphologists interested in dietary variability, given the wealth of skeletal and life history data available for this population (Shannon McFarlin, personal communication). In sum, this research represents an effort to embrace the spirit of Washburn's "New Anthropology" (Washburn, 1951) through the integration of multidisciplinary methodologies and the synthesis of laboratory and comparative based knowledge, so as to advance our understanding of the complex interactions between behavior, functional morphology, and evolution in primate and mammalian lineages.

# APPENDIX I: PRIOR EXPERIMENTAL STUDIES OF DIETARY VARIABILITY

| STUDY | SPECIES | STRAIN, SEX | DIET | STUDY DURATION | STARTING AGE | ENDING AGE | MORPHOLOGY |
|---|---|---|---|---|---|---|---|
| Bouvier and Hylander, 1984 | *Rattus norvegicus* | Long-Evans, males | pellets, dry meal[1] | 4 weeks | 4 weeks (weaning) | 8 weeks (adolescence) | Temporomandibular condyle and cartilage |
| | | | | 8 weeks | 6 weeks (prepubescence) | 14 weeks (subadulthood) | |
| Bouvier and Zimny, 1987 | *Rattus norvegicus* | Sprague-Dawley, males and females | pellets, moistened meal[2] | 4 weeks | 4 weeks (weaning) | 8 weeks (adolescence) | Temporomandibular condyle and cartilage |
| | | | | 12 weeks | ? weeks (mature) | ? weeks (mature) | |
| Yamada and Kimmel, 1991 | *Rattus norvegicus* | Unknown strain, females | pellets, moistened meal[3] | 8 weeks | 4 weeks (weaning) | 12 weeks (adolescence) | Mineral apposition rates and bone volume in the mandibular corpus and ramus; histmorphometry of condylar cartilage |

[1] Variable diet: meal-to-pellet only; 2/3 weeks (weaning-to-adolescence) or 4/4 weeks (prepubescence to subadulthood).
[2] Variable diet: meal-to-pellet only; 2/2 weeks (weaning-to-adolescence) or 4/4 weeks (mature).
[3] Variable diet: meal-to-pellet and pellet-to-meal; 4/4 weeks.

# APPENDIX II: EXPERIMENTAL SCHEDULE

| WEEK | AGE (DAYS) | | | | | | |
|---|---|---|---|---|---|---|---|
| **4** | **22** | **23** | **24** | **25** | **26** | **27** | **28** |
| | | CT Scan | CT Scan | Calcein Injection | | | |
| **5** | **29** | **30** | **31** | **32** | **33** | **34** | **35** |
| | | CT Scan | | CT Scan | | | |
| **6** | **36** | **37** | **38** | **39** | **40** | **41** | **42** |
| | | CT Scan | CT Scan | | | | |
| **7** | **43** | **44** | **45** | **46** | **47** | **48** | **49** |
| | | | | CT Scan<br>Calcein Injection | | | Blood Draw |
| **8** | **50** | **51** | **52** | **53** | **54** | **55** | **56** |
| | | CT Scan | CT Scan | | | | |
| **9** | **57** | **58** | **59** | **60** | **61** | **62** | **63** |
| | | | | CT Scan | | | |
| **10** | **64** | **65** | **66** | **67** | **68** | **69** | **70** |
| | **Diet Shift** | CT Scan<br>Calcein Injection | CT Scan<br>Calcein Injection | | | | Blood Draw |
| **11** | **71** | **72** | **73** | **74** | **75** | **76** | **77** |
| | | CT Scan | CT Scan | | | | |
| **12** | **78** | **79** | **80** | **81** | **82** | **83** | **84** |
| | | CT Scan | CT Scan | | | | |
| **13** | **85** | **86** | **87** | **88** | **89** | **90** | **91** |
| | | CT Scan | CT Scan<br>Calcein Injection | Blood Draw<br>Calcein Injection | | | |
| **14** | **92** | **93** | **94** | **95** | **96** | **97** | **98** |
| | | CT Scan | CT Scan | | | | |
| **15** | **99** | **100** | **101** | **102** | **103** | **104** | **105** |
| | | CT Scan | CT Scan | | | | |
| **16** | **106** | **107** | **108** | **109** | **110** | **111** | |
| | | CT Scan<br>Calcein Injection | CT Scan<br>Calcein Injection | Blood Draw | Sacrifice | Sacrifice | |

# APPENDIX III: RANDOM SORTING ANALYSIS

## Aims

In order to establish the morphological similarity of weanling rats across treatment groups at the outset of this experiment, this analysis tests the hypothesis that the experimental individuals were randomly sorted into dietary cohorts during the initial receiving process in the AALAC-accredited Office of Animal Resources facilities at the Harry S. Truman VA Hospital/University of Missouri.

> **Hypothesis 1:** Randomly sorted cohorts will be similar in craniomandibular dimensions at the start of the experiment.

## Methods

In order to test for random sorting at the outset of the experiment, 3D landmark data was collected from the initial μCT scans at weeks 4. 3D landmark data was also collected for weeks 10 and 16 in order to determine whether statistically significant differences in craniomandibular dimensions observed during week 4 persisted through later ontogenetic stages. 3D landmarks for the cranium and mandible (Table A1) were collected using the landmark placement plugin for eTDIPS (Mullick et al., 1999). A repeatability study (n=4, trials=4) was conducted to ensure precision in craniomandibular landmark placement with resulting standard errors (0.04-1.60 mm) below 5% of mean skull length during week 10 (mean = 44.0 mm, 5% of mean = 2.2 mm). Visual inspection of landmark

accuracy was also performed on individual wireframe models after Procrustes superimposition in Morphologika v2.5 (O'Higgins and Jones, 1998). To quantify gross craniomandibular dimensions, linear measurements were calculated between pairs of 3D landmarks using Euclidean distance (Table A2). Where $landmark_a$=a and $landmark_b$=b, this was calculated as: $\sqrt{((a_x-b_x)^2 + (a_y-b_y)^2 + (a_z-b_z)^2)}$.

## Statistics

Kruskal-Wallis tests ($\alpha$=0.05) were used to statistically compare raw cranial and mandibular dimensions among cohorts for each ontogenetic point. When a statistically significant difference was detected among cohorts within a given longitudinal point, individual pairwise comparisons were made using the Mann-Whitney *U* test with Bonferroni-adjusted *p*-values ($\alpha$=0.0083, 6 inter-cohort comparisons).

To test for true random sorting of weanling rats at the start of the experiment (week 4), all individuals were resorted into new cohorts using a list randomizer function (http://www.random.com/lists). Statistical analyses (described above) were then performed on the new randomly sorted cohorts to test hypothesis 1.

## Results

At the outset of the experiment (week 4), significant differences ($p \leq 0.05$) were found in several mandibular dimensions (Table A3). Pairwise comparisons (Table A4) indicate that jaw length ($p<0.001$) and diastema length ($p=0.003$) were greater in cohort 4 (M/P)

compared to cohort 1 (P) at this time point. Cohort 1 (P) demonstrated greater TMJ length compared to cohorts 2 (P/M) ($p$=0.003) and 4 (M/P) ($p$=0.003), and cohort 3 (M) also demonstrated greater TMJ length compared to cohort 2 (P/M) ($p$=0.006). Finally, cohort 2 (P/M) demonstrated greater coronoid height as compared to cohorts 1 (P) ($p$=0.007) and 3 (M) ($p$=0.006) during week 4. However, a random sorting analysis revealed that when individuals were randomly resorted into new cohorts, no significant differences in linear dimensions were detected during week 4 (Table A5).

While Kruskal-Wallis analyses suggested significant differences in linear dimensions among cohorts during week 10 (Table 3.6), this was not borne out by pairwise comparisons (Table 3.7). This is also the case for linear dimensions during week 16 (Tables 3.8 and 3.9).

## Conclusions

Statistical differences in mandibular dimensions at the beginning of the experiment (week 4) suggest that individuals were not randomly sorted into the four dietary cohorts ($H1_0$). Linear measurements during week 4 seem to indicate that cohort 1 (P) is problematic, possibly because too many individuals from a single litter were sorted into this cohort. However, the statistical differences observed during week 4 due to non-random sorting disappear by week 10, the mid-point of the experimental period.

**Table A1.** 3D landmarks collected in eTDIPS. Cranial landmarks 1-9 are located on the midline, 10-26 were collected on the right side. All mandibular landmarks were collected on the right side.

| **Cranial landmarks** | |
|---|---|
| **1** | Nasale |
| **2** | Nasion |
| **3** | Bregma |
| **4** | Parietal-Interparietal suture |
| **5** | Opisthocranion |
| **6** | Caudal nasal spine |
| **7** | Basisphenoid-Presphenoid synchondrosis |
| **8** | Opisthion |
| **9** | Basion |
| **10** | Premaxilla-nasal suture |
| **11** | Premaxilla-maxilla suture (inferior aspect) |
| **12** | Zygomatic-temporal suture (superior aspect) |
| **13** | Paracondylar process |
| **14** | Infraorbital foramen |
| **15** | External auditory foramen |
| **16** | Superior aspect of anterior root of zygoma |
| **17** | Inferior aspect of anterior root of zygoma |
| **18** | Superior aspect of posterior root of zygoma |
| **19** | Inferior aspect of posterior root of zygoma |
| **20** | Maxillary molar 1 |
| **21** | Maxillary molar 2 |
| **22** | Maxillary molar 3 |
| **23** | Foramen ovale |
| **24** | Hypoglossal foramen |
| **25** | Basioccipital-Basisphenoid synchondrosis (lateral aspects) |
| **26** | Pterygoid hamulus |
| | |
| **Mandibular landmarks** | |
| **1** | Posterior point on the temporomandibular condyle |
| **2** | Anterior point on the temporomandibular condyle |
| **3** | Coronoid process |
| **4** | Angular process |
| **5** | Mandibular notch |
| **6** | Subcondylar notch |
| **7** | Preangular notch |
| **8** | Superior aspect of incisal alveolus |
| **9** | Inferior aspect of incisal alveolus |
| **10** | Ramus-alveolar rim intersection |
| **11** | Mandibular molar 1 |
| **12** | Mandibular molar 2 |
| **13** | Incisal ramus |

**TABLE A2.** Linear measurements calculated from 3D landmarks.

| **Measurement** | **Landmark$_a$** | **Landmark$_b$** |
|---|---|---|
| Skull length | Opisthocranion | Nasale |
| Skull width | Left zygomatic-temporal suture | Right zygomatic-temporal suture |
| Face length | Bregma | Nasale |
| Midface height | Posterior nasal spine | Nasion |
| Neurocranial length | Opisthocranion | Bregma |
| Neurocranial width | Left external auditory foramen | Right external auditory foramen |
| Neurocranial height | Opisthocranion | Basion |
| Jaw length | Angular process | Inferior aspect of incisal alveolus |
| TMJ length | Posterior point on the temporomandibular condyle | Anterior point on the temporomandibular condyle |
| Coronoid height | Coronoid process | Preangular notch |
| Mandibular width | Left angular process | Right angular process |
| Alveolar height | Incisal ramus | Mandibular molar 1 |
| Diastema length | Superior aspect of incisal alveolus | Mandibular molar 1 |

**Table A3.** Linear cranial and mandibular measures (mm) for week 4.

| Cohort | Skull Length | | Skull Width | | Face Length | | Midface Height | | Neurocranial Length | | Neurocranial Width | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P)** (n=10) | 33.218 | 0.812 | 16.714 | 0.99 | 21.233 | 0.52 | 12.707 | 0.433 | 12.769 | 0.418 | 12.728 | 0.869 |
| **2 (P/M)** (n=9) | 33.366 | 0.588 | 16.642 | 1.451 | 21.26 | 0.53 | 12.335 | 0.414 | 12.893 | 0.436 | 13.04 | 0.285 |
| **3 (M)** (n=11) | 33.216 | 0.968 | 17.247 | 0.335 | 21.264 | 0.718 | 12.32 | 0.326 | 12.634 | 0.373 | 13.12 | 0.197 |
| **4 (M/P)** (n=11) | 33.601 | 0.806 | 17.293 | 0.221 | 21.583 | 0.795 | 12.416 | 0.353 | 12.803 | 0.301 | 13.006 | 0.175 |
| ***p*** | 0.789 | | **0.041*[A]** | | 0.712 | | 0.127 | | 0.492 | | 0.744 | |

| Cohort | Neurocranial Height | | Jaw Length | | TMJ Length | | Coronoid Height | | Mandibular Width | | Alveolar Height | | Diastema Length | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| **1 (P)** (n=10) | 8.406 | 0.138 | 17.902 | 0.384 | 2.533 | 0.162 | 7.812 | 0.402 | 14.211 | 0.376 | 5.012 | 0.138 | 5.455 | 0.418 |
| **2 (P/M)** (n=9) | 8.544 | 0.185 | 18.261 | 0.333 | 2.206 | 0.17 | 8.351 | 0.249 | 14.226 | 0.45 | 5.077 | 0.218 | 5.953 | 0.276 |
| **3 (M)** (n=11) | 8.618 | 0.202 | 18.377 | 0.417 | 2.412 | 0.128 | 7.942 | 0.287 | 14.414 | 0.383 | 5.144 | 0.193 | 5.964 | 0.333 |
| **4 (M/P)** (n=11) | 8.522 | 0.161 | 18.636 | 0.434 | 2.239 | 0.209 | 8.098 | 0.454 | 14.408 | 0.379 | 5.165 | 0.279 | 6.16 | 0.416 |
| ***p*** | 0.056 | | **0.004*[A]** | | **0.001*[A]** | | **0.019*[A]** | | 0.525 | | 0.374 | | **0.009*[A]** | |

[A] See Table A4 for pairwise comparisons. * $p \leq 0.05$

**Table A4.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of linear dimensions during week 4.

| **Skull Width** | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.568 | X | X | X |
| **3 (M)** | 0.041 | 0.160 | X | X |
| **4 (M/P)** | 0.017 | 0.053 | 0.768 | X |
| **Jaw Length** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.018 | X | X | X |
| **3 (M)** | 0.041 | 0.569 | X | X |
| **4 (M/P)** | **0.001*** | 0.074 | 0.178 | X |
| **TMJ Length** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | **0.003*** | X | X | X |
| **3 (M)** | 0.029 | **0.006*** | X | X |
| **4 (M/P)** | **0.003*** | 0.518 | 0.023 | X |
| **Coronoid Height** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | **0.007*** | X | X | X |
| **3 (M)** | 0.324 | **0.006*** | X | X |
| **4 (M/P)** | 0.139 | 0.210 | 0.375 | X |
| **Diastema Length** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.011 | X | X | X |
| **3 (M)** | 0.017 | 0.909 | X | X |
| **4 (M/P)** | **0.003*** | 0.305 | 0.341 | X |

***** Bonferroni-adjusted $p \leq 0.008$

**Table A5** Results from the Kruskal-Wallis test (α=0.05) performed for the random sorting analysis using linear dimensions from week 4.

| **Variable** | ***p*** |
|---|---|
| Skull length | 0.366 |
| Skull width | 0.941 |
| Face length | 0.263 |
| Midface height | 0.807 |
| Neurocranial length | 0.758 |
| Neurocranial width | 0.682 |
| Neurocranial height | 0.825 |
| Jaw length | 0.940 |
| TMJ length | 0.236 |
| Coronoid height | 0.972 |
| Mandibular width | 0.552 |
| Alveolar height | 0.916 |
| Diastema length | 0.972 |

**Table A6.** Linear cranial and mandibular measures (mm) for week 10.

| Cohort | Skull Length | | Skull Width | | Face Length | | Midface Height | | Neurocranial Length | | Neurocranial Width | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. |
| **1 (P)** (n=8) | 43.253 | 1.184 | 22.546 | 0.321 | 28.679 | 1.147 | 15.544 | 0.378 | 15.017 | 0.301 | 16.005 | 0.502 |
| **2 (P/M)** (n=8) | 43.163 | 1.042 | 22.424 | 0.392 | 28.693 | 0.886 | 15.421 | 0.569 | 14.929 | 0.408 | 15.955 | 0.575 |
| **3 (M)** (n=9) | 43.550 | 0.547 | 22.541 | 0.210 | 29.069 | 0.698 | 15.492 | 0.376 | 14.920 | 0.341 | 15.726 | 0.937 |
| **4 (M/P)** (n=11) | 43.836 | 0.649 | 22.726 | 0.306 | 29.182 | 0.809 | 15.423 | 0.456 | 15.165 | 0.364 | 15.908 | 0.315 |
| ***p*** | 0.240 | | 0.286 | | 0.405 | | 0.829 | | 0.475 | | 0.928 | |

| Cohort | Neurocranial Height | | Jaw Length | | TMJ Length | | Coronoid Height | | Mandibular Width | | Alveolar Height | | Diastema Length | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. |
| **1 (P)** (n=8) | 10.054 | 0.439 | 23.250 | 0.917 | 3.159 | 0.222 | 12.843 | 0.454 | 17.994 | 0.349 | 7.164 | 0.362 | 7.063 | 0.414 |
| **2 (P/M)** (n=8) | 9.950 | 0.236 | 23.026 | 0.650 | 2.751 | 0.416 | 12.883 | 0.310 | 17.968 | 0.402 | 7.182 | 0.122 | 7.045 | 0.468 |
| **3 (M)** (n=9) | 9.645 | 0.235 | 23.029 | 0.541 | 2.827 | 0.189 | 12.493 | 0.339 | 18.019 | 0.402 | 7.080 | 0.193 | 6.927 | 0.305 |
| **4 (M/P)** (n=11) | 9.558 | 0.415 | 22.986 | 0.561 | 2.753 | 0.467 | 12.618 | 0.211 | 18.084 | 0.675 | 7.309 | 0.261 | 7.109 | 0.470 |
| ***p*** | **0.014*** [A] | | 0.857 | | **0.039*** [A] | | **0.033*** [A] | | 0.528 | | 0.184 | | 0.795 | |

[A] See Table A7 for pairwise comparisons. * $p \leq 0.05$

**Table A7.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of linear dimensions during week 10.

| **Neurocranial Height** | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.248 | X | X | X |
| **3 (M)** | 0.027 | 0.021 | X | X |
| **4 (M/P)** | 0.017 | 0.039 | 0.849 | X |
| **TMJ Length** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.027 | X | X | X |
| **3 (M)** | 0.016 | 0.564 | X | X |
| **4 (M/P)** | 0.021 | 0.457 | 0.732 | X |
| **Coronoid Height** | | | | |
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.961 | X | X | X |
| **3 (M)** | 0.043 | 0.027 | X | X |
| **4 (M/P)** | 0.083 | 0.039 | 0.305 | X |

**Table A8.** Linear cranial and mandibular measures (mm) for week 16.

| Cohort | Skull Length | | Skull Width | | Face Length | | Midface Height | | Neurocranial Length | | Neurocranial Width | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. |
| **1 (P)** (n=10) | 47.439 | 0.88 | 23.822 | 0.462 | 32.043 | 0.815 | 16.766 | 0.587 | 15.924 | 0.288 | 16.883 | 0.202 |
| **2 (P/M)** (n=10) | 47.052 | 0.831 | 23.769 | 0.455 | 31.643 | 0.496 | 16.496 | 0.469 | 15.86 | 0.475 | 16.102 | 1.236 |
| **3 (M)** (n=11) | 47.033 | 0.726 | 24.112 | 0.256 | 31.589 | 0.759 | 16.601 | 0.56 | 15.859 | 0.509 | 16.863 | 0.402 |
| **4 (M/P)** (n=11) | 47.409 | 0.508 | 24.189 | 0.442 | 32.084 | 0.559 | 16.694 | 0.371 | 15.813 | 0.279 | 16.747 | 0.335 |
| ***p*** | 0.352 | | 0.113 | | 0.198 | | 0.666 | | 0.742 | | 0.311 | |

| Cohort | Neurocranial Height | | Jaw Length | | TMJ Length | | Coronoid Height | | Mandibular Width | | Alveolar Height | | Diastema Length | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. |
| **1 (P)** (n=10) | 10.142 | 0.132 | 25.212 | 0.645 | 3.369 | 0.216 | 14.238 | 0.377 | 21.849 | 0.637 | 7.978 | 0.191 | 7.792 | 0.27 |
| **2 (P/M)** (n=10) | 10.163 | 0.241 | 24.497 | 0.642 | 3.32 | 0.26 | 14.229 | 0.412 | 21.989 | 0.612 | 8.013 | 0.187 | 7.692 | 0.222 |
| **3 (M)** (n=11) | 10.096 | 0.168 | 24.977 | 0.396 | 3.348 | 0.158 | 14.274 | 0.321 | 22.43 | 0.590 | 8.223 | 0.495 | 7.78 | 0.411 |
| **4 (M/P)** (n=11) | 10.052 | 0.214 | 25.293 | 0.416 | 3.221 | 0.224 | 14.353 | 0.37 | 22.298 | 0.600 | 8.187 | 0.313 | 7.673 | 0.305 |
| ***p*** | 0.499 | | **0.011*[A]** | | 0.475 | | 0.917 | | 0.212 | | 0.279 | | 0.739 | |

[A] See Table A9 for pairwise comparisons. * $p \leq 0.05$

**Table A9.** Results (*p*-values) from Mann-Whitney *U* tests (α=0.008) of linear dimensions during week 16.

| Jaw Length | | | | |
|---|---|---|---|---|
| **Cohort** | **1 (P)** | **2 (P/M)** | **3 (M)** | **4 (M/P)** |
| **1 (P)** | X | X | X | X |
| **2 (P/M)** | 0.041 | X | X | X |
| **3 (M)** | 0.139 | 0.049 | X | X |
| **4 (M/P)** | 0.622 | 0.011 | 0.279 | X |

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# VITA

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